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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Abstract

Introduction Fibromyalgia syndrome (FMS) is a chronic pain syndrome. Previous analyses of untargeted metabolomics data indicated altered metabolic profile in FMS patients.

Objectives We report a semi-targeted explorative metabolomics study on the urinary metabolite profile of FMS patients; exploring the potential of urinary metabolite information to augment existing medical diagnosis.

Methods All cases were females. 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 chromatography-mass spectrometry (GC-MS) analysis and data processing and analyses were performed using Matlab, R, SPSS and SAS software.

Results 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 significantly increased metabolites. These metabolites are associated