

A metabolomics investigation of Fibromyalgia Syndrome

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DEDICATION

I dedicate this degree my biggest cheerleader, my late father. It saddens me that you never got to see me receive this degree but I know that where you are, you are smiling down on me with pride at my achievement.

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I would like to thank my supervisor, Prof Carools Reinecke, for all his help and guidance during my studies.

To Dr Shayne Mason, the best co-supervisor a student can ask for, thank you for all your help since my arrival in Potchefstroom and for always having an ear to listen.

To my mother and my sisters, thank you for being there and motivating me through this academic journey.

To my husband, you have been with me since the start of my tertiary academic career, thank you for allowing me to take this path, your motivation during my journey and always being my comfort. To my baby boy, thank you for adding that extra inspiration and motivation in the latter part of my studies

And finally, to the great man upstairs, God, through Him all things are possible. Just allow Him to take His time, you will be in awe when he reveals His hand.

ABSTRACT

This thesis, entitled: “A metabolomics investigation of Fibromyalgia Syndrome”, deals with Fibromyalgia Syndrome (FMS), a chronic widespread pain disorder with an estimated prevalence of 3.2% in the South African general population. Currently, the pathophysiology of FMS is uncertain, and is difficult to diagnose because diagnosis is based, almost completely, on patient feedback. No putative biomarkers have been described for this disorder, as of yet. The quest to identify reliable biomarkers for definitive diagnosis and monitoring of disease progression forms an important aspect of FMS research.

I present here an extensive metabolomics investigation using a clinically, well described FMS group in a thesis structured into three sections.

Section one contains three chapters that primarily cover my study and the literature. Chapter 1 gives an overview of the thesis and describes the content of each chapter. Chapter 2 is the review on FMS from a clinical aspect. Here I define pain, from a biochemical view, and discuss the different kinds of pain associated with FMS. Chapter 2 also includes the detailed information on the clinical profile of the FMS patients, as well as information on the controls. Against this background I formulated my biological question: *“Is there a metabolic perturbation in FMS that may subsequently be used to establish a pain profile for the disorder and to identify a biomarker or biosignature for FMS?”*. Chapter 3 is a review of the genetic component of FMS and I also introduce the investigative method employed in this study. Chapter 3 also reviews the three, key publications of the only other investigations that, likewise, studied FMS from a metabolomics aspect, during the course of my study.

Section two (Chapter 4) I present an untargeted proton magnetic resonance (^1H NMR) spectroscopy study on the urine of FMS patients and controls. This holistic ^1H -NMR metabolomics approach proved to be useful in that the findings revealed that my FMS cohort was metabolically distinguishable from my controls on the basis of their urinary metabolic profiles.

Section three (Chapter 5) focuses on a semi-targeted gas chromatography-mass spectrometry (GC-MS) study of the same FMS patients and control cohort as that in Chapter 4, with urine once more being used as the sample material. This was a follow-up study that was conducted on the basis of the findings from the ^1H -NMR study in Chapter 4. Outcomes of this GC-MS study revealed further insights on the disorder, FMS, and we speculated a further mechanism that may underlie the pathophysiology of FMS.

In the last section (Chapter 6), I discuss the achievements of this thesis. Here, I address the aims and objectives of this thesis and discuss the new mechanism we hypothesize to play a role in FMS pathophysiology, that may give rise to the phenotype observed in FMS. I conclude this study by speculating that 2-hydroxyisobutyric acid may be a potential putative biomarker for the metabolic perturbation occurring in FMS, as well as other diseases, as discussed in a brief overview.

Keywords: Fibromyalgia syndrome (FMS), pain, central sensitization, irritable-bowel syndrome (IBS), dysbiosis, gut-brain axis, metabolomics, proton magnetic resonance (¹H-NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), 2-hydroxyisobutyric acid (2-HIBA)

Format: This thesis is presented in article format and meets the requirements set out by the North-West University, Potchefstroom campus. Thus, the following full, peer-reviewed papers (1 published and 2 submitted for review) form part of this thesis:

- 1) Malatji, B.G., Meyer, H., Mason, S., Engelke, U.F., Wevers, R.A., Reenen, M. and Reinecke, C.J., 2017. A diagnostic biomarker profile for fibromyalgia syndrome based on an NMR metabolomics study of selected patients and controls. *BMC Neurology*, 17(1), 88–102.
- 2) Malatji, B.G., Mienie, L.J., Wevers, R.A., Meyer, H.P., Mason, S., van Reenen, M. and Reinecke, C.J. The GC-MS metabolomics signature in patients with Fibromyalgia Syndrome directs to dysbiosis as an aspect contributing factor of FMS pathophysiology (submitted to *BMC Neurology*).
- 3) Malatji, B.G., Mason, S., Wevers, R.A., Engelke, U.F.H., van Reenen, M and Reinecke, C.J. Alpha-hydroxyisobutyric acid: An overview and focus on fibromyalgia syndrome (submitted to *Biomarker Research*).

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CHAPTER 1: INTRODUCTION TO STUDY

Fibromyalgia (FM), also known as Fibromyalgia Syndrome (FMS), is recognized to be a chronic pain syndrome. Professor Helgard Meyer, primary clinician and co-supervisor of this study, states that “the primary care doctor, having a better understanding of the biopsychosocial background of the patient, is in the best position to manage patients with FM” (Meyer 2006). This view of Meyer’s concurs with those expressed at the recent Congress of the European Pain Federation (EFIC), held in Copenhagen, 6–9 September 2017 (Perrot 2017). Until 1980, FMS was mainly regarded as a psychological condition, but things changed. In 1990, the American College of Rheumatology (ACR) first approved criteria for fibromyalgia — “The American College of Rheumatology 1990 criteria for the classification of fibromyalgia” (Wolfe *et al.*, 1990), although the ACR endorsed in 2010 the Symptoms Severity Scale as an alternative approach to identify FMS (Wolfe *et al.*, 2010), generally known as the 2010/2011 criteria. Increased new insights on FMS again led to revisions, resulting in the 2016 revision of criteria to diagnose and classify FMS (Wolfe *et al.*, 2016). Virtually no information on chronic pain epidemiology was, until recently, available for South Africa as is common in many developing countries (Chopra & Abdel-Nasser 2008; Derman *et al.*, 2011). However, things changed in South Africa: a validated South African Pain Catastrophizing Scale (SA-PCS) recently became available and proved to be a valuable tool to assess FMS in a multicultural population, as prevails in South Africa (Morris *et al.*, 2012).

At the time when the present study was designed, the research consortium opted for application of the 1990 criteria of the ACR, which was the most widely accepted international practice at that stage, for the diagnosis of FMS. These criteria emphasized chronic and widespread musculoskeletal pain (including pain in the axial skeleton) in the presence of pain on at least 11 of 18 specified tender point sites with digital palpation of 4 kg/cm² (Wolfe *et al.*, 1990). Within this framework, our FMS patient population was homogeneous with regard to gender (female) and ethnicity (white). All patients were selected by Professor Helgard Meyer, Head of the Department of Family Practice, Faculty of Health Sciences, University of Pretoria.

The initiative for the present study on FMS was taken by a consortium under the auspices of the Nuclear Technologies in Medicines and Bioscience Initiative (NTeMBI) of the South African Nuclear Energy Corporation Limited (NECSA). The consortium consisted of NECSA, Biosequences (Pty) Ltd, the Centre for Genomic and Proteomic Research (CPGR), the

Clinicians Group and North-West University (NWU–Potchefstroom Campus). The Consortium Agreement was approved by NECSA on 26 October 2011 and provided, amongst other benefits, for an MSc bursary, managed on behalf of the National Research Foundation (NRF), by NTeMBI [Consortium Agreement, Clause 14]. The bursary was allocated to me, then Miss Bontle Molusi, aimed to conduct a “prototype project (fibromyalgia) as a focused approach” to consider required elements towards the ultimate aim of the consortium. The ultimate aim was to develop imaging biomarkers using radiolabels, to formulate and validate novel key candidate genetic (genomics investigation by the CPGR) or biochemical biomarkers (metabolomics investigation reported on here) and infer additional markers for routine diagnostic workup. The original research approach consisted of a pilot study, covering several aspects related to FMS. The flow diagram, patient and control groups, and initial outcomes at November 2012 are shown in Figure 1.1.

The genotyping analysis was conducted by Dr H.P. Mbongwa, but no clear indications of FMS-specific polymorphisms — (1) 5-HT_{2A} receptor polymorphism and (2) the catecholamine O-methyl transferase (COMT) polymorphism — could be detected in the present FMS patient group. My original amino acid metabolomics analysis, likewise, did not prove to give clear indications of diagnostic biomarkers (results not shown in this thesis), although three cases were suspected to be related to asymptomatic inborn errors of metabolism (IEM). A neurotransmitters analysis (e.g. involving gamma-aminobutyric acid) was subsequently performed in collaboration with Dr Nico Abeling of the Laboratory for Genetic and Endocrine Diseases (LGMD) at the Academic Medical Centre (AMC) of the University of Amsterdam (25 March to 6 April 2012), using a high performance-liquid chromatography (HPLC) separation procedure coupled to a fluorescence detector. Bioinformatics analysis on the complete normalized data set did not indicate a clear perturbation in the DOPA pathway, and any indications of dysregulated neurotransmitter function most probably reflected a stress-related profile, not necessarily specific for FMS. The outcome of the organic acid metabolomics analysis, done at the Metabolomics Platform of the Technological Innovation Agency (TIA, previously BioPAD), hosted in our laboratory at NWU, proved to present potential important markers for FMS (Figure 1.2). The content and volume of results obtained thus warranted its presentation for an MSc thesis, which I submitted to NWU in 2012. Owing to the depth of the potential information that could be extracted further from my MSc data, I was advised to apply for upgrading of the MSc to a PhD, which was approved by the Faculty Board of NWU. I was notified on 21 October 2013 by NTeMBI that they likewise approved the upgrading of the NRF bursary to the doctoral level.

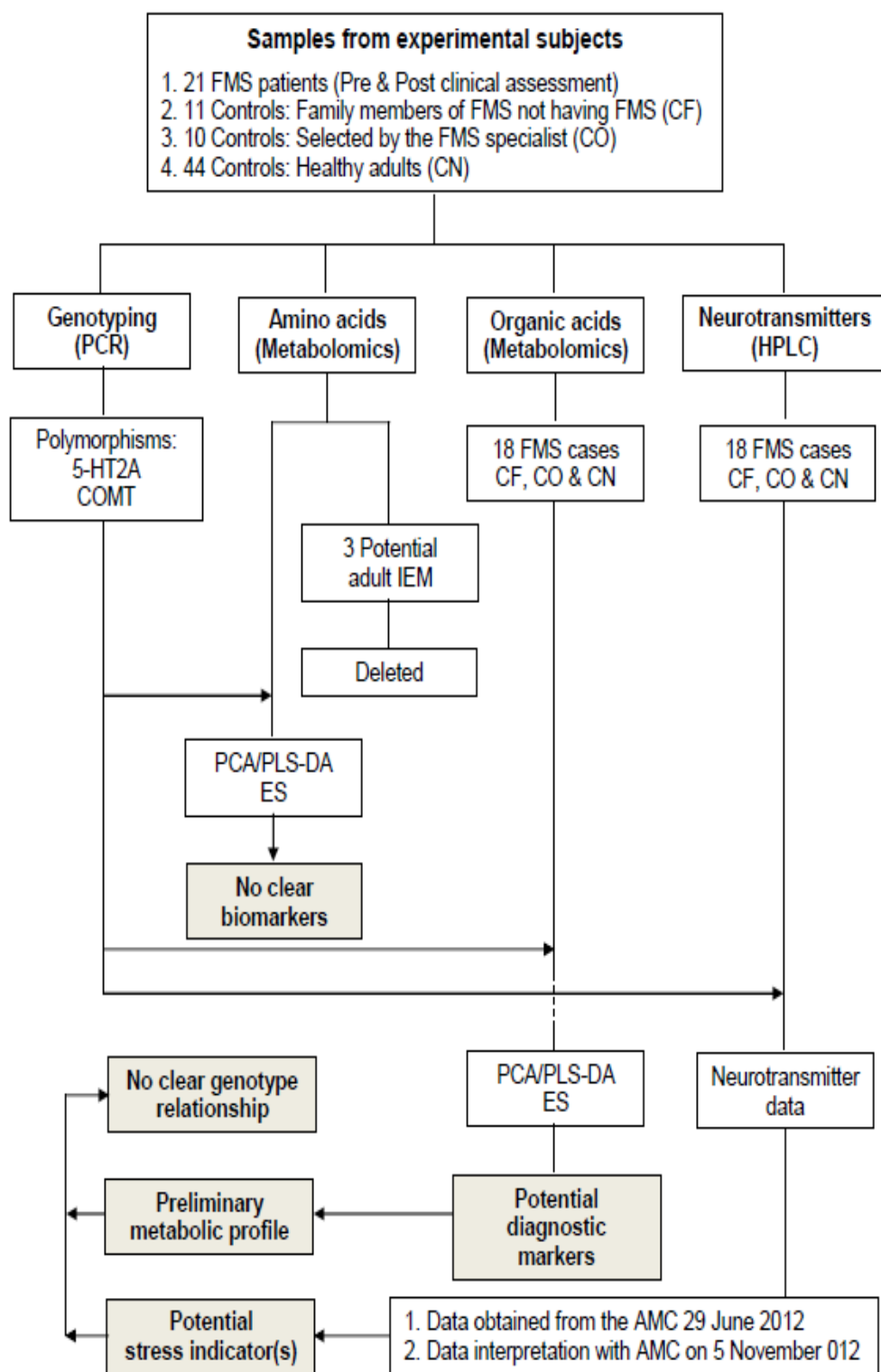


Figure 1.1: Flow diagram of the original experimental procedure followed to identify potential biomarkers for FMS, drafted November 2012.

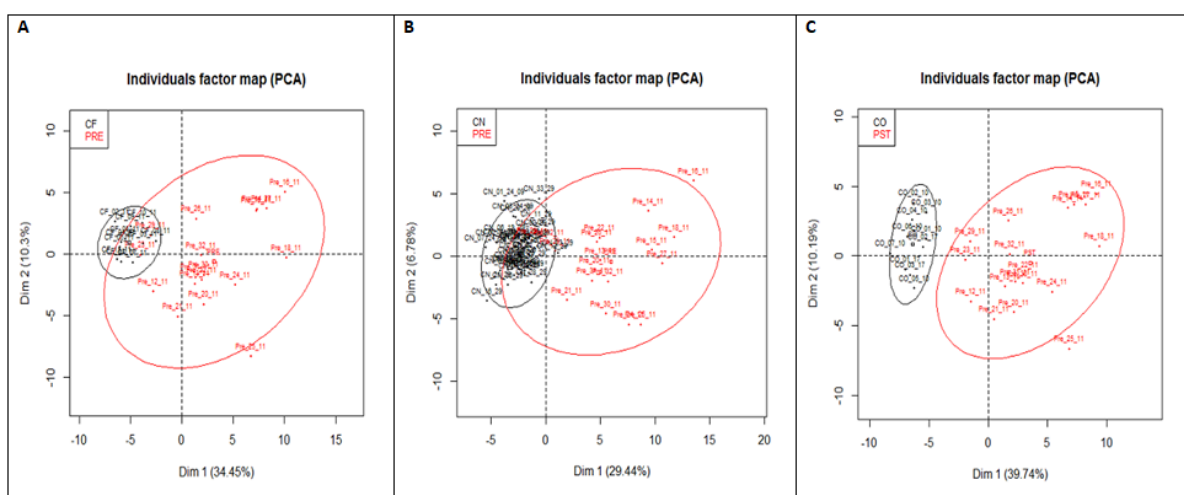


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Once the upgrade of the thesis to a PhD was approved, it was agreed to add a new technology – nuclear magnetic resonance (NMR) spectroscopy – to my study. This was done to analyse the urine metabolome in a holistic non-biased manner. Analyses using NMR were conducted by myself under the supervision of Professor Ron Wevers and Dr Udo Engelke at the Translational Metabolic Laboratory of the Medical Faculty of Nijmegen University, in the Netherlands.

The biological question, formulated in 2012, for this study, was: “*Is there a metabolic perturbation in FMS that may subsequently be used to establish a pain profile for the disorder and to identify a biomarker or biosignature for FMS*”. As a result, the aims and objectives of the thesis were thus as follows:

- Aim: The application of metabolic profiling to the disorder, FMS.
- The objectives of the study were to:
 1. Perform an explorative NMR metabolomics study (1) to elucidate the global urinary metabolite profile of patients suffering from FMS, and (2) to explore the potential of this metabolite information to contribute to improving diagnosis of FMS.
 2. Obtain complementary information on the metabolic profile of FMS patients. This was done by comparing affected individuals (cases) against those who were not affected (controls) through an semi-targeted study using GC-MS technology.
 3. Compare data from the GC-MS and NMR studies and identify a set of markers common to both studies that can be proposed as probable markers of the disorder.
 4. Formulate a hypothesis as to what the metabolic effects, if any, FMS has on an affected individual.

This thesis is presented in article format. The results from each of the analyses will be presented as an article that has been, or will be, published in a peer-reviewed journal. The following chapters of the thesis are structured as follows:

Chapter 2 provides a literature review of the mainline clinical aspects of FMS. It highlights those aspects of this chronic pain syndrome and what is currently circulating in the literature about FMS and how far studies have come in the elucidation of a probable cause of the disorder. From this review, my biological question was then formulated.

Chapter 3 comprises the genetic aspects of the disorder. Here a concise overview on the genetic basis of FMS is presented and also the chosen scientific method, being metabolomics, is introduced. A broad overview of metabolomics is presented followed by a discussion on the limited number of peer-reviewed studies on metabolomics applications on FMS. Subsequently, the aims of my study are defined along with my objectives.

Chapter 4 entails the NMR study that was conducted on a select group of FMS cases and controls. A brief overview of NMR technology in biofluids, with the main focus on proton NMR (^1H -NMR), is discussed and the outcomes of the study conducted are presented in the form of a peer-reviewed, published article (Malatji *et al.*, 2017). In this article we confirm that

FMS is indeed distinguishable from healthy counterparts and suggest a plausible biosignature for FMS. A poster presentation of the preliminary results, showing FMS to be distinguishable from its non-pain counterparts, was presented by myself at the MetaboMeeting Congress 2014 in London, United Kingdom (10–12 September).

Chapter 5 consists of the follow up GC-MS study conducted on the same group of subjects as in Chapter 4. A brief overview on the background of GC-MS technology is discussed and the outcomes of the study are presented in a scientific paper intended for submission to *BMC Neurology*. In this paper we highlight that dysbiosis is present in FMS and may have a role in the pathophysiology of the disorder. These results were also presented at the EFIC Congress 2017 by Professor Helgard Meyer in the form of a poster presentation.

Chapter 6 is the concluding chapter of this thesis. It includes a general discussion and conclusion on the overall contributions and results achieved from this investigation as a whole. I also reflect on the aims and objectives articulated previously in Chapter 1. The future prospects of this PhD research are also touched on. Lastly, a manuscript is presented on a metabolite, alpha-hydroxyisobutyric acid (2-HIBA), previously deemed a contaminant metabolite through environmental exposure. In our publication (Malatji *et al.*, 2017), 2-HIBA is identified as a distinguishing metabolite that was discarded due to the former reason. Several scientific publications have proven the contrary, thus implying that 2-HIBA has a probable role in the pathophysiology of FMS. As such, in this manuscript we report on 2-HIBA as a possible biomarker for diseases such as FMS.

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CHAPTER 2: LITERATURE REVIEW — CHARACTERISTICS OF FIBROMYALGIA SYNDROME

2.1 Introduction

FMS has been the subject of clinical studies since the 1980s. However, it still lacks one particular definition, mainly due to disagreements regarding its aetiology and pathogenesis (Häuser & Wolfe 2012). FMS is clinically defined and distinguished by a blend of perturbations in the autonomic, neuroendocrine, immune and nociceptive systems (Hackshaw *et al.*, 2013) and is the most common cause of widespread musculoskeletal pain (Jahan *et al.*, 2012). The mechanism of the pain experienced in this disorder is not yet fully understood and has been the focus of intense research, be it in regard to FMS or to other chronic pain syndromes associated with central sensitization such as migraine, irritable bowel syndrome (IBS), temporo-mandibular joint disorder and others (Park *et al.*, 2000; Chung 2004). As such, the quest to identify reliable biomarkers for the disorder for definitive diagnosis and monitoring of disease progression remains an important aspect of contemporary FMS research. Such a biomarker will aid in early diagnosis and appropriate management of the disorder with a reduction in both the direct and indirect financial burden (Greenberg *et al.*, 2009; Hackshaw *et al.*, 2013). The search for a specific biomarker for FMS is thus still unresolved, in part due to an overlap of potential biomarkers for FMS with other co-morbidities such as chronic fatigue syndrome (CFS) (Breeding *et al.*, 2012), on the one hand, and on the other, due to the large variation in FMS phenotypes. A preliminary investigation (Bazzichi *et al.*, 2009) — using a proteomics approach to detect potential markers for FMS — showed some potential for identifying biomarkers and clarifying some of the pathophysiological aspects of the disorder, although the authors agreed that “*no laboratory tests have been appropriately validated for the diagnosis and the prognostic stratification of the disease*”.

An ideal biomarker should be cost-effective and easy to assay, highly sensitive and specific to the particular disorder and should also adequately provide information and ideally allow quantification of the condition. Moreover, it should ideally be in a source material that is easily attainable, for example plasma or urine (Greenberg *et al.*, 2009). From the literature we see that many studies have investigated the elucidation of FMS on the genetic level. Metabolomics, on the other hand, is the study of the metabolome, which is the “complete set of metabolites in a cell or tissue” (Fiehn 2002; Brown *et al.*, 2005) and the final products of gene expression. From this we can tell that what occurs at the level of the gene will

ultimately have effect on what occurs at the metabolome level, thereby providing us with a biochemical perspective of a disease or disorder (Brown *et al.*, 2005; Kaddurah-Daouk & Krishnan 2009). For this reason, metabolomics is an attractive approach used for the identification of biomarkers of disease.

At the start of this study no comprehensive metabolomics investigations on FMS had been reported. In the interim, there have been three metabolomics studies that have been published, by Hackshaw *et al.*, in *Analyst*, 2013, Caboni *et al.*, in *PloS One*, 2014 and Hadrevi *et al.*, in *Scientific Reports*, 2015. These articles are discussed later in this thesis in Chapter 3. This review will thus focus mainly on summarizing the information obtained from the literature of the current clinical perspectives of FMS on a genetic and biochemical level. This review will serve to introduce the potential of a metabolomics approach in the study of FMS, but it should be noted that the established views on FMS as a specific disorder fall into the realm of medical science, whereas the focus of this dissertation is on a biochemical aspect, by being a metabolomics study of FMS. The inclusion of an overview including clinical aspects of this pain disorder is relevant because: (1) the clinical aspects are important for the appropriate selection of FMS patients and controls, which is a crucial aspect of metabolomics studies; and (2) in order to relate the subsequent biochemical findings to the characteristics of FMS.

2.2 Definition of Fibromyalgia Syndrome

2.2.1 Clinical definition of Fibromyalgia Syndrome

FMS is a syndrome of chronic widespread musculoskeletal pain associated with other symptoms such as fatigue, cognitive impairment and insomnia, for which no other cause can be identified. It is characterized by widespread pain, increased pain sensitivity, muscle and joint stiffness, disturbance in sleep, fatigue and depression (Bondy *et al.*, 1999; Buskila & Sarzi-Puttini 2008) and cognitive impairment (relating mainly to concentration and short-term memory impairment). There is currently strong evidence from brain imaging and other techniques that FMS has an organic basis, although psychosocial and behavioural factors may play a role in some patients (Nelson *et al.*, 2010; Clauw 2015). FMS affects 2–3% of the general population in the United States (Buskila *et al.*, 1996; Ablin *et al.*, 2008; Tander *et al.*,

2008) and an estimated 3.2% in South Africa (Lyddell & Meyers 1992). The condition occurs 10 to 20 times more frequently in women than in men and tends to affect individuals in their most productive years, being the years 35–60 (Matsuda *et al.*, 2010). The literature shows an occurrence of 0.5% for men and 3.4% for women (Clayton & West 2006; Silverman *et al.*, 2010).

Table 2.1: Table of clinical disorders which are often associated with FMS (adapted with permission from Jahan *et al.*, 2012.).

Musculoskeletal	Genitourinary	Gastrointestinal	Miscellaneous
	Primary dysmenorrhea	Irritable bowel syndrome	Tension type headaches
Temporomandibular joint disorder	Interstitial cystitis	Oesophageal dysmotility disorders	Migraine
Hypermobility syndrome	Vulvodynia		Mitral valve prolapse
Restless legs syndrome	Female urethral syndrome		Vestibular disorders (e.g. Menière's disease)
Rheumatoid arthritis	Vulvar vestibulitis		
Systemic lupus erythematosus	Premenstrual syndrome		Mood disorders
Sjögren's syndrome			Raynaud's phenomenon
Osteoarthritis			
			Lyme's disease
			Chronic fatigue syndrome
Myofascial pain syndrome			

Patients with FMS present with the symptoms mentioned above as well as tenderness in predetermined regions of the body, known as tender points (TPs). FMS is also known to present with a wide range of other chronic co-morbid disorders such as those summarized

in Table 2.1; which include IBS, migraine, restless leg syndrome and mood disorders (Bennett *et al.*, 2010; Lee *et al.*, 2012).

Individuals with FMS process pain differently from their healthy counterparts due to a dysfunction in the processing of pain by their central nervous system (CNS), which results in pain amplification (Ortancil *et al.*, 2010; Gracely *et al.*, 2002). Clayton and West (2006) also backed this theory by taking biopsies of the tender points and their findings showed no signs of peripheral pathology. Shah *et al.*, have demonstrated a low grade increase in certain cytokine levels (sub-nanogram quantities) in trigger points associated with myofascial pain and dysfunction (MPD) and FMS. These peripheral pain generators may contribute to the pathophysiology of some FMS patients (Shah *et al.*, 2005). Studies in the past have failed to demonstrate large-fibre neuropathy in FMS (Ersoz 2003), but, more recently, small-nerve fibre function has been proposed to be impaired in FMS patients (Üçeyler *et al.*, 2013). In a subset of FMS subjects, small-fibre neuropathy (SFN) was identified in skin biopsy material (Giannoccaro *et al.*, 2014), indicating that SFN may contribute to the sensory and autonomic symptoms in these FMS patients. Although the involvement of SFN in FMS is still not unequivocally established, it does appear that SFN plays a significant role in pain response in these patients (Caro & Winter 2014) and it has been proposed that a skin biopsy should be considered in the diagnostic work-up of FMS (Giannoccaro *et al.*, 2014).

Research has shown that there is a high familial aggregation of the disorder. There is approximately an 8-fold increase in risk to develop FMS in first-degree relatives of affected individuals when compared with healthy controls. From this it has been deduced that a genetic component is involved in the aetiopathology of FMS (Buskila *et al.*, 1996; Lee *et al.*, 2012) and it is currently regarded as a polygenic disorder (Buskila *et al.*, 2007; Rodriguez-Revena *et al.*, 2014).

The main aim of the 1990 American College of Rheumatology (ACR) criteria for FMS, was to standardize research populations and were not intended for clinical diagnosis (Wolfe *et al.*, 1990). These criteria stated that a patient should present with widespread pain, that it had occurred for longer than 3 months and should test positive for pain in at least 11 of the 18 predetermined TPs (Figure 2.1) (Silverman *et al.*, 2010). The term “widespread pain” refers to pain in all four quadrants of the body and includes pain in the axial skeleton (cervical, thoracic, and lumbar sacral spine) (Liu & Patterson 2009). TPs are assessed using a dolorimeter, with the amount of pressure applied being 4 kg/cm². To be considered a tenderpoint, pain should be experienced only where the pressure is applied and no referred pain should be experienced by the patient (Jahan *et al.*, 2012). The threshold of tenderness

is also assessed during examination. This is done by applying pressure at an increasing rate of about 1 kg/s. At this stage patients are told to indicate when the sensation changes from a feeling of pressure to a definite pain (Neumann *et al.*, 2008), which is usually associated with “wincing”, or withdrawal of that part of their body.

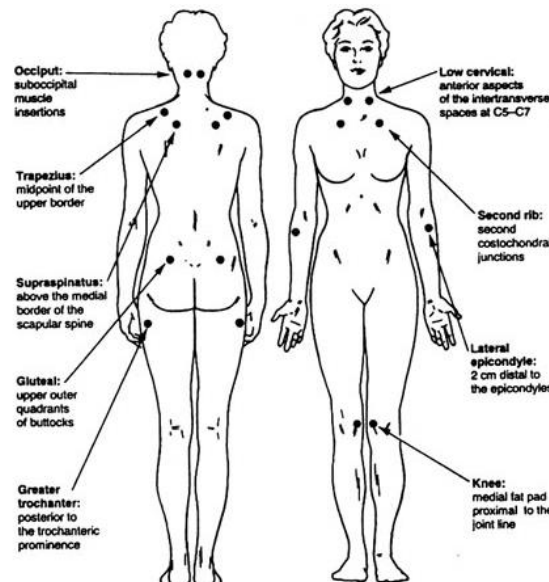


Figure 2.1: The location of the 18 predefined tender points (indicated with black dots) according to the 1990 American College of Rheumatology criteria (Reproduced with permission from Leskowitz 2008.)

The revised ACR criteria of 2010 for the diagnosis of FMS (Garg & Deodhar 2012) were implemented, as a barrier was found in the primary care setting due to the examination of TPs being a pivotal requirement for diagnosis. Also, these TPs gave the impression that FMS is a peripheral musculoskeletal disease whose pathology is centred solely on the presence of these TPs. The primary care setting is where most of the diagnoses of FMS is conducted, however, the examination of these TPs does not usually occur there and is often not performed to the prescribed standards (Garg & Deodhar 2012). Moreover, it was found that when assessing the TPs, results show a relationship with distress as the patient–examiner relationship come into play during consultation. The examination of TPs is normally ignored by general practitioners, pain and mental health specialists as these are the alternative routes of diagnosis that patients take when rheumatologists are not available. This examination will duly be avoided as it is time-consuming and training in the examination of TPs was mostly not provided during the residency years of most practitioners (Häuser & Wolfe 2012). For these reasons, revised criteria were needed for diagnosis of FMS. The new diagnostic criteria aim to simplify the process of FMS diagnosis in the primary care setting by

excluding the TP examination. Another reason for the change in criteria was for the recognition of the importance of including the other non-pain symptoms of FMS when diagnosing the disease. These symptoms include fatigue, sleep disturbance and cognitive problems (Table 2.2). The new criteria assess the severity of the disease and also allow patients, who may not fulfil the old criteria for FMS classification, to be monitored (Garg & Deodhar 2012; Jahan *et al.*, 2012).

In the new criteria the examination of TPs was eliminated and replaced by a widespread pain index (WPI). The WPI is a count of the number of bodily areas affected by pain as indicated by the patient on a scale of 0–19. An additional scale was added to assess the characteristic symptoms of FMS on a scale of 0–12. These include “fatigue, non-refreshed sleep, problems with cognition and the extent of somatic symptom reporting”. These are all assessed on a 0–3 scale and combined to a symptom severity (SS) scale and hence the 0–12 scale previously mentioned (Häuser & Wolfe 2012; Jahan *et al.*, 2012). The new criteria are expected to hold some advantages over the previous ones. First, they are easier to apply in the primary care setting than the previous criteria, which required the examination of TPs. Also, using these new criteria delivers a homogeneous group of patients for entrance into clinical trial studies. Second, application of the new criteria came to a correct classification of about 83% of new patients without having to examine them physically for the presence of TPs. This rate agrees with that achieved by diagnoses via a physician. Third, severity assessment or monitoring of patients who were previously diagnosed with FMS was not a part of the initial criteria whereas the new criteria capture the clinical essence of FMS (Garg & Deodhar 2012).

Advantages often come with some disadvantages. In the case of the new criteria for FMS, these include not being able to be applied to patients with other diseases, e.g. rheumatoid arthritis (RA) and systemic lupus erythromatosus (Garg & Deodhar 2012). Moreover, assessment of the SS scale and WPI necessitates an attentive interview with the patient that can be time-consuming (Häuser & Wolfe 2012). Furthermore, validation of these new criteria in the primary care setting has not been done by means of prospective studies and has not yet been accepted for routine use in clinical practice (Garg & Deodhar 2012).

Table 2.2: New criteria developed by the ACR for the diagnosis of FMS (Reproduced with permission from Jahan *et al.*, 2012).

Fatigue	Waking unrefreshed	Cognitive problems
0 = No problem	0 = No problem	0 = No problem
1 = Slight or mild problems; generally mild or intermittent problems	1 = Slight or mild problems; generally mild or intermittent problems	1 = Slight or mild problems; generally mild or intermittent problems
2 = Moderate; considerable problems, often present and/or at a moderate level	2 = Moderate; considerable problems, often present and/or at a moderate level	2 = Moderate; considerable problems, often present and/or at a moderate level
3 = Severe: pervasive, continuous. Life-disturbing problems	3 = Severe: pervasive, continuous. Life-disturbing problems	3 = Severe: pervasive, continuous. Life-disturbing problems

A study conducted by Egloff and colleagues investigated how these changes to the diagnostic criteria would impact diagnosis of other functional pain syndromes. The authors believe that the new ACR criteria are not accurate enough to differentiate the different types of functional pain syndromes, thus misclassifying other functional pain syndromes as FMS. Egloff *et al.*, used a cohort of 300 patients diagnosed with different types of functional pain syndromes, of whom 25 of these patients were diagnosed with FMS according to ACR 1990. After application of the new ACR 2010 criteria the number of patients classified with FMS increased to 130. This resulted in 109 new FMS patients of whom 21 were the existing FMS patients. Of the initial 25 diagnosed with FMS, four did not meet the 2010 criteria. This study showed that the new criteria are not specific enough for FMS and change the clinical profile of FMS by not taking into account the tender point count and widespread pain. The authors believe that new criteria could oversimplify FMS diagnosis, resulting in misclassification of other pain syndromes as FMS with a potential “over-diagnosis” of FMS (Egloff *et al.*, 2015).

Taken together, a comment on FMS diagnosis made at the EFIC-2017 congress seems applicable: “Diagnosis of FMS conveys information but little insight. Currently we *know what can work* but not *how* and in *whom*” (Cedraschi 2017). Thus, some reflection on pain, its associated clinical principles and their relation to FMS is needed.

2.2.2 Clinical principles of pain

2.2.2.1 Types of pain

Pain is something we experience in our daily lives, be it emotional or physical. Pain is a sensory and emotional experience, unpleasant in nature and is experienced in a variety of manifestations (Loeser & Melzack 2017; Diatchenko *et al.*, 2007; Little *et al.*, 2012). Pain can be *transient* in nature, which alerts the individual by an offending stimulus on the skin or other part of the body, not caused by tissue damage. The function of transient pain is related to the speed of the onset of the stimulation and its offset, indicating to the individual that the physical disturbance became resolved. The perception of *acute pain* allows us to recognize events that could be life-threatening and therefore this enables us to find ways to escape the danger, recognizing that we have an injured region that should be “immobilized” to reduce its use. *Chronic pain* is usually defined as pain that lasts longer than one would expect (according to the extent of the initial tissue damage) and is the type of pain associated with FMS. Chronic pain is dysfunctional and has mostly lost its warning function. It may have a significant impact on the individual's quality of life and activities of daily living. Due to this kind of human suffering, decreased quality of life and ultimately even a shortened life expectancy may follow (Diatchenko *et al.*, 2007; Loeser & Melzack 2017).

2.2.2.2 Pathophysiological mechanisms of pain

Pain has distinct cognitive and emotional aspects but has been shown to be primarily a multidimensional sensory experience. It is not the purpose of this review to present a comprehensive discussion of the pathophysiological mechanism of pain, but to focus on two main categories of sensitization resulting in pain: central sensitization and peripheral sensitization — as well as the concept of functional pain — all three are relevant to FMS.

1. Central sensitization

Central sensitization is the mechanism whereby stimulation that normally would not cause pain, such as movement or a gentle touch, can stimulate a low threshold level of mechanoreceptors to elicit an experience of pain by the individual. In his review (Woolf 2011) of 25 years of research on central sensitization, South African-born Clifford Woolf, an alumnus of the University of the Witwatersrand, reflects on his pre-clinical research of 1983 at University College London when he and his colleagues observed that “*a brief (~10–20 second), low frequency (1–10 Hz) burst of action potentials into the CNS generated by*

electrical stimulation or natural activation of nociceptors” [receptors at the end of a sensory neuron's axon that responds to stimuli of a potential or real damaging nature] “*increased synaptic efficacy in nociceptive neurons*” that lasted several minutes after the stimulus. These observations contributed to what became known as central sensitization causing nociceptive pain.

Central sensitization can thus be described as the state of increased excitation of the spinal cord involving the dorsal horn neurons responsible for nociceptive transmission in response to a distinct or subtle stimulation. This state of excitation is sustained as a result of these neurons being overly sensitive to certain nociceptive stimuli. This culminates in oversensitivity to both noxious and non-noxious stimuli and is clinically characterized by hyperalgesia and allodynia, respectively (Woolf 2004; Recla & Sarantopoulos 2009; Little *et al.*, 2012; Cervero 2014). There are two types of pain conduction fibres implicated in the process of central sensitization, namely, A-beta and C-fibres (Nielsen & Henriksson 2007). The myelinated A-fibres transmit a first pain that is promptly directed to the CNS at very high speeds. The C-fibres transmit second pain, are unmyelinated and direct pain signals to the CNS at slower speeds (Staud 2006). Central sensitization plays a role in inflammatory, neuropathic and functional pain. Nociceptor central terminals release transmitters that activate the dorsal horn neurons and ultimately modify the transmission of pain by changing the receptor density, threshold, kinetics and activation levels. Glutamate-activated N-methyl-D-aspartate (NMDA) receptors are fundamental to this process. They are upregulated from intracellular stores to the synaptic membrane by phosphorylation, which also causes an increased sensitivity to glutamate by elimination of the voltage-dependent Mg^{2+} ions on the receptor. This culminates in stimuli that are normally not painful, being perceived as painful and noxious stimuli. Ketamine can be used to inhibit this NMDA receptor action (early-phase central sensitization) but it has side effects that may bring about the induction of a psychotic state and therefore has limited clinical application. Humoral factor secreted by inflammatory cells after peripheral tissue injury stimulates the endothelial cells to secrete interleukin-1 β (IL-1 β), which penetrates the cerebrospinal fluid (CSF) to stimulate the IL-1 receptor to express cyclooxygenase-2 (COX-2) in the CNS neurons. This also causes an increase in circulating prostaglandin E₂ that plays a role in the late-onset phase of central sensitization. COX-2 expression plays a role in widespread pain, appetite loss, as well as mood and sleep cycle changes (Woolf 2004; Staud 2006).

Nociception is the ability to perceive pain through stimulation of the pain receptors (called nociceptors). This process occurs when a pre-synaptic neuron, after responding to a specific painful event, releases neurotransmitters into the synapse. Examples of these

neurotransmitters are glutamate and substance P. The post-synaptic neuron possesses 1-amino-3-hydroxyl-5-methyl-4-isoxazole (AMPA) receptors and they are implicated in this nociceptive process. Glutamate binds to these receptors, causing an inflow of sodium (Na^+) resulting in the depolarization of the neuronal membrane and subsequent action potential initiation (Figure 2.2 A). During prolonged exposure to a pain stimulus, more of the neurotransmitters are released into the synaptic space, causing increased activation of the AMPA and neurokinin receptors. Neurokinin receptors are activated via attachment of substance P. The increased activation of these receptors causes an enhanced depolarization of the neuronal membrane, subsequently inducing the elimination of the magnesium (Mg^{2+}) obstruction of the NMDA receptor. The unblocking of this receptor allows the inflow of calcium ions (Ca^{2+}), from the intracellular space, subsequently causing the upregulation of the AMPA receptors thereby strengthening the nociceptive signal (Figure 2.2 B). This prolonged exposure process is termed “enhanced nociception” and is the process employed in central sensitization (Recla & Sarantopoulos 2009; Little *et al.*, 2012), which is thought to play a role in the pathogenesis of FMS.

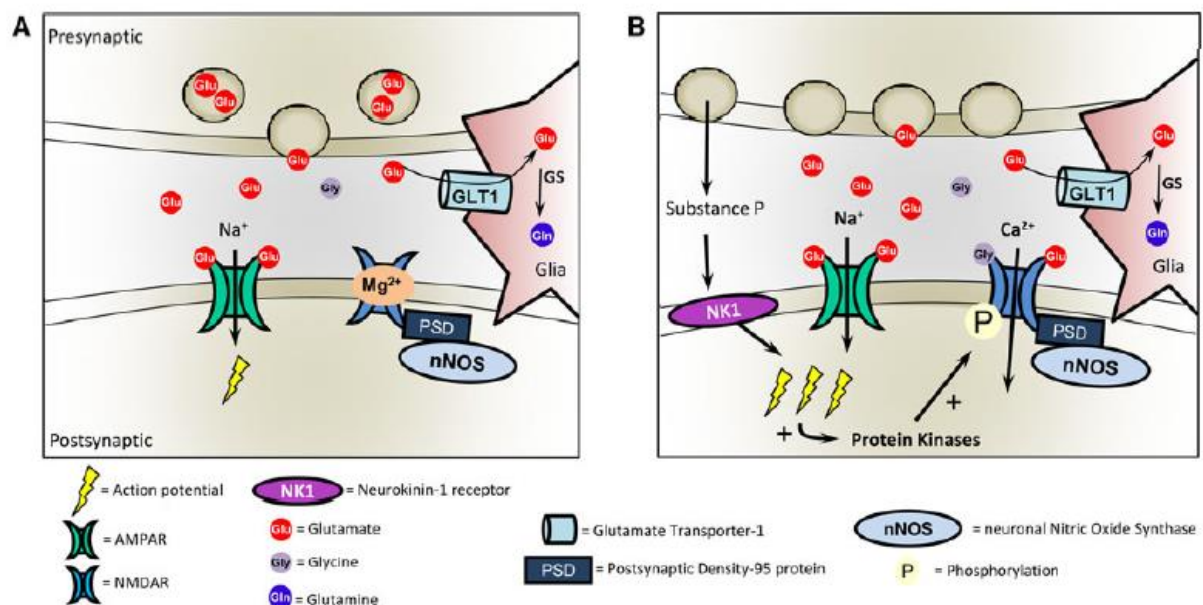


Figure 2.2: Biochemical representation of the process of nociception. Figure A shows the normal nociceptive transmission occurring at a synapse. Neurotransmitters arrive at the synapse due to nociceptive stimulation and are released into the synaptic space. Glutamate binds to AMPA and allows the outflow of sodium from the synaptic space, causing depolarization of the membrane. Figure B shows the transmission in prolonged nociception. Excess neurotransmitters are released in the synaptic space, bringing with it substance P, which causes the activation of the neurokinin receptor. This subsequently causes enhanced membrane depolarization effecting a magnesium block removal on the NMDA receptor,

allowing calcium to flow out of the synaptic space (Reproduced with permission from Little *et al.*, 2012).

One of the early studies on FMS observed a decrease in mechanical pressure thresholds to be a key clinical feature in FMS patients (Gibson *et al.*, 1994). Gibson and co-workers interpreted these findings to mean that greater activation of CNS pathways following noxious input occurred in a group of 10 FMS patients relative to 10 controls studied. These authors, however, cautioned that “*mechanisms of peripheral nociceptive sensitization and the role of psychological factors might contribute to their findings*”. In his review on central sensitization, Woolf proposed an operational definition of central sensitization: central sensitization is “*an amplification of neural signalling within the CNS that elicits pain hypersensitivity*” (Woolf 2011). Reflecting on FMS, he concludes that central sensitization contributes to the symptoms of FMS, although it is not the prime mechanism of FMS pathophysiology.

Reactive oxygen species (ROS) have been thought to play a role in persistent pain syndromes. ROS have already been shown to play a role in degenerative neurological diseases like Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS) (Chung 2004). ROS are produced as a by-product of many enzymatic reactions in the body. An increase in the production of ROS or an error in their removal can cause cellular damage via cytoplasmic swelling and can even cause cell death. There are different types of ROS that can cause cellular damage. Superoxide (SO) is produced by the mitochondrion via oxidative phosphorylation and is converted to hydrogen peroxide by the enzyme superoxide dismutase (SOD). Ultimately, this can be converted further to a noxious hydroxyl radical. Nitric oxide (NO), another form of ROS, and SO are both generated in the cytoplasm. Increased cytoplasmic levels of Ca^{2+} activate the production of SO and NO. These two compounds can react with one another to form the toxic peroxynitrite. ROS are hypothesized to play a role in the central sensitization phenomenon by initiating factors that have been identified to operate in this process (Kim *et al.*, 2004).

A study was conducted by Cordero and colleagues to assess the role of oxidative stress in the pathogenesis of FMS. Their sample material was blood mononuclear cells (BMC) and plasma. Their study found increased levels of SO along with decreased levels in both the mitochondrial coenzyme Q_{10} (CoQ_{10}) and the mitochondrial membrane potential in FMS patients as compared to controls. Moreover, increased signs of mitophagy, which is the

elimination of defective mitochondria by the process of autophagocytosis, were observed (Cordero *et al.*, 2010).

The mitochondria are essential in the role of energy production. Energy is produced in the form of the high energy molecule adenosine triphosphate (ATP). The ATP synthase pump forms part of the electron transport chain (Figure 2.3) implicated in oxidative phosphorylation. CoQ₁₀ is involved in the transport of electrons from complexes I and II to complex III of the electron transport chain. Decreased levels of this enzyme results in the failed transportation of these electrons between the complexes thereby, causing a proton gradient imbalance across the mitochondrial membrane. This gradient is required for the ATP synthase pump to produce ATP. This culminates in decreased ATP synthesis. Subsequently, ROS levels increase and mitophagy of the defective mitochondria occurs. From these results the authors noted that increased ROS levels are observed in FMS and that a perturbation in the bioenergetics of the cell could be implicated in FMS too (Cordero *et al.*, 2010).

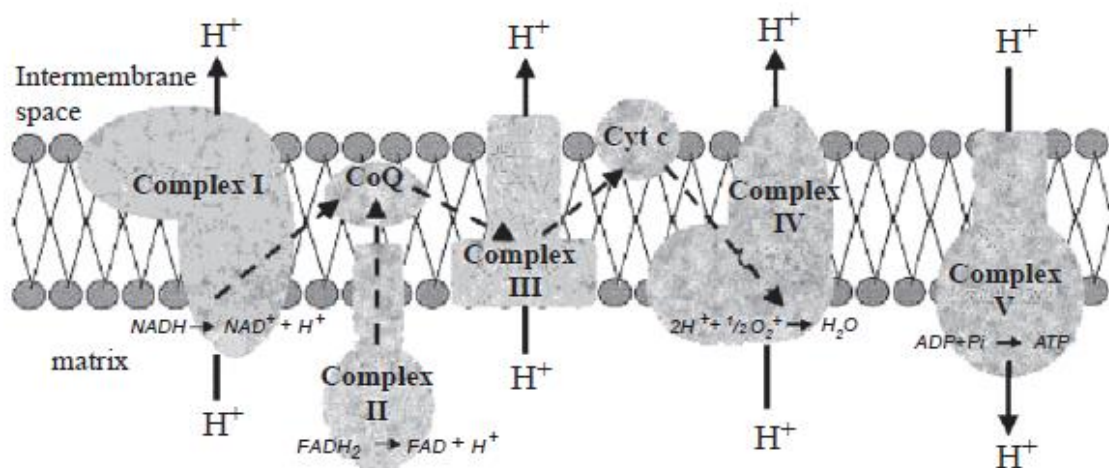


Figure 2.3: Electron transport system, involved in the production of energy via oxidative phosphorylation, present in the mitochondrion (reproduced with permission from Gardner & Boles 2011).

Nociceptive pain is the pain felt in response to a harmful (noxious) stimulus; it is the most important type of pain mechanism as it prevents an individual from further harm that can lead to tissue damage. In the event of tissue damage, the inflammatory pain system is activated. Inflammatory pain occurs when non-noxious stimuli are now perceived as noxious by the damaged area, typically causing inflammation. Once the damaged area is healed, the inflammatory pain response fades. Neuropathic pain is a form of maladaptive pain that does

not require a noxious stimulus for activation. It is associated with injury to the nervous system. Functional or “central” pain is also a form of maladaptive pain, which is associated with irregular functioning of abnormal processing of noxious stimuli in the nervous system. In this form of pain no specific cause of the pain can be detected in the nervous or muscular musculoskeletal systems. Fibromyalgia falls under this latter type of pain (Woolf 2004).

2. Peripheral sensitization

Peripheral sensitization is an increased sensitivity to stimuli in afferent neurons due to an injury or cell damage, which elicits an extensive response due to neuropeptides that affect nociceptors. Patients showing symptoms presently seen as FMS were formerly diagnosed as suffering from fibrositis – a term introduced in 1904 by the British neurologist Sir William Gowers (Inanici & Yunus 2004). Fibrositis is a term that implies a notable contribution of peripheral inflammation to the condition. In time, peripheral sensitization was recognized to arise through several means of which three distinct and different forms of stimuli became defined, as represented in Figure 2.4.

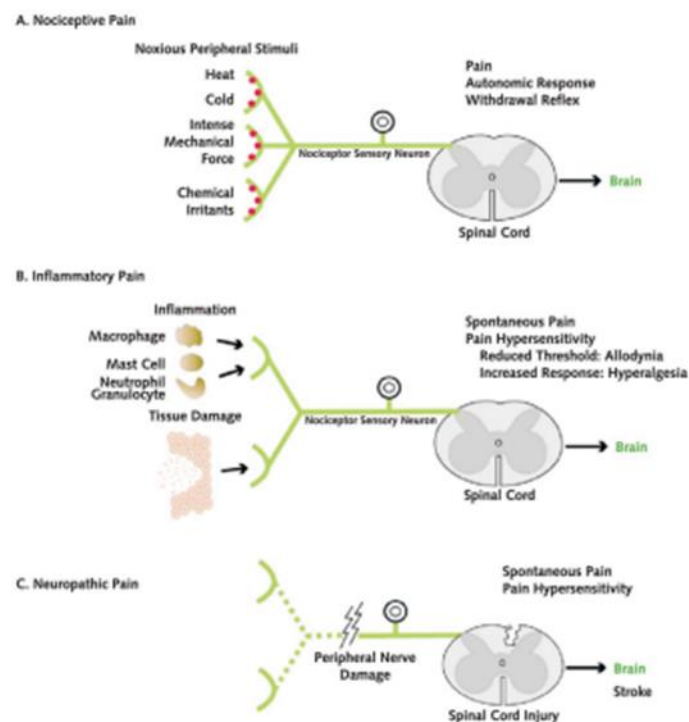


Figure 2.4: Three primary classes of stimuli that may act as peripheral sensitization in pain generation (adapted with permission from Woolf 2004).

Noxious peripheral stimuli include the effect of extreme temperatures (heat or cold), mechanical force (e.g. pressure or bruises) and chemical irritants (e.g. from strong acids). Inflammatory pain is a spontaneous response due to hypersensitivity associated with tissue damage and inflammation. Neuropathic pain occurs spontaneously as a consequence of damage or of a lesion to a nerve fibre. Inflammatory and neuropathic pain is a consequence of a decrease in the pain threshold of nociceptors causing an induction in pain at areas of injury or inflammation. In areas where cellular injury has occurred, cellular contents are spilled out and the recruited inflammatory cells release cytokines, chemokines and growth factors in response. The nociceptor terminal activity is altered by prostaglandin E₂ (PGE₂) and nerve growth factor binding to prostaglandin E and tyrosine kinase A receptors, respectively, whereas bradykinin binds to the B₂ receptors and activates the nociceptor. PGE₂ causes a reduction in the activation threshold of the nociceptor by binding to the aforementioned receptor and causes adenylyl cyclase activation. This in turn causes an increase in cyclic adenosine monophosphate activation of protein kinase A. Protein kinase C is activated by calcium released by calcium stores. Protein kinases A and C phosphorylate proteins at amino acid sites of serine and threonine, causing changes in the activity of receptors and ion channels. Phosphorylation also alters the threshold of voltage-gated sodium ion channels, causing an increase in membrane excitability by producing more action potentials than usual (Woolf 2004; Staud 2006).

Inflammatory pain disorders include RA, osteoarthritis (OA) and Crohn's disease (CD). These disorders tend to be co-morbid with FMS, in that they present with pain, but are not the same as FMS. The one distinguishing feature that they possess, on clinical examination, is inflammation. Although current research has demonstrated "subtle" inflammatory markers ("neurogenic inflammation") in some FMS patients, it is not considered an inflammatory disease in the true sense of the definition (Littlejohn 2015). Inflammation is the body's response to local tissue injury or invasion by harmful pathogens (O'Neill & Hardie 2013). The cells that mediate this process are the inflammatory immune cells, macrophages, dendritic cells (DCs) and T cells (Palsson-McDermott & O'Neill 2013). In this section we will discuss the metabolic changes that occur in these cells to bring about the process of inflammation and inflammatory pain in response to pathogen invasion and tissue injury.

The process of inflammation is an energy (in the form of ATP) demanding process. In normal cells at rest, the energy requirements are met through the standard progression of glycolysis in the cytosol. The end product of glycolysis is then decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) and enters the tricarboxylic acid (TCA) cycle followed by oxidative phosphorylation (OXPHOS) in the mitochondria. These cycles occur under normal

levels of circulating oxygen (normoxia). However, in conditions of low circulation oxygen (hypoxia), the cellular ATP is generated primarily by glycolysis and the TCA and OXPHOS cycles will be shut down. The pyruvate generated by glycolysis is then ultimately converted to lactate by lactate dehydrogenase (LDH) in the cytosol, when the generation of NAD⁺ for glycolysis starts again and does not enter the mitochondria. In the case of a tumour, cells are in a state of rapid growth and therefore their energy requirements are altered. They change from a low to a higher rate of glycolysis, with the production of lactic acid and an even lower rate of OXPHOS. When all these changes take place under aerobic conditions, this change in metabolism is named the “Warburg effect”, or aerobic glycolysis. This phenomenon in tumor cells was discovered by Otto Warburg in 1923. This change in metabolism can also take place in cells under anaerobic conditions, for example during short bursts of muscular energy (Palsson-McDermott & O’Neill 2013).

The Warburg effect creates an increased need for glucose and other biological building blocks including nucleotides, amino acids and NADPH by making use of the intermediates from glycolysis and generating these building blocks via the pentose phosphate pathway (PPP). Concomitantly, fatty acids are also required to produce membrane lipids from cytosolic citrate to generate acetyl-CoA. This reaction is catalysed by ATP-citrate lyase (ACL). Hypoxia-inducible factor 1 α (Hif 1 α) is the key regulator of the Warburg effect. It responds to hypoxia within cells, but can also be activated under normoxic conditions by succinate, and also activates enzymes involved in glycolysis, namely, hexokinase 2 (HK2), triose phosphate isomerase, glucose-6-phosphate isomerase and pyruvate kinase M2 (PKM2) (Palsson-McDermott & O’Neill 2013).

Immune cells like macrophages and DCs are able to alter their metabolic state to that corresponding to the Warburg effect when activated by invading pathogens. These pathogens stimulate the release of pro-inflammatory cytokines and stimulation of membrane-bound toll-like receptor (TLR) ligands. Glucose transporter 1 (GLUT1) becomes expressed in active DCs by stimulation of TLR4, TLR 2 and TLR9 ligands, which cause an increase in the glycolytic rate and the need for glucose (O’Neill & Hardie 2013; Palsson-McDermott & O’Neill 2013). Macrophages are able to take on two forms when activated, namely, the M1 or M2 form. The M1 (classically activated) form comes about when activated, in acute inflammation or anti-bacterial action, by interferon- γ or certain TLR ligands in which bacteria are removed by release of inflammatory cytokines and ROS. The M2 (alternatively activated) form comes about when activated by cytokines like IL-4 and IL-13. Only activation of the M1 form involves a switch in metabolism to the Warburg effect. The M2 form makes use of

OXPHOS for tissue repair or in anti-parasitic immunity (Palsson-McDermott & O'Neill 2013; O'Neill 2015).

Enhanced ATP production during inflammation by a shift in metabolism of inflammatory cells is clearly a hallmark change employed in inflammatory diseases. Inflammatory pain is brought on by the production of prostaglandins. Although FMS may not be classified as an inflammatory condition, the several links observed between FMS and inflammatory responses should be explored to further elucidate the cause or the effect of these responses on the suffering FMS patient.

3. Functional pain

Functional pain — the form of pain currently regarded as the main pathophysiological basis for pain in FMS — is defined as hypersensitivity to pain resulting from abnormal central processing of normal input, as represented in Figure 2.5 (Woolf 2004).



Figure 2.5: A representation of peripheral sensitization in pain generation without any identifiable trauma (physical or inflammatory) (adapted with permission from Woolf 2004).

To recapitulate: FMS is currently characterized as a chronic disorder of widespread pain and tenderness, accompanied by other symptoms such as disturbed sleep and chronic fatigue. The pathogenesis of FMS involves abnormalities in the pain-regulating mechanisms at various levels in the peripheral and central nervous system; FMS is considered a disorder of abnormal pain processing and pain amplification. It is also regarded as the “prototype” of a group of disorders characterized by sensitization of the nervous system (Ablin & Hauser 2016; Clauw 2015; Fitzcharles *et al.*, 2014).

Functional pain syndromes, also commonly referred to as functional somatic syndromes (FSS), are a cluster of pain disorders in which no anatomical or laboratory perturbations can be linked to the cause of the debilitating pain. Diseases of this class include FMS, IBS, CFS, migraine, and tension type headache; they present with common clinical symptoms of which

chronic pain is the most noticeable. These syndromes are more prevalent among females, their symptoms tend to be worsened by stress and menstruation and they are associated with depression and anxiety (Goodman 2016; Afari *et al.*, 2014). No specific abnormalities have yet been found at the level of laboratory testing, a known characteristic of FMS diagnosis that partly contributed to the present study.

Each of these disorders has their own clinical criteria on which they are diagnosed. The FMS ACR criteria of 1990 were modified in 2010, which elicited both criticism and approval (Garg & Deodhar 2012). The 1990 criteria were essentially developed to standardize research populations and not for diagnostic purposes in a clinical setting, whereas the modified 2010 criteria were designed for use in epidemiological studies (Clauw 2014). Since the 2010 criteria create a grey area with poor distinction between FMS and other functional pain syndromes (Egloff *et al.*, 2015), it demonstrates further the critical need for a diagnostic biomarker for this disorder. Since there is no definite perturbation that can be pointed out as the cause of the pain and other symptoms which FMS patients experience, they are often told by healthcare providers that their symptoms are “all in their head”, that they are suffering from a primary mental disorder and that they “should pull themselves together” (Wolfe *et al.*, 2014).

FMS patients have a high co-morbidity rate of psychiatric disorders, which include depression, anxiety, obsessive-compulsive disorder and posttraumatic stress disorder (PTSD) (Clauw 2014; Afari *et al.*, 2014). It has been suggested that FSS are more prevalent in patients with a history of stressful events. Afari *et al.*, in a meta-review, assessed the association between psychological trauma and PTSD with the presentation of FSS. These traumatic experiences included psychological, physical and emotional stressors or sexual abuse that occurred during childhood or adulthood and combat exposure. The authors identified 71 articles that met their search criteria. Their study found that people who reported exposure to some form of trauma were 2.7 times more likely to present with FSS. They also analysed the association of the types of trauma and FSS. At least 49 of the 71 studies reviewed showed that physical stressors had the smallest association with presentation of FSS. Of the different types of FSS included in the study, CFS was the most common example that was associated with reported trauma whereas IBS was the least associated with reported trauma (Afari *et al.*, 2014).

FMS and other FSSs are often viewed as symptoms of somatoform disorder (“somatic symptom disorder”), where unresolved or suppressed emotional trauma may manifest as an FSS, such as FMS (Wolfe *et al.*, 2014). With the results from Afari *et al.*, 2014 mentioned, one can understand this reasoning. A traumatic event may precipitate the onset of the FSS,

leading to avoidance of certain situations associated with the trauma and consequently with distorted thoughts about the symptoms experienced (Afari *et al.*, 2014). Wolfe and his colleagues analysed how a mental disorder may affect the severity of the symptoms experienced by patients suffering from FMS, RA and OA. The American Psychiatry Association defines somatic symptom disorder (SSD), according to their *Disease and Statistical Manual-5 (DSM-5)*, as “mental disease” that manifests with somatic symptoms such as pain. Diagnostic criteria of this disorder include “*disproportionate and persistent thoughts about the seriousness of one’s symptoms, persistently high level of anxiety about health or symptoms; or excessive time and energy devoted to these symptoms or health concerns*”. The crucial characteristic of this disease is not so much the presence of the symptoms but that they are “disproportionate” or “excessive”. Wolfe *et al.* conducted their study by asking FMS, RA and OA patients to complete the Patient Health Questionnaire-15 (PHQ-15), to quantify patient symptoms. This is a 15-symptom checklist according to which the disorder “*would consist of one or more physical symptoms currently present, not fully explainable by another medical or psychiatric disorder (with the exception of functional somatic syndromes), causing functional impairment. Duration must be at least 6 months, and severity could be graded as mild, moderate, or severe.*” They then used the *DSM-5* criteria to identify patients who qualified as having “mental illness”, according to the criteria. Overall, what they found from their study was that 51.4% of patients with FMS and 14.8% with RA had fatigue, sleep or cognitive problems that were severe, continuous, and life disturbing, related to the *DSM-5* criteria of “mental disease” (Wolfe *et al.*, 2014). This could classify FMS as a “mental disease” in which symptoms are precipitated by a traumatic event and then perpetuated as a syndrome of widespread pain due to “disproportional” or “excessive” thoughts about the seriousness of one’s symptoms. However, the authors are doubtful as to whether the *DSM-5* criteria can reliably differentiate patients that do or do not have “mental disease” and thus they warn that this tool should be used with caution.

In an extensive literature search by Hauser and Henningsen (2014) on the classification of FMS as a somatoform disorder, it was concluded that FMS is not similar to somatoform pain disorder, although both disorders are associated with unresolved emotional and psychosocial conflict. These authors also warn against “over-psychiatration” of patients with somatic disorders and “mislabelling” these patients as “mentally ill”.

Although there is a significant overlap and co-morbidity between FMS and psychomatic disorders, it is important to remember that most FMS patients do not have a definable psychiatric disorder. Although there is a possible overlap between the genetic susceptibility and neurotransmitters involved in both FMS and psychopathology, taken together, FMS is

not currently classified in the broad category of functional pain syndromes (discussed below) and is not regarded as a “mental disorder”, because the augmented pain processing of FMS can occur in the absence of psychopathology (Clauw 2015).

2.3 Co-morbid disorders associated with Fibromyalgia Syndrome

As previously mentioned, FMS does not present on its own. There are a number of co-morbid disorders that exist with FMS. These disorders tend to have overlapping symptoms and one disorder can give rise to the other if not attended to medically, as in the case of myofascial pain syndrome, giving rise to FMS (Gerwin 2010). Figure 2.6 shows the overlap, with the main perturbation common to these disorders being the presence of pain and its central nervous amplification. There are also some psychiatric conditions that overlap with FMS (figure 2.5). This overlap can possibly be attributed to the fact that FMS and certain psychiatric disorders (e.g. mood disorders) share similar neurotransmitters (e.g. serotonin and noradrenaline) and they also share neuro-anatomy in the limbic system (Smith *et al.*, 2011).

FMS has been classified under a number of groups over the years as new information of the disorder has emerged. These groups include idiopathic pain disorders (IPDs) (Diatchenko *et al.*, 2006), affective spectrum disorders (Hudson *et al.*, 2003), functional somatic syndromes, somatoform disorders, medically unexplained symptoms, chronic multi-symptom illnesses, central sensitivity syndromes (Smith *et al.*, 2011; Hackshaw *et al.*, 2013) and functional pain syndromes (Goodman 2016). Functional pain syndromes and functional somatic syndromes are used interchangeably but the most commonly used term is functional somatic syndromes. However, according to Yunus, *central sensitivity syndromes* is the most appropriate term to classify FMS and its co-morbidities, based on a biopsychosocial model (Yunus 2008). According to this model, FMS is regarded as the “prototype” of the central sensitivity disorders. These disorders share a common genetic predisposition (in the form of a polygenic polymorphism) and affected individuals have increased pain sensitivity due to imbalances in various pain modulating neurotransmitters, e.g. increased substance P levels and decreased serotonin and nor-epinephrine levels in the central nervous system. In this section we describe three of the most common co-morbid disorders with FMS that fall within the same category and also what recent scientific investigations on the disorders have

revealed with respect to each. In addition, some comments will be made on Complex Regional Pain Syndrome (CRPS) and chronic widespread pain (CWP) syndrome. Although FMS and CRPS have distinct clinical phenotypes, they do share many other features, which thus warrant some reflection.

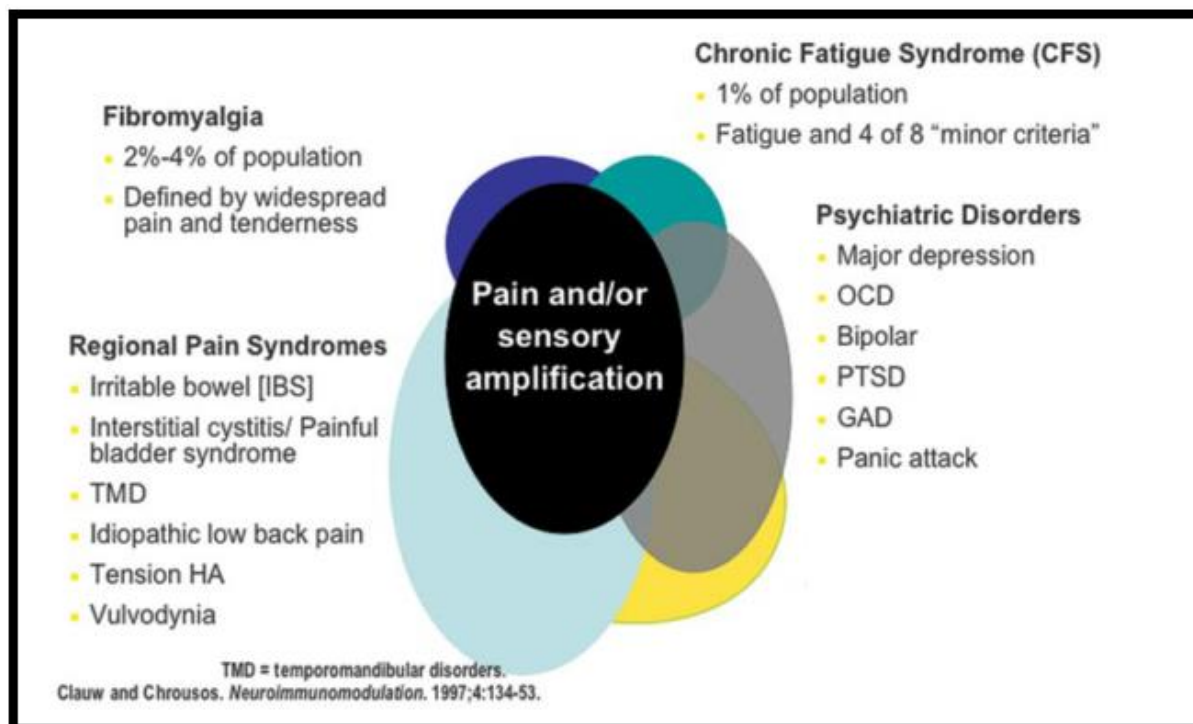


Figure 2.6: Pain and sensory amplification is the main underlying pathophysiological mechanism identified in co-morbid conditions with fibromyalgia (adapted with permission from Smith *et al.*, 2011).

Myofascial pain syndrome (MPS)

MPS is a chronic regional pain syndrome that falls under the same larger classification group of central sensitivity syndromes with FMS (Smith *et al.*, 2011; Hackshaw *et al.*, 2013). MPS presents with regional musculoskeletal pain, weakness, limited range of movement and referred pain (Saxena *et al.*, 2015). MPS pain arises from hypersensitive areas in taut bands found in skeletal muscle. The hypersensitive areas are known as myofascial trigger points (Desai *et al.*, 2013). These trigger points are hard to the touch, very tender on palpitation and located in a discrete muscle-band. Diagnosis is made on the history and palpation of the trigger point (Gerwin 2010).

MPS is classified as a subtype of FMS with regional and widespread pain being the distinguishing factor between the two disorders (Chandola & Chakraborty 2009). MPS is difficult to diagnose as there is no set of validated diagnostic criteria that can be used in a clinical setting. The current morphological and electrodiagnostic methods used are time-consuming, unreliable and expensive (Desai *et al.*, 2013) and emphasize the need for a biomarker.

The current accepted hypothesis for the formation of a trigger point is the integrated one of Mense and Simons (Mense *et al.*, 2001). According to this hypothesis, an episode (or episodes) of muscle trauma leads to an increased production and release of acetylcholine. This is associated with an increased release of calcium ions, which leads to non-physiological contraction of sarcomeres and local hypoxia. This then triggers the release of inflammatory substances and increased levels of pro-nociceptive substances (e.g. bradykinin, substance P, interleukin-1 beta) have been demonstrated in a micro-dialysis study where the biochemical milieu of trigger points was analyzed (Shah *et al.*, 2005).

Central sensitization is also known to play a role in MPS by means of the referred pain induced by trigger point stimulation (Gerwin 2010; Saxena *et al.*, 2015). From this we can identify that there are many common characteristics that FMS and MPS share and the nociceptive input via untreated myofascial trigger points of MPS may contribute to the maintenance of central sensitization in FMS patients (Meyer 2002).

Chronic fatigue syndrome (CFS)

CFS is a poorly understood disorder in which patients complain of unrefreshing sleep, inexplicable prolonged fatigue and musculoskeletal pain (Sheedy *et al.*, 2009; Armstrong *et al.*, 2015). It affects about five individuals in 1000 people (Wessely 1995). As with FMS, no underlying pathology can be detected as the cause of the disorder. According to the Canadian Consensus Criteria, a patient is diagnosed with CFS if they present with the following symptoms for at least six months: fatigue (including substantial reduction in activity levels), post-exertional malaise or fatigue, sleep dysfunction, pain, neurologic/cognitive manifestations and autonomic, neuroendocrine, or immune manifestations (Carruthers *et al.*, 2003).

In a 2015 study published by Armstrong and colleagues, a metabolomics approach was used to study and compare the blood serum and urine of patients diagnosed with myalgic

encephalomyelitis/chronic fatigue syndrome (ME/CFS) to that of healthy controls. The analytic method used was ^1H -NMR. What their study revealed was that there is a perturbation in the energy metabolism and oxidative stress pathways of patients with ME/CFS. Results for the blood serum analyses showed substantial increased levels of glucose and aspartate with decreased levels of acetate, glutamate, hypoxanthine, lactate and phenylalanine in the patient group. PCA score plots were significantly able to discriminate patients from controls. The urine analyses showed significantly increased allantoin and creatinine levels along with decreased levels of acetate, alanine, formate, pyruvate, valine and serine in the patient group. PCA score plots were, however, able only moderately to discriminate patients from controls. The results of Armstrong *et al.* show signs of glycolysis inhibition leading to a reduction in acetyl-CoA availability for the citric acid cycle. The reduction in amino acid levels indicates that the use of an alternative source of carbohydrate metabolites for utilization in the citric acid cycle. Hypoxanthine and allantoin serve as indicators of ROS production. These findings offers substantial information in understanding the pathogenetic mechanism of ME/CFS (Armstrong *et al.*, 2015) and also possibly that of FMS.

Irritable bowel syndrome (IBS)

IBS is a disorder of the gastrointestinal tract with its distinguishing features involving persistent abdominal pain and diarrhoea or constipation. It affects 10–20% of the adult population of whom females are most affected (Saito *et al.*, 2002). IBS is often associated with stress, depression and anxiety and is diagnosed using the Rome III criteria, which state that a patient should present with recurrent abdominal pain/discomfort for more than three days per month over the previous three months associated with two or more of the following: improvement with defecation, onset associated with change in frequency of stool, and onset associated with change in form/appearance of stool (Shih & Kwan 2007). As with FMS, diagnosis is challenging as it is based on the presenting patient's symptoms and no discernible tissue or structural damage is present. For this reason, the quest for the identification of plausible biomarkers is vital (Ponnusamy *et al.*, 2011; Baranska *et al.*, 2016).

A recent metabolomics study conducted by Baranska and colleagues sought to analyze the breath samples of IBS patients with age- and sex-matched healthy controls. The analytical method used for this study was gas chromatography in conjunction with time-of-flight mass spectroscopy. Baranska *et al.* analyzed volatile organic compounds (VOC) in the breath

samples and used these VOCs to obtain discriminatory profiles through the use of multivariate statistics. These authors were able to identify 16 VOCs that could discriminate patients from controls using a random forest classification model. The receiver operating characteristic (ROC) curve reported an area under the curve (AUC) value of 0.83. PCA score plots based on the proximity matrix, obtained from the random forest model, showed separation between patients and controls. A Kruskal–Wallis test was also conducted revealing no confounding factors that may have impacted the study negatively. These results correlated positively with the gastrointestinal (GI) tract symptoms indicated by the patients.

The study by Baranska *et al.*, demonstrates the use of metabolomics for biomarker identification without the use of conventional sample material — being blood, urine or CSF — to distinguish patients from controls in a non-invasive manner. It also demonstrates the potential for use in monitoring the gastrointestinal symptoms of patients with central sensitivity disorders.

Complex regional pain syndrome (CRPS)

CRPS is a rare chronic neuropathic pain syndrome. As with FMS, CRPS was initially known by many names including causalgia, minor causalgia, algodystrophy, shoulder-hand syndrome and Sudeck's atrophy, with reflex sympathetic dystrophy (RSD) being the most commonly used term. There are two types of the disorder, namely CRPS1 (replacing RSD) and CRPS2 (replacing causalgia) (Coderre 2011). The disorder types are distinguished by the presence (CRPS2) or absence (CRPS1) of major nerve injury. CRPS is diagnosed by using the 1993 International Association for the Study of Pain (IASP) criteria; Table 2.3 summarizes these criteria for diagnosis. In 2003 the diagnostic criteria were revised at a workshop in Budapest, Hungary, with the aim of validating the criteria and diagnosis of patients as the old criteria tend to over-diagnose patients with the disorder due to poor specificity. These new criteria were then dubbed the 2003 Budapest criteria (Harden *et al.*, 2007).

The prevalence of CRPS is estimated to be around 26.2 per 100 000 person-years (de Mos *et al.*, 2007; Lee *et al.*, 2015) with females being three times more likely to be affected than males. In contrast to FMS, CRPS tends to affect women in the age group 61–70 years; the upper extremity of the body is more often afflicted than the lower extremities (de Mos *et al.*, 2007). Clinical features of CRPS usually present after some form of tissue trauma with

fractures being the most common trigger (Sandroni *et al.*, 2003; de Mos *et al.*, 2007). Clinical features include neurogenic inflammation, allodynia, hyperalgesia, fluctuations in sweating, hair and nail growth, swelling, changes in skin colour and temperature of the affected limb (as compared to the healthier limb) and muscle weakness (Littlejohn 2015; Marinus *et al.*, 2011). Inflammation is prominent in the early stages of CRPS diagnosis whereas in FMS it tends to fluctuate over a longer period. Pain is localised in the injured limb in the early stages of CRPS diagnosis but as the disorder persists, pain then spreads to other areas of the body (Littlejohn 2015; Marinus *et al.*, 2011) in contrast to FMS in which pain is widespread from the onset. There is no definite pathophysiological explanation for CRPS although peripheral and central sensitization is also thought to play a role. Neurocognitive function is also found to be impaired in CRPS. In a study conducted by Lee *et al.* (2015), the authors investigated the brain cortical thickness for evaluation of executive function and response inhibition. Their study showed that indeed cortical thickness was decreased and cognitive function impaired as compared to healthy controls.

Table 2.3: Diagnostic criteria of the 1994 International Association for the Study of Pain used to diagnose CRPS (Reproduced with permission from Harden *et al.*, 2007).

<p>CRPS I (reflex sympathetic dystrophy)</p> <ol style="list-style-type: none"> 1) The presence of an initiating noxious event or a cause of immobilization 2) Continuing pain, allodynia or hyperalgesia to which the pain is disproportionate to any inciting event 3) Evidence at some time of oedema, changes in skin blood flow, or abnormal sudomotor activity in the region of pain 4) This diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction <p>Note: Criteria 2 to 4 must be satisfied</p>
<p>CRPS II (causalgia)</p> <ol style="list-style-type: none"> 1) Presence of continuing pain, allodynia or hyperalgesia after a nerve injury, not necessarily limited to the distribution of the injured nerve 2) Evidence at some time of oedema, changes in skin blood flow, or abnormal sudomotor activity in the region of pain 3) This diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction <p>Note: All 3 criteria must be satisfied.</p>

Chronic widespread pain (CWP) syndrome

CWP is not a disorder in its own right, but rather a symptom of disease. It is the hallmark symptom of FMS but it can also present in a number of other diseases and thus FMS is commonly misdiagnosed in patients that present with CWP. It is therefore for the diagnosing physician to rule out other diseases before diagnosing FMS. This is difficult, however, as no clear clinical diagnosis is available for CWP but patients are classified as having CWP when they do not meet the requirement of 11/18 TPs when being assessed for FMS (Häuser *et al.*, 2017). A thorough medical, psychosocial history and clinical examination is essential for the assessment of CWP. In a review, Hauser *et al.* (2017) described a number of disorders that mimic FMS in that they too present with CWP but they highlight critical features that can be used to distinguish them from FMS, thereby ensuring that over-diagnosis of FMS does not occur.

2.4 Conclusion

From the above we can deduce that FMS is a complex chronic pain disorder. It has no exact aetiology and can be precipitated (“triggered”) by a number of stressors probably in genetically vulnerable individuals. FMS shares many common characteristics with its overlapping disorders, making it difficult to diagnose on first presentation. It is also not a monogenic disorder in that no specific set of gene mutations can be implicated in its characteristic profile. Many studies conducted on the condition over the years provided evidence that FMS is mainly a CNS disorder of abnormal pain processing. As previously mentioned, diagnosis of FMS is based off of patient feedback. No structural or tissue damage is observed in the disorder thus creating the need to identify a biomarker for FMS.

In view of this information, the biological question for this thesis states: “*Is there a metabolic perturbation in FMS that may subsequently be used to establish a pain profile for the disorder and to identify a biomarker or biosignature for FMS?*”. Through the use of metabolomics, more scientific insight into FMS and its pain mechanisms may set us in the right direction to elucidate all forms of chronic pain syndromes.

In the following chapters I will review the genetic associations in FMS and also the current applications of the “omics” fields of study and technology, to understand the progression and possibly identify an accessible, reliable and cost-effective biomarker for this disorder.

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CHAPTER 3: LITERATURE REVIEW — METABOLOMICS AND GENOMICS OF FIBROMYALGIA SYNDROME

3.1 Introduction

The pathogenesis of FMS is not yet fully understood but some early studies have suggested that there exists a high familial aggregation of this disorder (Pellegrino *et al.*, 1989; Park *et al.*, 2015). A genetic predisposition has therefore been perceived to play a role in FMS. In their study, Pellegrino and colleagues suggested an autosomal dominant mode of inheritance from their findings. Buskila *et al.*, (1996) continued by suggesting a gender dependency on the mode of inheritance. Subsequent studies by other researchers then sought to identify the genes involved in pain and consequently the genes responsible for a genetic predisposition in FMS pathogenesis. Against this brief background, FMS appears to be a candidate disorder for investigations using contemporary 'omics' technologies. Among these, metabolomics is a relatively new field on the 'omics' research agenda (Wishart 2007; Smilde *et al.*, 2005). The other 'omics' technologies are genomics, transcriptomics and proteomics — respectively dealing with the characterization of organisms on the gene, transcript, and protein levels (Wang *et al.*, 2011). Together with metabolomics, these 'omics' technologies form part of an 'omics' cascade in which metabolomics falls at the most distal end (Dettmer & Hammock 2004).

In this chapter I give a brief background on the genetics of FMS and introduce the investigative method of choice, being metabolomics, used in my study.

3.2 Genetics of Fibromyalgia Syndrome

Many studies have attempted to understand the genetic link in FMS. The resultant candidate genes identified centred on pain processing and transmission (Ablin & Buskila 2015). Numerous markers associated with a genetic context have been identified as abnormal in FMS patients as compared with controls, and show symptom-based changes; these suggest a potential biomarker for the disorder (Ablin *et al.*, 2009). These biomarkers include polymorphisms in the genes of certain pathways, namely, of the serotonin, dopamine and catecholamine systems (Buskila 2009). According to Buskila (2009), the polymorphisms in these genes all seem to affect the monoamines' mode of transportation and their metabolism. It has to be noted, though, that Buskila also emphasized that these

polymorphisms are not specific to FMS but are also associated with other diseases overlapping with FMS. Thus, Buskila ruled out that a distinct biomarker for FMS emerged from these studies.

There are a large number of genes that are presumed to play a role in pain (Figure 3.1). Only about ten of these genes have been identified along with the body of information each contributes towards a pain profile. Discovery of these genes came about from murine models of pain and many more have yet to be discovered (Mogil 2012).

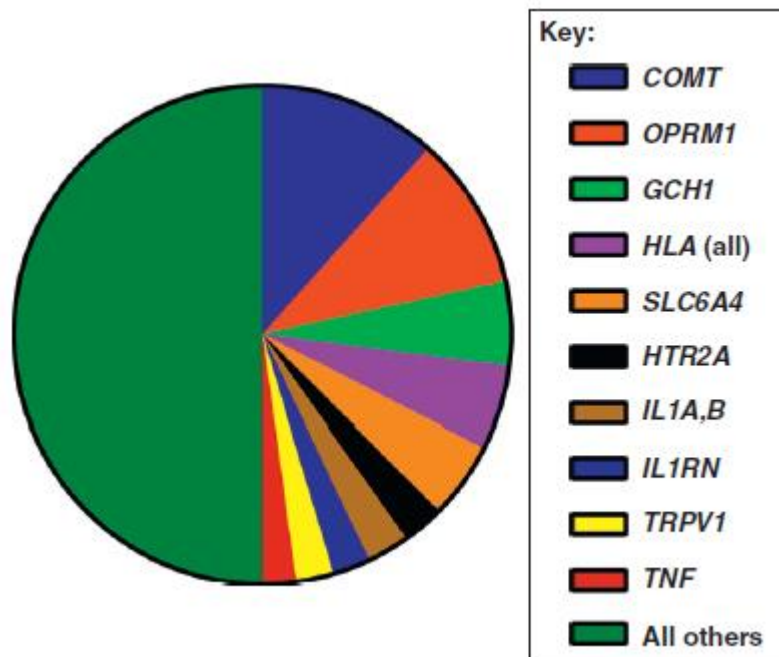


Figure 3.1: Pie chart of all known pain genes and how much information they contribute to a pain profile. The ten known pain-associated genes contribute only 50% of the pain genes known today. The other 50% consist of genes whose discovery is still pending (Reproduced with permission from Mogil 2012).

To date, most of the research conducted in pain studies has been based on traditional genetic analyses. However, the application of contemporary genomics technologies emerged to provide novel information that may further contribute to the understanding of the syndrome, and will be discussed below. In this section I give a brief overview of the three important systems and their gene polymorphisms known to play a role in FMS.

3.2.1 Dopaminergic system and the polymorphisms associated with FMS

Dopamine is a catecholamine, that is, it has a 3,4-dihydroxyphenyl (catechol) nucleus neurotransmitter that is derived from the breakdown of tyrosine. It is synthesized in the

kidneys by the adrenal glands and also in the sympathetic neurons. Dopamine has two functions: 1) in the brain it serves as a neurotransmitter, and 2) in the circulatory system it serves as a hormone. These two systems, however, operate separately because of the blood–brain barrier (Garret & Grisham 2005).

The polymorphism identified to play a possible role in the pathogenesis of FMS and related to the dopaminergic system is the 7-repeat allele found in exon III of the gene of the D₄ receptor (DRD₄). The chromosomal location of the polymorphism is found at 11p15.5 (Lee *et al.*, 2012). The frequency of this repeat allele has been shown to occur less in patients that have FMS and these patients subsequently display a personality of low novelty-seeking (Buskila & Sarzi-Puttini 2006). This 7-repeat polymorphism is a 48 base pair (bp) variable number tandem repeat (VNTR) polymorphism. There is also a 4-repeat form of this polymorphism but it will not be discussed as it is not associated with FMS (Lakatos *et al.*, 2002).

3.2.2 Serotonergic system and the polymorphisms associated with FMS

Serotonin is a biogenic amine derived from tryptophan, whose main function is that of a neurotransmitter. Serotonin stimulates the contraction of smooth muscle and simultaneously acts as a potent vasoconstrictor in blood vessels (Murray *et al.*, 2009).

There are two polymorphisms found in the serotonergic system that are implicated in the possible pathogenesis of FMS. These polymorphisms are found in the serotonin transporter (5-HTT) and in the serotonin receptor subtype 2A (5-HT_{2A}).

The 5-HTT gene is encoded by the SLC6A4 gene and can be found on the 17p11.1-p12 locus in the 5' flanking region (Williams *et al.*, 2003). The polymorphism occurs in the transcriptional region of the gene. Two forms of the polymorphism have been described in which a 44-bp insertion results in a “long allele” (L) and a deletion of the same 44-bp length results in a “short allele” (S). The L-variant of this polymorphism has been connected with transcriptional activity that increases threefold (Offenbaecher *et al.*, 1999). FMS patients have been identified to possess the S-variant of the polymorphism at higher frequencies than their healthy counterparts. This variant of the polymorphism is associated with higher incidences of depression and “psychological distress” (Offenbaecher *et al.*, 1999), both of which are associated with FMS.

There are seven serotonergic receptors (5-HT₁₋₇). The type 2 receptors are divided further into three sub-categories, namely, A, B and C. Of these, the 5-HT_{2A} receptor has been

associated with FMS (do Prado-Lima *et al.*, 2004). The gene for the 5-HT_{2A} receptor is located on the long arm of chromosome 13 (do Prado-Lima *et al.*, 2004; Bondy *et al.*, 1999; Myers *et al.*, 2007). The polymorphism is that of T102C, resulting in cytosine (C) being replaced by thymine (T) at codon 102 (Bondy *et al.*, 1999). This mutation does not result in the alteration in the amino acid sequence of the gene, making it a “silent mutation”; it does, however, alter the expression of the gene causing lesser production of these 5-HT_{2A} receptors in cells (do Prado-Lima *et al.*, 2004).

3.2.3 Catecholaminergic system: catechol-O-methyltransferase (COMT) enzyme and the polymorphisms associated with FMS

COMT (EC 2.1.1.6) is an enzyme that facilitates the metabolism of catecholamines. As such, it modulates the neurotransmission of dopamine and adrenaline/noradrenaline (van Esch *et al.*, 2011; Vossen *et al.*, 2010). A decrease in the activity of COMT causes the dopaminergic neurons to become activated, while levels of enkephalin in the neurons decrease and μ -opioid receptors increase in the region where pain is experienced. The induction of these μ -opioid receptors is in response to stressors and pain stimuli. Through this, pain and the stress responses are typically reduced. The total inhibition of the enzyme causes pain sensitivity to be increased using the $\beta_{2/3}$ -adrenergic system (van Esch *et al.*, 2011).

The COMT gene is located on the long arm of chromosome 22 at the 22q11.2 locus (van Esch *et al.*, 2011). There are many single nucleotide polymorphisms (SNPs) that affect the COMT gene but the one implicated in FMS is the Val158Met locus (Buskila & Sarzi-Puttini 2006). The SNP occurs at the genomic level, causing an alteration in the protein sequence of the transcriptome. The mutation occurs at position 158 where valine is altered to methionine. The genotypes are described as follows: H/H is val/val (normal, wildtype), H/L is val/met (defective, heterozygous) and L/L is met/met (defective, homozygous) (Vossen *et al.*, 2010; Matsuda *et al.*, 2010). The brackets indicate the kind of enzyme the alteration gives rise to and the genotype, respectively. This polymorphism affects the activity and thermostability of the enzyme with the H/H type being of highest activity and L/L being of lowest activity. It is also linked with a pain tolerance reduction as enzyme activity decreases in healthy counterparts (van Esch *et al.*, 2011; Desmeules *et al.*, 2012). FMS patients with the L/L genotype experience more severe pain compared with those with the H/H genotype (Matsuda *et al.*, 2010; Desmeules *et al.*, 2012). These L/L homozygotes also have increased neurotransmission of dopamine and decreased activation of the μ -opioid system due to the

low activity of COMT, causing them to have increased sensitivity to nociceptive stimulation (Vossen *et al.*, 2010).

In my study, blood samples were collected from our patients and controls for genetic studies based on the three systems and their polymorphisms. These genotyping results, however, did not produce conclusive information — see Addendum A1

3.2.4 Genomics studies on FMS

In a review by Ablin and Buskila (2014), the question was posed whether it is possible to predict future development of FMS. Due to familial aggregation and advances in scientific research on the disorder, the genetic aspect has become an attractive angle with which to identify individuals with a predisposition to FMS. In this section two recent scientific papers are reviewed that attempt to answer the question posed by Ablin and Buskila.

In 2013, Arnold *et al.* conducted an autosomal genome-wide linkage scan (also known as a genome-wide association study or GWAS) to detect all possible chromosomal loci that can be determined as markers associated with FMS susceptibility. The authors used members of 116 families from the Fibromyalgia Family Study who were then genotyped with 341 microsatellite markers.

Arnold *et al.* were able to identify two markers linked with FMS susceptibility. The markers were located on chromosome 17 in the p11.2–q11.2 region of the chromosome. One of the markers identified coincides with a well-known FMS candidate gene for SLC6A4 (see section 3.2.2 above). The other marker was a lesser known candidate gene for transient receptor potential vanilloid channel 2 (TRPV2). The role of TRPV2 is not fully understood yet, but it is thought to play a role in pain mediation. Arnold *et al.* acknowledge that the region where the SLC6A4 and TRPV2 genes were identified contains more than 100 other genes and that the specific role that this chromosomal region plays in the pathogenesis of FMS needs to be elucidated. However, their current findings warrant further investigation as some overlaps were seen with respect to earlier research.

In another GWAS-type study in 2014, Docampo *et al.* aimed to elucidate genetic susceptibility factors for FMS. This was done through two objectives: 1) by a GWAS study, and 2) using copy number variants (CNV) by using genotyping data (SNP) and array comparative genomic hybridization experiments (aCGH). Their study cohort comprised 313 samples from females diagnosed with FMS having low co-morbidities and 220 females with no signs of FMS, who served as controls.

The GWAS analysis conducted by Docampo *et al.* did not yield SNPs of importance with regard to FMS susceptibility. Docampo *et al.* therefore conducted a secondary study (Docampo *et al.*, 2014) in which 21 of the most associated SNPs were earmarked for replication in a larger cohort of 952 cases and 644 controls. From this secondary study, a SNP (rs11127292) in the myelin transcription factor 1-like (MYT1L) gene was identified as being associated with FMS susceptibility in the low-comorbidity cohort. MYT1L plays a role in neuronal differentiation and is associated with neuropsychiatric disorders. The aCGH study revealed an intronic 8-kb CNV deletion in NRXN3. NRXN3 also plays a role in neuronal development and signal transmission and may explain the pain dysfunction characteristic in FMS. These findings therefore implicate CNS involvement in FMS, which is consistent with reports highlighting a neurocognitive component in the disorder.

In both studies (Arnold *et al.*, 2013; Docampo *et al.*, 2014), the FMS group was characterized using the ACR 1990 criteria, as was done in my study. Arnold *et al.*, however, included males in their study, whereas only female subjects were used in the study by Docampo *et al.* In both studies a new genetic discrepancy was presented in FMS patients, not previously described in the literature. With further validation of these findings, it could possibly lead to a means of identifying individuals with a predisposition to the disorder, thereby allowing physicians to curb the effects at an earlier stage.

3.3 Metabolomics and its applications in FMS

Metabolomics encompasses the identification and quantification of small molecules called metabolites (<1500 Da) that are both exogenous and endogenous in nature (Zhang *et al.*, 2012a; Roessner & Bowne 2009). The functional use of the term metabolomics was first introduced in 2002 by Oliver Fiehn, who described it as “*a comprehensive analysis in which all the metabolites of a biological system are identified and quantified as needed*”. Metabolomics thus aids researchers in the comprehension of the intricate molecular interactions of all known biological systems (Bino *et al.*, 2004). Using ‘omics’ technology enables one to examine biological systems in response to perturbations (disease related or environmental) or genetic alterations, on various levels. These include biological material from cellular, tissue, organ and whole organism origin (Lin *et al.*, 2006). Eventually, the use of metabolomics information in a systems biology approach (van der Greef *et al.*, 2006) opens up an understanding of biology, culminating in personalized medicine (Ramautar *et*

al., 2013). A major goal of metabolomics is therefore the measurement of as many metabolites as possible encountered in a biological system (Smilde *et al.*, 2005).

Many scholarly reviews have been published on metabolomics (Goodacre *et al.*, 2004; Goodacre *et al.*, 2007; Fiehn *et al.*, 2007), metabolomics technology (Weljie *et al.*, 2006; Koek *et al.*, 2011; Zhou *et al.*, 2012) and on its applications (Spratlin *et al.*, 2009; Bundy *et al.*, 2009; Zhang *et al.*, 2012b). It is therefore beyond the purpose of this thesis to contribute to a deeper understanding of metabolomics and its technologies. It is, however, evident that a broad outline, as well as terminological information, is required as a prerequisite for my aim to use metabolomics in the study of FMS. The focus will thus be on the broad outline. There are many terms associated with metabolomics and these terms and their descriptions are summarized in Table 3.1, most of which will be used throughout this thesis.

Table 3.1: Table of terms used in metabolomics, and their definitions (reproduced with permission from Oldiges 2007).

Term	Definition
Metabolite	Bio-reactive small molecule involved in a biochemical network
Metabolome	Set of all metabolites present in a biological system (e.g. the cell)
Exo-metabolome	Metabolites present in the extracellular surroundings of the cell (e.g. cell supernatant)
Endo-metabolome	Metabolites present inside the cell (sometimes separated by compartmentalization)
Metabolic quenching	Immediate “freezing” (termination) of all metabolic activity in a sample (preferably during the sample collection procedure)
Metabolomics	Quantification of the metabolome (and keyword for this scientific endeavour)
Target analysis	Quantitative analysis of specific substrate and/or product metabolites
Metabolic profiling	Quantitative analysis of a set of pre-defined metabolites belonging to a class of compounds, or members of particular pathways or a linked group of metabolites (e.g. sugars, sugar phosphates, lipids, organic acids)
Metabolic fingerprinting	Semi-quantitative analysis of the endo-metabolome
Metabolic footprinting	Semi-quantitative analysis of the exo-metabolome

Metabolites offer us the information to bridge the gap between the genotype and the phenotype, by providing indicators (or eventually biomarkers) that represent the functional phenotype at a given point in time. Analysis of these low-molecular-weight substances could also provide a deeper understanding of how lifestyle and dietary factors impact the manifestation of certain diseases (Roessner & Bowne 2009; Zhang *et al.*, 2012a). Examples of these metabolites include amino acids, organic acids, phenolic acids, carbohydrates, lipids and alkaloids. Metabolites are not encoded in the genome like their RNA and protein counterparts, but are the expression products that are involved in the biochemical pathways present in the human body, as well as of all other organisms, depending on the source of the material being analysed. Metabolites are also the key substances of, or may be required for, metabolism (Dettmer & Hammock 2004). Collectively, these metabolites are called the *metabolome* (see Table 4.1) (Vinayavekhin *et al.*, 2010).

There are two main categories of platforms most generally used in metabolomics investigations for the identification of metabolites: (hyphenated) mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.

MS is the metabolite identification technique used in conjunction with a separation method, which can be mainly gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE), designated as hyphenated MS, e.g. GC–MS. MS can, however, be used on its own (not coupled to a separation device) by using direct-injection MS (DIMS). DIMS leads to a resultant spectrum in which a single sample is represented (Lin *et al.*, 2006). DIMS constitutes a rapid technique but allows for only a limited number of metabolites to be analysed. DIMS possesses some disadvantages, because it has a low ionization efficiency and co-suppression occurs. Hence, it is preferred to use MS in conjunction with a chromatographic separation technique — the hyphenated MS (Zhang *et al.*, 2012a). Other disadvantages of MS include the fact that samples are destroyed and cannot be re-analysed as in NMR analysis. It also usually calls for sample preparations that result in the loss, or chemical modifications, of metabolites. Key advantages are that analysis times are not long, its selectivity and sensitivity are very high and that it can be coupled to separation technologies (Wang *et al.*, 2011).

The most commonly used hyphenated MS platform for metabolite analysis is GC–MS (Wang *et al.*, 2011), which is mostly used in non-targeted or semi-targeted analyses (described in detail later in this review), especially for metabolites that are hydrophilic in nature (Zhang *et al.*, 2012a; Lin *et al.*, 2006). GC separation is ideal for the analysis of volatile compounds, for example organic acids. Since temperatures reach extreme heights during GC separation,

the analytes need to be thermally stable and volatile. This is achieved by derivatizing the samples before actual analysis on the mass spectrometer, but this can also result in metabolite loss or chemical modification of artifacts, which is one of the major disadvantages of GC–MS.

NMR is an analytical method used for metabolic profiling in which a sample is subjected to a magnetic field for compound identification, resulting in a collection of spectral peaks with each compound rendering a distinctive pattern (Wang *et al.*, 2011). It can be used for samples in a solid or liquid state. For the scope of this thesis, only liquid state NMR will be referred to. NMR is a non-discriminative (Wang *et al.*, 2011) method in which complex samples, like biofluids, can be analysed without destruction of the sample, which is the case with most other metabolomic techniques (Smolinska *et al.*, 2012). There are different forms of NMR that can be performed, with proton (^1H -NMR) and carbon-13 (^{13}C -NMR) being the most commonly used forms — my study focuses on ^1H -NMR. There are certain disadvantages to NMR as compared to chromatographic metabolomic techniques with the main drawback being that the sensitivity of NMR is low. This means that analyses using NMR can only be used on samples with sufficiently high physiological concentrations of the metabolites of interest (Smolinska *et al.*, 2012; Breau & Cantor 2003; Zhang *et al.*, 2012a). Second, the spectra produced from NMR are highly complex, such that rapid analysis of samples is not possible (Breau & Cantor 2003; Wang *et al.*, 2011). Lastly, compounds without protons cannot be detected in ^1H -NMR. Advantages of this method are that sample preparation is minimal and the sample does not get destroyed (as mentioned above) during analyses, thereby making NMR more reproducible than other techniques (Smolinska *et al.*, 2012; Breau & Cantor 2003).

3.3.1 Different approaches used in metabolomics investigations

Metabolomics experiments are conducted in either a targeted or untargeted fashion. This is done depending on the kind of information one is seeking to obtain from an analysis.

The targeted approach is a type of analysis in which the goal of the experiments is to focus on only certain metabolites (Figure 3.2) (Vinayavekhin *et al.*, 2010). These metabolites (targets) are all known (predetermined classes of molecules), hence no new metabolites are identified with these analyses. With this type of analysis only one type of analytical technique is used that is best suited for the analysis of these specific compounds (Roessner & Bowne 2009). This approach is also known as “biased” or “directed” metabolomics (Wang *et al.*,

2011). Targeted approaches normally make use of internal standards that are isotope-labelled for absolute quantitation (Vinayavekhin *et al.*, 2010; Wang *et al.*, 2011). As a hypothesis-driven approach, it is often used for the confirmation of an untargeted study and also for the validation of important biological pathways. The information generated from this approach is interpreted by means of known biological pathways and physiological interactions. However, the one disadvantage of this approach is that it is not seen as a universal or “omics” approach (Dettmer & Hammock 2004).

3.3.2 What is metabolic profiling?

Metabolic profiling (also see Table 3.1) is the “*identification and quantitation of compounds in the metabolome*” (Dunn *et al.*, 2011). This high-throughput method is applied to biofluids, like blood and urine, to measure and interpret their metabolic parameters (Kuhara 2005). By doing this we are able to track the changes in the levels of the metabolites, the end products of cellular metabolism. This in turn allows us to quantify gene function.

Studies using metabolic profiling are conducted using different analytical techniques including chromatography coupled to MS, as was the case for my PhD study where GC–MS was used.

As opposed to the targeted approach, the untargeted approach aims to measure as many metabolites as possible that are present in a given sample (Lommen *et al.*, 2007) and to compare how, if any, changes occur in the level of metabolites in response to a certain disease, exposure to toxins or any other applicable perturbation (Dettmer & Hammock 2004). If the untargeted analysis focuses on a certain class of metabolites, e.g. organic acids, steroids or bile acids, the approach is often typified as semi-targeted metabolomics. These metabolites that are being analysed can be either identified or unknown, based on their chemical make-up (Roessner & Bowne 2009). If the variables are not chemically identified by name or structural formula (e.g. as intensity or area values at a distinct retention time (RT) in a GC spectrum or of a spectral amplitude at a specific chemical shift position, in units of parts per million (ppm), of an NMR spectrum), this approach is termed “metabolic fingerprinting”. Both metabolite profiling and fingerprinting allow us to make use of a resultant profile from an analysis to classify a phenotype (Wang *et al.*, 2011). Both approaches were applied in the NMR metabolomics of urine samples from the FMS patients and controls studied in Malatji *et al.*, 2017. Metabolic fingerprinting can be seen as a true “omics” approach as the extensive amount of original data is compared (e.g. those of

patients with controls) using unsupervised statistical tools such as principal component analysis (PCA) and cluster analyses. If sample segregation occurs from these analyses, then further investigations are initiated to identify (annotate/absolute identification and quantification) these compounds/metabolites, leading to a metabolic profiling result. This may then be further analysed to identify a limited number of metabolites, which may become potential biomarkers or a biosignature for the perturbation under investigation (Dettmer & Hammock 2004). This untargeted approach is often an exploratory or hypothesis-generating process and is most commonly used in cases where biomarker identification is the goal of the study. Hence this is the approach that was largely followed in the experimental part of this project.

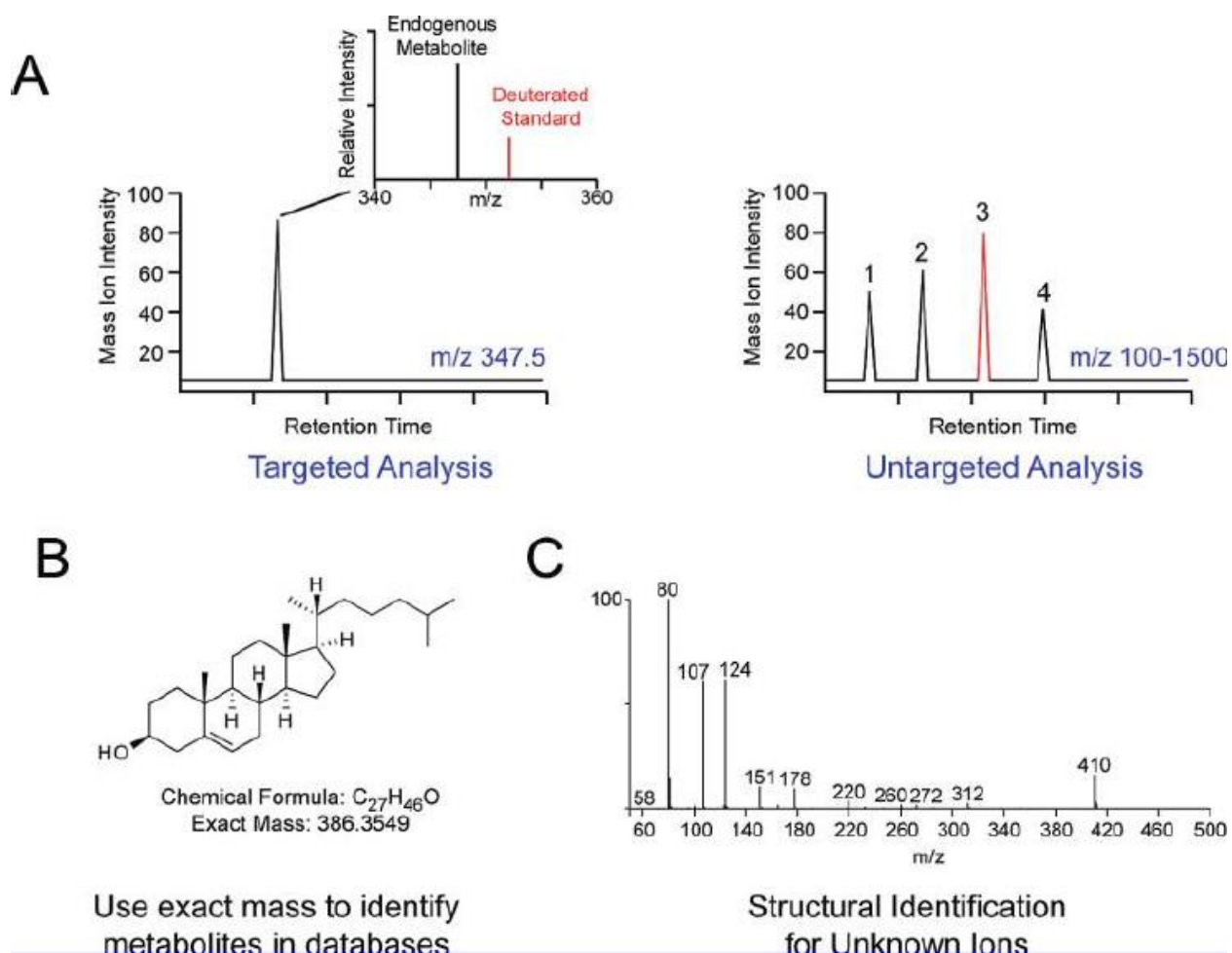


Figure 3.2: The differences between targeted and untargeted analyses, and their uses, as utilized in MS-based metabolomics investigations. A) Isotopic standards are used for absolute quantitation of metabolites in targeted analyses, whereas in an untargeted analysis variations in all metabolites are measured by scanning a broad mass range (e.g. m/z 100–1500). B) Knowing the exact mass of a compound can aid in identification of the metabolite

in question by comparison against other metabolites of the same mass. C) Structural characterization leads to identification of novel metabolites, which can be validated with the use of additional analytical experiments such as tandem MS (shown in figure) and NMR (Reproduced with permission from Vinayavakhin *et al.*, 2009.)

3.4 Metabolic indicators of Fibromyalgia Syndrome

When this project was initiated in 2011, no publications or investigations using metabolomics as the investigative method for identification of biomarkers in FMS had been published. However, after completion of the experimental aspects of the present investigation, three articles, by Hackshaw *et al.*, Caboni *et al.*, and Hadrévi *et al.*, were published online in the journals *The Royal Society of Chemistry* in April 2013, *PLOS ONE* in September 2014, and *Scientific Reports* in November 2015, respectively. The article by Hackshaw *et al.*, is entitled “A bloodspot-based diagnostic test for fibromyalgia syndrome and related disorders”, the article by Caboni *et al.*, is entitled “Metabolomics analysis and modelling suggest a lysophosphocholines-PAF receptor interaction in Fibromyalgia”, and the paper by Hadrévi *et al.*, is entitled “Systemic differences in the serum metabolome: a cross sectional comparison in woman with localized and widespread pain and controls”, with FMS being a special form of CWP.

For the remainder of this chapter, these three specific articles will be dissected and critically assessed based upon their: 1) novelty, 2) contribution to the subject, and 3) limitations, as offered as metabolomics studies of FMS.

3.4.1 Metabolomics of FMS from dried blood samples

Hackshaw and colleagues used a partly metabolomics approach in their investigation of FMS. The analytical protocol included mid-infrared microspectroscopy (IRMS) and a metabolomics (multivariate) analysis of data obtained from three platforms: ultra-high-performance liquid chromatography with tandem MS (UPHLC–MS/MS) and GC–MS using non-targeted metabolic profiling, with two UPHLC–MS/MS platforms being used, namely, one optimized for an acidic analysis and the other optimized for a basic analysis. The aim of their investigation was to identify a “rapid biomarker-based method” to help diagnose FMS (n = 14) and to distinguish it from two other inflammatory disorders, namely, osteoarthritis (OA) (n = 12) and rheumatoid arthritis (RA) (n = 15), using dried blood samples.

For metabolomics analyses, the punches from the bloodspot cards were subjected to the solvent extraction method using methanol; 100 μ l of this supernatant was used for analysis. Equal aliquots were made for each of the three analyses, respectively. The supernatant was then further evaporated to dryness under nitrogen and vacuum-desiccated. The samples were then reconstituted in different solvents for the different analyses to be performed. With regard to UHPLC–MS/MS, for acidic conditions 50 μ l of 0.1% formic acid in water was used, for basic conditions 50 μ l of 6.5 mM ammonium bicarbonate in water was employed. For the GC–MS analysis, the samples were derivatized using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and a solvent mixture of acetonitrile, dichloromethane and cyclohexane with trimethylamine to a final volume of 50 μ l.

Through using this approach, Hackshaw and colleagues were able to detect and identify 166 metabolites. By applying multivariate analysis, they were able to identify 30 metabolites as the top-ranking metabolites to contribute to the separation between FMS and RA. A PCA based on these 30 metabolites was insufficient to provide a natural separation between the FMS and the RA and OA cases (see Figure 3.3A). A natural separation could be obtained, however, for the FMS cases using eight variables which were identified as having high discriminatory value (see Figure 3.3B).

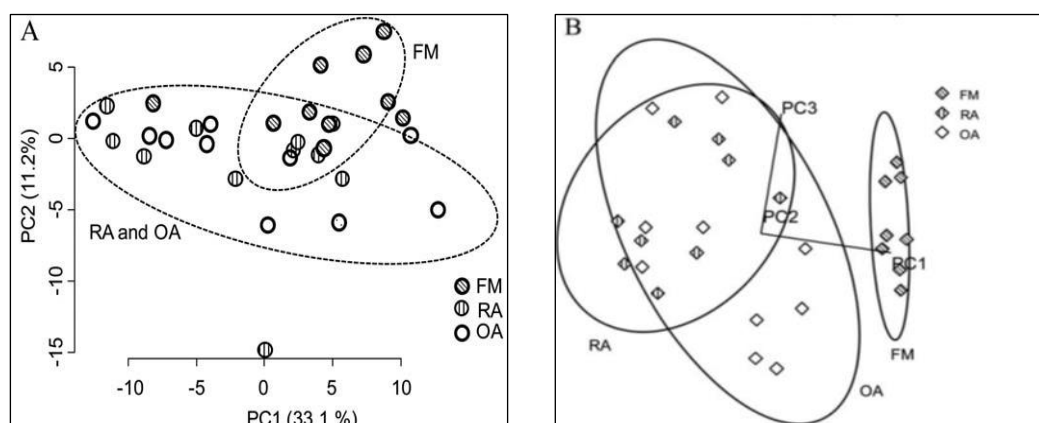


Figure 3.3: Results obtained from a PCA score plot created in a metabolomics experiment conducted by Hackshaw and colleagues. (A) PCA using the top 30 metabolites, identified through random forest analysis, shows that a natural separation could not be obtained for FMS versus the inflammatory disorders. (B) PCA showing the natural separation between FMS and the inflammatory disorders, based on the eight selected metabolites. The analysis was performed using SIMCA (adapted with permission from Hackshaw *et al.*, 2013.)

Amidst the 30 significant markers identified, *trans*-urocanate, kynurenine, bradykinin, sarcosine, ornithine and asparagine (all involved in metabolism of peptide/amino acids), heme, cysteine-glutathione disulfide, oxidized glutathione and opthalamate (oxidative stress markers), nicotinamide adenine dinucleotide (NAD⁺), fumarate and phosphoenolpyruvate (PEP) (all involved in energy metabolism), choline and glycerol-3-phosphate (all involved in membrane remodelling) were identified as the metabolites that were chiefly responsible for the observed separation. Similar results were obtained for the separation between FMS and OA cases. Kynurenine, *trans*-urocanate, cysteine-glutathione disulfide, PEP, pyruvate and NAD⁺ were all found in elevated concentrations in FMS sufferers. The observed important metabolites were not quantified, but the spectral information was correlated with the symptom severity of the patients, using pattern recognition software for the partial least squares regression (PLSR). Loadings on the PLSR factors provide information on the most influential explanatory variables, which turned out to be asparagine, heme and *trans*-urocanate, in descending order. Heme, along with other hemoproteins, was significantly elevated in FMS sufferers.

The novelty of this pioneering metabolomics study lies in the analytical advantage of using samples from blood spot cards — dried and transported to the laboratory for analysis elsewhere — which enabled a clear separation between samples from RA and OA relative to FMS cases. Furthermore, a notable biochemical contribution is the prediction by Hackshaw *et al.*, from the results of their untargeted metabolomics study, that changes in the tryptophan catabolic pathway differentiated FMS patients and those identified with RA and OA. Third, the fact that the discriminatory variables were identified via a regression algorithm and not through relative or absolute qualified values, limits the applicability of the results for future comparative studies.

3.4.2 Metabolomics of FMS through plasma lipidomic analysis

Caboni and colleagues used a modified Folsch method to extract plasma lipids, for their study on FMS, for the identification of potential biomarkers and better understanding of the pathogenesis of the disorder. In their study they utilized liquid chromatography–quadrupole time-of-flight–mass spectroscopy (LC-QTOF-MS) to analyse the plasma of 22 FMS patients and 21 matched controls. The metabolome of choice in this study was that of lipids, with compounds of interest being lysophosphochilines (lysoPC), phosphocholines and ceramides, based on the observation that oxidative stress with lipid peroxidation induced by ROS has been proposed as a relevant contributing factor to FMS pathogenesis.

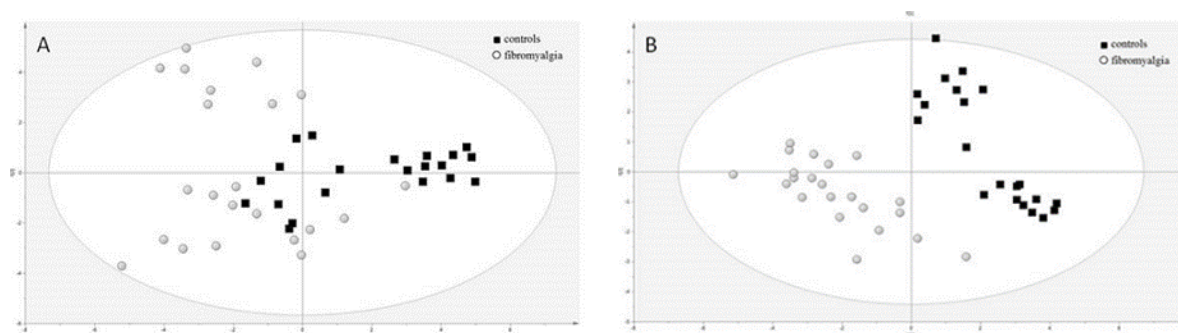


Figure 3.4: Unsupervised PCA (A) and supervised PLS-DA (B) analysis results obtained from the study by Caboni and colleagues on FMS patients (denoted by black squares) versus matched controls (denoted by grey circles). No natural separation or outliers were observed in the PCA, whereas a separation was observed in the PLS-DA. The analyses were based on the data of the metabolites identified using LC–QTOF–MS. All analyses were performed using SIMCA software. (Reproduced with permission from Caboni *et al.*, 2014.)

Caboni's findings showed elevated amounts of lysoPCs, which are produced by lipid oxidative fragmentation, in FMS patients as compared to controls. Statistical analyses showed no separation of the two groups, on the basis of the metabolites identified by LC–QTOF–MS, on an unsupervised PCA (Figure 3.4A), with subsequent separation on a supervised PLS-DA (Figure 3.4B). Using the numerical values of the loadings from a PCA plot, seven metabolites were identified as the discriminant molecules responsible for the separation between FMS and controls. Of these seven metabolites, PC(14:0/0:0) and PC(16:0/0:0) were noted to have a platelet activating factor (PAF)-like structure and their binding affinity for the PAF receptor (PAFr) was examined. This was done as the PAF/PAFr system has been linked to the modulation of pain signalling. These two phosphocholines possessing a PAF-like structure therefore suggests that they can bind to and activate the PAFr receptor, thereby eliciting pain signalling and enhancing pain sensitivity (Caboni *et al.*, 2014).

The novelty of this study was the application of metabolomics information to direct a molecular dynamics analysis of an endogenous metabolite–PAFr interaction that may play a role in the clinical manifestation of FMS. Second, this study, like the dried blood spot analysis by Hackshaw *et al.*, indicated the capability of a metabolomics approach to generate distinct metabolic profiles that distinguish between FMS and controls; here, through a targeted lipidomic analysis. Third, the same limitation as in Hackshaw *et al.* occurred here, as no relative or absolute qualified values of the relevant discriminating variable from the loadings plot were calculated.

3.4.3 Metabolomics of CWP/FMS through analysis of serum

The aim of the study by Hadrévi *et al.*, (2015) was to use metabolomics to explore the metabolite content and profile in patients with widespread and localized pain and controls. The blood serum of females was the sample material utilized in their study. The cases studied were female FMS patients ($n = 16$) with CWP (indicated as CWP/FM). These cases were identified through the sensitivity of 11 tender points out of 18, as for FMS. There were two sets of controls, namely, those with pain ($n = 32$) that had a localized nonspecific manifestation of pain (neck–shoulder pain, indicated by NP) and normal healthy controls ($n = 39$) without pain (indicated as CON).

Serum samples were extracted with methanol:water (9:1) followed by methoxymation and analysed by gas chromatography–time-of-flight–mass spectrometry (GC–TOF–MS). A total of 244 metabolites were detected by GC–TOF–MS but only 110 of those detected were identified. The metabolites were identified through hierarchical multivariate curve resolution (H-MCR). All 244 metabolites, however, were used for multivariate analyses. Group separation, through PCA, differentiated CON from undifferentiated CWP and NP, but an orthogonal partial least squares analysis (OPLS-DA) separated all three groups in a model using two predictive components (Figure 3.5). Metabolites (up- or down-regulated) unique to CWP were identified through a shared and unique structure plot (SUS-plot), shown in Figure 3.6.

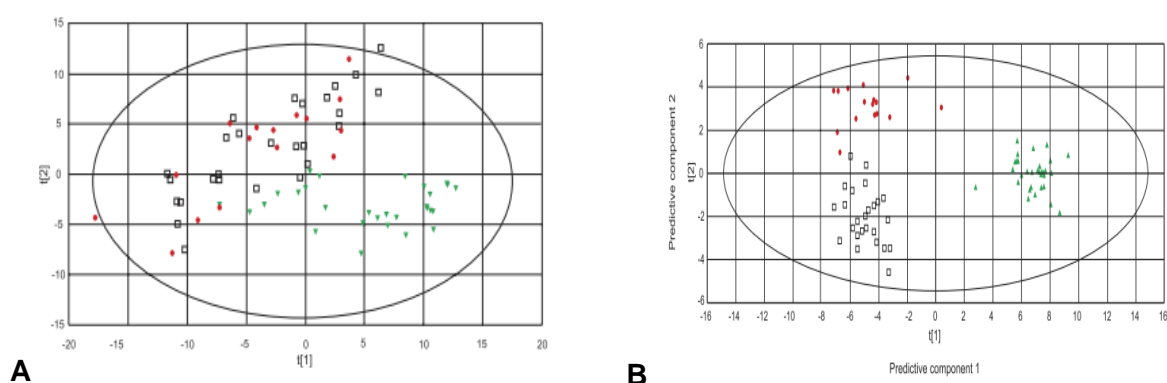


Figure 3.5: Unsupervised PCA (A) and supervised OPLS-DA (B) conducted by Hadrévi *et al.*, In both (A) and (B) FMS/CWP is denoted by red circles, NP by green triangles and CON by black squares. The PCA shows the initial multivariate analysis in which a clear natural separation of NP was observed from FMS/CWP and CON, which did not separate from each other. The OPLS-DA shows a clear separation of NP from the CON and FMS/CWP, with lesser separation observed between FMS/CWP and CON. (Reproduced with permission from Hadrévi *et al.*, 2015.)

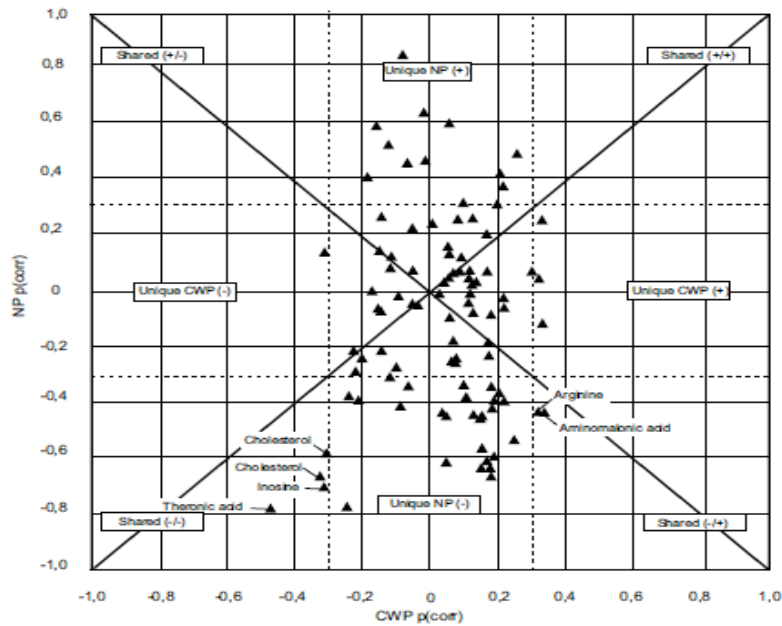


Figure 3.6: A SUS plot showing the shared and unique correlations between CON-NP, and the CON-CWP OPLS-DA model. Correlation coefficients of 0.3 are indicated by the dashed lines (Reproduced with permission from Hadrévi *et al.*, 2015.)

A metabolite profile, consisting of metabolites from several metabolic pathways, showed a differentiation between subjects with NP and the CON but only to a weak extent between subjects with CWP and CON. In the final instance the authors regard the outcome as a systemic difference of processes related to energy utilization, which may be central aspects of the mechanisms maintaining CWP.

The novelty of this publication is that Hadrévi *et al.* were able to show that there are systemic differences that enable a metabolomics study to distinguish widespread pain from localised pain and non-pain controls. Second, the scientific contribution is that their findings point to perturbations in the lipid metabolism (section 3.4.2: observed in the study by Carboni *et al.*) and energy utilization pathways (generally accepted to be a perturbation in the closely related FMS). Third, limitations of the publication are that Hadrévi *et al.* were not clear in how they distinguished CWP from FMS cases (e.g., they provided no detail on pressure point estimations as done for CWP and FMS patients) and the link between these two disorders was not addressed in the paper. Moreover, a similar limitation of Hackshaw *et al.* and Carboni *et al.* applies here in that the discriminating variables were not quantified.

In conclusion

These three investigations clearly substantiate the potential power of a metabolomics approach to identify biomarkers, and possibly the underlying pathophysiology, in perturbations as complex as FMS. From the results, of the respective studies, we can clearly see that the neurobiological pathways, as well as the energy metabolism pathways, are important in the pathophysiology of FMS, as intermediates associated with these pathways are found to be perturbed (increased or decreased) in FMS. Moreover, slight modifications in monosaccharide metabolites were also observed, lending further support to the recognition of energy disruption. Evidence of elevated oxidative stress biomarkers shows that oxidative stress does indeed play a role in the pathogenesis of FMS. Changes in amino acids are shown to occur in FMS but inconsistent results have been observed in this regard. Heme was shown to be present in highly elevated amounts in FMS patients but the reason as to why this occurred was not elucidated by Hackshaw *et al.* Through the latter's investigation we can see that FMS possesses distinguishing features from inflammatory diseases such as RA and OA. Moreover, the authors of all three publications are generally convinced that their results — and by implication the outcome of validated metabolomics studies on FMS — can be used to confirm possible biomarkers of disease diagnosis and progression.

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CHAPTER 4: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF FIBROMYALGIA SYNDROME

4.1 Brief NMR theory

Proton nuclear magnetic resonance (^1H -NMR) spectroscopy is an analytical platform employed in metabolomics for the analysis of biofluids such as blood, serum, plasma or urine. ^1H -NMR is a highly reproducible method in which a magnetic field is applied to the sample under investigation in order to manipulate the orientation of the hydrogens in the nuclei. There are many different kinds of NMR experiments that can be done but for the purposes of this thesis only proton (^1H) NMR will be referred to. The protons inside the nucleus of the particles have a random orientation when outside a magnetic field. Once they are placed in a static magnetic field they align in two possible orientations. These orientations can either be north–south or south–north. A radio frequency (RF) is then applied to the sample to “knock over” the aligned protons. As the protons return to their original state they release the energy applied by the RF pulse, which is called free induction decay (FID). Fourier transformation (FT) is then used to convert FID into the generally known NMR spectrum. Figure 4.1 shows this conversion. Each peak of the spectrum represents the intensity of protons of a particular compound.

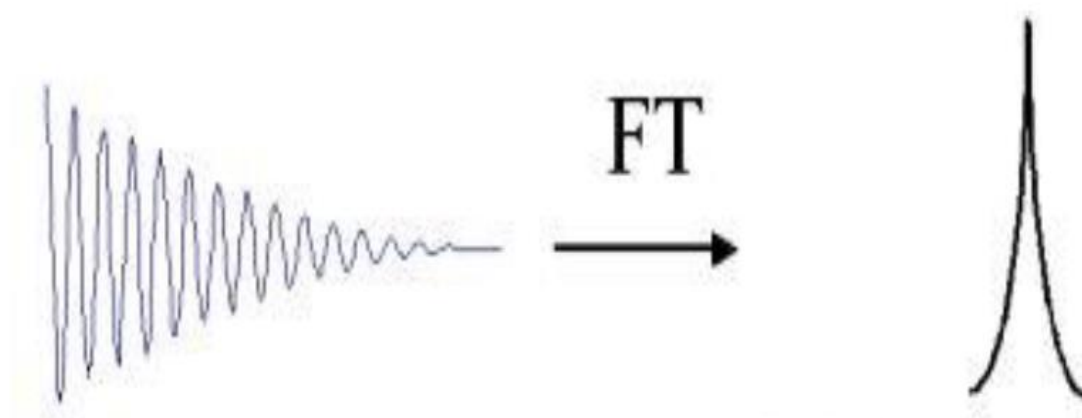


Figure 4.1: Transformation of free induction decay (time domain) to the NMR peak profile (frequency domain) by means of Fourier transformation. Each peak in the ^1H -NMR spectrum represents the intensity of free protons attached to carbons of a particular compound in a sample.

4.2 Advantages and limitations of NMR and sample preparation of biofluids

4.2.1 Advantages and limitations of NMR over MS

NMR and mass spectrometry are the most popular platforms that are used in metabolomics experiments. Both come with their own advantages and limitations. Each of their individual limitations can, however, be offset by the other. This essentially makes them complementary techniques. Numerous publications have made use of a combination of metabolomics platforms to investigate their research question. In fact, the Human Urine Metabolome Database (Bouatra *et al.*, 2013) was created with the aid of six different metabolomics platforms to elucidate the urine metabolome. Each method was able to identify a set of compounds unique to the method and compounds that overlap with each other. Figure 4.2 is a Venn diagram adapted from Bouatra *et al.* (2013), showing the number of compounds NMR and GC–MS can detect uniquely, and overlapping commonly found metabolites. Overall, GC–MS was able to identify 179 compounds with 89 of those compounds being unique to GC–MS analysis. NMR was able to identify 209 compounds with 108 of those compounds unique to NMR analysis. A total of 88 of the compounds identified were detected by both NMR and GC–MS. It should be noted that multiple extraction protocols were used to elucidate the urine metabolome and establish the urine metabolome database (Bouatra *et al.*, 2013). In this thesis, only NMR and GC–MS will be referred to as these are the two platforms used, as shown best by Bouatra *et al.*, to elucidate the urinary metabolic profile. GC–MS will be discussed in more detail in Chapter 5.

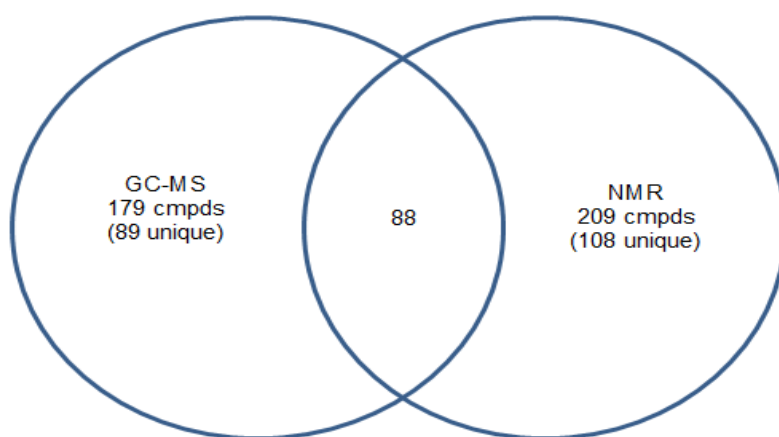


Figure 4.2: Venn diagram showing the number of compounds each metabolomics technique, being NMR and GC–MS, contributed to the elucidation of the human urine metabolome (adapted with permission from Bouatra *et al.*, 2013).

The main advantage of NMR is that no prior knowledge of the sample is needed, meaning that no tedious sample extraction needs to be conducted. This makes sample preparation simple and the whole metabolome can be studied at once. The sample is also not destroyed during analysis, allowing it to be analysed multiple times for NMR or to be used downstream in other experiments. NMR is, however, not as sensitive as MS, which can detect compounds in the pico- to femtomole range. That means NMR is not viable for the analysis of many secondary metabolites, which usually lie within that range. Although ^1H -NMR detects only metabolites with free hydrogens attached to carbons, only one technique is needed to analyse samples, unlike MS, where different extraction techniques usually need to be employed to target the different classes of metabolites. NMR is a highly reproducible method and analysis run times are typically short as compared to GC–MS (~15 minutes compared to ~45 minutes, respectively). Since minimal sample preparation and little prior knowledge of the sample is required for NMR analyses, it is best for untargeted metabolomics experiments (Emwas 2015). Moreover, the running costs of NMR are markedly lower than those of other platforms, although initial set-up is expensive. Minimal expertise is required to perform NMR analyses but it does require advanced expertise to interpret the spectra. Table 4.1 briefly outlines the comparative advantages and disadvantages of NMR and MS.

Table 4.1: Brief summary of the comparative advantages and disadvantages of NMR and MS (adapted with permission from Emwas 2015).

	NMR	MS
Sensitivity	Low	High
Specificity	High confidence in identification of compounds	Lower level of confidence in identification of compounds
Sample measurement	Only one measurement required for detection of metabolites	Different techniques are required for different classes of metabolites
Sample recovery	Non-destructive; multiple analyses can be performed on one sample	Sample is destroyed during analysis
Reproducibility	Very high	Moderate
Sample preparation	Minimal/simple preparation	More complex preparation
Targeted analysis	Yes	Yes

4.2.2 NMR sample preparation

As previously mentioned, the main advantage of NMR analyses is the simple preparation of samples. The protocol for sample preparation is a well-established Standard Operating Procedure (SOP) as used by the Translational Metabolic Lab at Nijmegen, in the Netherlands (Wortmann *et al.*, 2006; Engelke *et al.*, 2004; Moolenaar *et al.*, 2001). First, the urine is centrifuged to separate out any large particles as sediment, proteins in particular, present in the sample. This reduces the risk of broad resonance peaks that will obscure the presence of low concentration peaks in the spectra, and so give poor quantitative results. The resultant supernatant is then transferred to a clean tube and an internal standard (IS) is added. The IS in the case of this study was trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP), dissolved in a deuterated solvent, namely deuterium oxide (D_2O). The TSP serves as the source of a reference peak for quantification and scaling of the spectra, while the D_2O serves to lock the signal during analysis. It is important to standardize the pH of the samples as it affects the chemical shift of the metabolites in the spectra. The pH of urine varies quite considerably and so all samples were adjusted to $pH\ 2.5 \pm 0.05$, as per the Dutch protocol. The pH-adjusted samples were then transferred to a 5 mm NMR tube for analysis.

4.3 NMR profiling of Fibromyalgia Syndrome

4.3.1 Power of NMR metabolomics

Metabolomics is an emerging field in the “omics” cascade. It is of great value to systems biology as it allows us to study the distal alterations of gene and protein mutations, enabling us to define a phenotype of a particular disease. When coupled with NMR technology, it allows us even greater insight into disease analysis. Since little prior knowledge of the sample being analysed is needed, NMR makes for a valuable platform for untargeted metabolomics studies that are discovery based. This allows the metabolome to be studied untargeted, permitting one to identify the affected metabolites and pathways. From a foundation of these untargeted studies, one can then move on to more targeted studies using techniques such as MS.

Once the raw NMR spectra have been obtained, they have to be pre-processed so that statistical methods can be applied to identify class separation. As with any metabolomics investigation, normalization and scaling are applied to the data. This corrects for variability in metabolite concentrations between samples and within a sample (Powers 2014). Data pre-processing unique to NMR pre-processing involves binning. Binning constitutes separating

the spectrum into regions, or “bins”, of equal width (equidistant binning), usually around 0.01–0.04 ppm (Kim *et al.*, 2009; Wu *et al.*, 2012) width, or of variable size (Figure 4.3). This allows for correction of small variations in peak position and shape due to deviations in sample conditions such as pH, ionic strength and concentration (Powers 2009; Smolinska *et al.*, 2012). Variable-sized binning is generally preferred to equidistant binning because whole peaks of different compounds are incorporated into a single bin, whereas with equidistant binning peaks are potentially separated into different bins, depending on the bin width selected.

To elucidate the metabolites that differentiate a disease state from healthy controls, multivariate statistical techniques need to be employed, which are standard for any metabolomics investigation. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are the common multivariate statistical methods used to discriminate between experimental groups. Concurrently, univariate statistics help to describe the data.

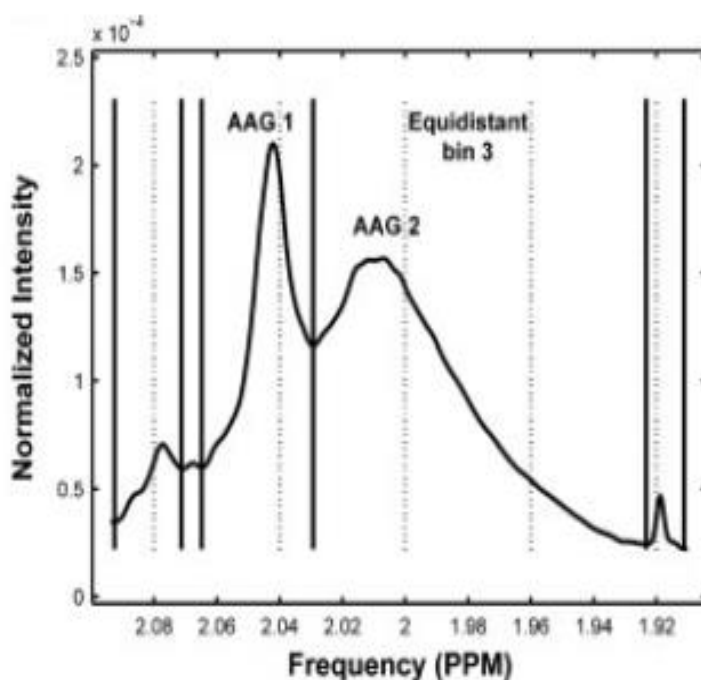


Figure 4.3: Equidistant and variable-sized binning methods used in the pre-processing of NMR spectra. Dotted vertical lines show the “bin” width for equidistant binning and how they could potentially cut peaks into different “bins”. Solid lines show variable-sized binning that allows a single peak to be incorporated into one bin. (Reproduced with permission from Powers 2009.)

4.3.2 A diagnostic biomarker profile for Fibromyalgia Syndrome

4.3.2.1 Background

FMS is a disorder of an unknown pathogenic mechanism. Criteria for diagnosis have been amended twice in the past two decades, yet, there is still no definitive way to diagnose FMS. Although we know far more about the disorder, today than before, the discovery measurable biomarkers for objective identification of affected individuals would substantially aid in the better and earlier diagnosis of FMS.

In this section I present the results of an explorative NMR study conducted on FMS patients and controls, published in *BMC Neurology*. The primary objective of this untargeted study was to determine if FMS patients could be differentiated from healthy controls on the basis of their urine metabolome, with the ultimate aim of identifying a possible diagnostic biomarker profile for FMS. This study is presented as a supplement to the current criteria used to diagnose the disorder. Note that the article is presented in the format that it was published in, as per the *BMC Neurology* journal's article submission guidelines.

4.3.2.2 A diagnostic biomarker profile for fibromyalgia syndrome based on an NMR metabolomics study of selected patients and controls

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Abstract

Background

Fibromyalgia syndrome (FMS) is a chronic pain syndrome. A plausible pathogenesis of the disease is uncertain and the pursuit of measurable biomarkers for objective identification of affected individuals is a continuing endeavour in FMS research. Our objective was to perform an explorative metabolomics study (1) to elucidate the global urinary metabolite profile of patients suffering from FMS, and (2) to explore the potential of this metabolite information to augment existing medical practice in diagnosing the disease.

Methods

We selected patients with a medical history of persistent FMS ($n = 18$), who described their recent state of the disease through the Fibromyalgia Impact Questionnaire (FIQR) and an in-house clinical questionnaire (IHCQ). Three control groups were used: first-generation family members of the patients ($n = 11$), age-related individuals without any indications of FMS or related conditions ($n = 10$), and healthy young (18–22 years) individuals ($n = 20$). All subjects were female and the biofluid under investigation was urine. Correlation analysis of the FIQR showed the FMS patients represented a well-defined disease group for this metabolomics study. Spectral analyses of urine were conducted using a 500 MHz ^1H nuclear magnetic resonance (NMR) spectrometer; data processing and analyses were performed using Matlab, R, SPSS and SAS software.

Results and discussion

Unsupervised and supervised multivariate analyses distinguished all three control groups and the FMS patients, and significant increases in metabolites related to the gut microbiome (hippuric, succinic and lactic acids) were observed. We have developed an algorithm for the diagnosis of FMS consisting of three metabolites — succinic acid, taurine and creatine — that have a good level of diagnostic accuracy (Receiver Operating Characteristic (ROC) analysis — area under the curve 90%) and on the pain and fatigue symptoms for the selected FMS patient group.

Conclusion

Our data and comparative analyses indicated an altered metabolic profile of patients with FMS, analytically detectable within their urine. Validation studies may substantiate urinary metabolites to supplement information from medical assessment, tender-point measurements and FIQR questionnaires for an improved objective diagnosis of FMS.

Keywords

Fibromyalgia syndrome, Proton nuclear magnetic resonance (^1H -NMR) spectroscopy, Metabolomics, Metabolite markers, Pain

Background

Fibromyalgia syndrome (FMS) is a common chronic pain syndrome characterized by widespread musculo-skeletal pain and associated with multiple other symptoms such as cognitive impairment, disrupted sleep and chronic fatigue. The American College of Rheumatology (ACR) first published criteria for FMS in 1990 [1] which emphasized chronic widespread musculo-skeletal pain (including pain in the axial skeleton) in the presence of pain on at least 11 of 18 specified tender point sites with digital palpation of 4 kg/cm².

The 2010 ACR updated criteria for FMS [2] are applied in a 2-part, self-administered questionnaire and do not require a tender point assessment. The first part assesses the presence of pain at 19 sites on a body diagram (widespread pain index) and part 2 measures the symptom severity score (0–3) of 3 core symptoms (insomnia, fatigue and cognitive impairment) and an average score (0–3) for additional somatic symptoms. FMS is the most common cause of widespread or generalized musculo-skeletal pain and affects 2–8% of the adult population with the highest prevalence in women between 30 and 55 years [3, 4].

FMS is currently viewed as a central sensitivity syndrome associated with abnormal pain processing. It is regarded as a “pain amplification syndrome” associated with increased sensitivity of the nervous system and decreased anti-nociception which results in the clinical phenomena of hyperalgesia and allodynia. Dysfunction in central mono-aminergic neurotransmission which involves serotonin, norepinephrine, nerve growth factor, substance P and others have been implicated in the pathophysiology of FMS. [5, 6, 7, 8] FMS patients often have associated comorbidities such as irritable bowel syndrome, interstitial cystitis and mood disorders [9, 10].

In the absence of an objective biomarker, the diagnosis of FMS is based on a comprehensive clinical assessment. Before 2010, the diagnosis was principally based on the 1990 ACR criteria of widespread pain (including in the axial skeleton) > 3 months and at least 11 painful “tender points” with digital palpation. Although the 2010 ACR criteria do not include a “tender point” count, a musculo-skeletal clinical examination remains mandatory, to exclude other causes of widespread pain and also to identify peripheral pain generators e.g. myofascial trigger points. Selective use of laboratory testing is used to exclude other causes of widespread pain such as polymyalgia rheumatica and hypothyroidism.

The pursuit of specific and measurable biomarkers that may assist in objectively identifying susceptible individuals, confirming disease diagnosis and facilitating treatment, is a continuing endeavour in FMS research. The development of high-throughput metabolic profiling and the study of the metabolome have proven to be particularly applicable in neurological research where small molecules are key in neurochemical metabolism and in performing a role as neurotransmitters, signalling modulators and osmolytes. It is now generally anticipated that metabolomics profiling methods, linked to systems biology approaches, will emerge with well-defined metabolic phenotypes, enhancing the understanding of brain metabolism in health and disease. Recently, a few metabolomics studies have been reported on fibromyalgia, potentially disclosing novel insights into metabolic perturbations in the brain that go beyond brain metabolic homeostasis beyond alterations of neurotransmission variations associated with neurological disorders [11].

In a pilot study, presented only as a poster at an Annual Meeting of the Rheumatologic Society of the UK [12], Richards and co-workers (2001) reported that muscle metabolites detected in the urine of fibromyalgia patients may suggest a prevailing muscle damage. Although not by definition a metabolomics study, their targeted metabolite analysis of urine by nuclear magnetic resonance (NMR) spectroscopy revealed significant levels of creatine in FMS patients and elevated (t-test $p < 0.05$)

urinary excretion of choline, taurine, citrate and trimethylamine N-oxide (TMAO) relative to matched controls.

The first metabolomics study on FMS, reported in 2013 [13], used 50 µl blood samples collected on blood spot cards (Whatman 903 Protein Saver Snap Apart Card, GE Healthcare, Westborough, MA, USA) from patients diagnosed with FMS ($n = 14$), osteoarthritis (OA; $n = 15$) and rheumatoid arthritis (RA; $n = 12$). Samples were dried and then transported to the laboratory for mid-infrared micro-spectroscopy (IRMS) and other analyses. The RA and OA groups appeared to be metabolically similar, but different from the metabolite profile of FMS. The IRMS approach did not conclusively identify the metabolites responsible for the diagnostic spectral differentiation, although changes in tryptophan catabolism seemed to be involved.

Another metabolomics approach to FMS involved liquid chromatography/quadrupole–time-of-flight/mass spectrometry (LC/Q-TOF/MS) with multivariate statistical analysis aimed at discriminating FMS patients ($n = 22$) and controls ($n = 21$) from blood plasma analysis [14]. Lysophosphocholine (lysoPCs), phosphocholine and ceramide lipids dominated the metabolite profile. The metabolites that discriminated the most between FMS patients and controls were identified as 1-tetradecanoyl-sn-glycero-3-phosphocholine [PC(14:0/0:0)] and 1-hexadecanoyl-sn-glycero-3-phosphocholine [PC(16:0/0:0)] — suggesting that lysoPCs may be potential biomarkers for FMS.

In addition to these metabolomics findings, a recent review on biomarkers of FMS included contributions from genetic and proteomic studies [15]. Although genetic factors have been shown to influence predisposition to FMS, no specific genes have been confirmed as being involved in this disease [16]. The review also listed several proteins of the immune response, cytoskeleton remodelling and the inflammatory process in FMS. Their role in FMS, however, is still controversial.

Thus the availability of biomarkers for unequivocal and objective diagnosis of FMS remains elusive in clinical practice. Yet, metabolites identified as being involved in the aetiology and pathogenesis of FMS could meanwhile contribute to insights into various presentations of FMS and provide ancillary diagnostic testing criteria to complement general diagnostic procedures. We thus present here the outcomes of a ^1H NMR metabolomics study on FMS. All experimental subjects were females and provided urine samples for the study. The investigation was designed as an untargeted approach and revealed metabolite information with predictive potential to discriminate between FMS patients and healthy young controls. The outcomes thus underscore the versatility of metabolomics to provide insights into disease pathophysiology, furthering potential novel approaches to supplement existing protocols proposed for the practising clinician to assess FMS and monitor its treatment [17].

Methods

Experimental subjects, physical characteristics, symptoms and clinical profiles

All the patients that were included in this study were previously diagnosed with FMS by the same specialist pain clinician from his chronic pain practice in Pretoria. This practice manages the full spectrum of chronic pain disorders, with a special interest in FMS and related pain disorders. The diagnosis was based on a comprehensive clinical assessment using the 1990 criteria. All patients in the study were confirmed with FMS before 2010 and all were on a comprehensive evidence-based management programme according to international guidelines. They were only included if they continued to complain of widespread musculo-skeletal pain (including in the axial skeleton) in the presence of >11 painful tender points with musculo-skeletal assessment.

Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form; ethical approval for the study was obtained as specified under Declarations. All participants in the study were female and the sample material investigated was urine. The experimental subjects consisted of one FMS patient group (Group 1) and three control groups (Groups 2 to 4). Clinical description and serum and urine sample collection on all experimental groups commenced from 2009 to 2011. Case definition and selection for the eventual study was done by clinical and scientific group of co-workers in 2010. Following scrutinizing of the records of patients with a medical history of FMS, a group of 18 FMS patients eventually selected based on the above selection criteria as well as after excluding outliers based on statistical analysis [see S5 in *Supplementary Information (SI) to Article 1*].

The socio-demographic, tender point and myofascial pain experience, awareness of gastro-intestinal symptoms, pain-specific medication and levels of emotional experience associated with FMS for the 18 patients was obtained through the FIQR [17] and the IHCQ. The questionnaires are presented in Table S1 and the response to the IHCQ are summarised in Table S2 of the SI. The IHCQ included 18 items that could be extended to a total of 30 sub-items. The questionnaire provided socio-demographic information on the patients (3 items), personal clinical experience of pain and their FMS condition (5 items) and use of medication against pain (2 questions), emotional experience (7 questions) and digestive functioning (1 item). The urine samples were provided by the patients prior to application of pressure to the tender-points (TPs). For a total of 16 of these FMS patients a complete set of data was available for the comparative analysis of the FIQR and metabolomics data, as some information on some patients had to be excluded because the data were incomplete. Some degree of comorbidity of conditions that overlap with FMS (e.g. chronic fatigue syndrome) could not be excluded, as the mean level of energy in the FIQR was rated at 7,0 and according to responses to the IHCQ, 94% of the patients experienced sleep disturbances and did not awoke refreshed. The responses to an experience of mood disturbances (58% answered “Yes”) and anxiety (52% answered “Yes”) for the FMS patients as a group were moderate. Responses on depression was inconsistent (mean FIQR-score = 5.1 with 84% “Yes” answers on the IHCQ) but 88% indicated discomforts with their gastrointestinal functions (Indicated as Irritable Bowel Syndrome (IBS) in the IHCQ). These scores were accepted as indications of the mental and physical profile of the FMS patient group and were not further clinically verified.

Three control groups were used: (1) a group of 11 subjects that were first-degree relatives of the patients, meaning that they were a mother, sister or daughter relation (Group 2: CF); (2) a group of 10 unrelated subjects, selected by physicians and defined as unrelated and age matched controls to the patients (Group 3: CO); (3) a control group of young and healthy individuals, comprising 20 randomly selected students (aged 18–22 years) of North-West University (NWU) (Group 4: CN). All individuals in the control groups showed no indications of FMS or related conditions and was not required to complete the FIQR or IHCQ.

This investigation used availability sampling on the clinically selected FMS patients and controls (CO, CF and CN). However, statistical analyses indicated that the sample sizes provided sufficient power to detect large effects at a univariate level in the FMS and CN comparison.

Sample preparation and ¹H NMR analysis

Spectral analyses were conducted according to the protocol at the NMR facility of the Translational Metabolic Laboratory at Radboud University Medical Centre in Nijmegen, the Netherlands [18, 19]. The urine samples were collected in South Africa, stored at –80 °C and transported to the Netherlands before being thawed at room temperature for analysis. A 1 ml volume of each sample was centrifuged at 3000 rpm for 10 min to remove any sediments or debris. A 70 µl volume of a deuterated solution

containing 20.2 mM of trimethyl-2,2,3,3-tetradeuteriopropionic acid (TSP, sodium salt; Sigma Aldrich) was added to 700 μ l of the supernatant and vortexed. This internal standard (IS) solution served to lock the signal during analysis and to provide a chemical shift reference of $\delta = 0.00$. The sample was then acidified to pH 2.5 ± 0.05 , with 37% concentrated hydrochloric acid (HCl). A 650 μ l aliquot of the acidified sample was then transferred to a 5 mm NMR tube (Wilmad Royal Imperial; Wilmad LabGlass, USA) and analysed on a 500 MHz Bruker Avance spectrometer (Bruker Analytische Messtechnik, Karlsruhe, Germany) (pulse angle 90° , delay time 4 s, number of scans 256, temperature 298 K). Water suppression was achieved by using gated irradiation focused on the water frequency. All samples were automatically shimmed prior to acquisition of data, using topshim from Bruker BioSpin. The resultant raw spectral data, in the form of free induction decay, were Fourier transformed. These transformed spectra were then manually corrected for phase and baseline. All the samples were normalized with reference to the creatinine CH_3 peak at 3.13 ppm. We opted for two methods of spectral analysis. The first method entailed equidistant binning [20] using a bin width of 0.02 ppm applied to the selected region of 0.5–10 ppm, which gave a total of 461 integrated units per NMR spectrum, excluding the water region, for each individual of the four experimental groups. The second method entailed variable-sized binning. The equal-binning procedure masks subtle chemical shift differences, hides potentially significant changes of low-intensity peaks and incurs the risk of splitting peaks or spectral features between bins [21]. To avoid these problems we also used variable bin sizes in areas of peaks above the noise level, preventing peak division between multiple bins. This approach was specifically applied for the identification and quantification of discernible and important known metabolites, generating data for univariate analysis.

Data and statistical analysis

The original normalized spectral data (presented in Additional file 1 as Table S4 in section S1 – *Supplementary information (SI) to Article 1* or Additional file 2 – Raw data matrix) were pre-processed by performing log transformation and auto-scaling. Outliers were detected through Hotelling's T^2 and PCA scores (using a 90% confidence region) analysis and resulted in the exclusion of 4 outliers from further analysis. Univariate statistical analyses, specifically the Mann–Whitney test p -values (MW) and associated effect sizes (ES), were generated for each feature. Multivariate analyses were performed using cluster analysis (Euclidean distance and Ward linkage) principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA), using a 90% confidence interval (CI). Data processing and analyses were performed using Matlab (MATLAB with Statistics and PLS Toolbox Release 2012b, The MathWorks, Inc., Natick, MA, USA); R (R version 3.2.3 downloaded from <https://www.R-project.org> with the corrplot package downloaded from <https://cran.r-project.org/web/packages/corrplot/>); the SPSS software package (SPSS Inc. (2015). IBM SPSS Statistics Version 22, Release 22.0.0, © IBM Corporation and its licensors - <http://www-01.ibm.com/software/analytics/spss/>) and SAS (SAS Institute Inc. 2016 The SAS System for Windows Release 9.4 TS Level 1 M3, SAS Institute Inc., Cary, NC, USA). A table containing all discriminant information, i.e. the power and VIP values as generated from the PCA and PLS-DA analyses, respectively, as well as the ES and MW p -values, was constructed.

We did not test for a normal distribution of the data, given the small number of cases and used Pearson's r and Spearman's ρ to assess correlations, analysed through SPSS version 12.0 (SPSS, Inc., Chicago, IL). All tests were one-tailed, given the positive fold changes (FC) observed for all metabolites.

Results

Characteristics of the FMS patient group

The age profile of the patients concurs with the general agreement of FMS being uncommon in young subjects (<25–30 years), increasing with age towards the prevalence peak in middle-aged individuals, and then declines [3, 4]. According to the feedback, 88% of our patients had stable relationships with a male partner, 89% had one form or another of day-filling or employment activities, and their emotional experience was not severely affected by their disease. The pain experience and medication used resembled that generally prescribed for FMS. The mean scores and ranges of the 21 FIQR questions obtained for our patient group and those (designated as the reference group) used for the standardization of the questionnaire [17] are compared in Table S3 (see *SI to Article 1*).

To characterize further the relationship between questions or variables making up the FIQR questionnaire, we calculated Kendall's tau correlation coefficients for the FMS patient group (Fig. 1). The correlation coefficients along with their associated significance levels are indicated in Table S3 (see *SI to Article 1*). The highest correlation ($r = 0.817$) was indicated for the relationship between pain and the symptoms for FMS. The function domain contains 9 physical functioning items related to the ability to perform relatively demanding but regular daily muscle tasks. Apart from the low score for 'brushing hair', all remaining items showed high correlation coefficients among each other, ranging from 0.399 to 0.778. These high values collectively substantiate the major signs and symptoms experienced by the FMS patients. The 'overall impact' domain contained 2 items that asked about the number of days individuals felt well (could reach their goals) and the corresponding number they were unable to work because of FMS symptoms. These again showed high correlation coefficients, ranging from 0.421 to 0.686, with the 8 items in the functional domain indicating the underlying negative impact of the FMS symptoms on the daily routine of the FMS patients. The symptoms domain contained 9 items on which patients had to rate work and physiological, psychological and environmental difficulties related to FMS. Lower correlations, ranging from 0.076 to 0.499 (mean = 0.25), were found between the 8 functional items and sleep patterns, memory, anxiety and depression, indicating little overlap within the patient group with other FMS-related conditions. Taking everything into account, we conclude the FMS patients represent a well-defined group for this explorative metabolomics study.

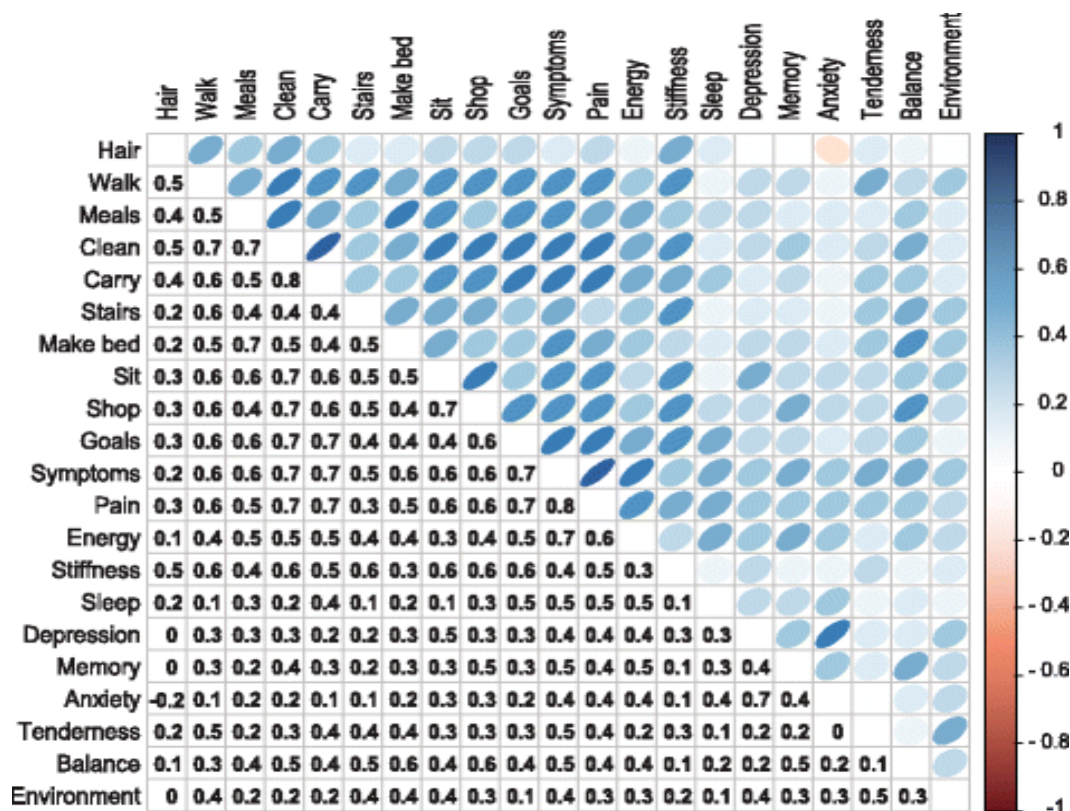


Fig. 1

Correlation matrix for all items on the FIQR questionnaire. Full details on the data analysis are included in the SI

Data generation and case selection

Representative scaled NMR spectra from an FMS patient and from the young control group (CN) is shown in Fig. 2 to illustrate some of the discernible qualitative NMR differences observed in these selected examples. Close inspection of the spectra indicates that there were no immediately discernible qualitative differences between the two representative examples, suggesting that FMS is not associated with distinctive metabolic aberrations, as otherwise observed in monogenetic disorders such as inborn errors of metabolism. Using the equal-bins spectral data, case reduction was first applied to all four experimental groups (see Figure S2 in *SI to Article 1*). Four outliers were identified using a 95% confidence region in a Hotelling's T^2 test in conjunction with the respective PCA score plots with 90% confidence regions. Cases that were identified as outliers by either method were removed. The outliers were: group 1 (FMS patients) – one outlier; group 2 (CF; family controls) – two outliers; group 3 (CO; matched controls) – no outliers; group 4 (CN; young controls) – one outlier.

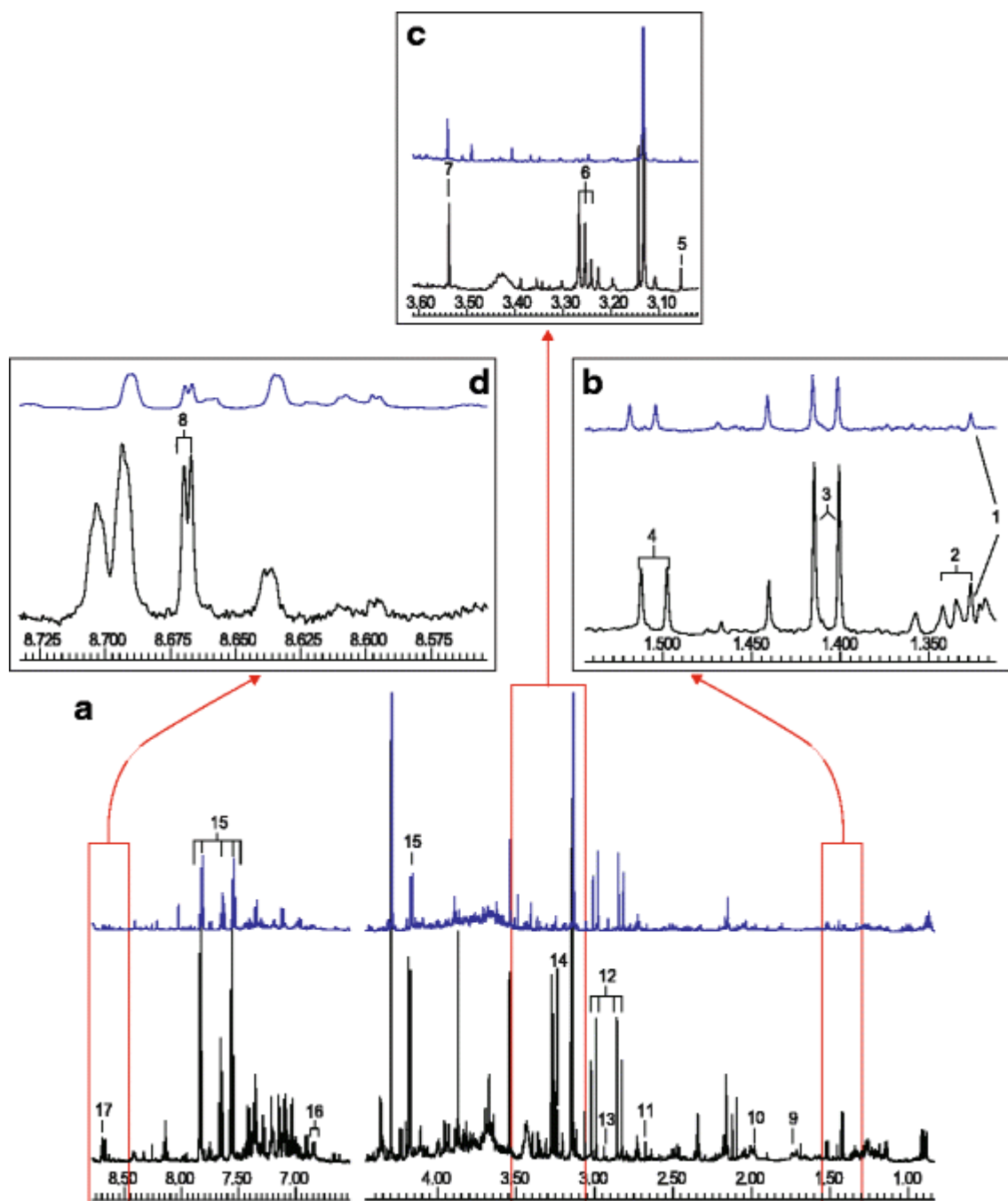


Fig. 2

Representative spectra from one FMS patient (**b**, *black*) and one young control subject (**a**, *blue*), both scaled according to the creatinine CH₃ peak at 3.13 ppm. Expanded regions (**c-e**), framed in *red* in the spectra, are the regions where variables important in projection (VIP) through the supervised PLS-DA are located. The labelled metabolites with their chemical shift (in ppm) and multiplicity, respectively, indicated in brackets are given numerically as follows: 1, 3-hydroxyisovaleric acid (1.33 s); 2, threonine (1.33 d); 3, lactic acid (1.41 d); 4, alanine (1.50 d); 5, creatine (3.05 s); 6, taurine (3.25 t, 3.42 s – *broad line*); 7, trimethylamineN-oxide (TMAO) (3.54 s); 8, histidine (8.68 d); 9, 2-hydroxyisobutyric acid (1.44 s); 10, N-acetyl-X (2.03 s); 11, succinic acid (2.67 s); 12, citric acid (2.91 AB); 13, N,N-dimethylglycine (2.93 s); 14, carnitine (3.22 s); 15, hippuric acid (4.18 d, 7.55 t, 7.64 t, 7.83 d); 16, tyrosine (6.89 d); 17, histamine (8.70 d); 18, creatinine (3.13 s, 4.29 s)

Group characteristics

Supposed changes in metabolite profiles from the FMS patients and the three control groups (excluding outliers) were established through three multivariate approaches: unsupervised Euclidian and Ward hierarchical cluster analyses presented as dendrograms, unsupervised PCA, and supervised PLS-DA models, applied to the original 461 ^1H NMR profiled bins for the four experimental groups.

Figure 3 shows the group separations based on the unsupervised cluster analysis, indicating the perceived closeness of spectral data encapsulated in the NMR bins. The main clusters formed between the CF family members group (Fig. 3a) and the CO age-matched group (Fig. 3b) relative to the FMS patients are heterogeneous in terms of case distribution. In contrast, two well-defined clusters were formed between the FMS patients and CN young controls (Fig. 3c), suggesting distinct differences in the spectral fingerprints between these two groups.

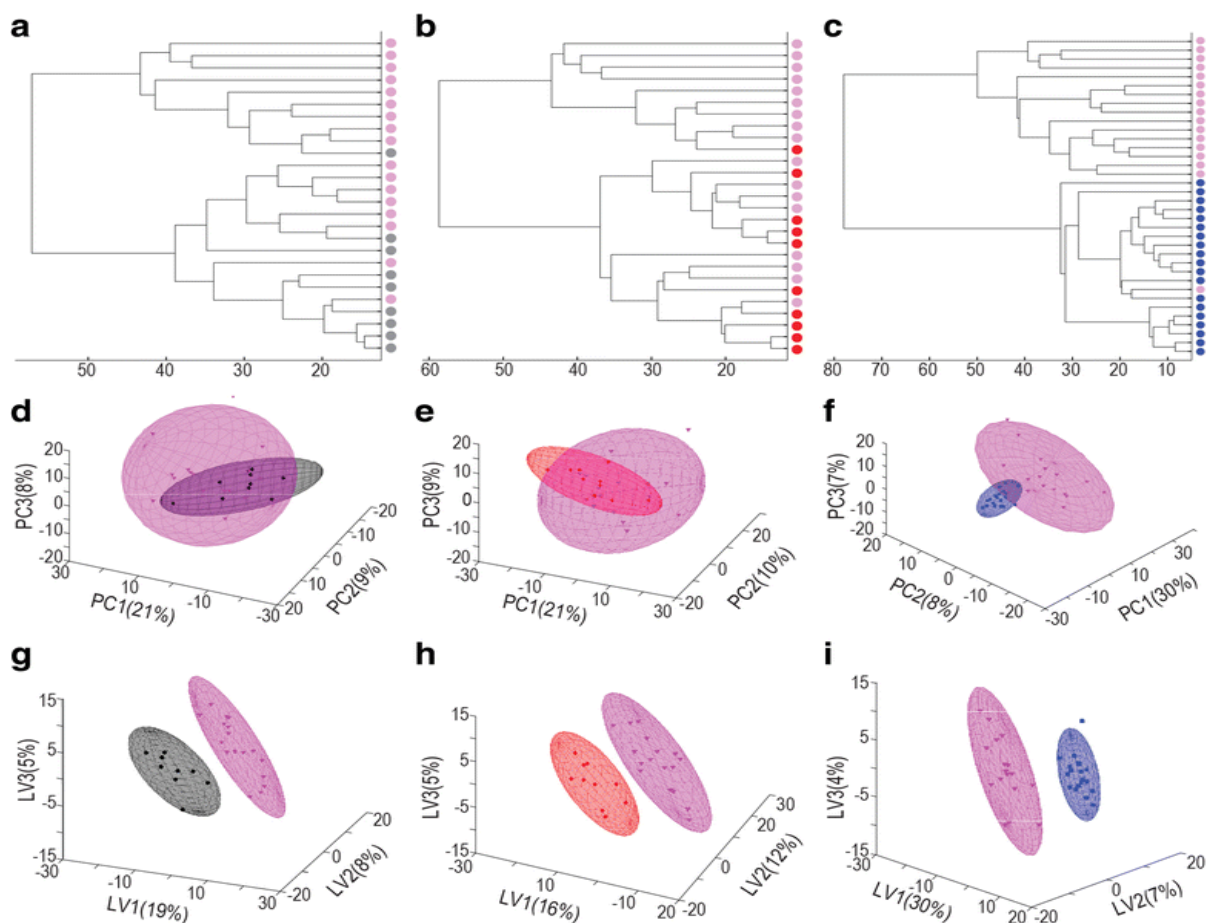


Fig. 3

Group separation between experimental groups through cluster and multivariate analysis based on equidistant binning data. (a–c): Dendrograms from cluster analysis are shown for the CF (a), CO (b) and CN (c) groups relative to FMS patients. Cases from the FMS patients are shown as *pink dots*, CF as *black*, CO as *red* and CN as *blue*. (d–f): PCA indicating the group separation between the FMS patients and CF (d), CO (e), and CN (f) groups respectively, with areas using the same colour code as the *dots* in the dendrograms. (g–i): PLS-DA indicating the separation between the FMS patients and CF (g), CO (h), and CN (i) groups respectively, with areas using the same colour code as in the PCA

Next, group separations based on unsupervised PCA and supervised PLS-DA were performed. The data were log transformed and auto-scaled. The PCA between the CF family members (Fig. 3d), CO matched controls (Fig. 3e) and FMS patients complemented results from the cluster analyses. A complete separation was obtained between all three control groups and the FMS patients (Fig. 3f–i) through supervised PLS-DA. Evaluation of the PLS-DA model shown in Fig. 3i (FMS vs CN) was performed by calculating the goodness-of-fit (R^2) and predictive ability (Q^2) parameters. These metrics confirmed the complete separation between the FMS and CN young control groups, with good model fit ($R^2 = 0.96$), however this model may not generalize well ($Q^2 = 0.29$).

From the equal binning analysis it is evident that there are bins or combinations of bins that can discriminate between our patient and control groups. However, since it is not clinically practical to measure bins, we did not investigate this data further. Instead, the metabolites potentially responsible for the separation of the FMS patients and the CN young controls were subsequently identified by

analyzing variable bins from the NMR spectra and converting these measures to concentration values of the identified metabolites.

Metabolite profile of the FMS patient group

Twenty-one metabolites could be identified and quantified from the NMR spectra. From this list we selected twenty endogenous metabolites (listed in Table 1), and also included 2-hydroxyisobutyric acid of exogenous origin [22], with high VIP, ES and ES values, despite being present in low concentrations. The endogenous metabolites include seven amino acids (tyrosine, leucine, valine, histidine, alanine, threonine and lysine), seven metabolites directly or indirectly associated with energy metabolism (lactic acid, succinic acid, citric acid, 3-hydroxyisovaleric acid, creatine, carnitine and formic acid), three osmolytes (taurine, TMAO and dimethylglycine), a major mammalian detoxification product (hippuric acid), histamine and an N-acetyl-derivative. The N-acetyl-derivative showed a singlet at 2.03 ppm, possibly indicative of an N-acetyl group. One-dimensional spectral data suggested that aspartic acid (multiplet at 4.70 ppm) could be the moiety linked to the N-acetyl group, which, however, could not be substantiated as N-acetyl-aspartic acid by two-dimensional NMR spectral analysis (Additional file 1: Figure S4). We thus designated the variable as an N-acetyl derivative (N-acetyl-X).

Table 1

Univariate, multivariate and descriptive statistics for the 20 bins, comparing FMS and CN

Variable	CS and M[Ps]	VIP 3 LV	Mann-Whitney		Fold Change	Mean		StDev	
			p-value	Effect size		CN	FMS	CN	FMS
2-Hydroxyisobutyric acid	1.44 s [CH3]	6.26	0.0001	0.72	-1.56	0.01	0.02	0.0	0.00
Succinic acid	2.66 s [(CH2)2]	0.25	0.0001	0.61	-1.63	0.02	0.03	0.01	0.01
Taurine	3.25 t [CH2]	5.21	0.0007	0.52	-2.29	0.20	0.45	0.05	0.57
Tyrosine	6.89 dd [(CH)2]	0.37	0.0029	0.45	-1.70	0.03	0.06	0.03	0.06
Lactic acid	1.41 d [CH3]	2.83	0.0044	0.42	-1.81	0.06	0.11	0.03	0.07
Creatine	3.05 s [CH3]	4.40	0.0053	0.41	-2.08	0.05	0.09	0.04	0.08
TMAO	3.54 s [(CH3)3]	2.21	0.0062	0.41	-2.10	0.06	0.14	0.06	0.23

Variable	CS and M[Ps]	VIP 3 LV	Mann-Whitney		Fold Change	Mean		StDev	
			p-value	Effect size		CN	FMS	CN	FMS
Dimethylglycine	2.93 s [(CH3)2]	0.00	0.0127	0.36	-1.29	0.01	0.01	0.00	0.00
Leucine	0.95 t [(CH3)2]	0.00	0.0136	0.36	-1.11	0.01	0.02	0.00	0.00
Formic acid	8.25 s [CH]	0.01	0.0361	0.29	-1.15	0.03	0.03	0.01	0.01
Valine	1.04 d [CH3]	0.00	0.0436	0.28	-1.24	0.01	0.01	0.00	0.00
Histamine	8.70 d [CH]	0.08	0.0436	0.28	-1.29	0.06	0.07	0.06	0.05
N-acetyl-X	2.03 s [CH3]	0.02	0.0464	0.27	-1.28	0.01	0.02	0.00	0.01
Lysine	1.73 m [CH2]	0.61	0.0739	0.23	-1.03	0.11	0.12	0.03	0.06
Hippuric acid	4.18 d [CH2]	1.61	0.0966	0.21	-1.55	0.22	0.35	0.10	0.24
Citric acid	2.89 AB [(CH)4]	1.36	0.1070	0.20	-1.21	0.39	0.47	0.16	0.17
Alanine	1.51 d [CH3]	0.13	0.1785	0.15	-1.16	0.06	0.07	0.02	0.03
Histidine	8.68 d [CH]	0.85	0.1942	0.14	1.19	0.07	0.06	0.04	0.04
Carnitine	3.22 s [(CH3)3]	0.02	0.2107	0.13	-1.24	0.02	0.02	0.01	0.01
Threonine	1.33 d [CH3]	0.04	0.2648	0.10	-1.28	0.03	0.04	0.01	0.04
3-Hydroxyisovaleric acid	1.33 s [(CH3)2]	0.00	0.4942	0.00	-1.02	0.00	0.00	0.00	0.00

We subsequently performed multivariate (log and centred concentration values) and univariate (unscaled concentration values) analyses on the reduced bins (endogenous metabolites, converted to

their respective metabolite concentrations) to refine our identification of the key variables that discriminate between the FMS patients and the controls. All cases were retained for this analysis as none were identified as outliers based on the concentrations. Multivariate PCA (Fig. 4a) and PLS-DA (Fig. 4b) both indicated that the 20 metabolites contained information that differentiates, but did not separate, the FMS patients from the young controls. Model performance was evaluated using the goodness of fit (R^2) and goodness of prediction (Q^2) parameters, which were $R^2(X) = 0.52$ and $Q^2(Y) = 0.05$, respectively, indicating a reasonable (R^2) but not necessarily reproducible (Q^2) fit between the variation in the data and the components (quantified metabolites) comprising the model for the present FMS group. It thus appears that some metabolites below the sensitivity range for quantification from the NMR spectra might be required for reproducibility (Q^2) and for further differentiation between the FMS patients and young controls.

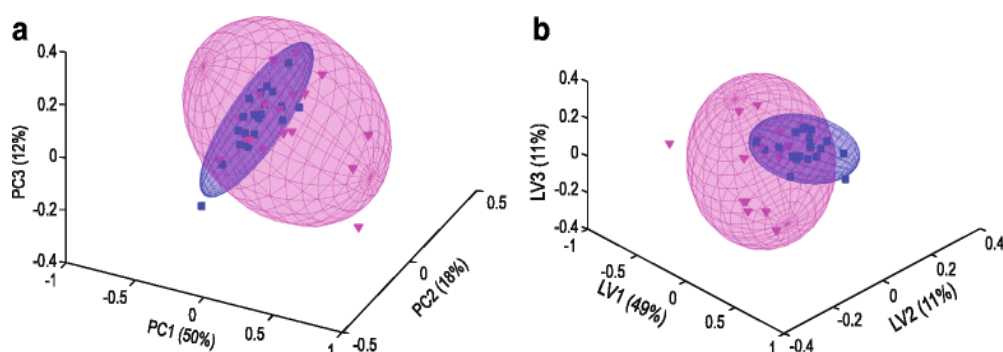


Fig. 4

PCA (a) and PLS-DA (b) for the FMS patients relative to the young controls, based on the quantified 20 metabolites

Univariate analyses using Mann–Whitney p -values and fold changes, as summarized in a volcano plot (Fig. 5a), point to important substances that cause group differentiation. The outcome of this analysis of the data set of 20 variables is presented in Fig. 5a, indicating which large-magnitude changes (fold change: $|\log_2 FC| > 1.5$) are also statistically significant (Mann–Whitney test: $p < 0.05$). Six informative metabolites complied with these measures, with their respective VIP values shown in brackets: succinic acid (0.246), taurine (5.214), tyrosine (0.365), lactic acid (2.832), creatine (4.402) and trimethylamine N-oxide (TMAO; 2.209).

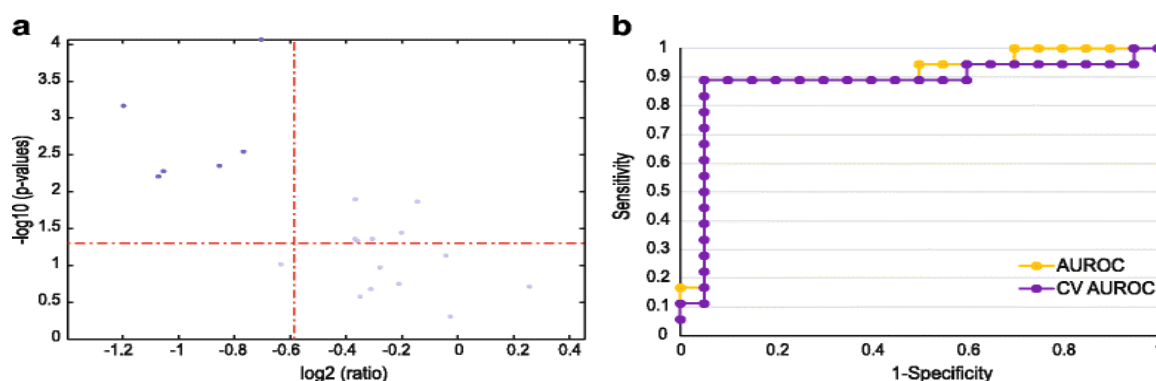


Fig. 5

Statistical assessments of three metabolites indicative of FMS: (a) Volcano plot mapped by the scaled fold change and p -values for the 20 metabolites observed for FMS patients and young controls. Metabolites with high FC and significant p -values among patients are indicated by *black squares*. (b)

ROC analyses for discriminating FMS patients from controls (AUROC) as well as leave-one-out cross validated ROC analysis (CV AUROC). The discriminator consisted of the three informative metabolites (succinic acid, taurine and creatine) identified by multivariate, univariate and metabolic pathway analyses

Important endogenous metabolites in the FMS patient group

A summary of the results for the univariate and multivariate statistical analyses is presented in Table 1, nine of which could be related to physiological functions that could be related to FMS.

The neurological functions of succinic acid, tyrosine and lactic acid are well known: the aerobic mitochondrial energy regeneration function, a precursor for neurotransmitters and a key metabolite in the astrocyte-neuron lactate shuttle [23], respectively. Taurine is an abundant β -amino acid in the mammalian brain [24] and has been shown to be a neurotransmitter in the substantia nigra (SN). It has been suggested from micro-dialysis experiments on Sprague-Dawley rats that osmoregulation of the nonsynaptic taurine pool of the SN could influence the nigral cell vulnerability, seen in the pathogenesis of Parkinson's disease [25]. Likewise, nutritional studies [26] suggest that TMAO may be involved in diet-induced variations in the balance of several osmolytes, including betaine, choline, creatinine and creatine, whereas creatine has also been proposed as being involved in pain experienced in FMS [25]. Thus, we subsequently evaluated the potential diagnostic value of these six metabolites on FMS by means of a logistic regression analysis, as indicated below.

Important exogenous metabolites in the FMS patient group

The pain intensity of patients with FMS has been reported to correlate with the degree of small intestinal bacterial overgrowth [9, 10]. This clinical observation may have pathogenetic relevance for FMS, because bacterial overgrowth leads to the exposure of immune cells to luminal antigens and consequent immune modulation. An untargeted NMR metabolomics study of celiac disease, a multifactorial immune-mediated enteropathy [27], suggested alterations of energy metabolism - a clinical characteristic in FMS - while urine data pointed to alterations of gut microbiota. At least three metabolites observed in the urine samples of our FMS patient group suggest perturbations in their gut metabolome (Fig. 6): (1) Hippuric acid is a normal and major component of urine and appear in humans as an increased excretory product from unnatural (detoxification) and natural (gastroesophageal reflux disease in children) sources. (2) 2-Hydroxyisobutyric acid, the most discriminatory variable between our FMS group and controls (VIP = 6.2 – Table 1), is an apparent catabolic from gut microbionics and was shown to be statistically linked to *Faecalibacterium prausnitzii* [28] an important commensal bacterium of the human gut flora proposed to be an indicator of the dynamic basis of host-microbiome symbiosis. (3) Lactic acid is a key intermediate in many biochemical processes and is a measure of critical illness in patients with poor prognosis. It may be of endogenous (L-lactate) or exogenous (D-lactate) origin and we recently proposed that the determination of its enantiomers in infectious conditions may provide a basis for substantiating the clinical significance of disease markers [29]. The presence of these exogenous markers of gut origin provides further indications of the connectivity between disturbances in the gut microbial populations and the metabolic consequences of the altered microbial-mammalian metabolic balance influencing host disease, which will be discussed below in the context of FMS.

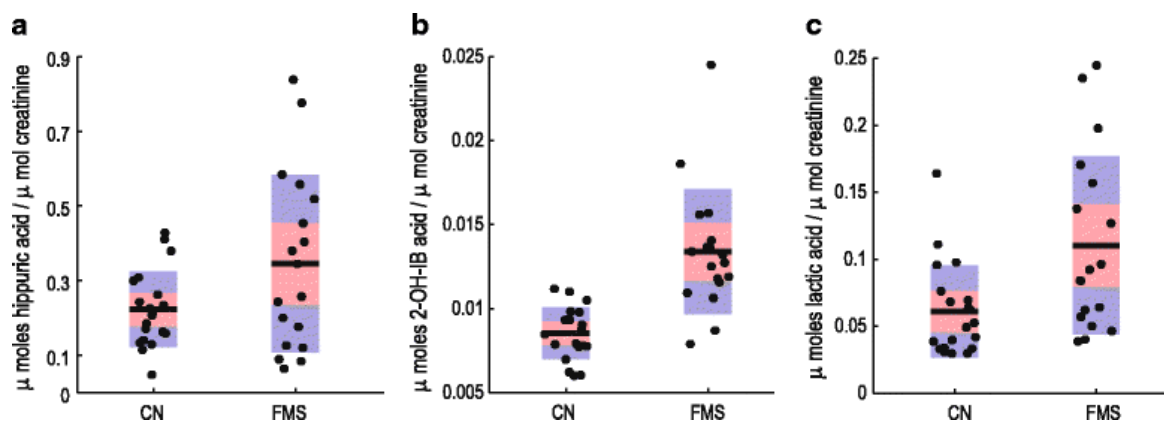


Fig. 6

Graphs showing important urinary metabolites related to the gut microbiome. Indicated in the figure are: FMS patients relative to young controls for hippuric (a), 2-hydroxyisobutyric (b) and lactic (c) acids. Values for all individual cases are shown as *dots*, while the squared area represents the 95% confidence interval (*orange*) and 1 standard deviation (*blue*) of the mean (*red line*)

A putative biosignature for FMS

A combination of three selection methods (Forward, Backward and Step-wise selection) was used to identify the best metabolite predictors. Instead of using one selection method, a combined approach was chosen since each method has its advantages and disadvantages [30, 31]. Although our aim was to explore a small set of highly discriminatory endogenous metabolites, we also investigated the potential of a combination of these metabolites to function as a biosignature for the FMS patient group. We followed a forced entry approach to evaluate the combination of metabolites. Table 2 lists the methods used as well as the preferred metabolite predictors selected from the six informative metabolites. The last model (Forced entry) entered succinic acid, taurine and creatine, and produced the best model based on -2 Log Likelihood ($-2LL$) from the present data. Table 2 also reports other model performance measures, but $-2LL$ was used to select the best model as it gives an indication of the variation not explained in the data, and gave the lowest $-2LL$ value compared to the other models. The Forced entry model was also well calibrated since the Hosmer Lemeshow (HL) statistic was not significant. The model fit is reported by using the Max Rescaled R-squared value and only the Forced entry model had a satisfactory value of above 0.6.

Table 2

Summary of logistic regression results for the six informative metabolites. The predictors used or selected by the logistic regression model are listed as *Predictors selected*. Other columns report the model fit results (Max Rescaled R-squared), the relative variance explained ($-2LL$), the calibration (HL p -value), and the classification ability (AUC and AUC (LOO CV)) of each model

Selection method	Predictors selected	-2LL	HL p -value	Max rescaled R-squared	AUC	AUC (LOO CV)
Forward	Creatine & succinic acid	36.15	0.0273	0.47	0.8917	0.8583
Backward	Taurine	40.16	0.6336	0.37	0.8056	0.7556

Selection method	Predictors selected	-2LL	HL <i>p</i> -value	Max rescaled R-squared	AUC	AUC (LOO CV)
Stepwise	Succinic acid	40.87	0.5496	0.35	0.8583	0.8306
Forced Entry	Creatine; succinic acid & taurine	29.66	0.0932	0.60	0.8972	0.875

Finally, the classification ability of each model was assessed by using a Receiver Operating Characteristic (ROC) analysis to the data mentioned. The values of the area under the ROC curve (AUC) provide a measure of how well this combination could distinguish between the two groups. A value of AUC = 1 represents a perfect test, while a cursory guide for classifying the accuracy of a diagnostic test is given by: AUC = 0.90–1 (excellent, i.e. high sensitivity and high specificity); 0.80–0.90 (good); 0.70–0.80 (fair); 0.60–0.70 (poor); 0.50–0.60 (fail). To provide some indication of how well the model would potentially generalize, the last column in Table 2 reports the classification ability when one sample is left out repeatedly — in other words, based on a leave-one-out cross-validation strategy (AUC (LOO CV)). Again the Forced entry model performed the best (AUC = 90% [0.8972]; AUC (LOOCV) = 88% [0.8750]).

Correlation between clinical and metabolic indicators

Pearson and Spearman correlation analysis was done to compare the bivariate relationships between responses to the FIQR and the three endogenous variables defining the biosignature of FMS. Specifically, correlations were assessed between the sum of all three FIQR domains as well as the sum of the functional, impact and symptoms domains and SUM-3, SUM-2, creatine, succinic acid and taurine. Finally we inspected the data for symptoms related to metabolism to be included in the bivariate correlation analysis. In this regard it should be noted: (1) The scores of the 21 questions of the FIQR corresponds to an average based on the subjective self-assessment of the FMS patients as used in the behavioural sciences (i.e., it is not empirically based). We therefore used the mean scores of fibromyalgia patients on the symptoms for experience of pain, low energy levels and tenderness to touch only as a directive to include these symptoms in the bivariate correlation analysis [30]. Their mean values did not differed in practice from the data of a reference group of the revised FIQR (see Figure S1 in SI to Article 1). (2) The number of FMS cases is relatively small for assessment of normality in the data distribution. We therefore included the Pearson and Spearman correlations in Table 3, but used only the Spearman's correlations for the interpretation of the bivariate correlation analyses, with guideline values for “small” ($r \geq 0.1$), “medium” ($r \geq 0.3$) and “large” ($r \geq 0.5$) as operational convention for the correlation coefficients [32].

Table 3

Relationship between the clinical information of the FIQR and the components of the FMS biosignature

Bivariate components for the correlation analysis	Pearson correlation		Spearman correlation	
	Coeff. (<i>r</i>)	<i>p</i> -value ^b	Coeff. (<i>r</i>)	<i>p</i> -value
Correlations of the biosignature (SUM-3) ^a with the FIQR domain categories				
SUM-3 vs Sum of 21 questions of the full FIQR	0.35	0.102	0.42	0.059

Bivariate components for the correlation analysis	Pearson correlation		Spearman correlation	
	Coeff. (r)	<i>p</i> -value ^b	Coeff. (r)	<i>p</i> -value
SUM-3 vs Sum of 9 questions of the functional domain	0.31	0.134	0.25	0.134
SUM-3 vs Sum of 2 questions of the impact domain	0.15	0.316	0.22	0.241
SUM-3 vs Sum of 10 questions of the symptoms domain	0.41	0.057	0.57	0.011*
Correlations of two components of the biosignature (SUM-2) with the FIQR domain categories				
SUM-2 vs Sum of 21 questions of the full FIQR	0.56	0.016*	0.53	0.021*
SUM-2 vs Sum of 9 questions of the functional domain	0.52	0.023*	0.41	0.008**
SUM-2 vs Sum of 2 questions of the impact domain	0.5	0.043*	0.51	0.039*
SUM-2 vs Sum of 10 questions of the symptoms domain	0.59	0.009**	0.57	0.011*
Correlations of components of the biosignature with the symptom of pain ^c				
SUM-3 vs pain experience	0.46	0.037*	0.64	0.004**
SUM-2 vs pain experience	0.52	0.02*	0.54	0.016*
Creatine vs pain experience	0.5	0.025*	0.5	0.024*
Succinic acid vs pain experience	0.08	0.384	0.18	0.249
Taurine vs pain experience	0.39	0.069	0.29	0.135
Correlations of components of the biosignature with the symptom of energy ^d				
SUM-3 vs energy loss	0.32	0.115	0.61	0.006**
SUM-2 vs energy loss	0.68	0.002**	0.72	0.001**
Creatine vs energy loss	0.65	0.003**	0.66	0.003**
Succinic acid vs energy loss	0.15	0.295	0.22	0.221
Taurine vs energy loss	0.22	0.204	0.14	0.307

^aBiosignature: SUM-3 = creatine + succinic acid + taurine; SUM-2 = creatine + succinic acid

^bStatistical significance: *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

^cPain: No pain = 0; Unbearable pain = 10

^dEnergy: Lots of energy = 0; No energy = 10

The results shown indicate a medium and borderline significant relationship between the SUM-3 biosignature and the sum of the FIQR, with insignificant correlations for its functional and impact domains. Sum-3 and the symptoms domain showed a large and significant correlation. The relationship between SUM-2 and the sum of the FIQR and its three domains improved significantly. Taken together these results directs to a more meaningful relationship between the metabolites which comprise the biosignature and clinical symptoms related to biochemical perturbations in FMS. This impression is substantiated by the strong and significant relationship between SUM-3 and SUM-2 on the experience of pain ($p = 0.004$ and 0.016 , respectively) and loss of energy ($p = 0.006$ and 0.001 , respectively) in the FMS patients as a group. Notably this relationship is not shared by succinic acid (a metabolite from the Krebs cycle) and taurine (an osmolite), but a good and significant relationship was shown between creatine and the symptoms pain and energy ($p = 0.024$ and 0.003 , respectively). The relationship between the biosignature components to tenderness to touch, the third clinical symptom evaluated, was statistically insignificant (not included in Table 2). All correlation coefficients were positive indicating that patients with high scores on the biosignature will likely also have high FIQR scores.

Discussion

The results of this metabolomics study lead to three main discussion points – whether FMS presents with a unique global metabolic profile which characterizes this disease, whether metabolomics studies contributed to the advancement of an objective clinical diagnosis of FMS in patients so affected and on gut microbial–host metabolic perturbations in FMS.

As the overall health status of individuals is captured in their metabolic state, there exists a prevailing view that metabolomics results embody global biochemical changes in an individual due to a disease and neurological conditions [33], and supported by our results and of two other NMR metabolomics investigations. The first NMR metabolomics study evaluated the diagnostic accuracy of biomarker profiles in three neurological conditions: idiopathic intracranial hypertension, multiple sclerosis, and cerebrovascular disease relative to controls with either no or combined neurological diseases [34]. It appeared that the metabolomics investigation identified differences in metabolite profiles in patients suffering from these three conditions. A related conclusion was drawn from the second NMR metabolomics study of FMS [14]. Although a relatively small number of patients formed the experimental group, the metabolomics approach was successful in identifying distinct metabolic profiles for FMS patients relative to controls, supporting the concept that the Platelet Activating Factor/Platelet Activating Factor Receptor (PAF/PAFr) system plays a role in modulating pain signalling. Our results furthermore indicated the differentiation of the three control groups used (family members, an age-matched group, and young individuals) and the FMS patients (Additional file 1: Figure S3). Statistical assessment of the outcome of a supervised PLS-DA model confirmed the complete separation between the FMS and young control groups. Good model fit values substantiated some unique differences between the global metabolic profiles of the FMS patients and the healthy young controls. The metabolites principally responsible for the differentiation between our FMS patients and controls included taurine and TMAO which were also reported to be significantly increased ($p < 0.05$) in an FMS patient group in a preliminary targeted NMR study [12]. In addition, we observed perturbed succinic acid suggesting altered energy metabolism in FMS. This result is linked to a study [13] where there was relatively elevated: glucose, the glycolytic intermediate phosphoenolpyruvate, pyruvate and nicotinamide adenine dinucleotide (NAD^+) seen in dried blood spots from FMS patients. This observation was previously reported for patients with chronic widespread pain [35].

A common thread in the metabolomics studies on FMS discussed here is the affirmation of the ability of metabolomics to identify distinct metabolic profiles for FMS patients relative to controls. Some

metabolites/biomarkers could therefore contribute to the disease phenotype by having a role in the pathogenesis of FMS. The biomarkers revealed in these metabolomics studies seemed, however, not to be metabolically closely linked, but may be due to the multi-factorial nature of FMS. Noteworthy also are the two main limitations of our own and the other two metabolomics studies: the FMS groups investigated and analytical methods used. Most metabolomics studies are limited by the number of experimental subjects available for investigation, and therefore ultimately call for follow-up validation studies with larger and better-defined experimental groups. Further, given the complexity of the human metabolome and the multi-dimensional nature of biofluids and other biological samples available for metabolomics studies, no single analytical technology can fully disclose and account for the information encapsulated in these samples. Nonetheless, metabolomics retains a promise well beyond the scope of standard clinical chemistry techniques, for affording detailed characterization of metabolic phenotypes and is believed, eventually, to lead to so-called precision medicine in which knowledge of their unique metabolic derangements explains the disease state of individual patients [36]. A third limitation in the present study is the use of the 1990 criteria for FMS (1, 14) as the patient selection was one before publication of the revised criteria in 2011. The use of the revised criteria is now standard practice in our pain clinic.

So, can metabolomics studies contribute to the advancement of objective clinical diagnosis of FMS? The results of the present and the two other metabolomics studies on the disease imply that they can, albeit with qualifications. The analyses of blood spots from FMS patients provided information using IRMS technology that differentiated samples from FMS subjects from those with RA or OA with zero misclassifications (100% accuracy). The accuracy of the metabolomics approach was 75%, but with the advantage of disclosing a prioritized list of metabolites that may underlie the differences identified [9]. The possible role of lysoPCs as biomarkers or as contributors to the FMS phenotype and function in the pathogenesis of this condition suggest they are potential new disease biomarkers and thereby open a new approach for the treatment in FMS [10]. Likewise, the predictive potential of the combination of succinic acid, taurine and creatine proved to be excellent for discriminating between our cases of FMS and controls (AUC = 90%). The combination of creatine and succinic acid also showed a significant correlation with the characteristic symptoms of pain and fatigue in FMS. The inclusion of this predictive information on these three metabolites could in time be considered to form part of the initial evaluation of patients suspected of suffering from the disease, in anticipation of validation of FMS diagnostic markers.

Finally, the involvement of gut microbial–host metabolic perturbations in FMS may prove to contribute significantly in defining the clinical profile in FMS. In health, brain-gut interactions are crucial in the maintaining of homeostasis [37]. It appears that neuroplasticity-related systems and neurotransmitter systems are influenced by the gut–brain axis regulation and perturbed homeostasis may contribute to risk of disease through alterations in gastrointestinal tract, central nervous, autonomic nervous and immune systems [38]. The frequent comorbidity of fibromyalgia with stress related disorders, such as chronic fatigue and irritable bowel syndromes and some CNS related abnormalities, suggests that gut–brain axis regulation may at least be a partial common denominator for these disorders. This view may well be revealed by data from a follow-up targeted metabolomics investigation of high sensitivity, like through mass spectrometric-based technologies.

Conclusions

An untargeted ¹H NMR metabolomics analysis of urine samples obtained from a group of clinically well-defined female FMS patients with no psychiatric co-morbidity could be fully differentiated from a group of young healthy women. The presence of metabolic indicators of perturbations in the gut microbiome (hippuric, 2-hydroxyisobutyric and lactic acids) supports the paradigm that regulation of the gut-brain axis becomes affected in stress related disorders, like FMS. Three metabolite markers

(taurine, creatine and succinic acid) were important for the differentiation between FMS patients and controls and were significant indicators of the pain and fatigue symptoms in FMS. ROC analysis and odds ratios substantiated the good predictive potential of a combination of these three metabolites for FMS in the present patient group. Follow-up metabolomics research on a larger number of urine samples, including those from individuals at high risk of developing the disease, as well as longitudinal studies on FMS patients during treatment, are needed to validate the findings presented here and to potentially detect effects which would require greater statistical power. These markers may in time provide objective supplementary information together with tender-point measurements and FIQR questionnaires used to confirm FMS.

List of abbreviations used

ACR: American College of Rheumatology
AUC: area under the ROC curve
AUROC: area under the ROC curve
CF: group of first-degree relatives of the patients
CN: group of healthy young subjects
CO: group of age-matched subjects but unrelated to the patients
CNS: central nervous system
CV AUROC: cross-validated AUROC
CWP: chronic widespread pain
ES: effect size
FC: fold change
FIQR: Fibromyalgia Impact Questionnaire
FM: fibromyalgia
FMS: fibromyalgia syndrome
IHCQ: In-house clinical questionnaire
IRMS: mid-infrared micro-spectroscopy
LOO CV: leave-one-out cross-validation
LC/Q-TOF/MS: liquid chromatography/quadrupole–time of flight/mass spectrometry
LV: latent variable
lysoPCs: lysophosphocholines
MW: Mann–Whitney test
N-acetyl-X: N-acetyl-derivative
¹H-NMR: proton nuclear magnetic resonance
NTeMBI: Nuclear Technologies in Medicine and Biosciences Initiative
NWU: North-West University
OA: osteoarthritis
OR: odds ratio
PAF/PAFr: Platelet Activating Factor/Platelet Activating Factor Receptor
PC: principal component
PCA: principal components analysis
PLS-DA: partial least squares discriminant analysis
Q²: predictive ability parameter
R²: goodness-of-fit parameter
RA: rheumatoid arthritis
ROC: Receiver Operating Characteristic
SN: substantia nigra
SUM-3: numerical sum of the concentrations of taurine, creatine and TMAO
TMAO: trimethylamine-N-oxide
TPs: tender-points
VIP: variable important in projection

Declarations

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Authors' contributions

This investigation required a multidisciplinary approach and the inputs of all authors were essential to produce the concept and final manuscript. CR and HM defined the aim of the study, developed the experimental design and obtained ethical approval for the study; they are, respectively, the promoter and co-promoter for BM. HM performed the selection and clinical description of the FMS patients and CF and CO control groups and provided the urine samples for these individuals. CR provided it for the CN control group and was responsible for coordination and integration of inputs from the authors. BM conducted all experimental analyses and compiled the clinical information provided by HM. SM and UE gave guidance and assessment on ¹H NMR data generation and spectral analyses. RW was responsible for critical evaluation of the analytical aspects of the clinical chemistry data and for their interpretation. MvR performed all the statistical analyses. All authors have given their approval of the version of the manuscript as submitted and agreed to the accountability requirements.

Competing interests

There are no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form. Ethical approval for this study was obtained via the consortium under the Nuclear Technologies in Medicine and Biosciences Initiative (NTeMBI) (ethical approval by Pharma Ethics Pty, Ltd., reference number 11064365). Pharma Ethics confirmed the following: "The study has been accepted as complying to the Ethics Standards for Clinical Research with a new drug in participants based on FDA, ICH GCP and the Declaration of Helsinki guidelines. The Ethics Committee (IRB) granting this approval is in compliance with the Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (2006), ICH Harmonised Tripartite Guidelines E6: Note: for the Guidance in Good Clinical Practice (CPMP/ICH/135/95) and FDA Code of Federal Regulation Part 50, 56 and 312."

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As previously stated, multiple extraction protocols and annotation methods were used by Bouatra and colleagues for metabolite isolation and identification. By this means they were able to identify many metabolites in their study especially with reference to the NMR component of the investigation. Two-dimensional (2D) techniques, COrrrelation SpectroscopY (COSY) and J-resolved spectroscopy (J-RES) were also employed in their NMR experiment. In our study, as presented in the publication above, only one-dimensional ^1H -NMR experiment was used for metabolite isolation and identification. 2D NMR was briefly used for the purposes of metabolite confirmation, being the presence of *N*-acetyl aspartic acid in the urine of FMS cases (data shown in the supplementary material of Malatji *et al.*, 2017). Likewise, only one SOP was used as that is the method employed by the Nijmegen lab, and as such is limited in what can be detected.

4.4 Conclusion

NMR is a quantitative analytical method and is thus ideally suited for non-targeted profiling studies, as presented in the publication referred to above. It allows us a snapshot of the total complement of metabolites in a particular metabolome at one particular time. MS and NMR, the primary analytical techniques used in metabolomics investigations, are complementary to each other, based on their advantages and limitations. From our untargeted NMR study we were able to discriminate FMS from healthy young controls based on their urine metabolome. Furthermore, we also identified compounds that show a link to a perturbation in the gut metabolome of FMS patients that warrants further investigation. A further discussion on how these findings contribute to the phenotype that is FMS will be discussed in Chapter 6. For this reason, further targeted studies were conducted using an MS platform, namely GC–MS, to confirm this finding. Chapter 5 deals with this GC–MS study, the findings of which are presented in the form of an article.

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CHAPTER 5: GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) OF FIBROMYALGIA SYNDROME

5.1 Brief GC-MS theory

Mass spectrometry (MS) is an analytical technique commonly employed in metabolomics studies to analyse biofluids. MS is a detection technique, in which molecules are ionized and the resultant ions are categorized according to their mass-to-charge (m/z) ratio, where m is the mass of the ion in Daltons and z is the fundamental charge of the ion. The resulting output is a mass spectrum (Emwas 2015) (figure 5.1). This is done by bombarding the molecules with a stream of electrons, breaking them into small, or large, fragments. A quadrupole (made of four magnets) allows fragments of a certain m/z to pass through a slit, few at a time, to the detector. The quadrupole cycles through each of the m/z ratios, numerous times per second, until the whole range of m/z ratios are covered. A single range is called a scan. Figure 5.1 shows a typical mass spectrum which is recorded for each scan. The m/z ratio is plotted on the x-axis and the signal intensity (abundance) is plotted on the y-axis for each of the detected fragments.

There are a number of separation techniques that can be coupled to MS, for example, gas chromatography (GC) and liquid chromatography (LC). For the purposes of this thesis, only GC-MS will be referred to.

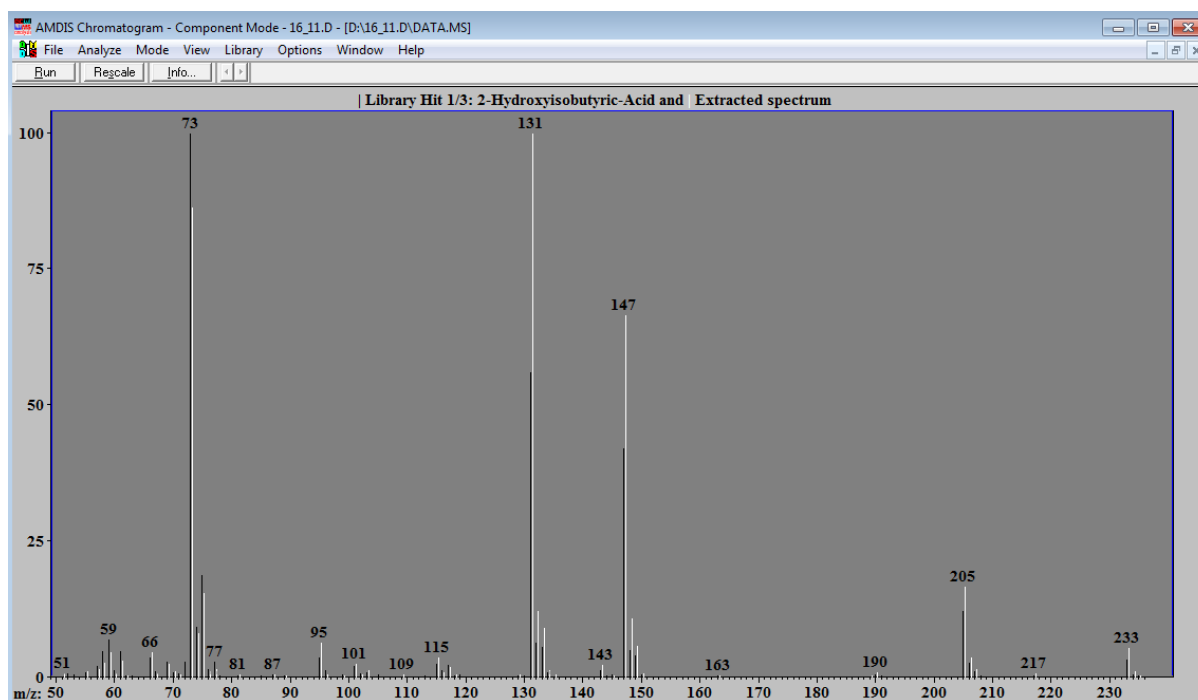


Figure 5.1: Example of a typical mass spectrum created by mass spectrometry representing one cycle of a range of m/z ratios of fragmented particles present in a sample. The plotted graph is of abundance (signal intensity; y-axis) versus the m/z ratio (x-axis). This particular mass spectrum shows the fragmentation pattern of the metabolite, 2-hydroxyisobutyric acid.

GC-MS is the most commonly used hyphenated MS platform for metabolite analyses (Wang *et al.*, 2011). Mostly used for non-targeted analyses, GC-MS is best used for the analyses of metabolites that are hydrophilic in nature (Zhang *et al.*, 2012; Lin *et al.*, 2006). GC is an analytical technique that separates complex mixtures into individual compounds. In all chromatography methods, there is a mobile phase and a stationary phase. In the case of GC, the mobile phase is an inert gas (e.g. helium), and the stationary phase is a column lined with a chemical that can selectively attract compounds in a sample. The sample under investigation is injected into the mobile phase and carried along the stationary phase, up until it elutes into the MS for detection. While being carried by the mobile phase, the compounds interact with the stationary phase at different speeds and the fastest to interact elute first while the slowest elute last. Retention time (RT) is the amount of time a compound takes, from time of injection to the time it elutes from the column, to enter the MS for detection. As the compounds elute from the column, they generate a signal that is captured on a graph of RT versus abundance (signal intensity) called a chromatogram (figure 5.2). The peaks represent the different compounds found in the sample. Unlike a NMR spectrum, each peak represents a compound. The RT is used against a reference

library to identify the compound. There are different parameters that have to be controlled in order to ensure the compounds always elute at, precisely, the same time on the graph. These parameters include: characteristics of the mobile and stationary phases, oven temperature ramp and type of GC column.

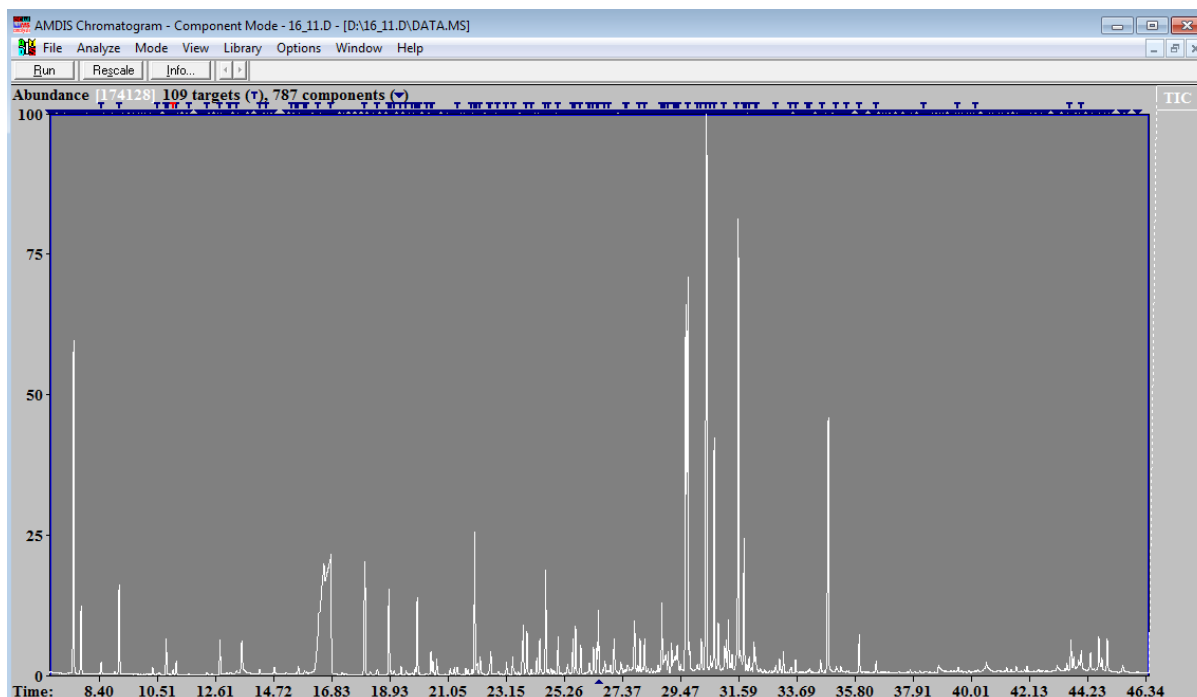


Figure 5.2: Typical ion chromatogram of retention time (RT; x-axis) versus abundance (y-axis) of a urine sample taken from one of the FMS patients, showing the compounds separated by gas chromatography. Each peak represents a compound as it elutes from the GC column. RT is the amount of time, from injection, each compound takes to elute from the column. Abundance indicates the amount of each compound present in the sample (i.e. high/tall peaks indicate a large presence of the compound in the urine).

5.2 Advantages and limitations of GC-MS

Coupled with the advantages of MS, as stated in chapter 4 (Table 4.1), GC-MS is a low cost, highly sensitive method, and the instrumentation is easy to use (Emwas 2015). Quantification of the data is much easier and less time consuming than that of NMR. Limitations include: laborious sample preparation that can incorporate experimental error, sample destruction and analysis that can only measure thermo-labile and volatile small molecules. Due to the latter, GC-MS is not widely used in global metabolic profiling studies, as is the case with NMR. Derivatization is a process whereby the chemical properties of

isolated metabolites are altered (in the case of my study, the organic acids). By doing this, the compounds become more “*thermally stable, chemically inert and volatile at temperatures below 300°C*” (Kuhara 2005). Derivatization alters compounds to make them more volatile; however, this process can cause non-volatile metabolites to form different forms of the same parent metabolite, resulting in erroneous detection, and quantification, of the metabolite. In samples with variable metabolite content, derivatization times of the different metabolites can vary, depending on the metabolite properties. This affects the results of the analysis (Emwas 2015). Lastly, batch sizes can also negatively impact the results of an analysis. Samples are queued for analysis, and the metabolites still continue to undergo derivatization. However, to help mitigate these problems, an internal standard (IS) is added for compound normalization to reduce incorrect quantification.

5.3 Organic acid extraction from urine for GC-MS analysis — standard operating procedure (SOP)

The protocol used for the extraction of organic acids was a Standard Operating Procedure (SOP) used by the Potchefstroom Laboratory for Inborn Errors of Metabolism (PLIEM) at the NWU. A detailed SOP, including reagents and equipment settings, for organic acid extraction has been included as Addendum A2 to this thesis.

Organic acids are the end products of metabolism excreted in the urine of humans. These chemical compounds are derived from host cellular metabolism, and also from the gut flora found in the host. As such, they can be used to monitor health and disease states of an individual. Organic acids primarily contain carbon and hydrogen, but oxygen, nitrogen, sulphur and phosphorus may be also present.

I was lead to do an organic acid extraction due to the findings from my explorative NMR study (Malatji *et al.*, 2017, Chapter 4). Here, abnormal metabolites were detected in the urine of the FMS patients, which pointed to a possible perturbation in the host gut metabolism. Since organic acid analyses can provide a snapshot on the state for the gut flora, yet still impart further information with regards to FMS, it was deemed feasible to expand my NMR study with organic acid extraction through GC-MS analysis. This study was conducted on the same experimental cohort as those used in the NMR study. For the remainder of this section I will briefly describe the SOP used for the organic acid extraction.

The creatinine content of each urine sample was determined by an external lab, namely Ampath Laboratories (Drs Du Buisson, Kramer, Swart, Bower Inc.). The creatinine content was subsequently used to determine the exact volume of urine to use in the extraction procedure (Table 5.1). This was done to correct for the concentration differences of metabolites in the urine between the individual samples, due to varying dilutions of urine. This creatinine normalization yields gas chromatograms that are comparable to each other. Correcting for creatinine content not only adjusts the urine volume to be used but also the amount of some of the reagents to be used during extraction and derivatization of the organic acids.

Table 5.1: Urine volume used for analysis with regards to creatinine content

Creatinine value (mmol/l):	Urine volume:
Creatinine > 8.8	0.5 ml
Creatinine < 8.8 and > 0.44	1 ml
Creatinine < 0.44 and > 0.18	2 ml
Creatinine < 0.18	3 ml

The calculated volume of urine samples were added to glass Kimax tubes, to which a standard amount of six drops of hydrochloric acid was added to each sample to acidify the urine to approximately pH=1. By adjusting the pH of the urine the organic acids become non-protonated and hydrophobic, facilitating their extractability to the organic phase (diethyl ether and ethyl acetate).

The IS used during all organic acid extractions was provided by PLIEM and was prepared by dissolving 26.25 mg of 4-phenylbutyric acid in a few drops of sodium hydroxide (NaOH). This was then diluted with distilled water to a volume of 50 ml.

The next step involved the liquid-liquid extraction of the organic acids using ethyl acetate (6 ml) and diethyl ether (3 ml) in two separate extraction steps. Ethyl acetate was added first to the preceding mixture, mixed by using a rotor torque for 30 min, and followed by centrifugation for 3 min at 40 000 rpm. The centrifugation step allows for separation of the mixture into the organic (upper) and aqueous (lower) phases. The organic phase was then transferred to a clean Kimax tube using a glass Pasteur pipette. The diethyl ether was then added to the remaining aqueous phase and, similarly, mixed for 10 min and centrifuged again. Following phase separation, the resulting organic phase was then transferred to the Kimax tube containing the previously transferred ethyl acetate organic phase. The aqueous

phase was then discarded. This second extraction step was incorporated to extract any remaining organic acids from the aqueous phase.

Sodium sulphate, two spatulas full in a powder form, was then added to the resultant organic phase solution. This step is for the removal of any remaining water in the organic phase. This was then vortexed and centrifuged for 1 min at 40 000 rpm. The anhydrous organic phase was then transferred to a smaller, clean Kimax tube and evaporated to dryness at 40°C under a flow of nitrogen for approximately an hour.

The dried sample was then derivatized using N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine according to a 5:1:1 ratio. BSTFA is the preferred silylation reagent due to the fact that it possesses the best reactivity, volatility and solvent properties. BSTFA is also found in its pure form, which is another good attribute of this reagent. The TMCS, together with BSTFA, acts as a catalyst, allowing for the full derivatization of compounds at 60°C within 10 min (Kuhara 2005).

Once all derivatizing reagents were added to the samples they were incubated in a sand bath at 80°C for 45min. An aliquot of the derivatized sample was then transferred to a 0.25 ml conical insert and placed in a 1.5 ml vial and capped. One microlitre of this derivatized sample was then injected by an auto-sampler onto the GC-MS.

The temperature program used for the GC was a standard program applicable to organic acid derivatives. The run-time for each GC-MS analysis was approximately 1 hour, and the reader is referred to Addendum A2 for more settings on the GC-MS run.

Once the raw data from the GC-MS machine had been generated for all the samples, it was analysed using AMDIS (Automated Mass Spectral Deconvolution and Identification System). This is a software programme used for the identification and spectral extraction of the GC-MS data for each compound in a sample (figure 5.3) (refer to Addendum A2 for the settings used in AMDIS). Deconvolution of peaks allows overlapping signals to be separated into single peaks. AMDIS extracts each compound's mass spectrum from the data file generated by the GC-MS and compares it to the chromatogram in the custom-made reference library developed by Prof. Mienie at PLIEM in order to identify the metabolite and assign it a name. This library for the organic acids partially consisted of spectra obtained from the NIST/EPA/NIH Mass Spectral Library and Search Software (NIST 11) (www.nist.gov). Spectra that were not available in the library were created by obtaining commercial, or synthesizing, standards, derivatizing them with BSTFA, and manually analysing on a GC-MS. This custom library was comparable to those used in other international laboratories for

the study of inherited metabolic diseases; but, unlike those, is not commercially available. The resultant AMDIS file was then opened with Microsoft Excel and for each of the identified metabolites their relative concentrations were calculated. This was done using the following formula: area of analyte/area of IS*262.5 (concentration of the IS). Using this formula expressed the concentration of the metabolites in mmol/mol creatinine. Data matrices were generated using these relative concentration values, and biostatistical analyses were carried out.

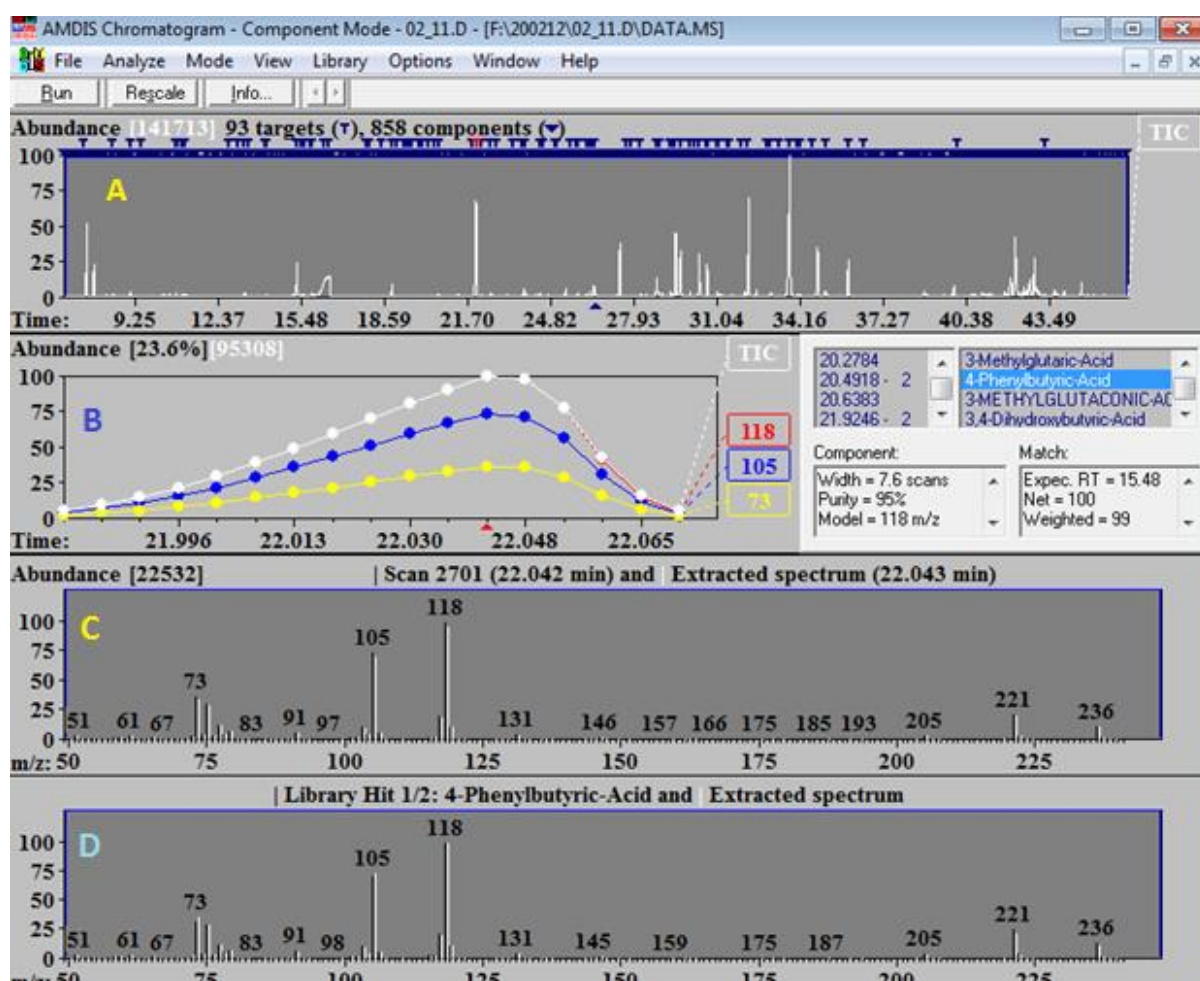


Figure 5.3: Example of the output window obtained in AMDIS when analyzing the raw data files. Graph A is the ion chromatogram of all the compounds in the mixture identified by the GC component. The 'T's' and triangles above each peak indicates named compounds identified by the library and unnamed compounds, respectively. Graph B indicates the abundance of the various fragments. Graph C is the MS profile of a selected compound, which in this case is 4-phenylbutyric acid. Graph D is the library match of the same identified compound in Graph C. Figures 5.1 and 5.2 are also part of the output window seen in AMDIS and correspond with D and A respectively.

5.4 GC-MS profiling of Fibromyalgia Syndrome

5.4.1 Statistical analyses of FMS data

This section will present results from a semi-targeted GC-MS study done on FMS patients and controls. The term semi-targeted, here, refers to the fact that a metabolome sub-class, namely organic acids, was extracted from the urine for analyses.

The resultant matrices that were generated from AMDIS analysis were subjected to some data pre-treatment steps, before statistical analyses. First, three patient cases were removed, as they were suspected of having a possible inborn error of metabolism from spectral analysis done by Prof Mienie. Second, an 80% zero filter was done on the variables to remove those with more than 80% zero concentration values across all four experimental groups, namely FMS, CF, CO and CN. Initially, a total of 357 variables were detected, from which 147 were deleted as a result of the zero filter; hence, 210 remained. Ten variables were further deleted as they were deemed as contaminant metabolites, and 200 variables remained. A further four metabolites were combined with their parent metabolites, as they were named differently but were essentially the same metabolite, and 196 metabolites remained. Zero replacement was done on the remaining variables from a Beta distribution truncated at the first non-zero value for each variable. Finally, the reduced data was log scaled and case outliers were removed based upon the Hotelling's T^2 method. Three outlier cases were removed as a result — two from the CN group and one from the FMS group — hence 80 cases remained.

5.4.2 Results and discussion

Unsupervised (PCA and cluster analyses) and supervised (PLS-DA) multivariate statistical analyses were then carried out on the matrix that resulted from these data pre-treatment processes. This was done for the patient group versus all three control group pairings; namely: FMS vs CF, FMS vs CN and FMS vs CO. Figure 5.4 shows the PCA results obtained for each of these pairings. A natural separation can be observed for all pairings. Figure 5.5 shows the PLS-DA results obtained for all pairings — a total separation was observed for all pairings, indicating that the FMS group is distinguishable from each control group. A Euclidean-Ward cluster analysis (figure 5.6) was also done on the data, further confirming that the FMS group is distinguishable from each control group. From these results it is possible to see that a unique metabolite profile exists in the FMS group. Subsequent univariate analyses (data not shown) identified a group of metabolites, namely monosaccharides and their derivatives, to be elevated in the FMS group. This indicated the

presence of a gut perturbation in FMS. These findings warranted further investigating to understand the biological implications.

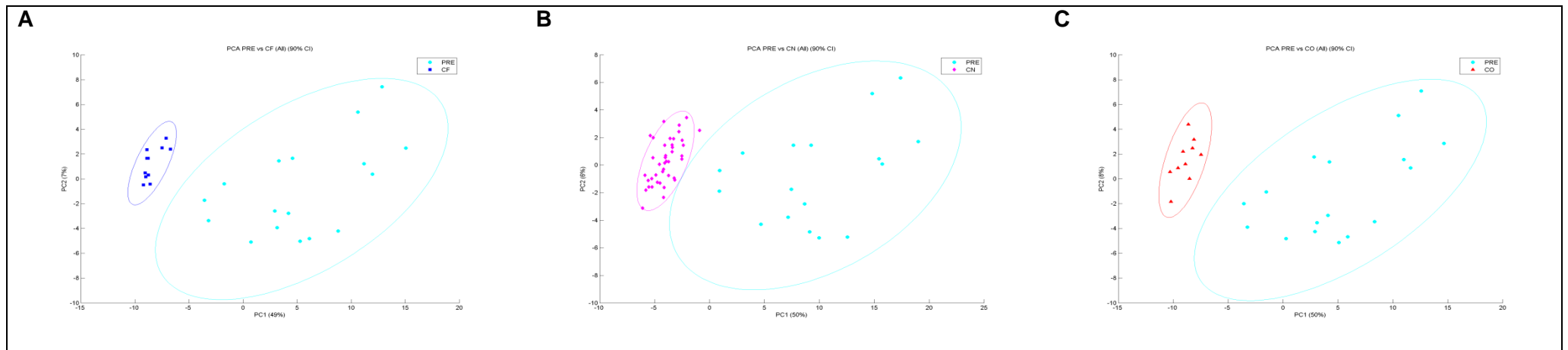


Figure 5.4: Unsupervised PCA analysis, at a 90% confidence interval (CI), of the patient (FMS) group versus each of the control groups, namely CF (A), CN (B) and CO (C). In each picture the FMS group is shown in blue and the control group is shown in their corresponding colour.

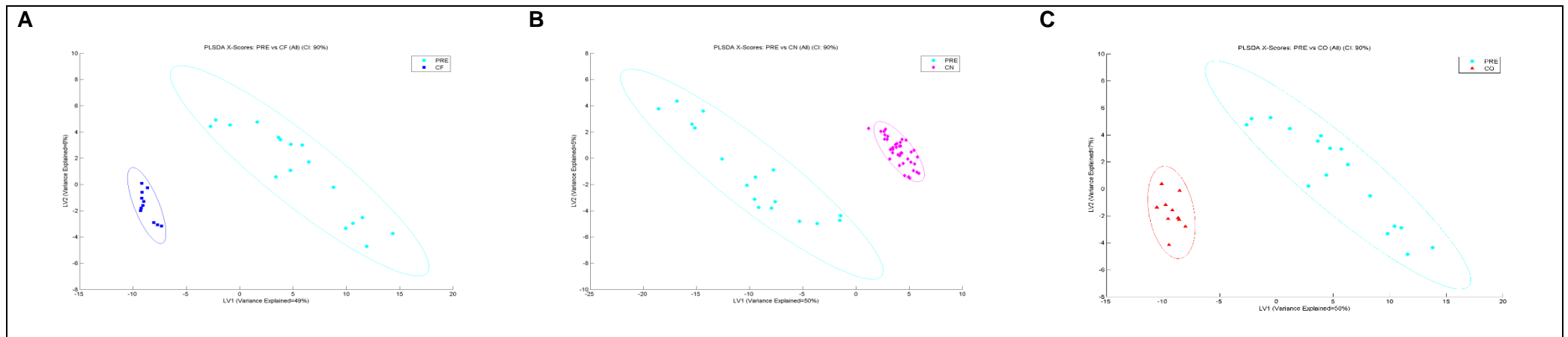


Figure 5.5: Supervised PLS-DA analysis, at a 90% CI, of the FMS group versus CF (A), CN (B) and CO (C). In each picture the FMS group is shown in blue and the controls groups in their corresponding colour. Analyses were done using 196 variables.

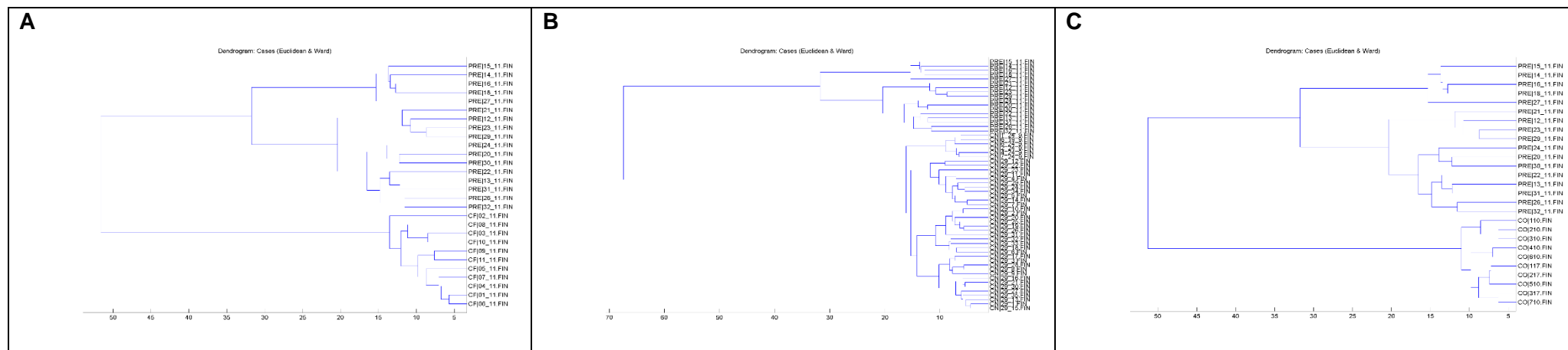


Figure 5.6: Euclidean-Ward cluster analyses of FMS versus CF (A), CN (B) and CO (C). These results reveal clear separations between FMS and the control groups, thus substantiating that there exists a unique metabolite profile in the FMS group.

5.4.3 A GC-MS metabolomics signature in patients with Fibromyalgia Syndrome

5.4.3.1 Background

FMS patients frequently complain of gastrointestinal discomfort. In our NMR study (Malatji *et al.*, 2017) we identified elevated metabolites in the urine of FMS patients that indicated a gut perturbation, in the form of dysbiosis. These findings warranted further investigation by means of a semi-targeted study. Thus organic acid analyses coupled with GC-MS metabolomics became an attractive option.

In the following section, I present a manuscript, submitted to *BMC Neurology*, documenting the results of this GC-MS study conducted on the FMS patients and controls. In this study, based on the findings, we speculated that dysbiosis, does indeed, seem to be present in FMS patients. Dysbiosis can be considered a feature underlying FMS pathophysiology, supporting the model that brain function in patients suffering from this disorder may be altered by the perturbed gut microbiota through the gut-brain axis, substantiating the notion that the gut may be a gateway to generalized pain. Note that the article is presented in the format as per *BMC Neurology* journal's article submission guidelines.

5.4.3.2 The GC-MS metabolomics signature in patients with Fibromyalgia Syndrome directs to dysbiosis as aspect contributing factor of FMS pathophysiology

The GC-MS metabolomics signature in patients with Fibromyalgia Syndrome directs to dysbiosis as an aspect contributing factor of FMS pathophysiology

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Submitted: *BMC Neurology*

Abstract

Background: Fibromyalgia syndrome (FMS) is a chronic pain syndrome. Comparative analyses of untargeted metabolomics data indicated an altered metabolic profile in patients with FMS. Our objective was to perform a semi-targeted explorative metabolomics study to (1) elucidate the global urinary metabolite profile of FMS patients and (2) explore the potential of this non-invasive metabolite information to augment existing medical practice in diagnosing the disease.

Methods: All cases were females. The patients had a medical history of persistent FMS (n = 18). Control groups were first-generation family members of the patients (n = 11), age-related individuals without indications of FMS (n = 10), and healthy young (18–22 years) individuals (n = 41). The biofluid investigated was early morning urine samples. Data generation was done through gas chromatographic-mass spectrometric (GC-MS) analysis and data processing and analyses were performed using Matlab, R, SPSS and SAS software.

Results and discussion: Quantitative analysis revealed the presence of 196 metabolites. Unsupervised and supervised multivariate analyses distinguished all three control groups and the FMS patients, which could be related to 14 highly significantly increased metabolites. These metabolites are associated with energy metabolism, digestion and metabolism of carbohydrates and other host and gut metabolites. The energy metabolites confirm interrupted energy utilization, often seen in FMS patients. The carbohydrate digestion products include components which are mostly very low to absent from normal urine and are known to be structural parts of complex dietary plant polysaccharides, digested by microbiota of the distal gut. In addition, the remaining abundant metabolites that differed between the FMS patients and controls are also gut-related, directing to dysfunction in the gut microbiome – dysbiosis.

Conclusion: The overall urinary metabolite profile observed in the FMS patients suggests that (1) energy utilization is a central aspect of this pain disorder, (2) dysbiosis seems to prevail in FMS patients, supporting the model that microbiota may alter brain function through the gut-brain axis, with the gut being a gateway to generalized pain and (3) screening of urine from FMS patients is an avenue to explore for adding non-invasive clinical information for diagnosis and treatment of FMS.

Keywords

Fibromyalgia syndrome, Gas chromatography- mass spectrometry (GC-MS), Metabolomics, Dysbiosis, Carbohydrate markers, Monosaccharides, Pain, Biomarkers

Background

Fibromyalgia syndrome (FMS) is currently viewed as part of the Functional Pain Syndromes (FPS) [1]: Central sensitization is associated with abnormal pain processing, increased sensitivity of the nervous system and decreased anti-nociception, which results in the clinical phenomena of hyperalgesia and allodynia. Dysfunction in mono-aminergic neurotransmission, which involves serotonin, norepinephrine, nerve growth factor, substance P and others, has been implicated to account for the central pathophysiology of FMS [2,3,4,5]. Peripheral pain generators may contribute to the pathophysiology of some FMS patients [6] and patients often manifest with multiple other symptoms such as cognitive impairment, disrupted sleep and chronic fatigue, including the association with comorbidities such as irritable bowel syndrome (IBS), small intestinal bacterial overgrowth (SIBO), interstitial cystitis and mood disorders [7,8,9].

The human gut microbiota, which functions symbiotically with the host, extensively affects the host through metabolic exchange and contributes to the risk of several human diseases [10]. A recent untargeted NMR metabolomics study of FMS [11] supported alterations of energy metabolism - a clinical characteristic of FMS - while hippuric, 2-hydroxyisobutyric and lactic acids observed in the urine samples of the patients suggested perturbations in the gut metabolome of the patient group. The metabolic associations of each of these metabolites have been shown to be associated with the *Clostridia* phylogenetic gut microbiotic group [12]. In health, host-gut microbiota metabolic [13] and brain-gut interactions are crucial in the maintenance of homeostasis [14]. It appears that neuroplasticity-related systems and neurotransmitter systems are influenced by the gut-brain axis regulation and perturbed homeostasis is proposed to contribute to disease aetiology through alterations in the gastrointestinal tract, central nervous, autonomic nervous and immune systems [15]. The frequent comorbidity of fibromyalgia with stress-related disorders, such as chronic fatigue [16] and IBS [17] and some CNS-related abnormalities [18], suggests that gut-brain axis regulation may at least be a partial common denominator for these disorders [19].

The presence of the exogenous markers of gut origin observed in the NMR metabolomics study [11] provides further indications of altered microbial-mammalian metabolic balance influencing FMS and may be significant in defining the clinical profile in FMS. To further investigate this view, we performed a semi-targeted GC-MS metabolomics study on the same samples used for the NMR study. The three gut metabolites observed in the NMR study were also significantly increased in the FMS patients relative to the controls, although not to the level of being responsible for group separations. Most noticeable was the presence of a wide array of metabolites which are associated with digestion of complex dietary plant polysaccharides by gut microbiota. Likewise, metabolites of carbohydrates associated with the metabolic pathways known to occur in gut microbiota were also present in the urine of FMS patients – observations that were not previously

reported. These results may provide an avenue for understanding the dynamic basis of host–microbiome perturbations in FMS, contribute to clinical information that distinguish FMS patients from related comorbidities and direct the development of a functional approach towards its treatment.

Materials and methods

The study population and sampling

We applied a sample collection and analysis pipeline (Fig. 1) for exploratory metabolic profiling of urine samples [20] from FMS patients and age-matched, healthy and non-related controls (CO) as well as two additional control groups: first-degree relatives of the patients (CF) and young (aged 18–22 years), healthy students of North-West University (CN). All cases were Caucasian females.

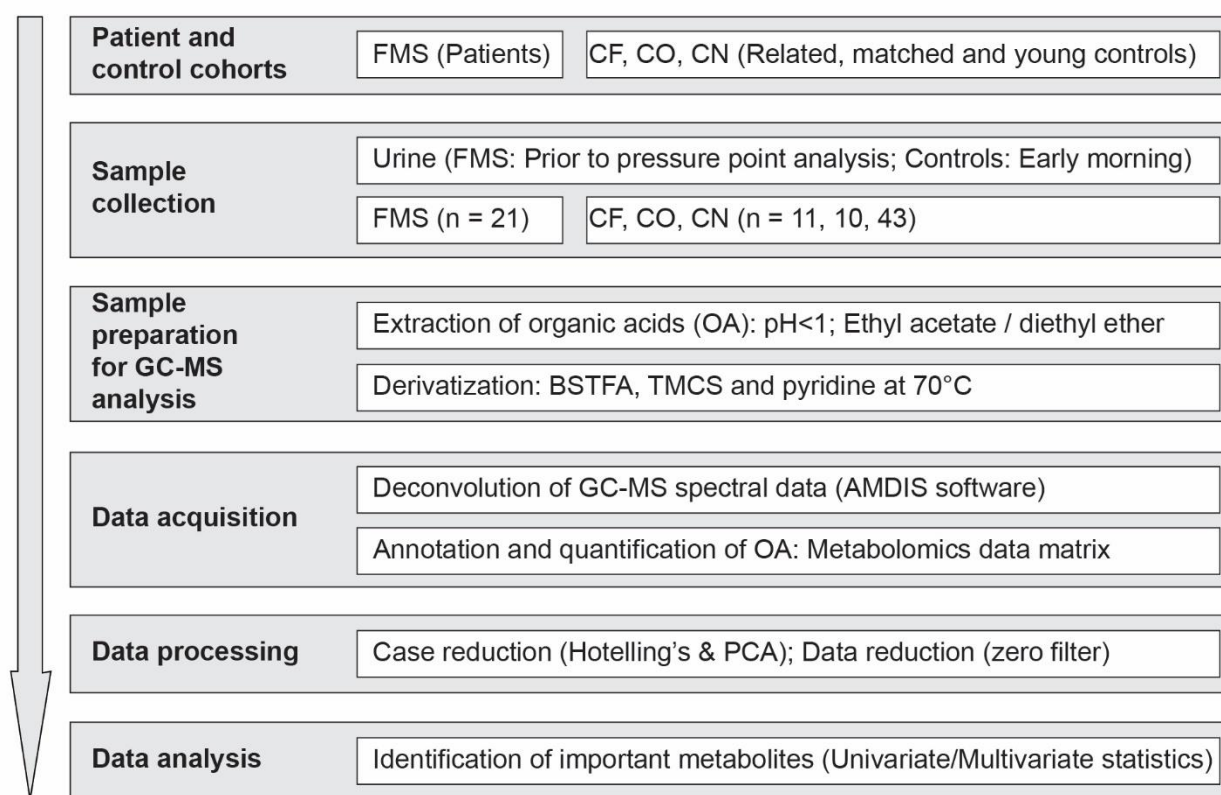


Fig. 1 Schematic pipeline applied for exploratory metabolic profiling of urine samples. The direction of the flow of the analytical procedures is shown by the arrow to the left. The samples were obtained from all cases prior to detection of outliers (4 FMS and 2 CN cases).

All the patients included in this study were previously diagnosed with FMS by the same specialist pain clinician from his chronic pain practice in Pretoria as previously described [11]. The diagnosis was based on a comprehensive clinical assessment using the American College of Rheumatology (ACR) criteria, first published for FMS in 1990 [21] and the 21 patients selected for this study (FMS group) were confirmed with FMS. All these patients were on a comprehensive evidence-based management programme according to international guidelines and were only included as they continued to complain of widespread musculoskeletal pain (including in the axial skeleton) in the presence of >11 painful tender points with musculoskeletal assessment.

The socio-demographic, clinical information, pain-specific medication and levels of emotional experience associated with FMS for the patients were obtained through the Fibromyalgia Impact Questionnaire (FIQR) [22] and an in-house clinical questionnaire (IHCQ) [11]. Further details are presented in the SI (see *SI to Article 2*). Clinical description, urine sample collection on all experimental groups commenced from 2009 to 2011. Case definition and selection for the eventual study was done by a clinical and scientific group of co-workers in 2010. Following scrutinizing of the records of patients with a medical history of FMS, a group of 17 FMS patients was eventually selected based on the above selection criteria as well as after excluding outliers based on statistical analysis. Taking everything into account, we conclude that the present FMS patients are representative of patients used in studies on FMS in general and also represent a well-defined group as required for metabolomics investigations. All individuals in the control groups showed no indications of FMS, or related conditions, were defined as healthy females and were not required to complete the questionnaires.

Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form; ethical approval for the study was obtained via the consortium under the Nuclear Technologies in Medicine and Biosciences Initiative (NTEMBI) (ethical approval by Pharma Ethics Pty, Ltd, reference number 11064365). Pharma Ethics confirmed the following: “The study has been accepted as complying to the Ethics Standards for Clinical Research with a new drug in participants based on FDA, ICH GCP and the Declaration of Helsinki guidelines. The Ethics Committee (IRB) granting this APPROVAL is in compliance with the Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (2006), ICH Harmonised Tripartite Guidelines E6: Note: for the Guidance in Good Clinical Practice (CPMP/ICH/135/95) and FDA Code of Federal Regulation Part 50, 56 and 312.” as specified under Declarations.

Analytical procedures and quality control

Organic acid profiles of urine collected from our sample groups were analysed by a standard GC-MS method (see section S2 in *SI to Article 2* for detailed descriptions), which was standardized to comply with the required levels of qualitatively and quantitatively repeatable and reproducible [23]. We observed that MS-spectra of some of the carbohydrates can be very similar when analysed as TMS-ethers and -esters. In addition, some carbohydrates may also be present in the linear or ring configuration, or can even be converted from one configuration to the other during the extraction and/or derivatization procedure. These characteristics of carbohydrates make the use of relative retention times (RRTs) in combination with EI MS spectra compulsory for final identification and are included in the standard operating procedure in our laboratory. RRTs using 4-phenylbutyric acid as an internal standard for all monosaccharides, sugar alcohols, aldonic acids, ulosonic acids, uronic acids and aldaric acids were extracted from a standard solution as described for urine organic acids. The dried product was derivatized with BSTFA, TMCS and pyridine and GC-MS analyses were done using the same GC operational settings and column as for organic acid analyses. Most of the monosaccharides and sugar acids produced at least 2 peaks and in some instances 3 peaks representing the linear structure, pyranose or furanose configuration and in some instances the pyranose as well as the furanose conformations for the same monosaccharide. The RRTs were calculated and the combination of RRTs and MS-spectra was added to the in-house spectral database for future use. For final identification, RRTs and MS-spectra from the in-house MS-spectra database as well as a commercially available database (National Institute of Standards and Technology (NIST) 17 Main EI MS Library) were applied.

Statistical analyses

Statistical analysis was performed on the organic acids data matrix, which consisted of 196 original features recorded from 85 original samples obtained from the four experimental groups, becoming 79 samples following outlier detection. The number of variables identified here compared well with the expanded urine metabolome of 179 metabolites (85 quantified), identified through GC-MS [25].

All variables in each group that did not contain values in at least 20% of the cases (i.e. more than 80% zero values) were removed from the original data matrix, a process known as zero filtering. Variables in the reduced data matrices were followed by manual curation and classification based on the Human Metabolome Database [24], with any non-biological variables (e.g. contaminants, medication and derivatization artefacts) being excluded from further analysis, leaving a biologically

heterogeneous group of 122 metabolites, related to energy metabolism (38), phenolic and benzene products from the gut microbiome (54) and carbohydrates (30).

Next, the data were scaled using a shifted log transformation with shift parameter equal to one. The Hotelling's T^2 statistic from a principal component analysis (PCA) model was used to detect outliers, after which outliers were excluded from further analysis. Univariate statistics, specifically the Mann-Whitney (MW) test (p values and effect sizes) and fold change (FC) ratios, were produced for the untransformed data.

Pairwise comparisons between groups identified features which differed for the three experimental groups. Zero replacement was performed for the untransformed data from the tail of a fitted beta distribution not exceeding the minimum observed value for each feature. After zero replacement, the data were again scaled using a shifted log transformation (with shift parameter equal to one) and mean centred. Unsupervised (PCA) and supervised (PLS-DA) models were fitted to the zero-replaced, transformed and centred data to identify combinations of features which differentiated between the groups. The next section describes the separations found between the groups and lists the features responsible for the separations.

The following statistical packages were used in the analysis of the metabolomics data:

- i. MATLAB with Statistics and PLS Toolbox Release (2012). The MathWorks, Inc., Natick, MA, USA; together with notBoxPlot.m developed by Rob Campbell (<http://www.mathworks.com/matlabcentral/fileexchange/26508-raacampbell13-notboxplot>).
- ii. SAS Institute Inc. (2015). The SAS System for Windows Release 9.3 TS Level 1M0, Copyright© by SAS Institute Inc., Cary, NC, USA.
- iii. SPSS Inc. (2015). IBM SPSS Statistics Version 22, Release 22.0.0, Copyright© IBM Corporation and its licensors (<http://www-01.ibm.com/software/analytics/spss/>)

Results

Data generation and case selection

Using the original variable data, case reduction was first applied to all four experimental groups (Fig. S1 in *SI to Article 2*). Outliers were identified firstly, based on the presence of suspicious metabolites (including those due to medication) and secondly, statistically by using a 95% confidence region in a Hotelling's T^2 test in conjunction with the respective PCA score plots with 90% confidence regions. Cases that were identified as outliers by either method were removed. The outliers were: FMS patients – four outliers (three on metabolite profiles and one through the statistical profiles); CF (family controls) – no outliers; CO (matched controls) – no outliers; CN (young controls) – two outliers through statistical profiles, yielding the final experimental groups: FMS patients (17), CF (11), CO (10) and CN (41) for the controls.

Group characteristics

Supposed changes in metabolite profiles from the FMS patients and the three control groups (excluding outliers) were first established through two unsupervised methods: unsupervised PCA and Euclidian and Ward hierarchical cluster analyses presented as dendrograms, based on all ($n = 196$) original metabolites. The data were log transformed and auto-scaled. Figure 2 shows the group separations based on these analyses. Differentiation between the FMS patients and all three control groups were found by the PCA (Fig. 2a to 2c) and complete separation by the cluster analysis (Fig. 2d to 2f). Both methods indicate a distinct difference between the metabolic profiles of the FMS patients and each of the control groups.

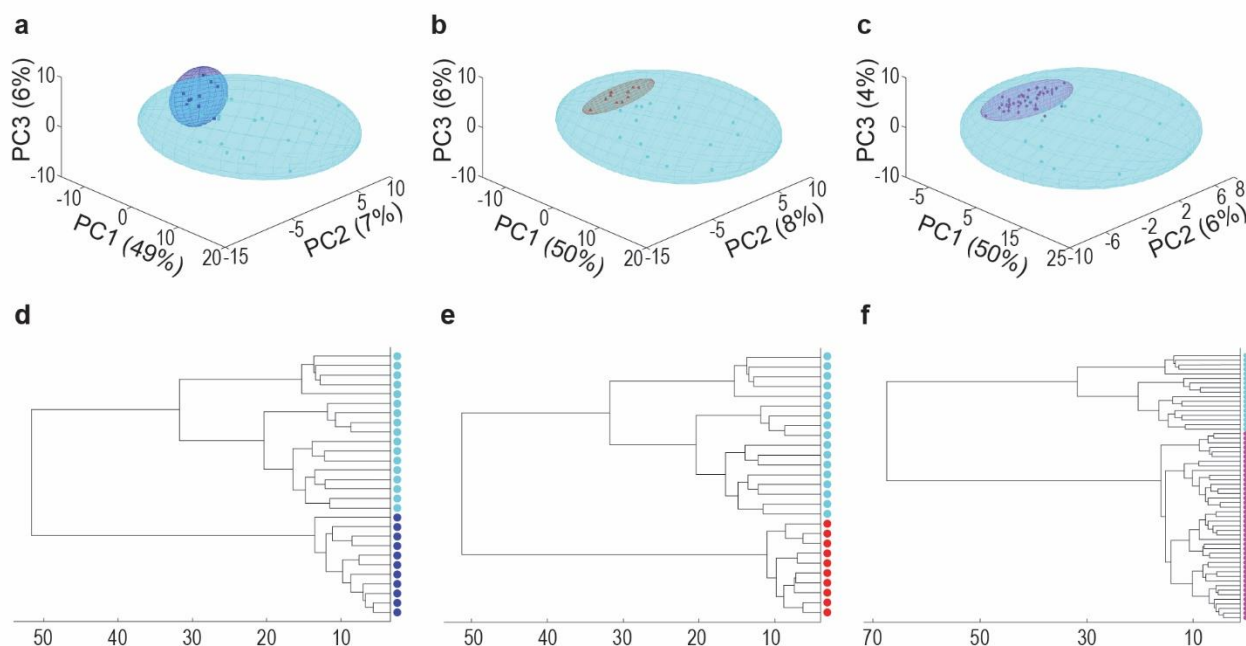


Fig. 2 Unsupervised analyses for the FMS patients relative to the three control groups based on all metabolites. PCA loading plots are shown for CF (a), CO (b) and CN (c) groups relative to FMS patients. Group separation between experimental groups through cluster analysis is shown in the dendrograms for the CF (d), CO (e) and CN (f) groups relative to FMS patients. Cases from the FMS patients are shown as light blue areas and dots, CF as dark blue, CO as red and CN controls as purple.

Next, group separations based on supervised PLS-DA and volcano plots were performed (Fig. 3) on the same data as for the unsupervised analyses. The PLS-DA between the CF (Fig. 3a), CO (Fig. 3b) and CN (Fig. 3c) against the FMS patients complemented results from the cluster analyses by indicating a complete separation between the three control groups and the FMS patients. The PLS-DA provided for calculating the goodness-of-fit (R^2) and predictive ability (Q^2) parameters. The outcome of these metrics is reported in the legend to Fig. 3, indicating a good model fit. Subsequently all three volcano plots for the FMS patients relative to the control groups (Fig 3d, 3e and 3f for CF, CO and CN respectively) indicate that a large number of variables ($n > 50$) differed significantly ($p < 0.05$) between the FMS and the control groups, manifesting with high up- or down-regulated FC-values.

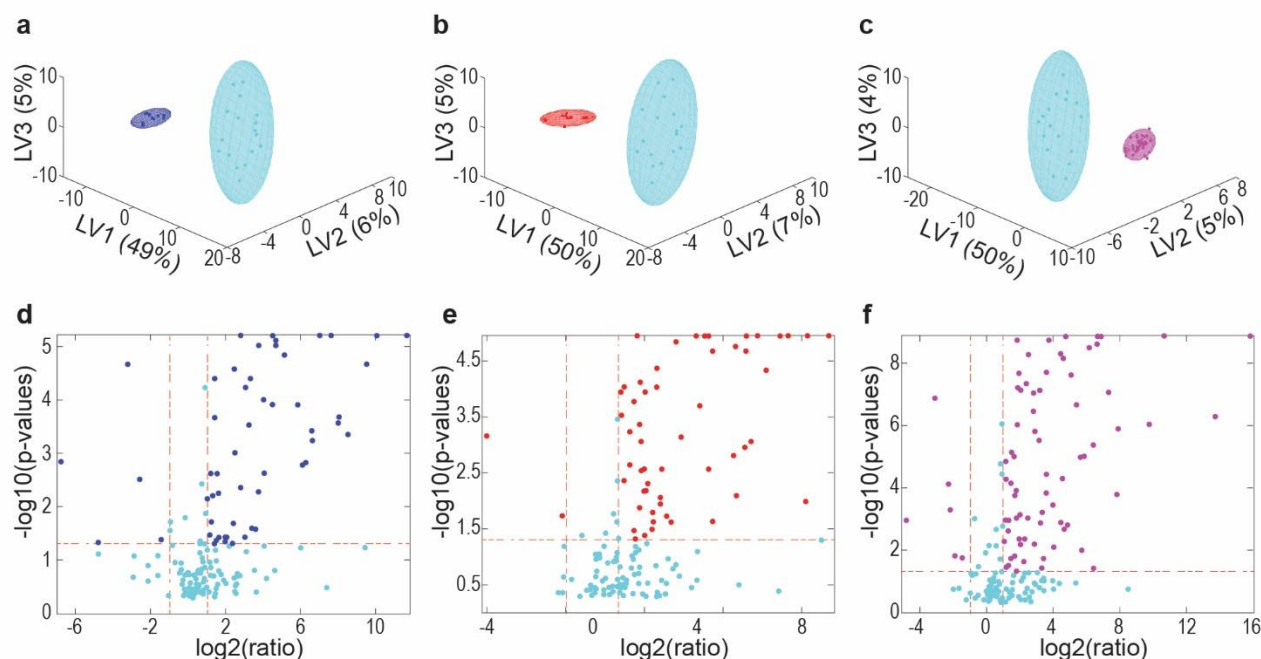


Fig. 3 Supervised (multivariate and univariate) analysis for the FMS patients relative to the controls, based on all metabolites. Upper panels: PLS-DA, with the 95% confidence areas indicated in light blue for the FMS group and in dark blue in (a) red in (b) and purple in (c) for the CF ($R^2 = 0.99$; $Q^2 = 0.49$), CO ($R^2 = 0.99$; $Q^2 = 0.58$) and CN ($R^2 = 0.99$; $Q^2 = 0.70$) controls, respectively. Lower panels: Red lines in the volcano plot (d to f) indicate the univariate boundaries of $p < 0.05$ and $|FC| > 2$, respectively.

Perturbed metabolite profile of the FMS patient group

Important metabolites that distinguish the FMS patients relative to the matched controls (CO) were identified from the values for variables important in projection (VIP) of the PLS-DA (Fig.3a). Additional inclusion criteria were statistical significance ($p < 0.5$ and smaller than the B-F 5% values), effect size > 0.8 and fold change > 5.0 . A total of 12 metabolites complied with these criteria, and were simultaneously common to all three control groups relative to, the FMS patients. The 12 metabolites are listed in Table 1, which also include oxalic acid and 4-hydroxybutyric acid, which were highly significant for the CO group and were also observed as such for one of the other control groups as well - the CF and CN groups, respectively. Tagatofuranose (common to CF and CN groups) is not included in Table 1, as tagatose is already included as common by all three groups. Of the two gut-related metabolites (hippuric and 2-hydroxyisobutyric acids) identified previously through the NMR study [11], 2-hydroxyisobutyric acid was significantly increased, but did not contribute to group separation ($p = 0.00012$; $FC = + 2.13$, and $VIP = 0.32$).

Table 1 Important metabolites in FMS patients relative to CO controls. Means and standard deviations (SD) for the metabolites listed are shown for the FMS patients and three control groups (CO, CF and CN). Abbreviations: VIP – Variables important in projection; M-W: Mann-Whitney p-values; B-F: Bonferroni-Holm test; ES: effect size; FC: fold change; 4-HBA: 4-hydroxybutyric acid; 2,3,4-trihydroxybutyl-L: 2,3,4-trihydroxybutyl-lactone; 2-D-3,5-DHPL: 2-deoxy-3,5-dihydroxypentanoic lactone. Twelve metabolites were important and common for all three controls groups relative to FMS, while two (no. 6 and 10) were common to only two groups, as indicated in brackets.

No.	Metabolite	VIP > 2.0	M-W < 0.5	B-F 5%	ES > 0.5	FC > 10	FMS Mean	FMS SD	CO Mean	CO SD	CF Mean	CF SD	CN Mean	CN SD
1	Sorbose	27.9	<0.00001	0.00026	0.82	>550	549	438	0	n/a	0	n/a	0.01	0.05
2	Phosphoric acid	11.2	0.000011	0.00028	0.82	59	147	111	2.47	2,39	0.05	0.09	5.42	6.95
3	Glutaric acid	10,6	0.000011	0.00028	0.82	144	77	60	0.53	0,59	0.39	0.33	0.76	0.77
4	Threonic acid	8,7	<0.00001	0.00027	0.82	298	39	28	0.13	0.40	0.04	0.09	1.78	5.69
5	Tagatose	7.3	0.00024	0.00031	0.67	>550	103	154	0	n/a	0	n/a	0.01	0.05
6	Oxalic acid (CO&CF)	7.2	0.000011	0.00027	0.82	21	46	24	2.11	2.69	6.79	5.32	12.13	6.56
7	Erythropentonic acid	6.9	<0.00001	0.00027	0.82	526	28	22	0,05	0,12	0.21	0.51	0.24	0.41
8	Rhamnose	4.7	0.000011	0.00027	0.82	81	15	9	0.19	0.17	0	n/a	0.01	0.05
9	Arabinose	3.9	0.000017	0.00028	0.80	44	19	17	0.44	0.64	0.56	0,51	0.27	0.46
10	4-HBA (CO & CN)	3.6	0.00109	0.00185	0.59	57	36	36	0.62	1,15	0.09	0.13	0.15	0.62
11	2,3,4-trihydroxybutyl-L	3.3	0.000021	0.00029	0.79	24	13	10	0.54	1.41	0.51	0.70	0.60	0.47
12	2-Keto-1-gluconic acid	2.8	<0.00001	0.00026	0.82	>550	19	15	0	n/a	0	n/a	0	n/a
13	2-D-3,5-DHPL	2.8	0.000199	0.00031	0.68	17	28	25	1.67	2.27	1.30	2.41	3.26	4.80
14	3-D-ribohexonic acid	2.6	0.000089	0.00029	0.72	>550	31	30	0	n/a	0	n/a	0	n/a

Assessment of the biological functions of these 14 metabolites indicates their association with energy metabolism, carbohydrate metabolism and gut-host associations. For further assessment of these observations, we divided the metabolites causing group separations (Fig. 2 and 3) into four groups with the metabolites relatively assigned as: (1) gut-host metabolites with a focus on benzene derivatives of poly-phenolic dietary origin (54 metabolites), (2) metabolites of energy and intermediary metabolism (36 metabolites), (3) carbohydrates and related metabolites (30 metabolites), and (4) the remaining metabolites. The lists of the assigned metabolites are included in the SI (section S4 in *SI to Article 2*). Note: We regard assignment as relative as a certain metabolite may actually be assigned to more than one group, while each metabolite was classified here in one group only.

For a qualitative visualization of the multitude of metabolites classified in the four groups, the unsupervised hierarchical clustering was applied for the FMS patients relative to the CO, CF and CN controls, using the quantitative concentrations of the four metabolite groups as basis for classification. The outcomes of these 15 analyses were visualized as heat maps as in Moon et al. [26]. Colour coding in the heat map indicates the metabolite concentrations in a range of six zones, from dark brown (high difference between FMS and controls), through lighter shades of brown to white (no differentiation), with the rows of subjects across the respective metabolites (columns). The outcome of these analyses is shown for the FMS and CO groups against the 54 gut-host metabolite (Fig. 4a), the 36 energy and intermediary metabolites (Fig. 4b) and the 30 carbohydrates and related metabolites (Fig. 4c). Incomplete cluster separation was observed from FMS relative to the CO for the gut-related metabolites (Fig. 4a), as well as for the CF and CN controls. Complete case separations occurred by application of the energy (Fig. 4b for FMS and CO) and carbohydrate (Fig. 4c for FMS and CO) metabolites. Subsequently, the heat maps were visually inspected to locate the distribution of the 14 important metabolites listed in Table 1 within the maps. First, four energy metabolites (phosphoric, oxalic, glutamic and 4-hydroxybutyric acid) showed very good differentiation between the FMS patients and CO controls (boxed areas 1 and 2 in Fig. 4b). Area 2 includes two highly increased intermediates of the Krebs cycle, which, however, did not comply with the criteria used for the selection of the most important metabolites shown in Table 1: malic acid ($p = 0.024$; ES = 0.38) FC = 24; VIP = 1.69) and 2-hydroxyglutaric acid ($p = 0.000013$; ES = 0.81; FC = 9.26; VIP = 1.62). We indicated these two metabolites as they are strong biological indicators of decreased energy efficiency in the FMS patients. Second, all ten carbohydrates listed in Table 1 clustered in two areas that showed excellent differentiation between the FMS patients and CO groups (boxed areas 1 and 2 in Fig. 4b). Area 2 again includes an additional metabolite (galactonic acid-lactone) which did not comply with the selection criteria for Table 1. Galactonic acid-lactone was significantly increased (VIP = 0.59; $p = 0.00084$; FC = + 66), but is a structural monosaccharide that abundantly forms part of pectin. Together, the combination of the visual inspection (Fig. 4) and the quantitative metabolite

concentrations (Table 1) points to the effectiveness of the monosaccharide metabolite signature of gut-host metabolites.

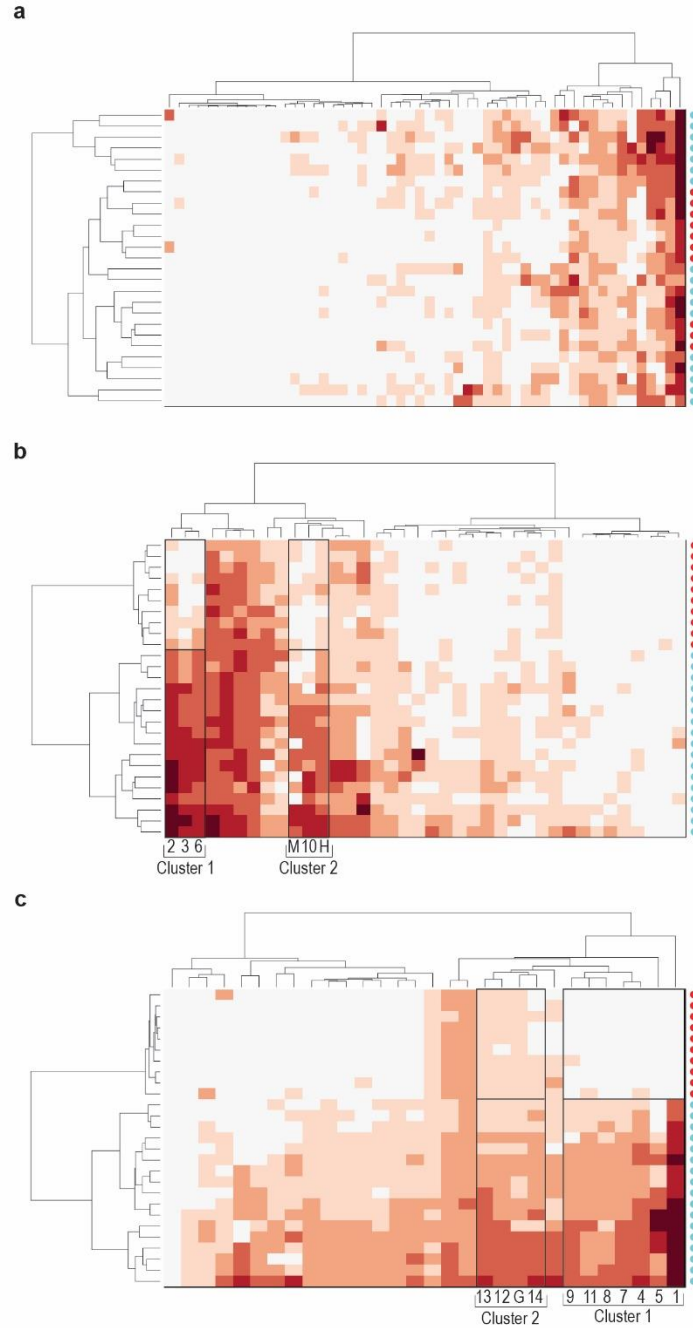


Fig. 4 Heat map analysis of FMS and CO controls. A clustered analysis of metabolites, expressed as quantified values, representing (a) 54 gut-host metabolites, (b) 36 energy-related metabolites and (c) 30 carbohydrates and their metabolites, determined in urine from the FMS patients and controls. Indicated clusters which differentiate between FMS (17 blue dots) and CO (10 red dots) controls are: (a) none; (b) Cluster 1: 2 = phosphoric acid; 3 = glutaric acid; 6 = oxalic acid; Cluster 2: M = malic acid; 10 = 4-hydroxybutyric acid; h = 2-hydroxy-glutaric acid. (c) Cluster 1: 1 = sorbose; 5 = tagatose; 4 = threonic acid; 7 = erythropentonic acid; 8 = rhamnose; 11 = 2,3,4-trihydroxybutyl-lactone; 9 = arabinose; Cluster 2: 14 = 3-deoxy-ribohexonic acid; G = galactonic acid-lactone; 12 = 2-keto-gluconic acid; 13 = 2-deoxy-3,5-dihydroxy-pentanoic acid-lactone.

An underlying possible relationship between the small number of metabolites that distinguish patients from controls was finally tested by a correlation analysis between the metabolites concentrations from Table 1. The outcome of the correlation analysis is shown in Fig. 5. The immediate observation is the broad correlation (~ 65% of correlation coefficients greater than 0.8) between the 14 metabolites. The very high correlation between sorbose and 2-ketogulonic acid ($r > 0.95$) indicates a possible link between a catabolic pathway of glucose, shared by sorbose. The high correlation between threonic acid and 2,3,4-trihydroxybutyryl lactone (tetronic acids) supports the co-metabolic destiny between the host and the microbiome of digestive products of plant origin [27].

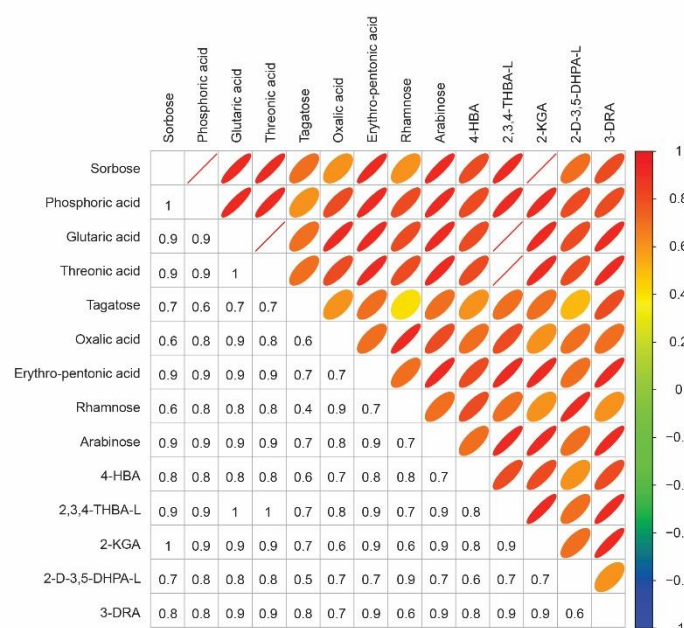


Fig. 5 Correlation matrix for the 14 significant metabolites discriminating between FMS patients and controls. Abbreviations used as in Table 1. Red: High up-regulation; blue: high down-regulation.

Discussion

Although FMS is presently better understood than ever before [28], there is still no consensus on the mechanisms leading to its pathogenesis. Recent genome-wide profiling studies identified at least 482 genes that differ between FMS patients and controls [29]. Untargeted metabolomics studies on FMS have revealed that tryptophan [30], lysophosphocholine [31] and gut metabolism [11] were perturbed in FMS patients. Based on the diverse genomic and metabolic findings, we hypothesize that systemic metabolic differences underlie FMS pathophysiology, which include host and gut microbiome interactions. To direct deductive reasoning, we present a conceptual representation that highlights three aspects that we regard as essential elements for the hypothesis (Fig. 6).

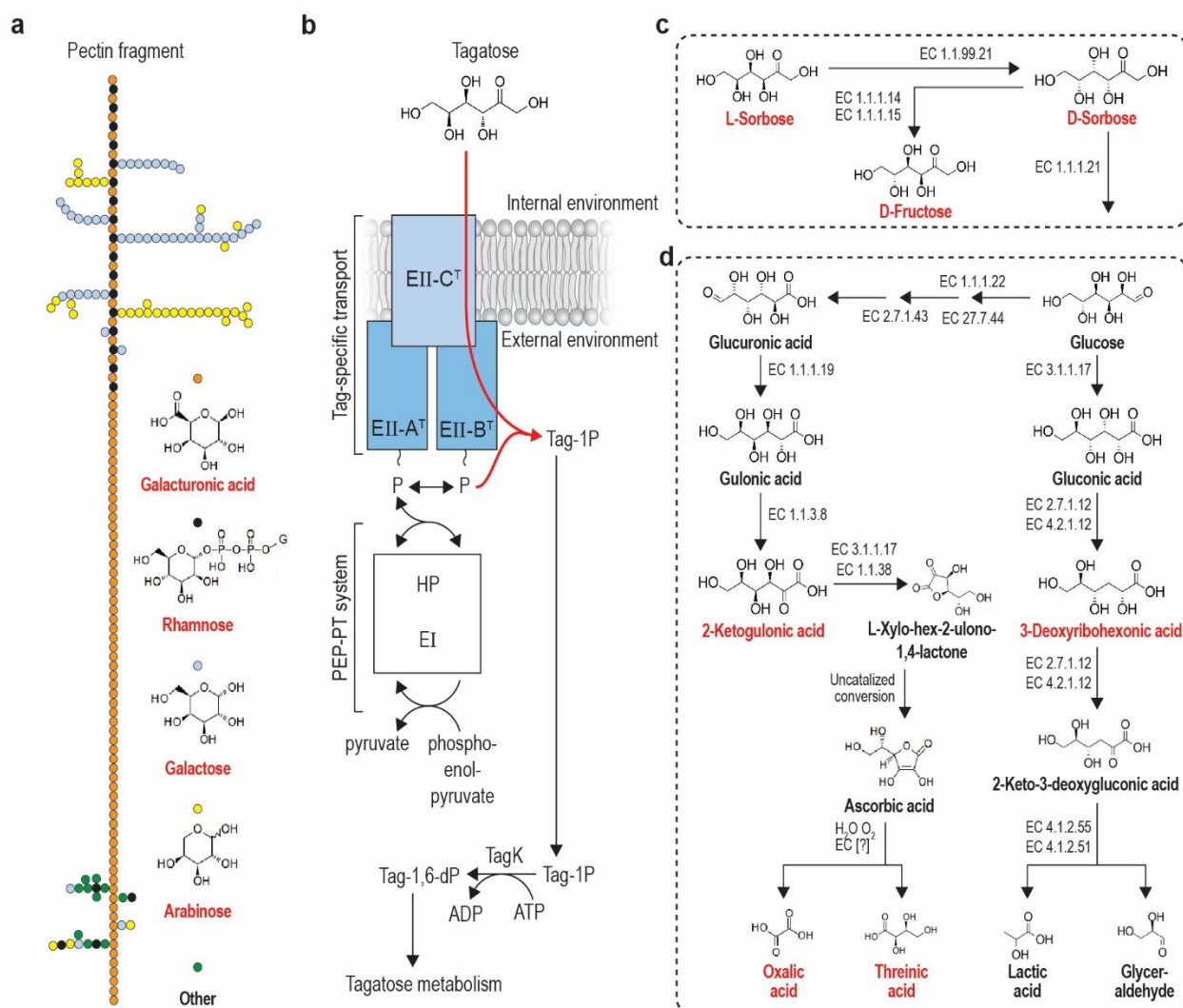


Fig 6 Conceptual representations of microbiome-host metabolic interactions proposed to be elemental in dysbiosis in FMS patients. (a) A model of the pectin (derived from [32]) with a colour code that combines the dietary polysaccharide structure and constituent monosaccharides from pectin digestion, observed in the urine of FMS patients. (b) A model (adapted from [39]) for the tagatose-specific membrane phosphor-transferase system (PTS) for the trans-membrane transport and for phosphorylation of tagatose, only detected in urine of the FMS patients, and the PTS proposed to be operative in *B. licheniformis*, a gut microbe. (c) Enzyme dependent conversions of sorbose (having the highest VIP in the multivariate analysis of FMS vs CO), with (d) a link to glucose and a catabolic pathway, including metabolites that were observed to be increased in FMS patients (names indicated in red). Abbreviations: EII-A^T, EII-B^T and EII-C^T: tagatose-specific *B. licheniformis* multi-domain membrane proteins; EI and HP: a general and a histidine-containing cytoplasmic phosphor-carrier bacterial protein system; Tag-1P and Tag-6P: tagatose-1-phosphate and tagatose-6-phosphate, respectively; ~P: high-energy phosphate; TagK: tagatose-1-phosphate kinase; ATP and ADP: adenosine-triphosphate and adenosine -diphosphate, respectively; EC: Enzyme Commission number (EC number) of the numerical classification scheme for enzymes.

First, the human genome encodes a limited number of intestinal saccharidases and pancreatic amylases for the digestion of plant reserve carbohydrates (starch) and the cell walls of plants, which are an enormous human nutrient source of chemically and structurally highly complex carbohydrates

(cellulose, xylan, and pectin) [32]. These macromolecular foodstuffs are intrinsically resistant to human enzymatic breakdown, but are substrates for digestion by the gut microbiome, with constituent structural monomers (e.g. arabinose, rhamnose, xylose and galactose) being the digestive products. The plant-derived monosaccharides are important precursors or co-factors in human metabolism and fulfil a protective function by repressing the overgrowth of harmful microorganisms and foster human immunological protection. Monosaccharides are normal constituents of the human urine metabolome [25] but exceed normal reference ranges in dysbiosis, the imbalance in the gut microbiome [33]. Metabolic profiling of urine provides a strategy to characterize metabolites from microbial origin and to define dysbiosis [13]. An example was the increase in arabinose observed in our FMS patients relative to the CO controls (arabinose: Ref. value: 0.8–19.4 $\mu\text{mol}/\text{mmol}$ creatinine (HMDB); FMS 19.60 and CO 0.44 $\mu\text{mol}/\text{mmol}$ creatinine), but the urinary profile also included monosaccharides which are constituents of dietary polysaccharides but which are not part of the normal urine metabolome and are rarely detected in human urine (sorbose, rhamnose and tagatose, Table 1). From these observations we postulate that dysbiosis is part of the FMS pathophysiology. Although the basis of dysbiosis in gastrointestinal disturbances is still unresolved, there is increasing evidence that a redistribution of the microbiota in specific gut *Firmicutes*, *Bacteroidetes* and *Faecalibacteria* of these patients does occur [34, 35]. It may thus be reasoned that the combination of gastrointestinal discomfort and an abnormal urinary monosaccharide profile provide biomarkers that a disturbed composition of the gut microbiome prevails in FMS patients.

Second, variation in the content of the gut lumen challenges the microbiota to detect these frequent changes and to regulate their metabolism according to these changes. In bacteria, membrane-bound transport systems are part of their sensing ability, which includes the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) [36]. Since its discovery in 1964 [37], the PTS has been shown to be a complex protein kinase system that regulates a wide variety of transport metabolic and mutagenic processes and the expression of numerous genes in bacteria [38]. The PTS has tight substrate specificity for the translocation and subsequent phosphorylation (formation of the monophosphate monosaccharide) process, shown schematically in Fig. 6b for tagatose [39]. Two factors of the PTS are key regarding our hypothesis: (1) The transport, phosphorylation and metabolism of the monosaccharides are tightly coupled, and no free monosaccharides reside within the bacteria, and (2) mutants of PTS of several microbial species indicated that they lost their capacity to utilize monosaccharide forage and cease to grow [40], while mutants of *E. faecium* displayed a colonization defect in antibiotic-treated mice [41]. These observations clearly linked an aspect of dysbiosis to potential genetic aberrations in the PST of a gut microbe, which may provide a second line of thinking about the basis of the gastrointestinal discomfort in patients suffering from FMS. Patients with increased urinary metabolites, not seen in normal urine, may best serve for metagenomic investigations of their gut microbiota.

Third, a number of the metabolites listed in Table 1 relate to the microbiome (4-hydroxybutyric acid - a short-chain fatty acid [42], the main end products of microbial metabolism), the host (glutaric acid - intermediate in lysine catabolism, and a biomarker of succinic semialdehyde dehydrogenase deficiency, in an inborn error of energy metabolism [43]) or to microbiome-host co-metabolism (sorbitol; see Fig. 6c and 6d). Sorbitol is a structural product of fruit polysaccharides [44]. Two metabolic sorbitol pathways reside in bacteria: (1) The PTS mediated pathway, of L-sorbitol \rightarrow L-sorbitol-1-P \rightarrow D-glucitol-6-P \rightarrow D-fructose-6-P \rightarrow energy release [45]; (2) The 2-keto-L-gulonate pathway (Fig. 6d), observed in a large number of bacterial strains, including the gut-linked *Escherichia coli* [46]. Next, sorbitol, an abundant osmolyte with a key role in regulating human cell volume homeostasis and cytoprotection, is produced from L-sorbitol (sorbitol reductase – EC 1.1.1.289). Sorbitol can be converted to glucose (aldehyde reductase – EC 1.1.1.21) and catabolized to 2-keto-L-gulonate, threonic and oxalic acid, all three highly elevated in the urine from the FMS patients. Against this background it can be predicted that the increased urinary sorbitol in FMS may result from dysfunction in the gut microbiome and/or the host metabolism as well as through their co-metabolism – alternatives to be considered in the dysfunctional systemic metabolism hypothesis on FMS pathophysiology.

In conclusion: The results and interpretation of this study are hampered by limitations. (1) Based on the clinical criteria used for selection of the FMS patients, we regard the group as representative of FMS in general. However, the observations should be validated through a metabolic study on a different group of FMS patients and the study should include healthy controls (as with the CO and CN controls in our study) as well as a patient group with a well-defined gastrointestinal disorder, like IBS. (2) As we did not validate the present metabolic profile over time the present observations actually are only a snapshot of a proposed metabolic profile that distinguishes FMS from controls. What is required is a longitudinal component in a future experimental design to confirm the claim of dysbiosis being a key clinical feature of FMS. (3) Furthermore, in the present study no analytical analysis on stereoisomer standards was included in the confirmation of the monosaccharides, which is a requirement for final interpretation of their functional implications which seems to be key in the FMS pathophysiology [e.g. the tetronic acids [(R*,s*)-2,3,4-trihydroxy-butanoate: threonic acid; (R*,r*)-2,3,4-trihydroxy-butanoate: erythronic acid] and their lactones (2,3,4-trihydroxybutyryl-lactone)]. Notwithstanding these limitations, the results from the present investigation provide a new insight into the gastrointestinal discomfort shared by 80% of FMS patients and, more importantly, provide a potential target for therapeutic benefit. Knowledge of FMS is gaining momentum. A present view sees effective treatment for fibromyalgia now to be achievable [28]. The challenge remains for a deep understanding on how our complex symbiotic gastrointestinal organs interact with our complex immune and nervous systems, as implied by gut-brain interactions [47]. This might provide the key to future management of FMS.

Competing interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Ethics approval and consent to participate

Ethical approval for the study was obtained via a consortium under the Nuclear Technologies in Medicine and Biosciences Initiative (NTeMBI) of South Africa and ethical approval by Pharma Ethics Pty, Ltd, reference number 11064365). Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form.

Author contributions

This investigation required a multidisciplinary approach and the inputs of all authors were essential to produce the concept and final manuscript. CR and HM defined the aim of the study. The urine samples from the FMS patients and age-matched and family-related controls were provided by HM, who also performed all relevant clinical aspects, and acted as assistant promoter to BM. CR developed the experimental design, acted as the promoter for BM and arranged with HM for ethical approval for the study and for the collection of samples from the young controls. BM conducted all experimental analyses and compiled the clinical information provided by HM. SM and UE gave guidance and assessment on NMR data generation and spectral analyses. RW was responsible for critical evaluation of the analytical aspects of the clinical chemistry data and for their interpretation. MvR performed all the statistical analyses. CR was responsible for coordination and integration of inputs from the authors who contributed to the manuscript as presented here.

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Additional information

1 Supplementary Information (SI)

2 Raw GC-MS data matrix

References

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5.5 Conclusion

GC-MS is a powerful hyphenated analytical technique employed in metabolomics studies that allows one to qualitatively and quantitatively analyse a chemical mixture of interest. It consists of gas chromatography, for chemical separation, and mass spectrometry, for compound identification. In this chapter, a semi-targeted metabolomics study, using GC-MS as the analytical platform, was presented; wherein the aim was to discriminate FMS patients from controls. The study revealed that, indeed, FMS does possess a unique metabolic profile, and thus substantiates the findings presented in Chapter 4. Subsequently, it was identified that a perturbation in the gut metabolome prevails which indicates extensive dysbiosis in the patient group. Although validation studies on this finding need to be conducted, a plausible set of biomarkers are presented, thus shedding insight on the complex disorder that is FMS.

5.6 References

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CHAPTER 6: DISCUSSION AND FUTURE PROSPECTS

6.1 Discussion: Addressing the aims and objectives of the investigation

To recapitulate: FMS has an estimated prevalence of 2% in the general population with an estimated 3.2% prevalence in South Africa (Lyddell & Meyers 1992). Diagnosis of this disorder may take several years to be firmly established as there is no pathological basis on which to diagnose the disorder. Therefore, initial diagnosis is usually done by exclusion. FMS was first described by Wolfe and colleagues in 1990 when a set of criteria, called the ACR 1990 criteria, was defined for the diagnosis of this disorder. In 2010, 2011 and recently in 2016, the criteria were refined for diagnosis as primary care clinicians' main issue with the 1990 criteria was that the process of palpation of the tender points was not feasible in the primary care setting. Diagnosis had to be simplified, which resulted in the alternative 2010 fibromyalgia criteria, which became the preferred manner of FMS diagnosis – with the input actually being an entirely self-report by the patient, interpreted by the physician (Häuser & Wolfe 2012).

Thus, currently no definitive biologically-based biomarkers for FMS have been identified. Much research has been conducted on the genetic facet of the disorder and numerous scientific papers have been published on this topic. Since metabolomics is synonymous with biomarker discovery, it became a very attractive method to address this question of biomarker discovery for FMS. The application of metabolomics technology to the study of FMS is still new. In fact, since the initiation of this investigation, only three research articles (reviewed in Chapter 3) have been published on FMS in which metabolomics was the investigative method of choice.

As such, in this section, I will reflect on the aims and objectives of this investigation with emphasis on whether each was achieved and also briefly discuss the outcomes of each.

6.1.1 Aim: The application of metabolic profiling to the disorder, FMS

This was the primary goal of this investigation as no metabolic information had been reported on FMS in the literature. As previously mentioned, three metabolomics investigations were since published after the start of this investigation. As such, the action of applying a metabolomics approach to FMS was achieved in that I was able to distinguish FMS from non-pain controls. However, no global metabolic profile could be established

through the use of NMR and GC-MS metabolomics. My interpretation is that this is due to the multifactorial nature of FMS. Hence, the perturbation in FMS is not of a singular origin, as is the case of other metabolomics studies done in our laboratory, for example, isovaleric academia [which is an endogenous monogenetic disorder (Dercksen 2014)] and tuberculous meningitis [which is an exogenous infectious disorder (Mason 2016)].

6.1.2 Objective 1: Perform an explorative NMR metabolomics study to (1) elucidate the global urinary metabolite profile of patients suffering from FMS, and (2) explore the potential of this metabolite information to contribute to improved diagnosis of FMS

The NMR study was an initial study conducted to identify if a metabolic discrepancy due to pain could be detected in the urine of patients with FMS versus controls, without the discrimination of pre-selecting a particular set of metabolites to look at - in other words, a global study. This NMR study was successful in obtaining this information as it was vital for progression of the investigation. The exploratory NMR metabolomics study revealed an elevated profile for metabolites that point to a disturbance in the gut microbiome. Using this outcome of an altered metabolic profile, it was then possible for me to execute the second part of this objective, being to offer supplementary information on a possible pathophysiology of FMS and also further to contribute to a strategy for improved diagnosis of the disorder. Detailed outcomes are presented in Chapter 4.

6.1.3 Objective 2: Obtain complementary information on the metabolic profile of FMS patients using GC-MS technology. This was done by comparing affected individuals (cases) against those that are not affected (controls), through a semi-targeted study using GC-MS technology

The follow-up on the explorative NMR study led to a semi-targeted GC-MS metabolomics study, with emphasis on organic acids. Outcomes of this study are presented in Chapter 5 in the form of a peer-reviewed publication. The main findings from the GC-MS study were that dysbiosis seems to underlie an important aspect of the pathophysiology in FMS: First, it related to the gastrointestinal discomfort complained about by the majority of FMS patients and very much so (81%) in the cohort studied by myself. Second, the gut-metabolites observed in the GC-MS study, confirmed the outcomes of the NMR study, that hippuric and 2-hydroxyisobutyric acids direct to a gastrointestinal disturbance in FMS. The significance of 2-hydroxyisobutyric acid will be discussed in detail in the final section of this chapter. These findings are not unfounded as the complaints of FMS patients on gastrointestinal problems are a common clinical feature in IBS, a comorbid condition in FMS. Moreover, alterations in

the gut-brain axis (discussed in detail later in this chapter) is a chief perturbation in IBS and from the findings of this GC-MS study, we can deduce that it also plays a role in FMS.

6.1.4 Objective 3: Compare data from the GC-MS and NMR studies and identify a set of markers common to both studies that can be proposed as probable markers of the disorder

The data from both metabolomics studies conducted were compared and only one metabolite marker was identified as common to both studies, namely 2-hydroxyisobutyric acid (2-HIBA). According to the human metabolome database (Wishart *et al.*, 2013), 2-HIBA is an exogenous, contaminant metabolite seen in urine due to environmental exposure. As a result, 2-HIBA was initially discarded from our findings in the NMR metabolomics study, albeit its contribution as a distinguishing metabolite in the separations, observed. However, several scientific publications emerged to prove this to the contrary. Moreover, urinary 2-HIBA may even be linked to altered microbial–host symbiosis, indicating perturbed host energy metabolism and mucosal integrity due to altered gut microbiota functioning. As such, this could also be linked to the phenotype observed in FMS as dysbiosis was identified to be present in the disorder and that 2-HIBA was identified as a discriminatory metabolite. Against this background, we present a manuscript (as a future prospect in section 6.2), for publication in *Biomarker Research* in which we suggest 2-HIBA as an emerging biomarker in disease conditions, like FMS.

6.1.5 Objective 4: Formulate a hypothesis as to the metabolic effects, if any, FMS has on an affected individual

From this investigation, I was able to identify that metabolic changes are present in FMS. However, these changes that are observed are not, *per se*, as a direct result of pain itself but rather as a result of a continuum of disorders that present together to form the phenotype that is FMS. As such, in true metabolomics fashion, I was able to formulate a hypothesis which I put forward in section 6.3.

6.2 Future prospect 1: 2-HIBA as a potential biomarker of FMS

6.2.1 Background

I have observed 2-HIBA as one of the discriminating metabolites in the NMR metabolomics study of FMS (Malatji *et al.*, 2017). Our interpretation of this observation was:

“2-Hydroxyisobutyric acid, the most discriminatory variable between our FMS group and controls, is an apparent catabolic metabolite from gut microbionota and was shown to be statistically linked to *Faecalibacterium prausnitzii*, an important commensal bacterium of the human gut flora proposed to be an indicator of the dynamic basis of host–microbiome symbiosis”.

Subsequently, it appeared that 2-HIBA occurs as a potential biomarker in several clinical conditions, unrelated to FMS. These observations from the literature prompted an overview and re-assessment of our findings on 2-HIBA in FMS. For this purpose, data from the NMR metabolomics (Chapter 4) as well as the GC-MS data (Chapter 5) were used. We regard the information that emerged from this study as an important future application for FMS research. Herewith I present a copy of the manuscript that we submitted to *Biomarker Research* (part of the BMC publishing group) as one of my suggestions for future research. Note that the manuscript is presented in the format as per *Biomarker Research* journal's article submission guidelines.

6.2.2 Alpha-hydroxyisobutyric acid: An overview and focus on fibromyalgia syndrome

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Submitted: *Biomarker Research*

Abstract

Background

2-Hydroxyisobutyric acid (2-HIBA) is of unknown metabolic origin and appears to be a non-metabolite. Several metabolomics studies, however, have revealed 2-HIBA as a potential biomarker in a variety of conditions in health and disease. Thus, we firstly present an overview of ten of these unrelated investigations, followed by an evaluation of 2-HIBA observed in fibromyalgia syndrome (FMS) relative to three control groups, using untargeted NMR and semi-targeted GC-MS metabolomics data.

Methods and results

An overview of clinical studies reveals that 2-HIBA is a measurable indicator in several disease states. 2-HIBA has also been discovered as being an acylation agent of histones through lysine 2-hydroxyisobutyrylation – a potential new histone mark. Results on 2-HIBA in FMS have indicated a significant difference ($p < 0.0001$) between the FMS group relative to unrelated controls. The difference between the FMS and family-related group ranged from significant ($p = 0.01$) to insignificant ($p = 0.27$). Receiver Operating Characteristic analysis for the FMS patients relative to unrelated controls indicated a very good ($AUC > 0.9$) classification of the FMS patients. The classification of the FMS patients and the family member group was distinctly found to be poor ($AUC = 0.5$ to 0.7).

Conclusions

Taken together, the overview indicates 2-HIBA to be an emerging biomarker, measurable in several kinds of biological samples from unrelated disease conditions, including FMS. Longitudinal metabolomics studies may suggest 2-HIBA to be a product from the gut microbiome, an indicator of metabolic allostasis, or histone dynamics.

Keywords: Alpha-hydroxyisobutyric acid, 2-HIBA, biomarker, fibromyalgia syndrome, metabolic allostasis, gut microbiome, histone marker.

Overview

Alpha-hydroxyisobutyric acid (2-hydroxyisobutyric acid; 2-HIBA) was listed as one of 155 important metabolites in the pioneering publication on potential biomarkers for inborn errors of metabolism (IEM) [1, 2]. Today, outcomes from multiple metabolomics platforms and technologies include 2-HIBA as a confirmed metabolite that constitutes the human urine [3] and serum metabolome [4]. Views on 2-HIBA in urine samples from normal and diseased individuals are ambiguous. Urinary 2-HIBA is reported to be an endogenous co-metabolite with lactic acid in ketone body metabolism observed in IEM [5]. The emphasis in the Human Metabolome Database (HMDB) is that 2-HIBA is a metabolite of methyl tertiary-butyl ether (MTBE; CAS 1634-04-4) and tertiary-butyl acetate (TBAC; CAS 540-88-5) – both present in humans through environmental exposure. MTBE may be encountered by breathing air contaminated with gasoline fumes, causing several symptoms of respiratory irritation [6]. Along the same line, the US Environmental Protection Agency lists TBAC as an exempt volatile organic compound [7], mostly derived from adhesive industrial coatings and cleaning applications.

From these ambiguous views, 2-HIBA seems to be a non-metabolite and regarded to be of lesser biological or diagnostic significance, but views are beginning to change. During the last decade, several clinical and metabolomics studies, using different technology platforms, have revealed 2-HIBA among metabolite predictors on a variety of conditions in health and disease, summarized in Table 1. Chinese individuals served to model the microbial–host metabolic connectivity, which indicated 2-HIBA to be a marker metabolite derived from *Faecalibacterium prausnitzii* (*F. prausnitzii*), the most significant n-butyrate-producing gut bacterium, having a known effect on host energy metabolism and mucosal integrity [8]. Moreover, 2-HIBA was reported to be associated with several diseases having metabolic consequences: diabetes mellitus [9], lung [10] and gastric [11,12] cancer, myocardial infarctions in men and women [3], chronic kidney disease [14,15] and fibromyalgia syndrome (FMS) [16]. Interestingly, 2-HIBA was identified through an NMR metabolomics study to be an important metabolite that was increased in obese individuals [17], but could be normalized in obese children following a treatment with the VSL#3[®] probiotic [18]. None of the studies listed in Table 1 assessed 2-HIBA as a classifier, which is a key requirement for its predictive and diagnostic value. Here we present such an assessment of 2-HIBA for FMS.

Of particular interest, but not included in Table 1, are metabolomics studies on 2-HIBA that were complemented by genome-wide association studies (GWAS). A significant negative association was revealed between urinary 2-HIBA levels and SNP rs830124, an intronic SNP of the WDR66 gene on chromosome 12, which is closely associated with mean platelet volume [19, 20]. The regulatory potential of histones is affected by even small chemical differences through modifications of amino acid side chains. These variations may lead to very different functional outputs. A project on chromatin structure identified lysine 2-hydroxyisobutyrylation as such a new form of histone

modification [21], thus defining a new type of histone mark. The histone mark is conserved, widely distributed and induces distinct structural changes, adding to the putative role of 2-HIBA as a measurable indicator of perturbations affecting health and disease.

Table 1 Association reported for discriminating levels of 2-HIBA in healthy and diseased groups

Date	Experimental group and disease condition	Analytical technology	2-HIBA biomarker pattern Response / Disease / Control (Statistical significance)	Interpretation	Reference
2008	Chinese family	NMR / PCR Metagenomics	Qualitative presence of 8 urinary metabolites including 2-HIBA	2-HIBA as an indicator of the dynamic basis of host-microbiome symbiosis.	[8]
2009	Diabetic patients Healthy controls	GC x GC–TOFMS Metabolomics	[↑] in plasma	2-HIBA as one potential biomarker for diabetes mellitus pathophysiology	[9]
2010	Obesity / Lean	NMR Metabolomics	[↑] 12.1±3.8 / 9.8±2.1 (μmol/mmol Cr) (<i>p</i> < 0.001)	Significant functional disturbance in metabolic activity of the microbiome of obese individuals	[17]
2011	Lung cancers Healthy volunteers	GC-MS Metabolomics	[↑] FC = 1.35 (<i>p</i> < 0.0001)	Indicator that metabolites in serum were changed by the pathogenesis of lung cancer.	[10]
2013	Chronic Kidney Disease Adult controls	NMR 1° spectroscopy	[↑] 32 ± 15 μM Reference range: 7 (0-9) μM	2-HIBA is a novel uremic retention solute and it negatively influences cell physiology in CKD patients	[14]
2015	Obese children Clinical trial	NMR Corr. Spectroscopy	Time 0/4: 6.98 / 5.20 (μmol/mmol Cr) (<i>p</i> < 0.005 / 0.023 / 0.021)	Decreased 2-HIBA following probiotic treatment indicates it as an indicator of metabolic dynamics	[18]
2015	Myocardial infarction Male/Female controls	UPLC/Q-TOF MS Metabolomics	[↑] 32.1 / 39.5 <i>m/z</i> intensity [Male MI] (<i>p</i> 0.003 - MM/CM)	Increased serum 2-HIBA in MI is likely to be related to oxidative stress, ROS and inflammation	[13]
2016	Gastric cancer cohort Normal controls	NMR Metabolomics	[↑] VIP: 1.26; FC = 1.02 (<i>p</i> : 0.000 – adjusted: 0.000)	2-HIBA as one of 48 tissue metabolites that distinguish various stages of gastric cancer	[11]
2016	Chronic Kidney Disease Prospective cohort	CE-TOF-MS Metabolomics	[↑] Hazard ratio ~ 4; (<i>p</i> = 0.008; 3 rd Tertile: <i>p</i> = 0.013)	2-HIBA has predictive value on the outcome of CKD, [15] and was detected in 67.5% of cohort patients.	[15]
2017	Fibromyalgia Patients and controls	NMR Metabolomics	[↑] VIP: 6.26; FC = 1.56 (<i>p</i> = 0.0001)	2-HIBA seems to be from the gut, but with high biomarker values despite low urinary concentrations	[16]
2017	Gastric cancer Patients and controls	GC-MS Metabolomics	[↓] VIP: 1.05; FH = - 1.059 (<i>p</i> = 0.00326)	2-HIBA as a differential from multivariate analysis but not so from univariate statistical analysis.	[12]

Abbreviations: NMR – nuclear magnetic resonance; GC-MS – gas chromatography-mass spectrometry PCR – polymerase chain reaction GC-TOFMS – gas chromatography-time-of-flight-mass spectrometry; GC x GC: two dimensional gas chromatography; UPLC Q TOF MS ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry; CE: capillary electrophoresis; VIP: variable important in projection; Corr.: correlation; FC: fold change; CKD: chronic kidney disease; ROS: reactive oxygen species; Cr: creatinine; MI: myocardial infarction

2-HIBA in FMS

Regardless of the interest in 2-HIBA, as follows from Table 1, still little insight has been gained regarding its biological origin, distribution and regulation [15]. We regularly observe 2-HIBA in urinary metabolomics studies, but not significantly up- or down-regulated, although we reported on quantitative urinary concentrations of 2-HIBA from an NMR metabolomics study on FMS in middle-aged women [16]. Despite being present in a relatively low concentration (mean = 0.008 and 0.014 μmol 2-HIBA/ mmol creatinine in controls and patients, respectively), multivariate analysis indicated 2-HIBA as the most important metabolite that distinguished the patients from matched controls (FC = 1.56; VIP = 6.2). We then interpreted 2-HIBA as an apparent catabolic metabolite from gut *F. prausnitzii*, an indicator of the host–microbiome symbiosis, and disregarded further attention to the presence of 2-HIBA in FMS. However, the recent observations on 2-HIBA (Table 1) alerted us to a possible link between the gut microbiome and dysbiosis in FMS, as has recently been highlighted in other extra-intestinal diseases like FMS [22].

We therefore extended our previous research [16], by reporting on 2-HIBA in additional controls and expanded our ^1H -NMR analytical study with a complementary GC-MC metabolomics study. The three control groups were: a group of first-degree relatives of the FMS patients, unrelated age-matched controls and a group of young and healthy females. The additional observations indicated 2-HIBA to be important in FMS and strengthened the apparent importance of 2-HIBA in defining pathophysiological conditions, as suggested in the overview and summarized in Table 1.

Materials and methods

Study participants and sample collection

All our patients regularly attend a chronic pain practice in Pretoria and were previously diagnosed with FMS by a specialist pain clinician. The diagnosis was based on a comprehensive clinical assessment using the American College of Rheumatology (ACR) criteria [21] and clinical questionnaires. The patients selected for this study were confirmed with FMS ($n = 17$). Full socio-demographic, clinical and medication information was previously reported [16]. The three control groups were: first-generation family members of the patients (CF; $n = 11$), age-related individuals without any indications of FMS or related conditions (CO; $n = 10$), and healthy young (18–22 years) individuals (CN; $n = 20$ and 41 in the NMR and GC-MS studies, respectively). All participants were female. All cases complied with selection criteria, following exclusion of outliers based on statistical analysis. Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form. Ethical approval for the study was obtained via the consortium under the Nuclear Technologies in Medicine and Biosciences Initiative (NTEMBI) (ethical approval by Pharma Ethics Pty, Ltd, reference number 11064365). The study has been performed in accordance with the Declaration of Helsinki.

Conventional metabolomics data generation

Untargeted NMR metabolomics data were generated as previously reported for the FMS and age-matched controls [16]. Targeted GC-MS metabolomics data were generated through standard analytical procedures, shown to be repeatable and reproducible [24]. Identification of 2-HIBA was done in the GC-MS study through our in-house as well as a commercially available MS database (MS database (National Institutes of Standards and Technology (NIST) 17 EI MS library), and from the NMR spectral data through the resonance singlet at 1.44 ppm from the combination of its iso-methyl groups $[(CH_3)_2]$. Quantification of 2-HIBA was expressed as $\mu\text{mol}/\text{mmol}$ creatinine in both methods of analysis.

Statistical analysis

Univariate statistical analyses, including the Mann–Whitney test p -values (MW), were generated for the 2-HIBA concentrations in each experimental group, using the NMR as well as GC-MS data. The classification ability of 2-HIBA was assessed by applying a Receiver Operating Characteristic (ROC) analysis to the data mentioned. The ROC analysis was based on a simple two-group logistic regression model with the FMS patient group compared to each control group. Our interest was primarily in the discriminatory ability of 2-HIBA with respect to FMS and not between control groups. The values of the area under the ROC curve (AUC) provide a measure of how well 2-HIBA could distinguish between the FMS and each of the three control groups, respectively. To obtain an indication of how well 2-HIBA per comparison would potentially generalize, we assessed the classification ability with one sample is left out repeatedly, i.e. leave-one-out cross validation [AUC (LOO CV)].

Results

Urinary 2-HIBA levels in FMS patients and controls

A representative ^1H -NMR spectrum from an individual from the young control group (CN) is shown in Fig. 1. The highlighted region (1.425–1.450 ppm) contains the 1.44 ppm singlet used for identification of 2-HIBA and the zoomed box illustrates the median peaks – scaled according to creatinine – for FMS, CO, CN and CF, indicating the overall increase of 2-HIBA in FMS; and to a small degree in family-related controls (CF).

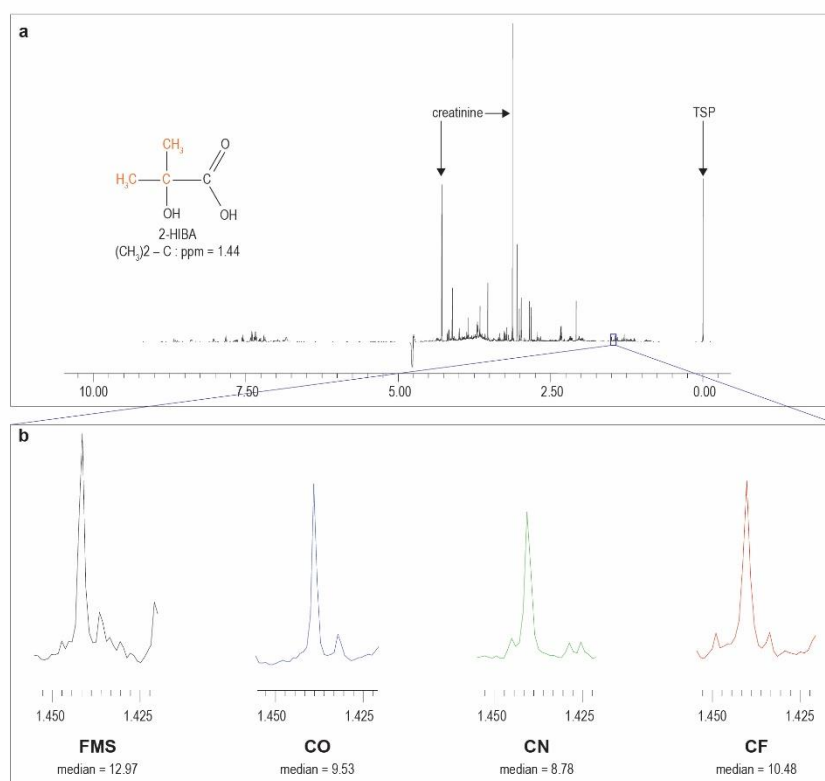


Fig. 1 A representative ¹H-NMR spectrum of human urine at pH 2.5 [TSP = 0.00s ppm; creatinine = 3.13s, 4.29s ppm]. Highlighted region (1.425–1.450 ppm) contains the 1.44 ppm singlet that represents the combination of the iso-methyl groups (indicated in red) from alpha-hydroxyisobutyric acid (alpha-carbon also in red). The Box indicates median peaks – scaled according to creatinine – for FMS, CO, CN and CF, with median values of 12.97, 9.53, 8.78 and 10.48 μmol 2-HIBA/mmol creatinine, respectively.

Concentrations of 2-HIBA for each individual in this study, arranged within their respective groups, are indicated in Fig. 2, as determined from the NMR (Fig. 2a), as well as the GC-MS analyses (Fig. 2b). The summary statistics of these comparisons are summarized in Table 2. For comparative purposes, we used the effect sizes (derived from the Mann-Whitney test statistic) as p-values are difficult to compare directly across applications of the Mann-Whitney test. Effect sizes greater than 0.5 are considered to represent practically significant group differences, while ES larger than 0.3 are considered practically visible. The urinary concentrations for 2-HIBA were higher as determined through the NMR analysis than through the GC-MS analysis. Such differences are not the exception, illustrated by extensive side-by-side platform comparisons on the serum metabolome which informed on the array of factors causing these differences [4]. Notwithstanding this, a significant difference between the values for 2-HIBA in the FMS group relative to the age-matched controls (CO) was found for the NMR (ES = 0.64), as well as the GC-MS (ES = 0.71) data. The respective differences relative to the young controls (CN) are likewise practically significant (NMR ES=0.73 and GC-MS ES=0.55, respectively). By contrast, the difference between the FMS and family-related group was

less significant, only practically visible, based on the NMR data (ES = 0.42) and insignificant (ES = 0.12) for the GC-MS data.

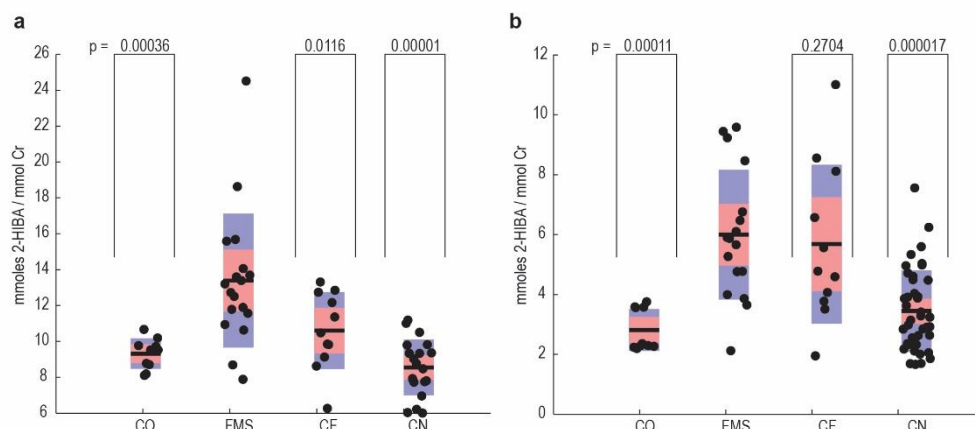


Fig. 2 Graphs showing urinary 2-HIBA for all individuals from all four groups. Concentration values were determined through NMR (a) and GC-MS (b) metabolomics, respectively. Values for all individual cases are shown as dots, while the squared area represents the 95% confidence interval (orange) and 1 standard deviation (blue) of the mean (black line). The *p*-values are indicated above the brackets between the respective groups, relative to the FMS group.

Table 2 Summary statistics on 2-HIBA determined for the FMS patients relative to all control groups.

Experimental Groups	M-W test p-value	Summary statistics		Mean FMS	SD FMS	Mean control	SD control
		B-H (5%)	ES				
¹ H NMR Quantified 2-HIBA data (μmol/mmol creatinine)							
FMS/CN	0.00001	0.05	9.73	13.390	3.733	8.548	1.550
FMS/CF	0.0116	0.05	0.43	13.390	3.733	10.600	2.145
FMS/CO	0.00036	0.05	0.64	13.390	3.733	9.138	0.847
GC-MS Quantified 2-HIBA data (μmol/mmol creatinine)							
FMS/CN	0.000017	0.00033	0.55	5.998	2.164	3.447	1.362
FMS/CO	0.000112	0.00029	0.71	5.998	2.164	2.814	0.698
FMS/CF	0.270426	0.00104	0.12	5.998	2.164	5.681	2.650

Classification analysis

The classification ability of 2-HIBA was based on a logistic regression model by using a Receiver Operating Characteristic (ROC) analysis for the FMS patients relative to each of the three control groups, as well as for data from both methods of analysis (NMR and GC-MS). The values of the area under the ROC curve (AUC) provided a measure of how well this combination could distinguish between the two groups. A value of $AUC = 1$ represents a perfect test, while a cursory guide for classifying the accuracy of a diagnostic test is given by: $AUC = 0.90-1$ (excellent, i.e. high sensitivity and high specificity); $0.80-0.90$ (good); $0.70-0.80$ (fair); $0.60-0.70$ (poor); $0.50-0.60$ (fail). To provide some indication of how well the model would potentially generalize, the classification ability was tested with one sample is left out repeatedly — in other words, based on a leave-one-out cross-validation strategy (AUC (LOO-CV)). The graphic outcomes of these analyses are shown in Fig 3. The classification ability of 2-HIBA for the FMS group relative to the age-matched controls was good ($AUC = 0.89$) to excellent ($AUC = 0.93$) for the NMR and GC-MS data, respectively. The respective classification values were in the same category for the FMS group relative to the young controls ($AUC = 0.93$ and 0.83 for the NMR and GC-MS data, respectively). These values were supported by the outcome of the LOO-CV analysis. The outcome on the classification of the FMS patients and their family members (CF-group) was distinctly different: the classification ability was fair ($AUC = 0.76$) for the NMR data but failed (0.57) for the GC-MS data. These observations were strengthened by the LOO-CV analysis. Importantly, the AUC statistic provides information on the classification ability of the associated logistic regression model. The validity of the model was also assessed based on the goodness-of-fit (i.e. will a different or more complicated model performs better). Goodness-of-fit statistics are provided and discussed in the SI (Table S1 in *SI to Article 3*).

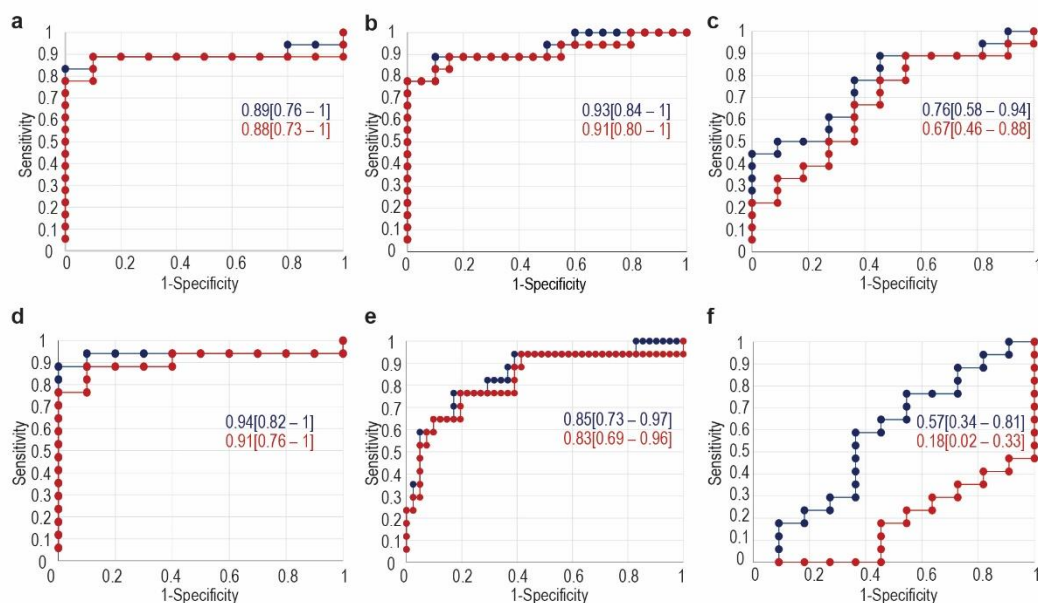


Fig. 3 ROC analyses of 2-HIBA to be indicative of FMS biomarker. (a to c) ROC analyses for discriminating FMS patients from CO (a and d), CN (b and e) and CF (c and f) controls based on NMR (a to c) and GC-MS data (d to f). Classification of cases and the respective AUC values are indicated in red, and for the LOO-CV analysis in blue, with their respective 95% confidence intervals in square brackets.

Discussion

2-HIBA has been found to be increased in several disease conditions (Table 1), including FMS in middle-aged females [16]. No classification ability for 2-HIBA was reported in these investigations. Here, we have indicated that in a potential biomarker model, 2-HIBA, could yield an AUC [95% Confidence Interval] for FMS of 0.89[0.76-1] and 0.94[0.82-1] relative to healthy, age-matched female controls through NMR, as well as GC-MS analysis, respectively. These observations demonstrate that the clinical applicability of metabolic profiling for FMS diagnosis shows great promise and should be explored further. From the information presented in the overview, it may be speculated that 2-HIBA is an emerging biomarker for a variety of disease conditions, although no common aetiology for such a biomarker role emerged from these reports. Nonetheless, at least three lines of thinking, on such a role, can be distinguished.

First, 2-HIBA was shown to be linked to *F. prausnitzii*, one of the symbiotic human gut microbes [8]. Additionally, the group of Miccheli [17] indicated through an NMR-based metabolomic analysis that a combination of 2-HIBA and other gut flora-derived metabolites contributed to a classification model that discriminate between obese and lean controls, which was later extended to inclusion of 2-HIBA in a group of urinary metabolites ('biomarkers'), which indicated clinical improvement in children

suffering from non-alcoholic fatty liver disease [18]. Lastly, our preferred interpretation of 2-HIBA, the most discriminatory variable between our FMS group and controls, was likewise for 2-HIBA being a metabolite from gut microbiota [16].

Second, 2-HIBA has not, hitherto, been identified as a biomarker for an IEM, but high concentrations of 2-HIBA, 3-hydroxyisovaleric and 2-methyl-3-hydroxybutyric acids were found in the urine from patients presenting with ketoacidosis, which was proposed to be associated with derangement of the metabolism of the three branched-chain amino acids [4]. Furthermore, urinary 2-HIBA is known to be observed in conditions like severe ketosis (highly increased lactic, 3-hydroxybutyric, acetoacetic and 2-hydroxyisovaleric acids), but not necessarily directly correlated with these markers in time – 2-HIBA seems to peak in the second phase following the severe clinical presentations [26, 27]. It thus appears that a distinction is warranted between a primary biomarker (diagnostic indicator) and a secondary biomarker (perturbation biomarker). We speculate that 2-HIBA might be a perturbation indicator, associated with allostasis due to metabolic stress. A recent perspective in this regard proposed the concept of ‘mitochondrial allostatic load’ [29] to define the deleterious structural and functional changes mitochondria undergo in response to perturbed metabolite levels and stress-related pathophysiology – a concept that could provide insight on the association between increased 2-HIBA seen in cancers, diabetes, obesity and related disease conditions, summarized in Table 1.

Third, the observation of 2-HIBA being a histone mark through lysine 2-hydroxyisobutyrylation, remains to be considered [21]. The histone mark is conserved and widely distributed, has high stoichiometry and apparently induces large structural changes. These findings suggest its critical role on the regulation of chromatin functions and place further emphasis of a putative role of 2-HIBA on metabolism in health and disease. In this regard the comparable levels of 2-HIBA between the FMS patients and their relatives (Fig. 2 and Table 2) as well as the poor value of the discrimination between the FMS and CF groups (AUC: 0.76[0.58-0.94]; 0.57[0.34-0.81], for the NMR and GC-MS data, respectively in Fig. 3), raise a different question: “Could 2-hydroxyisobutyrylated lysine produce increased 2-HIBA seen in some perturbed conditions?” A directive undertone for this question is the increasing evidence that alteration of the histone epigenome is one of the earliest steps in oncogenic transformation [30, 31], and strongly associated with cancer homeostasis [32].

Taken together, the overview presented here indicates 2-HIBA to be increased in faecal and urine samples [8], blood [9,13,14,15], serum [10], surgical tissues specimens [11] and urine [12,16,18] during several disease conditions. 2-HIBA is proposed to be a potential biomarker in diabetes [9], gastric cancer [12], non-alcoholic fatty liver disease [12], chronic kidney disease [14] and for FMS as shown here. We propose that 2-HIBA may be an exogenous product from the gut microbiome or may be of endogenous origin related to allostasis due to perturbed metabolic homeostasis. Longitudinal metabolomics studies may provide explorative insights to direct further studies on 2-HIBA and disease.

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Available data on the samples

The urine samples from the FMS patients and age-matched and family-related controls for the original research [16] were provided by Helgard Meyer. Raw NMR spectral data (Excel format) normalized relative to the CH₃ singlet of creatinine at 3.13 ppm, given as an electronic file (Additional File 1-NL_FMS_2.5 - Excl 3 patients.xls) is attached online as part of the Supplementary Information in Malatji, BG, Meyer, H, Mason, S, Engelke, UFH, Wevers, RA, van Reenen, M, and Reinecke, CJ (2017) A diagnostic biomarker profile for fibromyalgia syndrome based on an NMR metabolomics study of selected patients and controls, *BMC Neurology* 17:88-103 DOI 10.1186/s12883-017-0863-9. GC-MS data for 2-HIBA will be included in the Supplementary Information of this paper, once the paper is accepted for publication.

Ethics approval and consent to participate

Ethical approval for the study was obtained via a consortium under the Nuclear Technologies in Medicine and Biosciences Initiative (NTEMBI) of South Africa and ethical approval by Pharma Ethics Pty, Ltd, reference number 11064365). Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form.

Author contributions

This investigation required a multidisciplinary approach and the inputs of all authors were essential to produce the concept and final manuscript. CR and RW defined the aim of the study. CR developed the experimental design, acted as the promoter for BM and arranged for ethical approval for the study and for the collection of samples from the young controls. BM conducted all experimental analyses and compiled the clinical information provided by HM. SM and UE gave guidance and assessment on NMR data generation and spectral analyses. RW was responsible for critical evaluation of the analytical aspects of the clinical chemistry data and for their interpretation. MvR performed all the

statistical analyses. CR was responsible for coordination and integration of inputs from the authors who gave their approval of the version of the manuscript as submitted and agreed to the accountability requirements.

Competing interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Abbreviations

2-HIBA - 2-hydroxyisobutyric acid; IEM - Inborn errors of metabolism; HMDB - Human Metabolome Database; MTBE - methyl tertiary-butyl ether; TBAC - tertiary-butyl acetate; GWAS - genome-wide association studies; ACR - American College of Rheumatology; NTeMBI - Nuclear Technologies in Medicine and Biosciences Initiative (NTeMBI); NIST - National Institutes of Standards and Technology; MW - Mann–Whitney; ROC - Receiver Operating Characteristic; AUC - area under the ROC curve; AUC LOO CV – area under the ROC curve leave-one-out cross-validation; NMR – nuclear magnetic resonance; GC-MS – gas chromatography-mass spectrometry; VIP – variables important in projection; FC – fold change; Cr – creatinine; SNP – single nucleotide polymorphism

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6.3 Future prospect 2: FMS as a function of altered microbe gut-brain axis

The gut-brain axis (GBA) is the bidirectional communication system between the brain and the gut in humans (Mayer *et al.*, 2015; Raskov *et al.*, 2016) and regulates functions such as gut motility and appetite. It involves the CNS, enteric nervous system (ENS), autonomic nervous system (ANS) and the GI. GBA is a well-known phenomenon involved in disorders like IBS and functional GI disorders like small intestinal bacterial overgrowth (SIBO) (Mayer *et al.*, 2015) and is also speculated to play a role in chronic pain (Amaral *et al.*, 2008). When an individual is in a healthy state, the gut communicates with the CNS in an autonomous fashion, but when a pathological state is assumed, the somatic sensory system may be signalled and cause symptoms such as discomfort, nausea and pain as seen in FMS patients, while CNS output by means of the ANS can cause gastrointestinal dysfunction. When alterations (disruptions) in the symbiotic interactions between the host and microbiota occur, known as dysbiosis as discussed in Chapter 5, it can negatively influence the GBA (Raskov *et al.*, 2016; Slim *et al.*, 2015).

To recapitulate: Dysbiosis is a microbial imbalance in which, in the case of the gut, the normal (beneficial) dominating species of the gut flora become replaced with other gut microbial species not usually dominating in the gut, to fill the void (Tamboli *et al.*, 2004). Dysbiosis can occur as a result of overexposure to antibiotics and diet that cause changes in bacterial metabolism and allow overgrowth of potentially pathogenic microorganisms that release toxic products that underlie a cause of many chronic and degenerative diseases (Hawrelak & Myers 2004).

Dysbiosis is associated with IBS, a comorbid disorder of FMS, but can it be associated with pain generation or modulation? Amaral *et al.* (2008) showed, using a germ-free mouse model, that the gut-microbiota is necessary for the mice to develop inflammatory hypernociception, proving that indeed there exists a link between the gut and pain. This will further be elaborated on below, regarding a microbiome gut-brain axis model.

The GBA is comprised of a complex group of interacting systems, being the CNS, ANS, ENS and the GI tract. The CNS regulates the GI tract and ENS via the ANS, which is composed of the sympathetic and parasympathetic nervous systems (Mayer *et al.*, 2015). The ANS conducts afferent signals from the gut lumen to the CNS via the enteric, spinal and vagal pathways (of which the vagus nerve serves as the chief communication pathway between the gut and the brain). Stress is also a contributor to gut microbiota alterations though an increase in the parasympathetic output to the small and large intestine and a reduction in vagal output to the stomach. Neurotransmitters 5-HT, somatostatin, dopamine, neuropeptide Y, peptide YY, cholecystokinin and corticotropin-releasing factor are responsible for

signalling between bacteria and epithelial cells in the gut (Raskov *et al.*, 2016). The gut can also activate the immune system via action of the ANS in two ways. Direct activation occurs by means of gut immune cells (e.g macrophages and mast cells) reacting to luminal bacteria with antimicrobial peptide and indirect activation by means of modifying accessibility of the gut immune cells to the luminal bacteria (Mayer *et al.*, 2015). This action may account for the presence of inflammatory cytokines observed in the serum of FMS patients even though FMS is not an inflammatory disorder. The ENS functions autonomously and can produce more than 30 different neurotransmitters. The enterochromaffin cells (ECC) of the GI tract are the main producer of dopamine and 5-HT, while the remainder is produced by the myenteric neurons and mast cells. In the distal gut, production of 5-HT is increased by the gut microbiota by means of the action of the short-chain fatty acids (e.g propionate, acetate, butyrate and 4-hydroxybutyrate [observed through my GC-MS data]), which are end products of fermentable carbohydrates that have been broken down in the colon, on ECC (Raskov *et al.*, 2016; Mayer *et al.*, 2015). The levels of 5-HT (as well as nitric oxide and substance-P) can also be influenced by alterations in the gut microbiota (Raskov *et al.*, 2016), which may account for the unknown increase of substance P observed in the CSF of FMS patients in other studies.

Against this background, it is possible to see that there exists a large nervous system component to FMS, which can substantiate the central sensitization theory currently postulated as FMS pathophysiology. Yunus was one of the earliest authors to speculate, FMS as a dysfunction of various components of the CNS (Yunus 1992). Petzke and Clauw (2000) went on to show that specifically the sympathetic nervous system seems to underlie the pathophysiology in FMS. Our results from the study presented in Chapter 5 shows that through the GBA, the gut microbiota (as dysbiosis), also plays a role in the pathophysiology of FMS in synergy with the ANS. In a recent review by Martinez-Martinez *et al.* (2014), it was shown that 65% of the literature they reviewed support the hypothesis that the sympathetic nervous system is the predominant and common dysfunction in FMS and its comorbid syndromes.

From the discussion thus far, it seems that a bidirectional model (brain-gut axis / gut-brain axis) may actually be operative in the interaction between the nervous system and the digestive system (Mayer 2011). It has been proposed that in IBS the gut-brain axis may be dominating, with dysbiosis an important determinant in this model (figure 6.1)

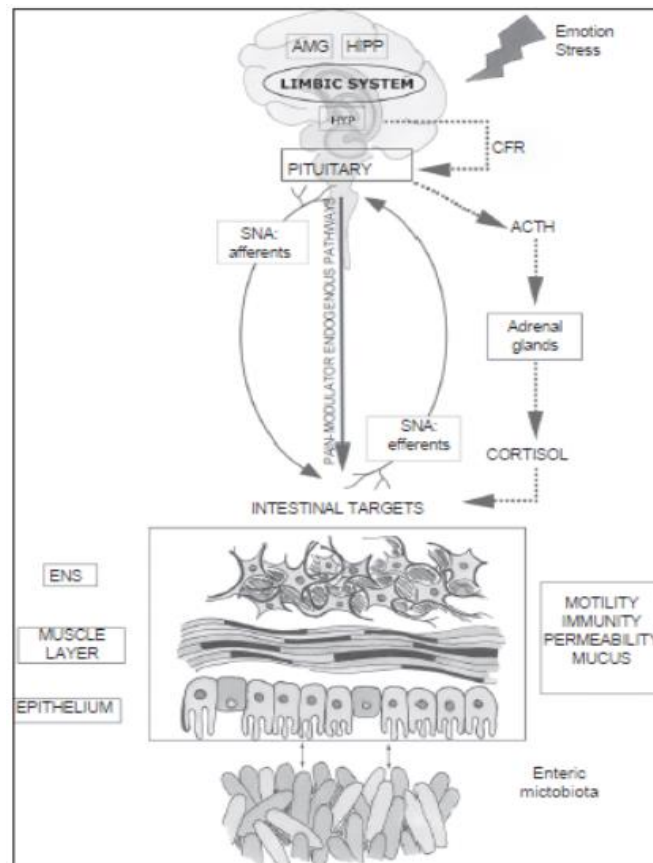


Figure 6.1: The structure of the bidirectional microbiome gut-brain axis. The central nervous system can be activated in response to environmental factors, such as emotion or stress. Hypothalamic (HYP) secretion of the corticotropin-releasing factor (CRF) stimulates adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland that, in turn, leads to cortisol release from the adrenal glands. In parallel, the central nervous system communicates along both afferent and efferent autonomic pathways (SNA) with different intestinal targets such as the enteric nervous system (ENS), muscle layers and gut mucosa, modulating motility, immunity, permeability and secretion of mucus. The enteric microbiota has a bidirectional communication with these intestinal targets, modulating gastrointestinal functions and being in itself modulated by brain-gut interactions (reproduced with permission from Carabotti *et al.*, 2015).

I speculate that a microbe gut-brain axis model may contribute to the pathophysiology underlying FMS, based on three points of view:

(1) Comorbidity with IBS

It is known that IBS is comorbid with FMS and it has been shown that the GBA alteration plays a key role in its pathophysiology (Mayer *et al.*, 2015; Kennedy *et al.*, 2014). Naturally, FMS should also possess a GBA malfunction element by association.

(2) Perturbed metabolic profile in FMS, largely influenced by excretion of urinary microbial metabolites (as presented in our manuscript in Chapter 5).

Both the NMR and GC-MS studies conducted revealed a perturbed metabolic profile in FMS versus controls, and a number of microbial metabolites were identified as discriminatory metabolites for the disorder. These show that dysbiosis, due to altered gut microbial activity, is present in FMS. 2-HIBA was identified as common to both studies and can thus be considered as a marker in FMS as postulated above in the provisional manuscript in section 6.2.

(3) Probiotics in the treatment of FMS-Future directive?

Supraha *et al.* (2013) suggested a protocol wherein administering probiotics (live organisms) in FMS may have a beneficial effect by alleviating the gastrointestinal symptoms, and thus the pain too, experienced in the disorder. They proposed this intervention (as opposed to many of the current interventions, which are mostly pharmacological) focus almost exclusively on the pain element of FMS (with limited to moderate positive effect), and all other symptoms being largely ignored. Moreover, manipulation of the microbiota has been confirmed to have a positive effect on alleviation of symptoms like abdominal pain and bowel movement habits in other conditions, like IBS (Kennedy *et al.*, 2014).

Against this background, I formulated a hypothesis as follows:

“A continuum in comorbidities (CFS-IBS-FMS-CRPS) may be defined by metagenomics of gut microbiome and metabolomics of host metabolites”

Accumulating evidence in the literature shows that the gut microbiome plays a critical role in the GBA. It has been speculated that the effects observed as a result of the alterations on the GBA may be strain specific (Carabotti *et al.*, 2015). Thus, if scientists were to identify the exact make-up of the gut microbiome – through metagenomic studies – this, together with metabolomics, could aid us in elucidating the exact microbe responsible for a dysregulated GBA.

Another future directive/suggested therapy for FMS, with regard to manipulation of the gut microbiota, is fecal microbiota transplantation (FMT). FMT is the infusion of fecal matter, in the form of a suspension, from a healthy individual into the GI tract of an individual to cure disease linked to dysbiosis (Aroniadis & Brandt 2013). This treatment has in fact been administered with success in IBS patients (Borody & Khoruts 2012; Smits *et al.*, 2017). FMT as a possible therapy may help alleviate the GI symptoms seen in FMS, thereby assisting with pain reduction and GBA communication.

A commentary published by Komaroff (2017) states: “*On one hand, it now is clear that there are objective abnormalities in patients with CFS: abnormalities that standard laboratory tests do not measure. On the other hand, not all of these reported abnormalities have yet been repeatedly confirmed. On one hand, it is clear that the underlying pathology involves the nervous system, energy metabolism, and the immune system. On the other hand, it is not clear what ties together the pathology seen in these different systems, nor which of the abnormalities came first, or what triggered that first abnormality*”. The same can be said for FMS. I speculate that in FMS pain is the onset symptom. Due to multiple doctor’s visits and no cause for the pain being identified, the patient feels discouraged and stress sets in. It has been proven that stress can affect and initiate GBA alterations (Carabotti *et al.*, 2015; Mayer *et al.*, 2015), thus affecting the microbiota leading to dysbiosis. This then feeds back to the brain as GBA is a bidirectional platform leading to exacerbation of the symptoms observed in FMS.

In conclusion: FMS is a complex disorder whose pathophysiology, to date, has still not been elucidated although ongoing research is providing more information to substantiate the phenotype that is FMS. In this investigation an altered metabolic profile was observed in FMS. No global urinary profile could be achieved due to the multifactorial nature of FMS in that there is not a singular origin for the perturbation that occurs in the disorder. No putative biomarkers were identified for FMS, however markers that gave additional information on the possible pathophysiology underlying the disorder were identified. We were able to develop an algorithm that we propose can be used together with the current diagnostic methods to classify patients. Dysbiosis was identified to be present in FMS and consequently a GBA element is also thought to play a role in FMS. This indicates that the CNS and its branches play a large role in the pathophysiology of FMS. The findings obtained in this investigation require follow up validation studies in a larger cohort as the one used in the present investigation was small.

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ADDENDUM

Addendum A1: Genotyping analyses conducted on blood samples from FMS patients and controls

Blood samples were taken from each of the participants for genotyping studies. From the literature, we know that there are several polymorphisms associated with the pathogenesis of FMS. For this reason, each participant was genotyped for the four polymorphisms referred to in the literature and in Chapter 3 (namely, the *5-HT_{2A}* serotonin receptor polymorphism, *SLC6A4* serotonin transporter polymorphism, *COMT* polymorphism and the *DRD4* dopamine receptor exon III repeat polymorphism). The genotyping studies were performed by Dr HP Mbongwa, a post-doctoral fellow formerly in the NWU Biochemistry Department and co-worker in this investigation. A conventional polymerase chain reaction (PCR) was used to execute these analyses. This method was not sensitive enough for the *DRD4* and *SLC6A4* polymorphisms, however, and the use of real-time PCR was suggested, by Dr Mbongwa, as an alternative method for characterizing these two polymorphisms, as it is more sensitive than conventional PCR. However, Dr Mbongwa left for the University of KwaZulu-Natal (UKZN) at the end of 2011, so further optimization of this PCR method for these polymorphisms could not continue. For this reason, all patients and controls were genotyped for only the *5-HT_{2A}* and *COMT* polymorphisms.

For each polymorphism there are three possible genotypes, depending on the alleles each person possesses. These genotypes are wild type (WT), heterozygous (He) and homozygous (Ho). Figures A1 and A2 show the agarose gel resolution results, depicting what each genotype should look like for classification after a digestion reaction using the restriction enzymes *MspI* and *NlaIII* for both *5-HT_{2A}* and *COMT*, respectively. These gels were produced by Dr Mbongwa.

Serotonin receptor 5-HT_{2A} (rs6313)

- ▶ 102T/C
 - T/T = 342bp (homozygous)
 - T/C = 342, 215 and 126bp (heterozygous)
 - C/C = 215 and 126bp (wildtype)

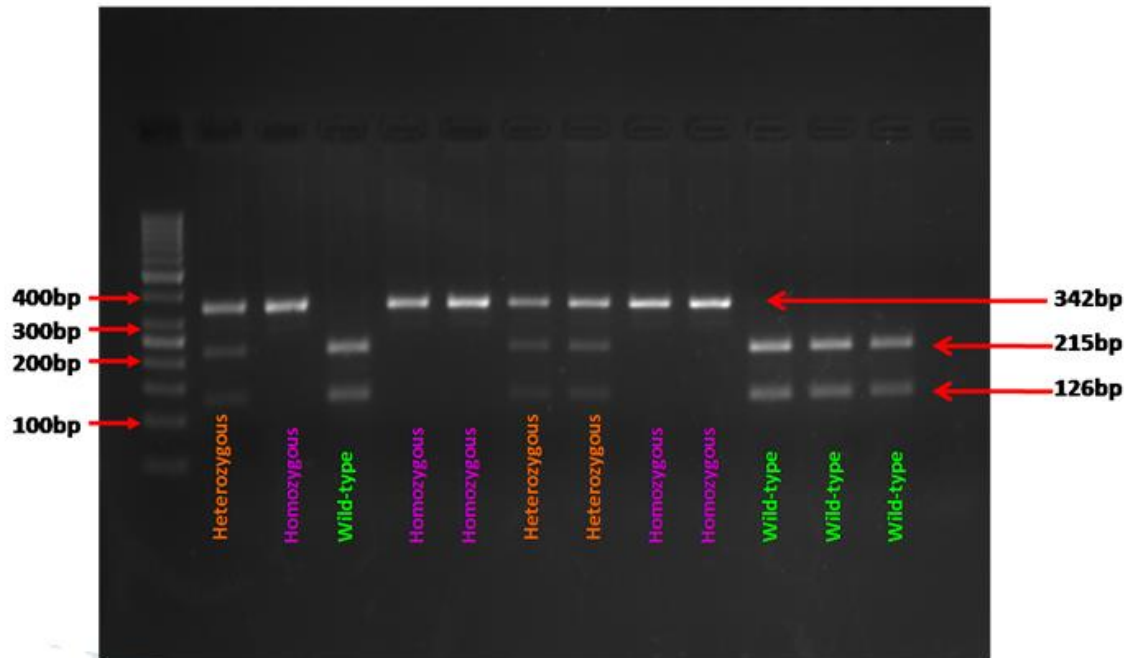


Figure A1: Agarose gel (3%) resolution, for the 5-HT_{2A} polymorphism, of 12 of the CN control group of experimental subjects after digestion with the *MpsI* enzyme. The size of the undigested band (which serves as a digestion control, not shown as it is similar to and lies at the same position as the homozygous genotype) is 342 base pairs (bp). If after digestion the band is intact, it means that the subject is homozygous for this particular polymorphism. If after digestion the sample contains 2 bands (namely, at 215 bp and 126 bp) or 3 bands (namely, at 342 bp, 215 bp and 126 bp), it means that the subject is classified as wild type or as heterozygous, respectively.

COMT (rs4680)

- ▶ 185 bp G/A
 - G/G = 114, 36 and 35bp fragments
 - G/A = 114, 96, 36, 35 and 18bp fragments
 - A/A = 96, 36, 35 and 18bp fragments

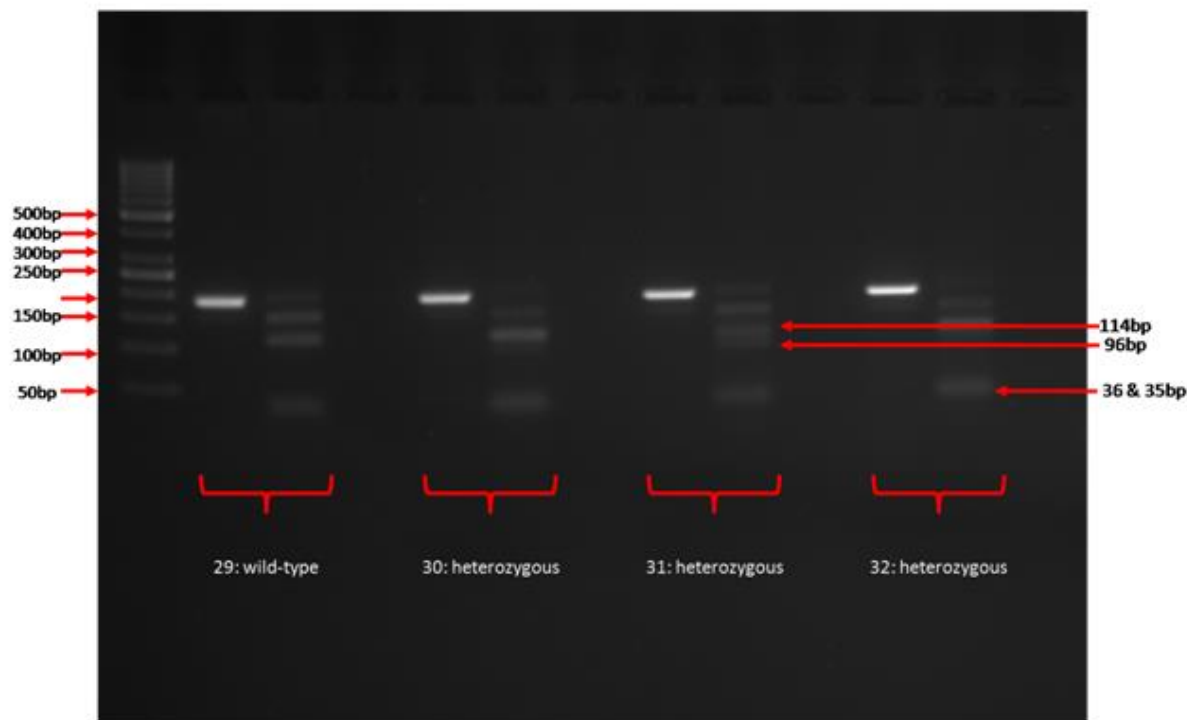


Figure A2: Agarose gel (3%) resolution, for the *COMT* polymorphism, of 4 experimental subjects from the CN group after enzymatic digestion with *Nla*III. The size of the undigested band is 185 bp (represented by the first lane of each sample). The 18 bp band is too small to be visualized on the gel and hence there is no band at the 18 bp mark for any of the samples. After digestion, a wild-type genotype will have bands at the 114 bp, 36 bp and 35 bp that correspond with those on the molecular ladder in the first lane. The latter two bands are very close in bp fragment size so they lie adjacent to each other in the gel. A slightly clearer view of these two bands can be seen in sample 32, which represents the heterozygous genotype. Heterozygotes, after digestion, will have bands at the 114 bp, 96 bp, 36 bp, 35 bp and 18 bp marks. No homozygous individuals were observed but, after digestion, should possess bands at the 96 bp, 36 bp, 35 bp and 18 bp marks.

In 2013, preliminary supervised (PLS-DA) and unsupervised (PCA) statistical analyses were conducted on the GC–MS data that were produced from the laboratory analyses. Pairwise comparisons were conducted for the FMS-pre (urine samples taken prior to pressure point analysis) and the controls (namely, CF, CN and CO). Subsequent results revealed that a partial natural separation could be observed in the PCA results of the FMS-pre vs CF and FMS-pre vs CN comparisons; a total separation for FMS-pre vs CO was observed (results presented in Chapter 1). The PLS-DA analyses further revealed a distinctive separation for

only the FMS-pre vs CO pairing. These statistical analyses were conducted using the mixOmics package from the R program. Figure A3 shows the outcome of the PLS-DA analysis for the FMS-pre vs CO comparison.

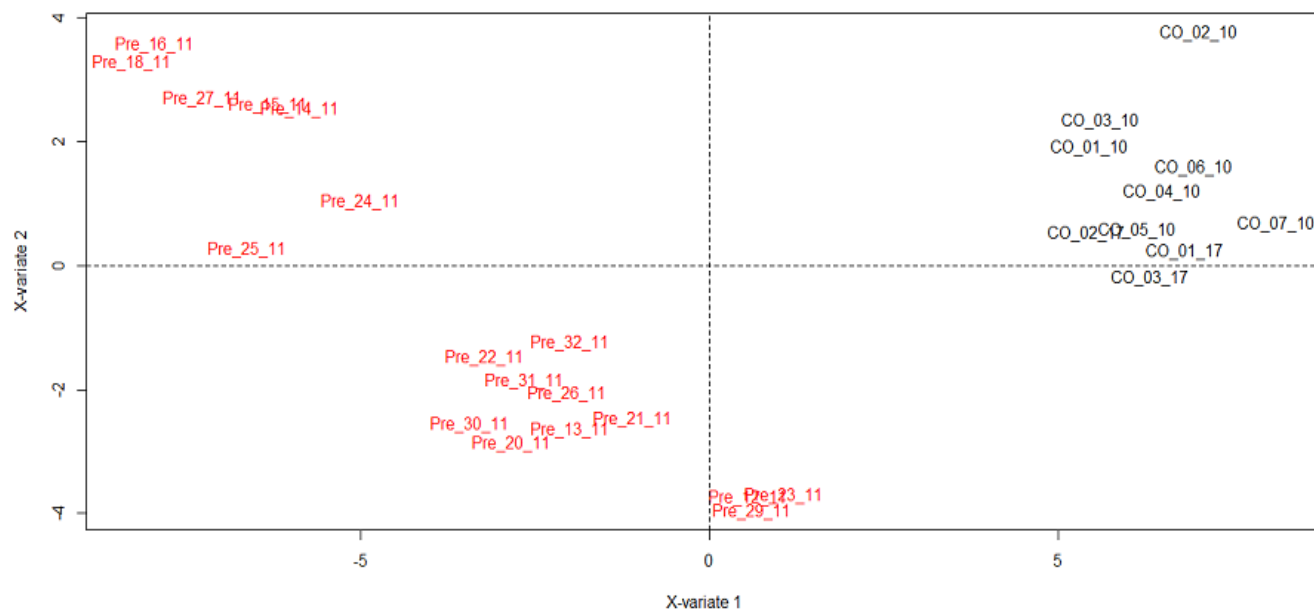


Figure A3: Two-dimensional PLS-DA representation of the urinary organic acids for the FMS patients Pre (red) versus the CO (black) control group.

On visual inspection of the PLS-DA, I observed that the patient (red) group results gathered into clusters. It was speculated that this phenomenon occurred as a consequence of the genotype polymorphism associated with FMS pathogenesis. This notion was investigated, as all experimental subjects were genotyped for the *5-HT_{2A}* and *COMT* polymorphisms, and the results of this endeavour are tabulated in Table A1. This table was constructed to determine if this clustering was due to the polymorphic identity of the patients

Table A1: Table of the polymorphic identities of the clusters observed in the PLS-DA score plot (see Fig. A3 above) for each of the genotyped polymorphisms, *5HT_{2A}* and *COMT*, associated with FMS. Abbreviations: W = wild type, He = heterozygote, Ho = homozygote.

Upper cluster	5HT _{2A}	COMT	Middle cluster	5HT _{2A}	COMT	Lower cluster	5HT _{2A}	COMT
Pre 16	W	He	Pre 32	He	W	Pre 12	W	W
Pre 18	He	W	Pre 22	W	He	Pre 23	He	W
Pre 27	Ho	W	Pre 31	W	He	Pre 28	W	W
Pre 15	He	He	Pre 26	He	He	5HT2A: W:45% He:33% Ho:22% COMT: W:61% He:39% Ho:0%		
Pre 14	He	W	Pre 21	W	W			
Pre 24	Ho	W	Pre 30	Ho	He			
Pre 25	W	He	Pre 13	Ho	W			
			Pre 20	W	W			

Table A1 shows the polymorphic identities of the patient samples for each of the polymorphisms, *5HT_{2A}* and *COMT*, associated with FMS. From this table we note that there was no uniform clustering as a consequence of the polymorphic identity. From this observation it can be noted that polymorphic identity does not appear to be related to the metabolomic profiles of the FMS patients. As a result, it was concluded that polymorphic identity does not appear to influence the metabolic profile of FMS patients and, therefore, in the pathophysiology of FMS. For this reason, further studies on polymorphisms, and their correlation with FMS, were stopped.

Addendum A2: GC-MS standard operating procedure

In this section, the laboratory standard operating protocol (SOP) for general organic acid analysis of urine by GC-MS is presented.

Overview

The 3 principal steps for this method are:

1. Isolation of the organic acids from physiological fluids
2. Formation of volatile derivatives
3. GC-MS analysis.

Organic acids are isolated from physiological fluids with ethyl acetate and diethyl ether extractions. The organic acid extract is evaporated to dryness under nitrogen; volatile trimethylsilyl (TMS) derivatives of the extracted organic acids are formed by heating with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The TMS derivatives are less than ideal products for some classes of compounds such as acylglycine, which form mono and di-TMS derivatives, yet they are the most useful and versatile compounds for the wide range of functional groups in organic acids. The derivatives are analysed on GC-MS.

GC-MS is able to separate the highly volatile organic acids using gas chromatography, followed by detection of individual components by means of mass spectroscopy. This procedure permits rapid identification and quantification of constituent organic acids with a high degree of sensitivity and chromatographic resolution.

Reagents

NOTE: refer to chemical information sheet below for descriptions and precautions/hazards of chemicals used.

Internal standard (4-phenylbutyric acid solution) prepared by measuring precisely 26.25 mg 4-phenylbutyric acid, adding 3 drops of 1M sodium hydroxide (NaOH) to dissolve and adding 50 ml distilled H₂O (dH₂O). Other reagents include: 5M hydrochloric acid (HCl); ethyl acetate (HPLC grade), distilled once to purify further; diethyl ether (HPLC grade), distilled once to purify further; anhydrous sodium sulphate (Na₂SO₄); bis(trimethylsilyl)-trifluoroacetamide (BSTFA); trimethylchlorosilane (TMCS); pyridine and hexane.

Instrument Settings

Gas chromatography (GC)

GC	Agilent 7890A
Auto-sampler	Agilent 7693
Oven program	50°C for 1 min; then 20°C/min to 60°C; then 5°C/min to 120°C; then 7°C/min to 280°C
Run time	40.35 min
Post run	1 min at 300°C
Injection volume	1 µl
Pre-injection washes	Solvent A: 2 x 4 µl Solvent B: 0
Post-injection washes	Solvent A: 1 x 4 µl Solvent B: 2 x 4 µl
Front inlet	Heater: 280°C Carrier gas: Helium Total flow: 15.29 ml/min Split ratio: 12:1 Split flow: 11.34ml/min
Column	DB-1MS 340°C: 30 m x 250 µm x 0.25 µm

Mass spectrometry (MS)

MS	Agilent 5975C VL MSD
Solvent delay	7 min
Acquisition mode	scan
Scan parameters	Low mass: 50.0 High mass: 600.0 Threshold: 15
MS source	230°C (max 250°C)
MS quad	150°C (max 200°C)

Organic acid extraction

1. Add 1 ml sample to large kimax test tube
2. Add 6 drops 5M HCl to adjust to pH 1 (using glass pipette)
3. Add creatinine-based calculated volume of internal standard

4. Add 6 ml ethyl acetate
5. Cap test tubes and check there is no leakage by inverting test tube (quality control step to ensure no sample is lost during next step)
6. Mix for 30 min in Roto-torque
7. Centrifuge at 3000 rpm for 3 min
8. Aspirate the organic phase into clean large kimax test tube (using glass pipette) and set aside
9. Add 3 ml diethyl ether to the aqueous (lower) phase
10. Cap test tubes and check there is no leakage by inverting test tube
11. Mix for 10 min in Roto-torque
12. Centrifuge at 3000 rpm for 3 min
13. Aspirate the organic phase and add to the ethyl acetate phase (using glass pipette)
14. Discard lower aqueous phase into appropriate organic waste container
15. Add two level spatula scoops of anhydrous Na_2SO_4
16. Cap and invert test tube several times (or vortex for 5 seconds) to ensure good mixing (proper dispersion of Na_2SO_4 ensures all water molecules removed from organic phase as water reverses chemical process of silylation, thereby reducing the efficiency of derivatization)
17. Centrifuge at 3000 rpm for 1 min
18. Pour/decant the organic phase into a clean small kimax tube
19. Evaporate to dryness in heating block at 37°C under nitrogen gas (~1 hour)
20. Use Hamilton glass syringe to add 5:1:1 BSTFA, TMCS and pyridine, respectively, based on creatinine-based calculated volume.
NOTE: Hamilton glass syringe is kept clean with pyridine and approximately 100 μl hexane is withdrawn into syringe and discarded (five times) between the addition of each reagent (quality control step to ensure syringe is clean and avoid cross-contamination)
21. Cap test tubes and incubate at 60°C for 1 hour (45 min at 70°C)
22. Set up and label GC-MS vials, with insert and cap
23. Transfer approximately 100 μl sample to GC-MS vial
NOTE: clean glass syringe with hexane (five times) after each transfer
24. Cap GC-MS vial and place in auto-sampler and process via GC-MS.

General AMDIS settings (organic acids)

- 80% minimum match factor
- Type of analysis: use internal standards for RI (show standards)
- Resolution: medium

- Sensitivity: medium
- Shape requirements: medium

Feature/metabolite identification is done by comparing each feature's/metabolite's MS-spectral pattern with customized spectral library specific to the urine sample under investigation.

Chemical information sheet

- 4-Phenylbutyric acid – $C_{10}H_{12}O_2$; Mw: 164.21 g/mol; supplier: Fluka (25 g) (index no.78243). Precaution/ hazard: avoid contact with skin and eyes.
- Sodium hydroxide – NaOH; Mw: 40.00 g/mol; supplier: Merck (500 g). Precaution/ hazard: corrosive (causes severe burns).
- Hydrochloric acid (32%) – HCl; Mw: 36.36 g/mol; supplier: Merck (2.5 l). Precaution/ hazard: corrosive (causes severe burns); irritating to respiratory system.
- Ethyl acetate – $CH_3COOC_2H_5$; Mw: 88.11 g/mol; supplier: Merck (2.5 l) (index no. 607-022-00-5). Precaution/ hazard: highly flammable; causes drowsiness/dizziness; causes eye irritation; repeated exposure causes skin dryness/cracking.
- Diethyl ether – $(C_2H_5)_2O$; Mw: 74.12 g/mol; supplier: Merck (2.5 l) (index no. 602-022-00-4). Precaution/ hazard: extremely flammable; harmful if swallowed; causes drowsiness/dizziness; repeated exposure causes skin dryness/cracking; may form explosive peroxides.
- Hexane – $CH_3(CH_2)_4CH_3$; Mw: 86.18 g/mol; supplier: Merck (2.5 l) (index no. 601-037-00-0). Precaution/ hazard: highly flammable; fatal if swallowed; causes skin irritation; toxic to aquatic life; causes drowsiness/dizziness; may cause infertility or damage to unborn child; may cause damage to organs through prolonged or repeated exposure.
- Sodium sulphate – Na_2SO_4 ; Mw: 142.04 g/mol; supplier: Merck (500 g).
- Chlorotrimethylsilane (TMCS) – C_3H_9ClSi ; Mw: 108.64 g/mol; supplier: Flukka Analytical (100 ml) (index no. 92360). Precaution/ hazard: highly flammable; corrosive (causes severe burns); reacts violently with water; harmful by inhalation/contact to skin; irritating to respiratory system.
- Pyridine – C_5H_5N ; Mw: 79.10 g/mol; supplier: Flukka Analytical (1 l) (index no. 82703). Precaution/ hazard: highly flammable; harmful if inhaled or swallowed; harmful to skin.
- Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) – $CF_3C=NSi(CH_3)_3OSi(CH_3)_3$; Mw: 257.40 g/mol; supplier: Supelco Analytical (25 ml) (index no. 3-3027). Precaution/ hazard: flammable; irritant to eyes and skin; causes burns.

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[3A%20a%20cross%20sectional%20comparison%20of%20women%20with%20localised%20and%20widespread%20pain%20and%20controls&volumeNum=5](#)

Chapter 4

Figure 4.2:

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Figure 4.3:

Order Summary

Licensee:	Mrs. Bontle Malatji
Order Date:	Oct 31, 2017
Order Number:	4219361152017
Publication:	Magnetic Resonance in Chemistry
Title:	NMR metabolomics and drug discovery
Type of Use:	Dissertation/Thesis
Order Total:	0.00 USD

<https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=745cd0ae-8299-4b2f-aa45-3c266c022c80>

Table 1:

Order Summary

Licensee:	Mrs. Bontle Malatji
Order Date:	Nov 15, 2017
Order Number:	4230070217620
Publication:	Springer eBook
Title:	The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research
Type of Use:	Thesis/Dissertation
Order Total:	0.00 USD

<https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=fc3315a2-d347-4df1-a90d-c2cb6225da78>

Chapter 6

Figure 6.1:

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Addendum A4: Supplementary information (SI) to publications

SUPPLEMENTARY MATERIAL TO ARTICLE 1

A diagnostic biomarker profile for Fibromyalgia Syndrome based on an NMR metabolomics study of selected patients and controls

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S1: Experimental subjects symptom assessment questionnaire

The Fibromyalgia Impact Questionnaire (FIQR) is an internationally derived questionnaire developed by Burckhardt and co-workers [1]. It was developed with the aim to evaluate and understand the effects of therapy on the broad range of symptoms that manifest in FMS. As such, the questionnaire has routinely been used, since its official release in 1991, as a means to assess the progression of the disorder and any therapeutic interventions applied [2].

Questionnaire A in Table S1 shows this FIQR questionnaire that was voluntarily completed by the FMS patients who took part in this study. The in-house clinical questionnaire, Table S1B, was drawn up to identify secondary data about the patients for use in conjunction with the FIQR questionnaire.

Table S1: Fibromyalgia Impact Questionnaire (FIQR) (A) and Clinical questionnaire (B). Questionnaire A was used by the clinicians to assess the severity of the symptoms experienced by the FMS patients. Questionnaire B was used to gather supplementary information on the FMS patients

A - Fibromyalgia Impact Questionnaire (FIQR)

1. Function domain

Directions: For each question, place an “X” in the box that best indicates how much your fibromyalgia made it difficult to do each of the following activities during the past 7 days

Brush or comb your hair	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Walk continuously for 20 minutes	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Prepare a homemade meal	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Vacuum, scrub or sweep floors	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Lift and carry a bag full of groceries	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Climb one flight of stairs	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Change bed sheets	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Sit in a chair for 45 minutes	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Go shopping for groceries	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult

2. Overall impact domain:

Directions: For each question, check the one box that best describes the overall impact of your fibromyalgia over the last 7 days:

Fibromyalgia prevented me from accomplishing goals for the week	Never	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Always
I was completely overwhelmed by my fibromyalgia symptoms	Never	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Always

3. Symptoms domain:

Directions: For each of the following 10 questions, select the one circle that best indicates the intensity of your fibromyalgia symptoms over the past 7 days

Please rate your level of pain	No pain	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Unbearable pain
Please rate your level of energy	Lots of energy	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	No energy
Please rate your level of stiffness	No stiffness	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Severe stiffness
Please rate the quality of your sleep	Awoke well rested	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Awoke very tired
Please rate your level of depression	No depression	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very depressed
Please rate your level of memory problems	Good memory	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very poor memory
Please rate your level of anxiety	Not anxious	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very anxious
Please rate your level of tenderness	No	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very tender

to touch	tenderness		
Please rate your level of balance problems	No imbalance	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Severe imbalance
Please rate your level of sensitivity to loud noises, bright lights, odors and cold	No sensitivity	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Extreme sensitivity

B - Clinical Questionnaire

1. Age:

Years

Months

2. Relationship status:

1. Married	2. Engaged	3. In a relationship, but not married or engaged	4. Single
------------	------------	--	-----------

3. Current employment status

1. Fulltime employed	2. Part-time employed	3. Home executive (housewife)	4. Retired	5. Disabled
----------------------	-----------------------	-------------------------------	------------	-------------

4. How long ago did your fibromyalgia symptoms start (e.g. widespread muscle pain, poor sleep, fatigue, headaches, etc.)

Years

Months

5. How long ago were you diagnosed with fibromyalgia?

Years

Months

6. (a) Were your fibromyalgia symptoms triggered by? Please tick (v)
(you may tick more than one block)

1. Neck injury

☐

2. Other injuries

☐

3. After surgical procedure

☐

4. Severe emotional stress

☐

5. Acute infection

☐

6. Spontaneous onset

☐

7. Uncertain

☐

(b)

Length

Bodyweight

7. Please rate your pain by circling the one number that best describes you pain at its **worst** in the last month. (A rating of 10 would indicate pain so severe as to prohibit all activity; the worst pain you can imagine.)



8. Please rate your pain by circling the one number that best describes you pain on the **average** in the last month. (A rating of 10 would indicate pain so severe as to prohibit all activity; the worst pain you can imagine.)



9. (i) Which medications are you receiving **for your pain**?

Please tick

Trepeline	<input type="checkbox"/>	Lyrice	<input type="checkbox"/>
Cymbalta	<input type="checkbox"/>	Syndol	<input type="checkbox"/>
Tramal (Tramahexal)	<input type="checkbox"/>	Myprodol	<input type="checkbox"/>
Tenston	<input type="checkbox"/>	Mypaid	<input type="checkbox"/>
Stilpayne	<input type="checkbox"/>	Cataflam	<input type="checkbox"/>
Other	<input type="text"/>		

- (ii) How often do you take pain killers (e.g. Tramal, Tramahexal, Tramacet, Panado, Syndol., Mypaid, Cataflam, Voltaren, etc.)

- ☐ Once a week
☐ Twice a week
☐ Three days a week
☐ Every second day
☐ Daily

<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>

10. During the past week how much did the state of your health, including any pain, interfere with the following things: choose the one number, from 0 to 4 below, that best describes your state and write them in the appropriate box (I to vi).

- 0 Not at all
 1 A little bit
 2 Moderately
 3 Quite a bit
 4 Extremely

i. Mood	<input type="checkbox"/>
ii. Relations with other people	<input type="checkbox"/>
iii. Walking ability	<input type="checkbox"/>
iv. Sleep	<input type="checkbox"/>
v. Normal Work (includes both work outside the home and housework)	<input type="checkbox"/>
vi. Enjoyment of life	<input type="checkbox"/>

11. Have you ever been diagnosed and treated for **depression**

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

12. Do you suffer from regular **headaches**?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

- 13 **Irritable bowel syndrome** (IBS) is known to commonly affect patients with fibromyalgia. It is characterized by abdominal pain and cramps as well as bloating, flatulence, diarrhea and/or constipation

Have you ever been diagnosed with IBS?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

- 14 **Restless leg syndrome (RLS)** is characterized by uncomfortable sensations in the lower legs and an uncontrollable urge to move them so as to provide relief. Some of the sensations felt in RLS include burning, creeping or a crawling feeling inside the legs.

Have you ever experienced symptoms of RLS?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

15. Have you suffered from **anxiety** since being diagnosed with fibromyalgia or thereafter? (Symptoms such as feeling nervous most of the time, not able to control worrying, etc.)

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

16. If yes, have you been diagnosed and treated for anxiety?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

17. Have you ever suffered from a **sleep disturbance**?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

If yes, please tick the appropriate block(s)
(you may tick more than one block)

Problem with sleep initiation
Problem with maintaining sleep
Early morning awakening
Waking up feeling unrefreshed

<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>

18. **Dysmenorrhoea** is defined as painful menstruation often associated with cramps for mostly 1–3 days after beginning of menstruation.

Were you treated before or are you currently being treated for dysmenorrhoea?

Yes	<input type="checkbox"/>
No	<input type="checkbox"/>

Table S2: Summary of the supplementary data collected based on the in-house questionnaire (Table S1B) completed by the FMS patient group.

Variable	Mean	%
1. Socio-demographic information		
Age (years)	45.5	n/a
Marital status		
Single	2	11
Separated/Divorced/Widow	0	0
In permanent relationship	2	11
Married	15	78
Employment		
Disabled/Retired	2	11
Housewife	6	32
Part-time	1	5
Full-time	10	52
2. Pain experience		
Worst pain experience	7,7	n/a
Recent past pain average	5,7	n/a
Pain specific medication		
Trepiline (antidepressant for neuropathic pain)	12	63
Cymbalta (antidepressant for chronic pain)	10	52
Tramal (analgesic for moderate to severe pain)	9	47
Myprodol (relief of pain of inflammatory origin)	6	32
Patients using also other medication against pain	11	58
3. Levels of emotional experiences affected by FMS		
Mood	2.3	n/a
Relations with other people	2.2	
Enjoyment of life	2.1	
Normal work	2.4	

0 = Not at all **1** = A little bit **2** = Moderately **3** = Quite a bit **4** = Extremely

S2: Comparison of FMS symptom severity with a published reference group

The mean scores of FMS patients' symptoms based on severity in relation to the published reference group [3] are shown in Figure S1.

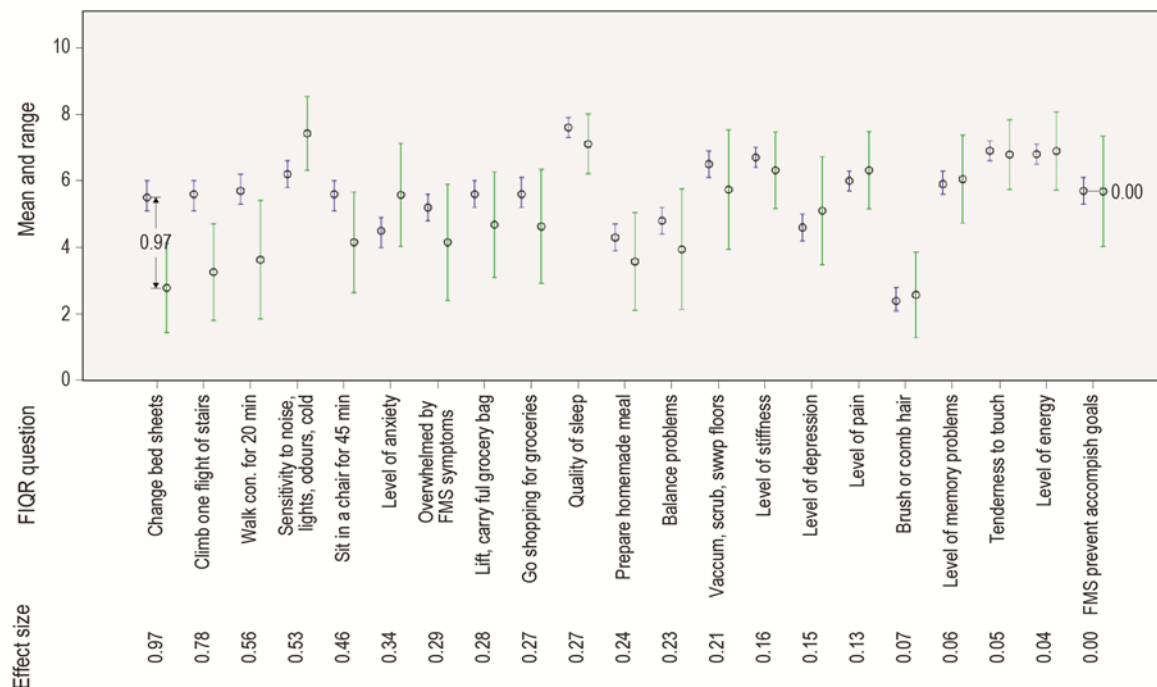


Figure S1: Comparison of minimum, mean and maximum scores of fibromyalgia patients' symptoms based on severity in relation to a published reference group. Every FIQR question is compared with the data from a reference group, presented for the revised FIQR. The questions are ranked according to the difference between the effect sizes (ES) ranked from high to low ($1.0 \geq ES \geq 0.00$), with the mean values shown as a circular point in the figure. The range of points scored for the reference group is shown in blue and for the present FMS group by green vertical bars.

Higher scores are indicative of greater dysfunction or symptom severity, while differences between the blue (previously published study) and green (current study) observed ranges show how the current patient group differs from another patient group. These differences were also quantified using Cohen's d-value as a measure of practical significance of differences (i.e. effect size). Effect sizes exceeding 0.5 are considered practically visible, whereas those exceeding 0.8 are considered practically significant [4]. The six highest scores reported for the reference group were: sleep quality (mean 7.61 ± 2.4 (standard deviation)), tenderness to touch (6.86 ± 2.5), energy level (6.80 ± 2.4), stiffness (6.72 ± 2.2), sensitivity to the environment (6.19 ± 2.9), and pain (6.01 ± 2.1). The mean values of the highest scores obtained for the present FMS group remarkably resemble the published observations:

sensitivity to the environment > sleep quality > energy level > tenderness to touch > stiffness and pain. Likewise, 'difficulty with combing hair' had the lowest score in both groups. The scores for the three FIQR domains did, however, indicate some clear differences between the two groups: Reliability was assessed using Cronbach's alpha coefficient (α) and indicated reliability for all domains. The mean values (reference group vs present group) obtained were 18 vs 36 for the 'Function domain' ($\alpha = 0.94$ and mean inter-item correlation = 0.62); 11 vs 10 for the 'Impact domain' ($\alpha = 0.89$ and mean inter-item correlation = 0.8) and 30 vs 60 for the 'Symptoms domain' ($\alpha = 0.88$ and mean inter-item correlation = 0.44). The total scores ($\alpha = 0.95$ and mean inter-item correlation = 0.5) were comparable: 55 vs 50. It is important, finally, to notice that the difference between the minimum and maximum scores obtained for all 21 questions involving the present group mostly exceeded the values for the reference group, clearly suggesting a greater diversity between the present FMS patients group than in the reference group.

S3: Correlation table based on the FIQR questionnaire

Table S3: Correlation table data, as based on the FIQR questionnaire, used to draw up Fig. 1 in the main article.

Comparison	Correlation coefficient	Correlation coefficient (As quoted in Fig. 1 of main article)	p-value
Hair correlation with Walk	0.466454	0.5	0.012
Hair correlation with Meals	0.418437	0.4	0.024
Hair correlation with Clean	0.460269	0.5	0.014
Hair correlation with Carry	0.421214	0.4	0.022
Hair correlation with Stairs	0.216187	0.2	0.247
Hair correlation with Make bed	0.177345	0.2	0.345
Hair correlation with Sit	0.307686	0.3	0.095
Hair correlation with Shop	0.269565	0.3	0.154
Hair correlation with Goals	0.335035	0.3	0.070
Hair correlation with Symptoms	0.195924	0.2	0.296
Hair correlation with Pain	0.261516	0.3	0.159
Hair correlation with Energy	0.068820	0.1	0.710
Hair correlation with Stiffness	0.497131	0.5	0.007
Hair correlation with Sleep	0.157715	0.2	0.407
Hair correlation with Depression	0.014139	0.0	0.940
Hair correlation with Memory	0.048174	0.0	0.795
Hair correlation with Anxiety	-0.151901	-0.2	0.414
Hair correlation with Tenderness	0.202921	0.2	0.279
Hair correlation with Balance	0.079710	0.1	0.676
Hair correlation with Environment	0.020921	0.0	0.911
Walk correlation with Meals	0.538961	0.5	0.003
Walk correlation with Clean	0.719570	0.7	0.000
Walk correlation with Carry	0.636686	0.6	0.000
Walk correlation with Stairs	0.646953	0.6	0.000
Walk correlation with Make bed	0.483494	0.5	0.008
Walk correlation with Sit	0.614890	0.6	0.001
Walk correlation with Shop	0.557361	0.6	0.002
Walk correlation with Goals	0.550165	0.6	0.002
Walk correlation with Symptoms	0.609392	0.6	0.001
Walk correlation with Pain	0.553749	0.6	0.002
Walk correlation with Energy	0.403911	0.4	0.025
Walk correlation with Stiffness	0.620928	0.6	0.001
Walk correlation with Sleep	0.149298	0.1	0.420
Walk correlation with Depression	0.281064	0.3	0.124
Walk correlation with Memory	0.312705	0.3	0.083
Walk correlation with Anxiety	0.078433	0.1	0.665
Walk correlation with Tenderness	0.549777	0.5	0.003

Walk correlation with Balance	0.342981	0.3	0.065
Walk correlation with Environment	0.389492	0.4	0.033
Meals correlation with Clean	0.666757	0.7	0.000
Meals correlation with Carry	0.546649	0.5	0.002
Meals correlation with Stairs	0.448906	0.4	0.014
Meals correlation with Make bed	0.718525	0.7	0.000
Meals correlation with Sit	0.569582	0.6	0.002
Meals correlation with Shop	0.443203	0.4	0.016
Meals correlation with Goals	0.563110	0.6	0.002
Meals correlation with Symptoms	0.563025	0.6	0.002
Meals correlation with Pain	0.508146	0.5	0.005
Meals correlation with Energy	0.469058	0.5	0.009
Meals correlation with Stiffness	0.411774	0.4	0.023
Meals correlation with Sleep	0.278236	0.3	0.133
Meals correlation with Depression	0.267680	0.3	0.143
Meals correlation with Memory	0.175897	0.2	0.331
Meals correlation with Anxiety	0.176474	0.2	0.330
Meals correlation with Tenderness	0.245081	0.2	0.180
Meals correlation with Balance	0.377279	0.4	0.042
Meals correlation with Environment	0.237656	0.2	0.193
Clean correlation with Carry	0.778044	0.8	0.000
Clean correlation with Stairs	0.416107	0.4	0.024
Clean correlation with Make bed	0.505193	0.5	0.006
Clean correlation with Sit	0.723825	0.7	0.000
Clean correlation with Shop	0.716828	0.7	0.000
Clean correlation with Goals	0.684344	0.7	0.000
Clean correlation with Symptoms	0.653202	0.7	0.000
Clean correlation with Pain	0.695425	0.7	0.000
Clean correlation with Energy	0.456994	0.5	0.012
Clean correlation with Stiffness	0.578102	0.6	0.002
Clean correlation with Sleep	0.241471	0.2	0.198
Clean correlation with Depression	0.326561	0.3	0.078
Clean correlation with Memory	0.377517	0.4	0.039
Clean correlation with Anxiety	0.186056	0.2	0.310
Clean correlation with Tenderness	0.316500	0.3	0.087
Clean correlation with Balance	0.474216	0.5	0.012
Clean correlation with Environment	0.248322	0.2	0.178
Carry correlation with Stairs	0.424982	0.4	0.019
Carry correlation with Make bed	0.399043	0.4	0.029
Carry correlation with Sit	0.576935	0.6	0.001
Carry correlation with Shop	0.645120	0.6	0.000
Carry correlation with Goals	0.685912	0.7	0.000
Carry correlation with Symptoms	0.656023	0.7	0.000
Carry correlation with Pain	0.703284	0.7	0.000
Carry correlation with Energy	0.541981	0.5	0.003
Carry correlation with Stiffness	0.543761	0.5	0.003

Carry correlation with Sleep	0.396545	0.4	0.031
Carry correlation with Depression	0.192205	0.2	0.290
Carry correlation with Memory	0.322608	0.3	0.073
Carry correlation with Anxiety	0.110047	0.1	0.541
Carry correlation with Tenderness	0.380494	0.4	0.036
Carry correlation with Balance	0.353276	0.4	0.056
Carry correlation with Environment	0.241913	0.2	0.182
Stairs correlation with Make bed	0.539328	0.5	0.004
Stairs correlation with Sit	0.473776	0.5	0.009
Stairs correlation with Shop	0.512020	0.5	0.006
Stairs correlation with Goals	0.421135	0.4	0.020
Stairs correlation with Symptoms	0.478117	0.5	0.010
Stairs correlation with Pain	0.344401	0.3	0.059
Stairs correlation with Energy	0.357647	0.4	0.050
Stairs correlation with Stiffness	0.598036	0.6	0.001
Stairs correlation with Sleep	0.075891	0.1	0.685
Stairs correlation with Depression	0.197297	0.2	0.285
Stairs correlation with Memory	0.245055	0.2	0.179
Stairs correlation with Anxiety	0.079738	0.1	0.663
Stairs correlation with Tenderness	0.397309	0.4	0.031
Stairs correlation with Balance	0.488164	0.5	0.009
Stairs correlation with Environment	0.382550	0.4	0.038
Make bed correlation with Sit	0.522093	0.5	0.004
Make bed correlation with Shop	0.437500	0.4	0.020
Make bed correlation with Goals	0.428384	0.4	0.019
Make bed correlation with Symptoms	0.561697	0.6	0.003
Make bed correlation with Pain	0.464860	0.5	0.011
Make bed correlation with Energy	0.410963	0.4	0.025
Make bed correlation with Stiffness	0.310924	0.3	0.092
Make bed correlation with Sleep	0.189484	0.2	0.315
Make bed correlation with Depression	0.304500	0.3	0.101
Make bed correlation with Memory	0.276221	0.3	0.133
Make bed correlation with Anxiety	0.202777	0.2	0.271
Make bed correlation with Tenderness	0.363048	0.4	0.051
Make bed correlation with Balance	0.581692	0.6	0.002
Make bed correlation with Environment	0.423270	0.4	0.022
Sit correlation with Shop	0.716204	0.7	0.000
Sit correlation with Goals	0.425806	0.4	0.018
Sit correlation with Symptoms	0.561206	0.6	0.002
Sit correlation with Pain	0.571441	0.6	0.002
Sit correlation with Energy	0.324682	0.3	0.072
Sit correlation with Stiffness	0.605892	0.6	0.001
Sit correlation with Sleep	0.142051	0.1	0.442

Sit correlation with Depression	0.466926	0.5	0.011
Sit correlation with Memory	0.266239	0.3	0.140
Sit correlation with Anxiety	0.293174	0.3	0.105
Sit correlation with Tenderness	0.297109	0.3	0.103
Sit correlation with Balance	0.369223	0.4	0.046
Sit correlation with Environment	0.401394	0.4	0.027
Shop correlation with Goals	0.602414	0.6	0.001
Shop correlation with Symptoms	0.568546	0.6	0.002
Shop correlation with Pain	0.633287	0.6	0.001
Shop correlation with Energy	0.384015	0.4	0.038
Shop correlation with Stiffness	0.567775	0.6	0.002
Shop correlation with Sleep	0.287735	0.3	0.129
Shop correlation with Depression	0.346023	0.3	0.064
Shop correlation with Memory	0.498545	0.5	0.007
Shop correlation with Anxiety	0.297406	0.3	0.108
Shop correlation with Tenderness	0.287698	0.3	0.124
Shop correlation with Balance	0.560411	0.6	0.003
Shop correlation with Environment	0.307212	0.3	0.099
Goals correlation with Symptoms	0.680049	0.7	0.000
Goals correlation with Pain	0.727288	0.7	0.000
Goals correlation with Energy	0.538972	0.5	0.003
Goals correlation with Stiffness	0.553772	0.6	0.002
Goals correlation with Sleep	0.473503	0.5	0.011
Goals correlation with Depression	0.266815	0.3	0.144
Goals correlation with Memory	0.311695	0.3	0.084
Goals correlation with Anxiety	0.234539	0.2	0.195
Goals correlation with Tenderness	0.343326	0.3	0.060
Goals correlation with Balance	0.376060	0.4	0.043
Goals correlation with Environment	0.144765	0.1	0.427
Symptoms correlation with Pain	0.817389	0.8	0.000
Symptoms correlation with Energy	0.651253	0.7	0.000
Symptoms correlation with Stiffness	0.360032	0.4	0.050
Symptoms correlation with Sleep	0.470726	0.5	0.012
Symptoms correlation with Depression	0.409578	0.4	0.027
Symptoms correlation with Memory	0.465180	0.5	0.011
Symptoms correlation with Anxiety	0.380034	0.4	0.039
Symptoms correlation with Tenderness	0.459459	0.5	0.013
Symptoms correlation with Balance	0.510802	0.5	0.007
Symptoms correlation with Environment	0.417511	0.4	0.024
Pain correlation with Energy	0.562092	0.6	0.002
Pain correlation with Stiffness	0.452461	0.5	0.013
Pain correlation with Sleep	0.510630	0.5	0.006
Pain correlation with Depression	0.443113	0.4	0.016
Pain correlation with Memory	0.411765	0.4	0.023

Pain correlation with Anxiety	0.400002	0.4	0.028
Pain correlation with Tenderness	0.365499	0.4	0.046
Pain correlation with Balance	0.392274	0.4	0.035
Pain correlation with Environment	0.251678	0.3	0.169
Energy correlation with Stiffness	0.288526	0.3	0.112
Energy correlation with Sleep	0.510630	0.5	0.006
Energy correlation with Depression	0.416257	0.4	0.023
Energy correlation with Memory	0.503268	0.5	0.005
Energy correlation with Anxiety	0.419674	0.4	0.021
Energy correlation with Tenderness	0.159490	0.2	0.384
Energy correlation with Balance	0.385392	0.4	0.038
Energy correlation with Environment	0.304662	0.3	0.095
Stiffness correlation with Sleep	0.136615	0.1	0.463
Stiffness correlation with Depression	0.303115	0.3	0.099
Stiffness correlation with Memory	0.078689	0.1	0.665
Stiffness correlation with Anxiety	0.118421	0.1	0.516
Stiffness correlation with Tenderness	0.313361	0.3	0.088
Stiffness correlation with Balance	0.138092	0.1	0.460
Stiffness correlation with Environment	0.179411	0.2	0.328
Sleep correlation with Depression	0.293735	0.3	0.118
Sleep correlation with Memory	0.299569	0.3	0.107
Sleep correlation with Anxiety	0.396184	0.4	0.033
Sleep correlation with Tenderness	0.083069	0.1	0.658
Sleep correlation with Balance	0.229404	0.2	0.230
Sleep correlation with Environment	0.055193	0.1	0.768
Depression correlation with Memory	0.416257	0.4	0.023
Depression correlation with Anxiety	0.734211	0.7	0.000
Depression correlation with Tenderness	0.157005	0.2	0.397
Depression correlation with Balance	0.219148	0.2	0.245
Depression correlation with Environment	0.380988	0.4	0.039
Memory correlation with Anxiety	0.386887	0.4	0.033
Memory correlation with Tenderness	0.166136	0.2	0.364
Memory correlation with Balance	0.536796	0.5	0.004
Memory correlation with Environment	0.344401	0.3	0.059
Anxiety correlation with Tenderness	-0.026669	0.0	0.885
Anxiety correlation with Balance	0.227852	0.2	0.222
Anxiety correlation with Environment	0.292373	0.3	0.110
Tenderness correlation with	0.104959	0.1	0.578

Balance			
Tenderness correlation with			
Environment	0.457915	0.5	0.013
Balance correlation with			
Environment	0.299872	0.3	0.111

Table S3 shows the correlation coefficient and associated p -value (rounded to 1 and 3 decimals, respectively, for display purposes) for each question with the remaining questions, as summarized in Fig. 1 in the main paper. We made use of Kendall's tau correlation coefficient as it is a non-parametric method better suited to small groups with multiple tied values [4]. Coefficients range between -1 and 1 are considered practically visible if above 0.3 or below -0.3 (indicating an inverse relationship) and as practically significant is above 0.5 or below -0.5 . Since we did not have a truly randomised sample, we focus more on practical significance than statistical significance; however, we also report the associated p -values indicating statistically significant associations if less than 0.05 .

S4: Normalized data of the original NMR spectral bins

Division of the original ^1H NMR spectrum of each analysed urine sample into 0.02 ppm equal-sized bins, between 0.5 ppm and 10 ppm, yielded 468 bins with spectral data, excluding the region of the water peak (4.66–4.90 ppm). To account for dilution differences common to urine samples, each spectral bin was made relative to the CH_3 singlet of creatinine at 3.13 ppm. The raw, normalized spectral data matrix of every analysed sample in this study is given as an electronic file (Excel format) in Table S4.

Table S4: Raw NMR spectral data (Excel format) normalized relative to the CH_3 singlet of creatinine at 3.13 ppm, given as an electronic file (See Additional File S2 – Raw data matrix) attached online as part of the SI.

S5: Outlier identification

Outliers were detected using equidistant binning data to identify cases with undue influences on the predictor models. This was done by assessing the principal components analysis (PCA) score plots for each of the experimental groups as shown in Fig S2(e–h). Cases presenting outside of the confidence interval boundary are classified as outliers. Similarly, a Hotelling's T^2 (Fig S2 a–d) analysis was also applied to the same data for supplementary outlier detection. Cases presenting above the solid horizontal line are classified as outliers. These two methods were then used in conjunction to identify outliers. Cases identified by either method were excluded from further analysis.

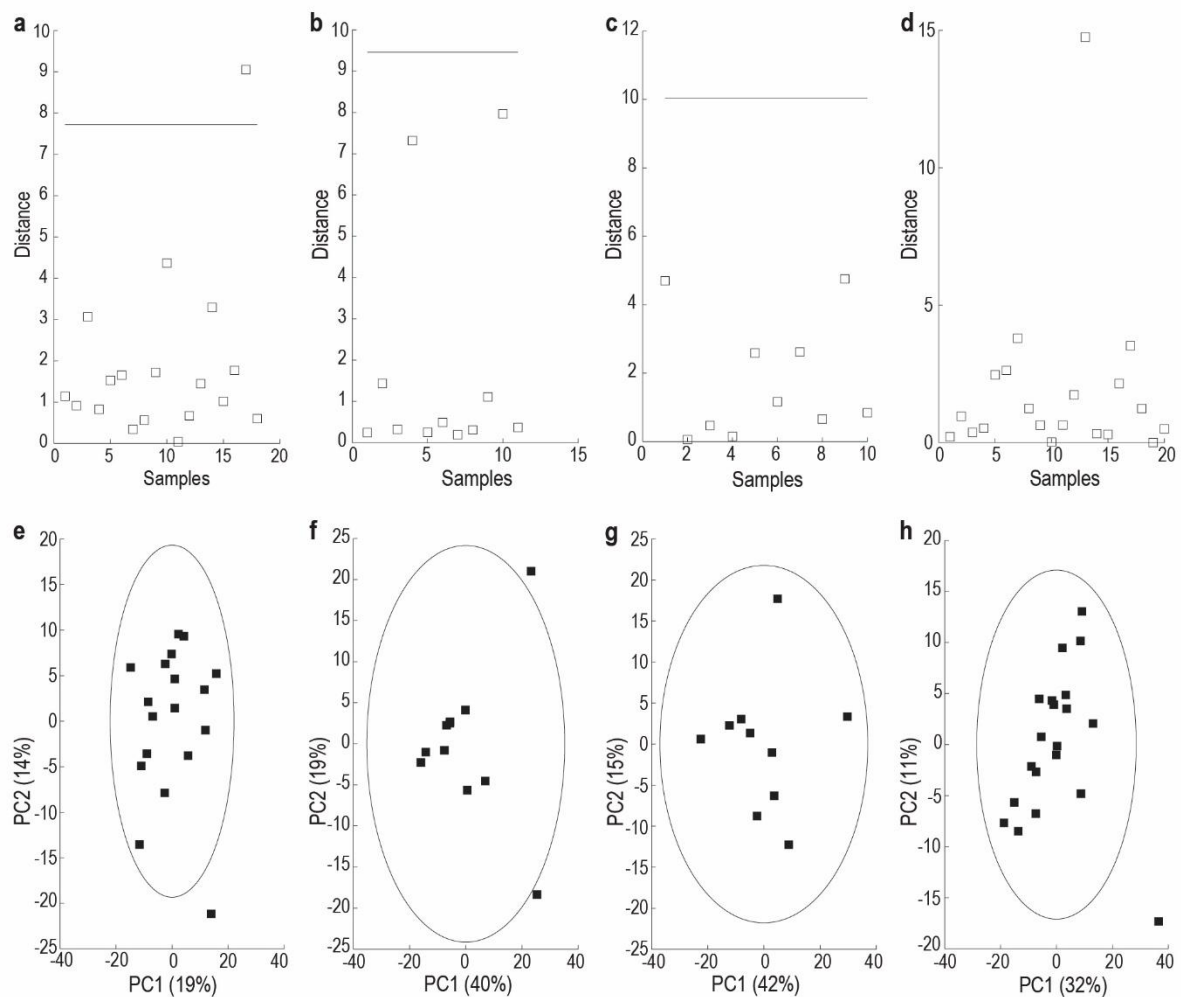


Figure S2: Outlier detection by means of a Hotelling's T^2 distance plot (a–d) and PCA scores plot (e–h) for each of the four experimental groups. Cases appearing above the red line (Hotelling's T^2 plot) or outside the blue confidence interval boundary (PCA scores plot) were earmarked as outliers.

S6: PCA Analysis

Principal component analysis (PCA) was also performed on the entire scaled dataset, including all four groups. This provides a more holistic view of the variation in the data relative to the groups.

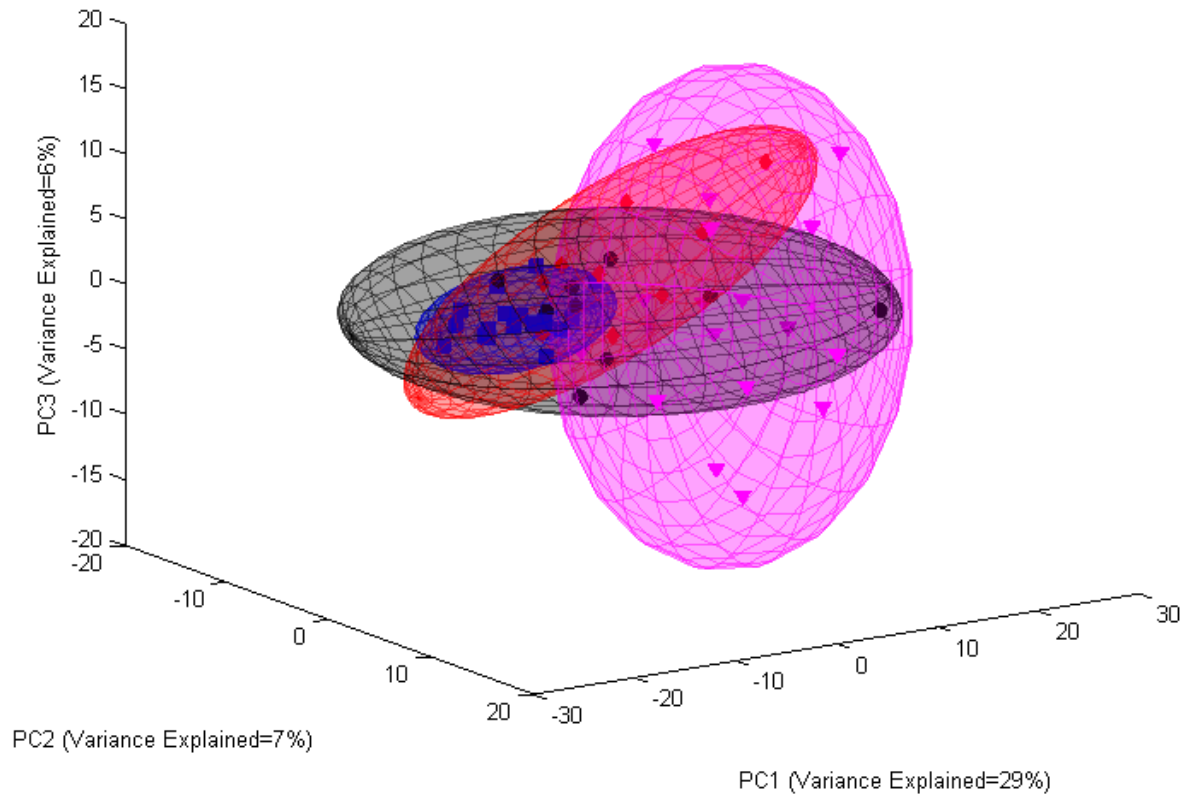


Figure S3: PCA of FMS (magenta); CF (black); CN (blue) and CO (red) groups

S7: *N*-acetyl aspartic acid (NAA) verification

Overview

Peak 9 in Fig. 2 of the main article was initially labelled as *N*-acetylaspatic acid. However, this peak is a characteristic moiety of most *N*-acetyl compounds and thus this result necessitated more detailed NMR analyses, by means of two-dimensional (2D) NMR, for verification. Of these 2D analyses we opted for correlation spectroscopy (COSY) and J-resolved spectroscopy (JRES).

Reagents

The reagents used for this analysis were three of the study's experimental subjects' urine, namely, two Pre patient samples (Pre 1 and Pre 2) and one control sample from the CN group. A 1 mM sample of pure NAA compound (Sigma-Aldrich) was prepared in MilliQ-water. The samples were then spiked with this pure compound for analysis.

Sample preparation and analysis

The two patients' urine samples and the pure NAA compound were prepared as per the protocol described in the main article. Only the CN sample was spiked with the pure NAA compound as the aim of this analysis was to identify and verify NAA in the patient samples; we also required a urine control to see where the spiked NAA peak would present in urine. As such, the preparation protocol was adjusted for the CN sample, to take into account the addition of the pure NAA, by adding 630 μ l of the centrifuged urine supernatant, 70 μ l pure 1 mM NAA compound and 70 μ l of internal standard (IS), which was TSP. the samples were adjusted to pH 2.5. All four samples were then analysed on ^1H NMR, COSY and JRES. For the purposes of verification, the ^1H NMR analysis was done at 512 scans to reduce the noise peaks to a minimum. The results of this analysis are shown in Fig. S4.

Results and discussion

Fig. S4 shows the ^1H NMR overlay of the four analysed samples. Red indicates the pure NAA compound, green indicates the spiked CN urine sample, and orange and light blue indicate the non-spiked Pre samples. The circled areas show the regions of interest where the pure NAA compound and NAA+ urine (spiked CN urine) peaks appear. Within the blue circled area in Fig. S4A, we can see that all the samples present with a singlet peak, which is characteristic of any *N*-acetyl compound, as also identified in the JRES (Fig. S4B) analysis

shown in the corresponding blue area. In the yellow circled area we can see that all samples peak in this area, with the red sample being the highest. This peak depicts the characteristic multiplet peak of NAA. In the JRES picture we see that only the red and green samples present with this multiplet peak as the peak patterns correlate. All other peaks presenting in this area of the other samples can be concluded as not including the characteristic NAA peak as the peak patterns do not match those of the CN (green) and pure NAA (red) peaks.

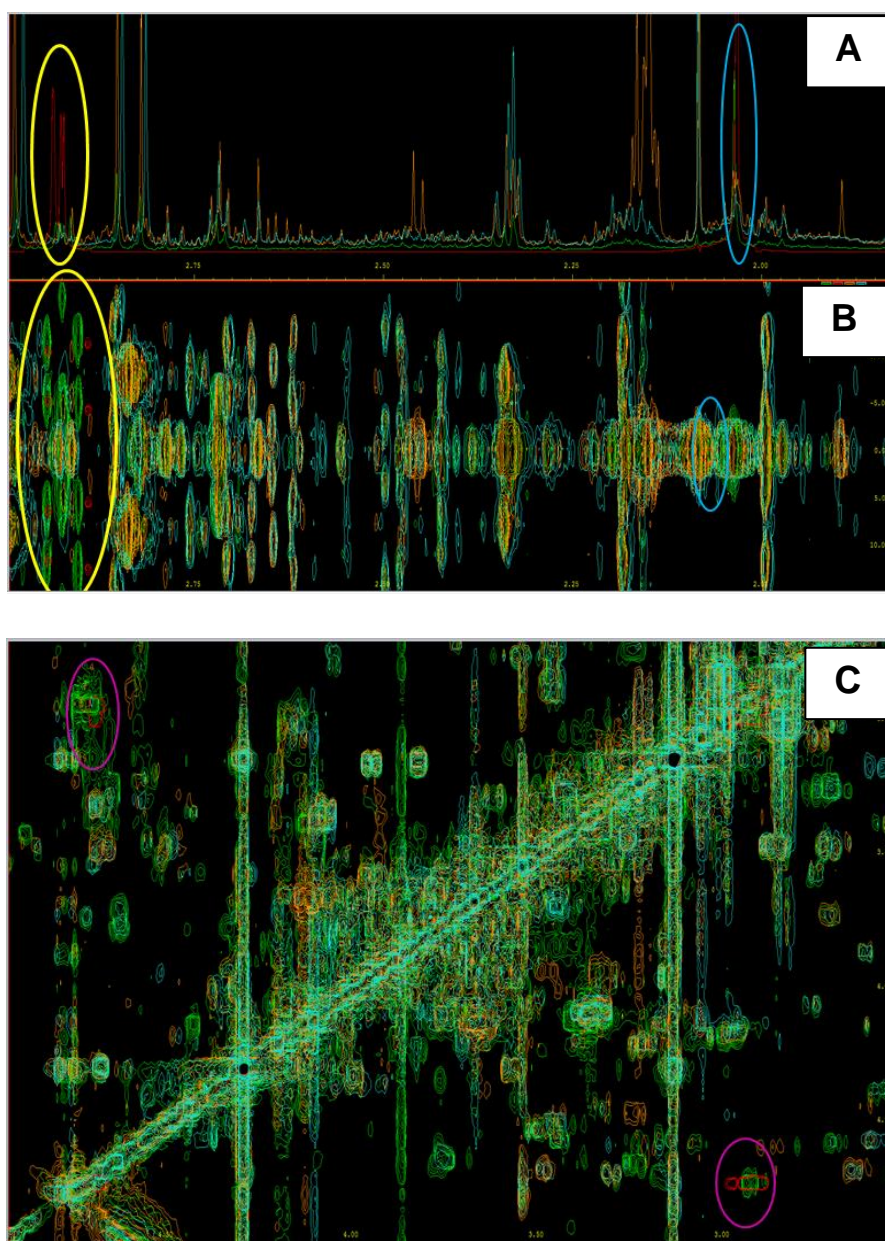


Figure S4: ^1H NMR overlay of three urine samples, namely, two PRE patient samples (orange and blue) and a CN control group sample (green) spiked with pure N-acetyl aspartic acid. Red indicates the pure NAA compound dissolved in MilliQ water. Picture A shows the one-dimensional NMR analysis whereas B and C show the two-dimensional JRES and COSY analyses, respectively. The circled areas show the regions of interest for the verification of the presence of NAA.

References:

1. Burckhardt CS, Clark SR, Bennett RM. The Fibromyalgia Impact Questionnaire: development and validation. *J Rheumatol*. 1991;18:728-733.
2. The overview of the FIQR and SIQR. <http://fiqrinfo.ipage.com/index.html>. Accessed on 22 January 2016.
3. Bennett RM, Friend R, Jones KD, Ward R, Han BK, Ross RL. The Revised Fibromyalgia Impact Questionnaire (FIQR): validation and psychometric properties. *Arthritis Res Ther*. 2009; 11(5):415.
4. Field, AP. *Discovering statistics using IBM SPSS Statistics: and sex and drugs and rock 'n' roll*. 3rd ed. London: Sage Publications; 2009.

SUPPLEMENTARY INFORMATION TO ARTICLE 2

The GC-MS metabolomics signature in patients with Fibromyalgia Syndrome directs to dysbiosis as an aspect contributing factor of FMS pathophysiology

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S1: Experimental subjects symptom assessment questionnaire - Malatji et al 2017 [1]

The Fibromyalgia Impact Questionnaire (FIQR) is an internationally derived questionnaire developed by Burckhardt and co-workers [2]. It was developed with the aim to evaluate and understand the effects of therapy on the broad range of symptoms that manifest in FMS. As such, the questionnaire has routinely been used, since its official release in 1991, as a means to assess the progression of the disorder and any therapeutic interventions applied [3].

Questionnaire A in Table S1 shows this FIQR questionnaire that was voluntarily completed by the FMS patients who took part in this study. The in-house clinical questionnaire, Table S1B, was drawn up to identify secondary data about the patients for use in conjunction with the FIQR questionnaire.

Table S1: Fibromyalgia Impact Questionnaire (FIQR) (A) and Clinical questionnaire (B). Questionnaire A was used by the clinicians to assess the severity of the symptoms experienced by the FMS patients. Questionnaire B was used to gather supplementary information on the FMS patients

A - Fibromyalgia Impact Questionnaire (FIQR)

4. Function domain

Directions: For each question, place an "X" in the box that best indicates how much your fibromyalgia made it difficult to do each of the following activities during the past 7 days

Brush or comb your hair	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Walk continuously for 20 minutes	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Prepare a homemade meal	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Vacuum, scrub or sweep floors	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Lift and carry a bag full of groceries	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Climb one flight of stairs	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Change bed sheets	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Sit in a chair for 45 minutes	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult
Go shopping for groceries	No difficulty	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Very difficult

5. Overall impact domain:

Directions: For each question, check the one box that best describes the overall impact of your fibromyalgia over the last 7 days:

Fibromyalgia prevented me from accomplishing goals for the week	Never	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Always
I was completely overwhelmed by my fibromyalgia symptoms	Never	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Always

6. Symptoms domain:

Directions: For each of the following 10 questions, select the one circle that best indicates the intensity of your fibromyalgia symptoms over the past 7 days

Please rate your level of pain	No pain	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	Unbearable pain
Please rate your level of energy	Lots of	<input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	No energy

<i>energy</i>		
Please rate your level of stiffness	<i>No stiffness</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Severe stiffness</i>
Please rate the quality of your sleep	<i>Awoke well rested</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Awoke very tired</i>
Please rate your level of depression	<i>No depression</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Very depressed</i>
Please rate your level of memory problems	<i>Good memory</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Very poor memory</i>
Please rate your level of anxiety	<i>Not anxious</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Very anxious</i>
Please rate your level of tenderness to touch	<i>No tenderness</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Very tender</i>
Please rate your level of balance problems	<i>No imbalance</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Severe imbalance</i>
Please rate your level of sensitivity to loud noises, bright lights, odors and cold	<i>No sensitivity</i> <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3 <input type="checkbox"/> 4 <input type="checkbox"/> 5 <input type="checkbox"/> 6 <input type="checkbox"/> 7 <input type="checkbox"/> 8 <input type="checkbox"/> 9 <input type="checkbox"/> 10	<i>Extreme sensitivity</i>

B - Clinical Questionnaire

7. Age: Years Months

8. Relationship status:

1. Married	2. Engaged	3. In a relationship, but not married or engaged	4. Single
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9. Current employment status

1. Fulltime employed	2. Part-time employed	3. Home executive (housewife)	4. Retired	5. Disabled
----------------------	-----------------------	-------------------------------	------------	-------------

10. How long ago did your fibromyalgia symptoms start (e.g. widespread muscle pain, poor sleep, fatigue, headaches, etc.)

Years

Months

11. How long ago were you diagnosed with fibromyalgia?

Years

Months

12. (a) Were your fibromyalgia symptoms triggered by? Please tick (v) (you may tick more than one block)

1. Neck injury

☐

2. Other injuries

☐

3. After surgical procedure

☐

4. Severe emotional stress

☐

5. Acute infection

☐

6. Spontaneous onset

☐

7. Uncertain

☐

(b)

Length

Bodyweight

8. Please rate your pain by circling the **one** number that best describes you pain at its **worst** in the last month. (A rating of 10 would indicate pain so severe as to prohibit all activity; the worst pain you can imagine.)



9. Please rate your pain by circling the **one** number that best describes you pain on the **average** in the last month. (A rating of 10 would indicate pain so severe as to prohibit all activity; the worst pain you can imagine.)



10. (i) Which medications are you receiving **for your pain**?

Please tick

Trepeline	<input type="checkbox"/>	Lyrice	<input type="checkbox"/>
Cymbalta	<input type="checkbox"/>	Syndol	<input type="checkbox"/>
Tramal (Tramahexal)	<input type="checkbox"/>	Myprodol	<input type="checkbox"/>
Tenston	<input type="checkbox"/>	Mypaid	<input type="checkbox"/>
Stilpayne	<input type="checkbox"/>	Cataflam	<input type="checkbox"/>
Other	<input type="text"/>		

(ii) How often do you take pain killers (e.g. Tramal, Tramahexal, Tramacet, Panado, Syndol., Mypaid, Cataflam, Voltaren, etc.)

- ± Once a week
- ± Twice a week
- ± Three days a week
- ± Every second day
- ± Daily

<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>

10. During the past week how much did the state of your health, including any pain, interfere with the following things: choose the one number, from 0 to 4 below, that best describes your state and write them in the appropriate box (I to vi).

- 5 Not at all
- 6 A little bit
- 7 Moderately
- 8 Quite a bit
- 9 Extremely

vii. Mood	<input type="checkbox"/>
viii. Relations with other people	<input type="checkbox"/>
ix. Walking ability	<input type="checkbox"/>
x. Sleep	<input type="checkbox"/>
xi. Normal Work (includes both work outside the home and housework)	<input type="checkbox"/>
xii. Enjoyment of life	<input type="checkbox"/>

13. Have you ever been diagnosed and treated for depression

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

14. Do you suffer from regular headaches?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

14 Irritable bowel syndrome (IBS) is known to commonly affect patients with fibromyalgia. It is characterized by abdominal pain and cramps as well as bloating, flatulence, diarrhea and/or constipation

Have you ever been diagnosed with IBS?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

- 15 Restless leg syndrome (RLS) is characterized by uncomfortable sensations in the lower legs and an uncontrollable urge to move them so as to provide relief. Some of the sensations felt in RLS include burning, creeping or a crawling feeling inside the legs.

Have you ever experienced symptoms of RLS?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

15. Have you suffered from anxiety since being diagnosed with fibromyalgia or thereafter? (Symptoms such as feeling nervous most of the time, not able to control worrying, etc.)

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

16. If yes, have you been diagnosed and treated for anxiety?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

19. Have you ever suffered from a sleep disturbance?

<input type="checkbox"/>	Yes	<input type="checkbox"/>	No
--------------------------	-----	--------------------------	----

If yes, please tick the appropriate block(s)
(you may tick more than one block)

Problem with sleep initiation
Problem with maintaining sleep
Early morning awakening
Waking up feeling unrefreshed

<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>

20. Dysmenorrhoea is defined as painful menstruation often associated with cramps for mostly 1–3 days after beginning of menstruation.

Were you treated before or are you currently being treated for dysmenorrhoea?

No

<input type="checkbox"/>
<input type="checkbox"/>

Table S2: Summary of the supplementary data collected based on the in-house questionnaire (Table S1B) completed by the FMS patient group.

Variable	Mean	%
1. Socio-demographic information		
Age (years)	45.5	n/a
Marital status		
Single	2	11
Separated/Divorced/Widow	0	0
In permanent relationship	2	11
Married	15	78
Employment		
Disabled/Retired	2	11
Housewife	6	32
Part-time	1	5
Full-time	10	52
2. Pain experience		
Worst pain experience	7,7	n/a
Recent past pain average	5,7	n/a
Pain specific medication		
Trepiline (antidepressant for neuropathic pain)	12	63
Cymbalta (antidepressant for chronic pain)	10	52
Tramal (analgesic for moderate to severe pain)	9	47
Myprodol (relief of pain of inflammatory origin)	6	32
Patients using also other medication against pain	11	58
3. Levels of emotional experiences affected by FMS		
Mood	2.3	n/a
Relations with other people	2.2	
Enjoyment of life	2.1	
Normal work	2.4	

0 = Not at all **1** = A little bit **2** = Moderately **3** = Quite a bit **4** = Extremely

S2: Standard Operating Protocol for organic acid extraction

In this section, the laboratory standard operating protocol (SOP) is presented.

General organic acid analysis of urine by GC/MS:

Overview

The 3 principal steps for this method are:

1. Isolation of the organic acids from physiological fluids
2. Formation of volatile derivatives
3. GC-MS analysis.

Organic acids are isolated from physiological fluids with ethyl acetate and diethyl ether extractions. The organic acid extract is evaporated to dryness under nitrogen; volatile trimethylsilyl (TMS) derivatives of the extracted organic acids are formed by heating with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The TMS derivatives are less than ideal products for some classes of compounds such as acylglycine, which form mono and di-TMS derivatives, yet they are the most useful and versatile compounds for the wide range of functional groups in organic acids. The derivatives are analysed on GC-MS.

GC-MS is able to separate the highly volatile organic acids using gas chromatography, followed by detection of individual components by means of mass spectroscopy. This procedure permits rapid identification and quantification of constituent organic acids with a high degree of sensitivity and chromatographic resolution.

Reagents

NOTE: refer to chemical information sheet below for descriptions and precautions/hazards of chemicals used.

Internal standard (4-phenylbutyric acid solution) prepared by measuring precisely 26.25 mg 4-phenylbutyric acid, adding 3 drops of 1M sodium hydroxide (NaOH) to dissolve and adding 50 ml distilled H₂O (dH₂O). Other reagents include: 5M hydrochloric acid (HCl); ethyl acetate (HPLC grade), distilled once to purify further; diethyl ether (HPLC grade), distilled once to purify further; anhydrous sodium sulphate (Na₂SO₄); bis(trimethylsilyl)-trifluoroacetamide (BSTFA); trimethylchlorosilane (TMCS); pyridine and hexane.

Instrument Settings

Gas chromatography (GC)

GC	Agilent 7890A
Auto-sampler	Agilent 7693
Oven program	50°C for 1 min; then 20°C/min to 60°C; then 5°C/min to 120°C; then 7°C/min to 280°C
Run time	40.35 min
Post run	1 min at 300°C
Injection volume	1 µl
Pre-injection washes	Solvent A: 2 x 4 µl Solvent B: 0
Post-injection washes	Solvent A: 1 x 4 µl Solvent B: 2 x 4 µl
Front inlet	Heater: 280°C Carrier gas: Helium Total flow: 15.29 ml/min Split ratio: 12:1 Split flow: 11.34ml/min
Column	DB-1MS 340°C: 30 m x 250 µm x 0.25 µm

Mass spectrometry (MS)

MS	Agilent 5975C VL MSD
Solvent delay	7 min
Acquisition mode	scan
Scan parameters	Low mass: 50.0 High mass: 600.0 Threshold: 15
MS source	230°C (max 250°C)
MS quad	150°C (max 200°C)

Organic acid extraction

1. Add 1 ml sample to large kimax test tube
2. Add 6 drops 5M HCl to adjust to pH 1 (using glass pipette)
3. Add creatinine-based calculated volume of internal standard
4. Add 6 ml ethyl acetate
5. Cap test tubes and check there is no leakage by inverting test tube (quality control step to ensure no sample is lost during next step)
6. Mix for 30 min in Roto-torque
7. Centrifuge at 3000 rpm for 3 min
8. Aspirate the organic phase into clean large kimax test tube (using glass pipette) and set aside
9. Add 3 ml diethyl ether to the aqueous (lower) phase
10. Cap test tubes and check there is no leakage by inverting test tube
11. Mix for 10 min in Roto-torque
12. Centrifuge at 3000 rpm for 3 min
13. Aspirate the organic phase and add to the ethyl acetate phase (using glass pipette)
14. Discard lower aqueous phase into appropriate organic waste container
15. Add two level spatula scoops of anhydrous Na_2SO_4
16. Cap and invert test tube several times (or vortex for 5 seconds) to ensure good mixing (proper dispersion of Na_2SO_4 ensures all water molecules removed from organic phase as water reverses chemical process of silylation, thereby reducing the efficiency of derivatization)
17. Centrifuge at 3000 rpm for 1 min
18. Pour/decant the organic phase into a clean small kimax tube
19. Evaporate to dryness in heating block at 37°C under nitrogen gas (~1 hour)
20. Use Hamilton glass syringe to add 5:1:1 BSTFA, TMCS and pyridine, respectively, based on creatinine-based calculated volume.
21. NOTE: Hamilton glass syringe is kept clean with pyridine and approximately 100 μl hexane is withdrawn into syringe and discarded (five times) between the addition of each reagent (quality control step to ensure syringe is clean and avoid cross-contamination)
22. Cap test tubes and incubate at 60°C for 1 hour (45 min at 70°C)

- 23.** Set up and label GC-MS vials, with insert and cap
- 24.** Transfer approximately 100 µl sample to GC-MS vial
- 25.** NOTE: clean glass syringe with hexane (five times) after each transfer
- 26.** Cap GC-MS vial and place in auto-sampler and process via GC-MS.

General AMDIS settings (organic acids)

- 80% minimum match factor
- Type of analysis: use internal standards for RI (show standards)
- Resolution: medium
- Sensitivity: medium
- Shape requirements: medium

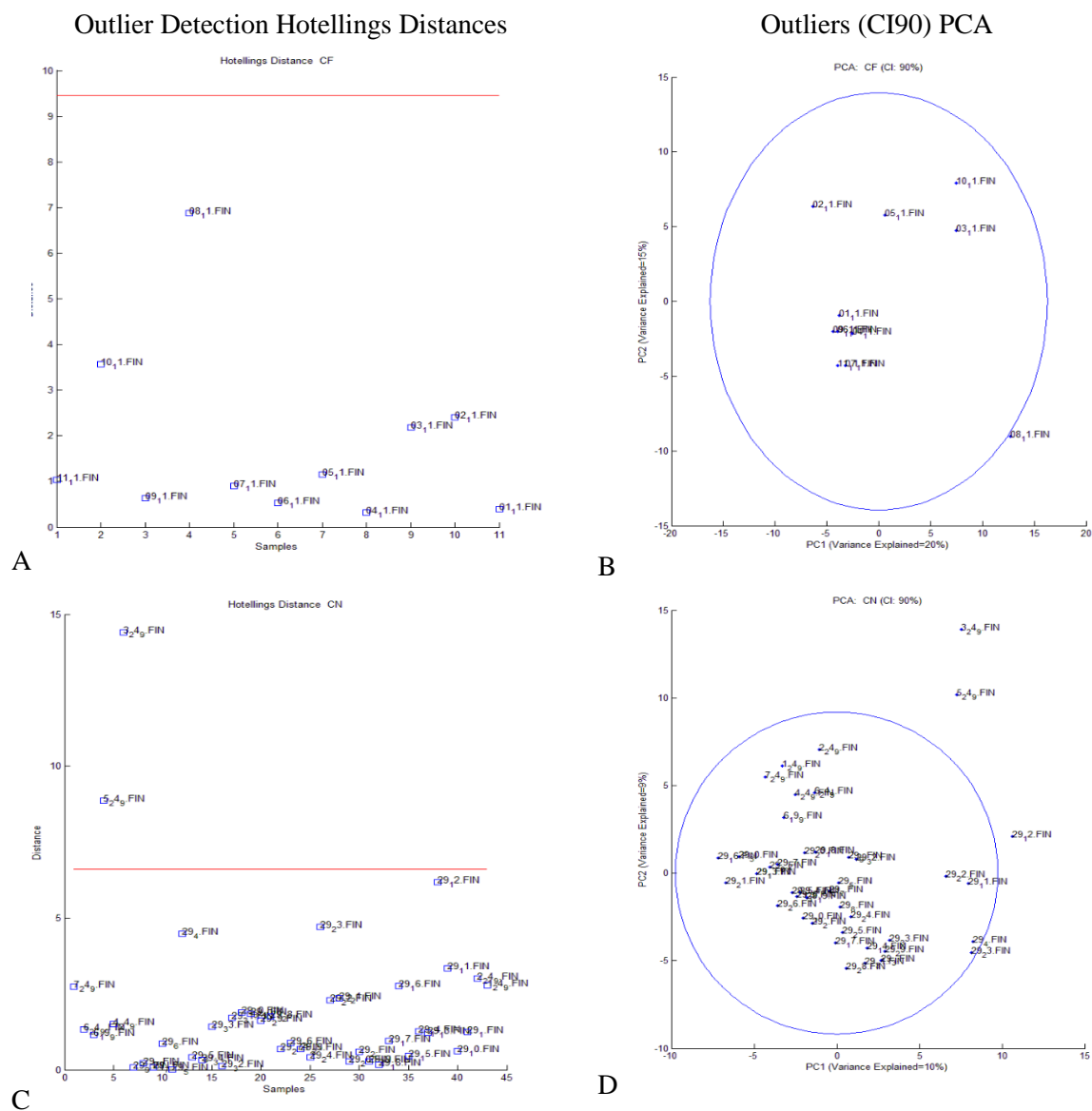
Feature/metabolite identification is done by comparing each feature's/metabolite's MS-spectral pattern with customized spectral library specific to the urine sample under investigation.

Chemical information sheet

- 4-Phenylbutyric acid – $\text{C}_{10}\text{H}_{12}\text{O}_2$; Mw: 164.21 g/mol; supplier: Fluka (25 g) (index no.78243). Precaution/ hazard: avoid contact with skin and eyes.
- Sodium hydroxide – NaOH ; Mw: 40.00 g/mol; supplier: Merck (500 g). Precaution/ hazard: corrosive (causes severe burns).
- Hydrochloric acid (32%) – HCl ; Mw: 36.36 g/mol; supplier: Merck (2.5 l). Precaution/ hazard: corrosive (causes severe burns); irritating to respiratory system.
- Ethyl acetate – $\text{CH}_3\text{COOC}_2\text{H}_5$; Mw: 88.11 g/mol; supplier: Merck (2.5 l) (index no. 607-022-00-5). Precaution/ hazard: highly flammable; causes drowsiness/dizziness; causes eye irritation; repeated exposure causes skin dryness/cracking.
- Diethyl ether – $(\text{C}_2\text{H}_5)_2\text{O}$; Mw: 74.12 g/mol; supplier: Merck (2.5 l) (index no. 602-022-00-4). Precaution/ hazard: extremely flammable; harmful if swallowed; causes drowsiness/dizziness; repeated exposure causes skin dryness/cracking; may form explosive peroxides.
- Hexane – $\text{CH}_3(\text{CH}_2)_4\text{CH}_3$; Mw: 86.18 g/mol; supplier: Merck (2.5 l) (index no. 601-037-00-0). Precaution/ hazard: highly flammable; fatal if swallowed; causes skin irritation; toxic to aquatic life; causes drowsiness/dizziness; may cause infertility or damage to unborn child; may cause damage to organs through prolonged or repeated exposure.
- Sodium sulphate – Na_2SO_4 ; Mw: 142.04 g/mol; supplier: Merck (500 g).
- Chlorotrimethylsilane (TMCS) – $\text{C}_3\text{H}_9\text{ClSi}$; Mw: 108.64 g/mol; supplier: Flukka Analytical (100 ml) (index no. 92360). Precaution/ hazard: highly flammable; corrosive (causes severe burns); reacts violently with water; harmful by inhalation/contact to skin; irritating to respiratory system.
- Pyridine – $\text{C}_5\text{H}_5\text{N}$; Mw: 79.10 g/mol; supplier: Flukka Analytical (1 l) (index no. 82703). Precaution/ hazard: highly flammable; harmful if inhaled or swallowed; harmful to skin.
- Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) – $\text{CF}_3\text{C}=\text{NSi}(\text{CH}_3)_3\text{OSi}(\text{CH}_3)_3$; Mw: 257.40 g/mol; supplier: Supelco Analytical (25 ml) (index no. 3-3027). Precaution/ hazard: flammable; irritant to eyes and skin; causes burns.

S3: Case reduction analyses

Using the original variable data, case reduction was first applied to all four experimental groups. Outliers were identified based on the presence of suspicious metabolites (including due to medication) and statistical by using a 95% confidence region in a Hotelling's T^2 test in conjunction with the respective PCA score plots with 90% confidence regions. Cases that were identified as outliers by either method were removed. Figure S1 shows the results of these case reduction analyses.



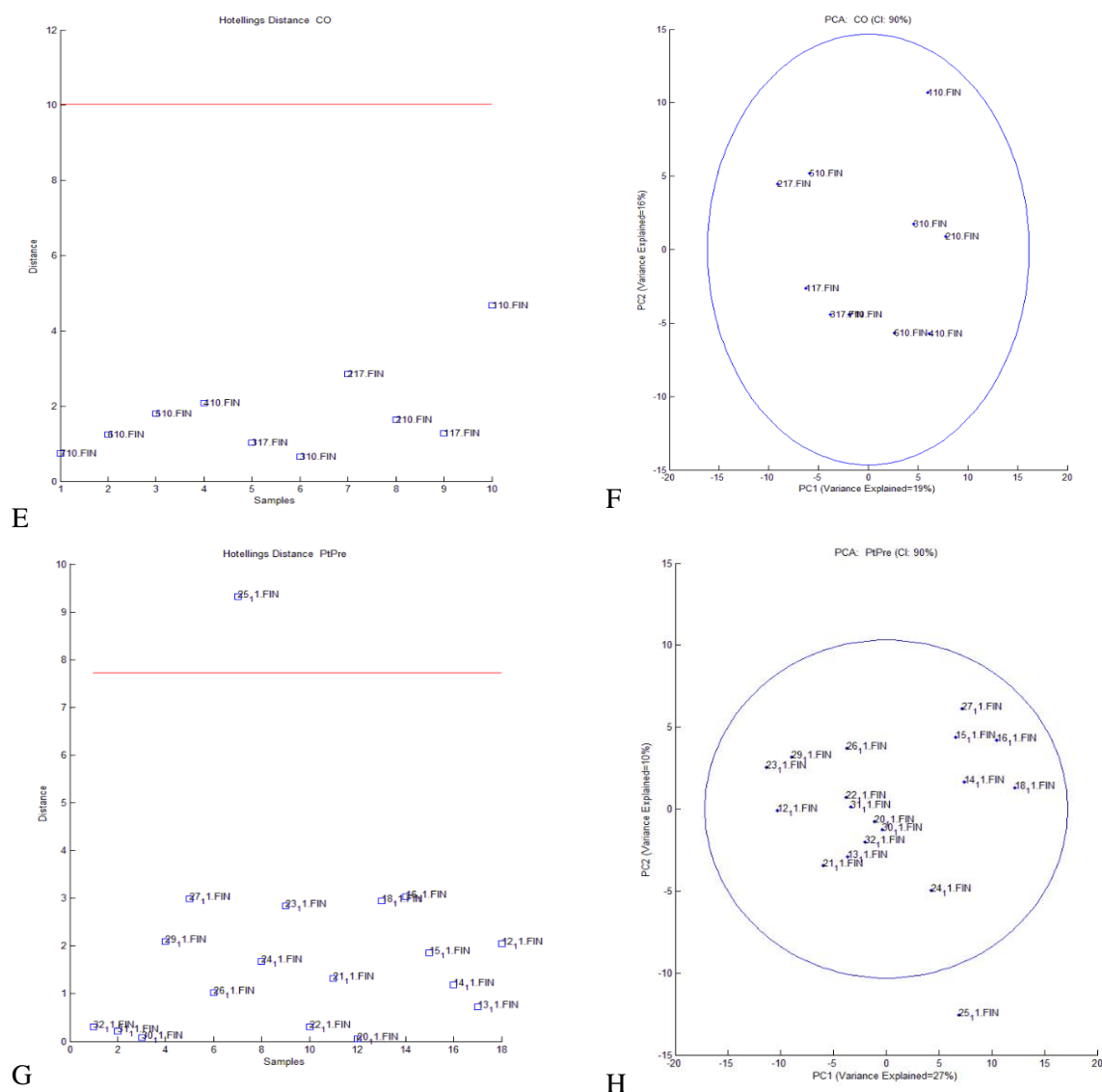


Fig. S1: Case reduction analyses, using Hotelling's T2 and a PCA test. These tests were used to detect outliers in the controls (CF (A to B), CO (C to D) and CN (E to F)) and patients (G to H). Red (Hotelling's) and blue (PCA) lines indicate the threshold where a sample is considered an outlier.

S4: Variable lists indicating metabolite groupings

In this section we show the metabolite lists used to obtain the results in Fig. 4 of the main text namely (1) gut-host metabolites with a focus on benzene derivatives of poly-phenolic dietary origin (54 metabolites), (2) metabolites of energy and intermediary metabolism (36 metabolites), (3) carbohydrates and related metabolites (30 metabolites), and (4) the remaining metabolites. Note: We regard assignment as relative as a certain metabolite may

actually be classified to more than one group, while each metabolite was classified here in one group only.

Benzenes:

1,2,3,5-Tetramethylbenzene
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)
ester
1,2-Benzenedicarboxylic-acid
1,2-Dihydroxybenzene
2,3,4-Trihydroxybenzoic-Acid
2,3-Dihydroxybenzoic-Acid
2,4-Dihydroxybenzoic-Acid
2,5-Dihydroxybenzoic acid
2,6-Dihydroxybenzoic-Acid
2-Aminobenzoic-Acid
2-Hydroxy-5-Methoxybenzoic-Acid
2-Hydroxybenzoic-Acid
2-Hydroxyhippuric-Acid
2-Hydroxyphenylacetic-Acid
3,4-Dihydroxybenzoic-Acid
3,4-Dihydroxycinnamic-Acid
3,4-Dihydroxyphenylacetic-Acid
3,4-Dihydroxyphenylpropionic-Acid
3,5-Dihydroxybenzoic-Acid
3-Hydroxybenzoic-Acid
3-Hydroxyhippuric-Acid
3-Hydroxyphenylacetic-Acid
3-Hydroxyphenylhydracrylic-Acid
3-Hydroxyphenylpropionic-Acid
3-Methoxy-4-hydroxycinnamic-acid
3-Methoxy-4-Hydroxyphenylhydracrylic-Acid
3-Methoxy-4-Hydroxyphenyllactic-Acid
3-Methoxy-4-Hydroxyphenylpropionic-Acid
4-Hydroxybenzoic-Acid
4-Hydroxybenzeneacetic-Acid
4-Hydroxybutyric-Acid
4-Hydroxycinnamic-Acid
4-Hydroxycyclohexylacetic-Acid
4-Hydroxyhippuric-Acid
4-Hydroxymandelic-Acid
4-Hydroxyphenyllactic-Acid
4-Methoxy-3-Hydroxycinnamic-Acid
4-Methylmandelic-Acid
4-Phenol
Benzamide, N-(trimethylsilyl)-

Benzoic-Acid
Butylated Hydroxytoluene
Hippuric-Acid
Homovanillic-Acid
Hydroxymethoxybenzoylglycine
Mandelic-acid
N-Acetyl-4-Phenol
N-ACETYLTYROSINE
Phenylacetic-Acid
Phenylacetylglutamine
Phenyllactic-Acid
p-Tolylglucuronide
Vanillic-Acid
Vanillylmandelic-Acid

Energy:

1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)
ester
1H-Indole-3-Acetic-Acid
2-HYDROXYGLUTARIC-ACID
2-Hydroxyphenylacetic-Acid
3-(4-Hydroxy-2,5-Dioxoimidazolidin-4-yl)propanoic-
Acid
3,4-Dihydroxybenzoic-Acid
3,4-Dihydroxycinnamic-Acid
3-Hydroxypyridine
3-Methoxy-4-Hydroxyphenylpropionic-Acid
4-Hydroxybenzeneacetic-Acid
4-Hydroxybutyric-Acid
4-Hydroxyphenyllactic-Acid
4-Phenol
5-Hydroxyindoleacetic-Acid
Aconitic-Acid
Butylated Hydroxytoluene
Dodecanoic-Acid
ETHYLMALONIC-ACID
Furoylglycine
GLUTARIC-ACID
GLYCOLIC-ACID
Hexanoic-Acid
Levulinic-Acid
Maleic-Acid
Malic-Acid
METHYLMALONIC-ACID
METHYLSUCCINIC-ACID

Monohexadecanoylglycerol
Monostearyl glycerol
N-ACETYLASPARTIC-ACID
N-ACETYLTYROSINE
N-TIGLYLGLYCINE
Octadecanoic-Acid
OXALIC-ACID
Palmitic-Acid
Phosphoric-Acid
SUCCINIC-ACID
Tiglic-Acid

Sugars:

1,2-Dihydroxyethane
2,3,4,5-Tetrahydroxypentanoic-Acid-1,4-Lactone
2,3,4-Trihydroxybutyric-Acid
2,3,4-Trihydroxybutyric-Acid-Lactone
2,4-Dihydroxybutyric-Acid
2-Deoxy-3,5-Dihydroxypentonic-Acid-G-Lactone
2-Keto-l-gluconic-Acid
2-Methyl,2,3-Dihydroxypropanoic-Acid
3,4,5-Trihydroxypentanoic-Acid
3,4,5-trihydroxyvaleric-Acid-Lactone
3,4-Dihydroxybutyric-Acid
3-Deoxy-erythro-Pentonic-Acid
3-Deoxy-ribohexonic acid
Arabinose
D-Erythronic acid τ -lactone
Erythro-Pentonic-Acid
Fructopyranose
Fucono-G-Lactone
Galactonic-Acid-Gamma-Lactone
Galactonic-Acidlactone
Galactopyranose-2-Deoxy
Galactopyranose-Alpha-D
Glycerol
Mannonic-Acid
Mannose
Rhamnose
Sorbose
Tagatofuranose
Tagatose
Threonic-Acid

Other

1,2-Butanediol
1,2-Dihydroxypropane
1,6-Dihydroxyhexane
1H-Indole-1-acetic-Acid
2-(Furan-2-yl)-2-Hydroxyacetic-Acid
2,2-Dihydroxyacetic-Acid
2,3,5-Trihydroxyvaleric-Acid-Lactone
2,3-Dihydroxybutane
2,3-Dihydroxybutanoic-Acid
2,5-Furandicarboxylic-Acid
2,6-Dihydroxy-4-Pyrimidinecarboxylic-Acid
2-Ethyl-3-Hydroxypropionic-Acid
2-Hexenoic-Acid
2-Hydroxy-3-Methylbutyric-Acid
2-Hydroxy-3-Methylvaleric-Acid
2-Hydroxyadipic-Acid
2-Hydroxybutyric-Acid
2-Hydroxyisobutyric-Acid
2-Hydroxysebacic-Acid
2-Keto-3-Methylbutyric-Acid
2-Ketobutyric-Acid
2-KETOGLUTARIC-ACID
2-Methyl-2-Hydroxybutyric-Acid
2-METHYL-3-HYDROXYBUTYRIC-ACID
2-Octenoic-Acid
3-HYDROXY-3-METHYLGLUTARIC-ACID
3-HYDROXYGLUTARIC-ACID
3-HYDROXYISOBUTYRIC-ACID
3-HYDROXYISOVALERIC-ACID
3-HYDROXYPROPIONIC-ACID
3-HYDROXYSEBACIC-ACID
3-Methyl-2-pentenedioic-Acid
3-Methyladipic-Acid
3-METHYLGLUTACONIC-ACID
3-Methylglutaric-Acid
4-Hydroxy-3-Penten-2-One
4-Hydroxycyclohexanecarboxylic-Acid
4-Ketovaleric-Acid
4-Pyridinecarboxylic-Acid
5-(Hydroxymethyl)Furan-2-Carboxylic-Acid
5-Hydroxyhydantoin
5-Hydroxyvaleric-Acid
6-Hydroxyhexanoic-Acid
ACETOACETIC-ACID

Acetylamino-phenylglucopyranosiduronic-Acid
ADIPIC-ACID
Alto-2-Heptulose
Azelaic-Acid
Citraconic-Acid
Citramalic-Acid
Citric-acid
Erythronic-Acid
FUMARIC-ACID
Glucopyranose
Glucopyranono-(6-1)Lactone
Glucuronic-Acid
Glutaconic-Acid
GLYOXYLIC-ACID
Hydantoinpropionic-Acid
Isocitric-Lactone
LACTIC-ACID
MALONIC-ACID
METHYLCITRIC-ACID
Methylmaleic-Acid
N-Acetylanthranilic-Acid,
N-Acetylisoleucine
N-Acetylthreonine
N-HEXANOYLGLYCINE
N-ISOBUTYRYLGLYCINE
N-ISOVALERYLGLYCINE
Nonanoic-Acid
Octenedioic-Acid
Oleic-Acid
Pantothenic-Acid
Parabanic-Acid
Pimelic-Acid
Pyroglutamic-Acid
Pyrrole-2-Carboxylic-Acid
Pyruvic-Acid
Quinolinic-Acid
Ribonic-G-Lactone
Sorbic-Acid
Suberic-Acid
Threitol
Uracil

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SUPPLEMENTARY INFORMATION TO ARTICLE 3

Alpha-hydroxyisobutyric acid: An overview and focus on fibromyalgia syndrome

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Logistic regression models for FMS patient and control groups

Table S1 summarizes the six logistic regression models fit to predict FMS from three different control groups (CF, CN and CO) utilizing two platforms (GC-MS and NMR). The first two columns provide details of the model being considered. The third and fourth columns consider measures of goodness-of-fit, two tests are considered Hosmer-Lemeshow and Stukel. Goodness-of-fit statistics are complimentary to the predictive ability, as reported in columns 7 to 11, as it provides an indication as to whether a more complex model may perform better. If the Hosmer-Lemeshow test is significant we can conclude that the current model is not acceptable. The change in the log likelihood (column four) from the fitted model to the Stukel model (described below) also indicates if a more complex model may perform better. A negative value indicates that the more complex Stukel model is better, but without penalizing the Stukel model for having more parameters.

Columns five and six consider the significance of the parameter estimates based on the Wald statistic. The first p-value relates to the overall significance of the model parameters actually in the model, if this p-value is significant it implies that the predictor contributes significantly to the predictive ability of the model. The second p-value is associated with a model adjusted based on Stukel's approach, where two terms are added to the model which allow for asymmetry and a different approach rate. If the p-value for the extended model is significant then the parameters should be considered individually and if the added effects are significant, a more complex model should be considered. This was the case for the logistic model

regressing against groups FMS and CN for the GC-MS data, here we may need to adjust the model to allow for asymmetry.

The predictive ability of each model is described in columns 7 to 11. The first statistic reported is Tjur's R-squared which is similar to the r-squared reported for linear models and is also a coefficient of discrimination. Values close to 1 for Tjur's R-squared are excellent and as we can see from the table, the values here are low to moderate indicating that some information may not be captured by the models.

The remaining predictive statistics are discussed in the main text. The final three columns provide the odds ratios, confidence intervals for the ratios and the unit of change.

Table S1 Outcome of six logistic regression models applied to data from FMS patients and controls

Platform	FMS vs ...	Goodness-of-Fit Significance Test		Significance of BETA (Wald)		Predictive Power					OR	OR CI	OR Unit (1 x SD)
		Hosmer-Lemeshow	Stukel (Change in -2logL)	Model	Stukel	Tjur R ²	AUC	AUC CI	LOO AUC	LOO AUC CI			
GC-MS	CF	0.69	1.65	0.6	1	0.01	0.57	0.34-0.81	0.18	0.02-0.33	1.27	0.52-3.24	0.4
	CN	0.70	0.39	0.0004	0.01	0.35	0.85	0.73-0.97	0.83	0.69-0.96	4.12	2.1-10.4	0.3
	CO	0.34	10.1	0.009	0.3	0.58	0.94	0.82-1	0.91	0.78-1	10.07	2.7-120.7	0.3
NMR	CF	0.78	1.24	0.04	0.36	0.18	0.76	0.58-0.94	0.67	0.46-0.88	2.6	1.17-7.9	0.2
	CN	0.07	4.23	0.002	0.27	0.59	0.93	0.84-1	0.91	0.8-1	10.22	3.2-62.1	0.2
	CO	0.57	9.07	0.008	0.39	0.49	0.89	0.76-1	0.88	0.73-1	10.15	2.56-90.45	0.2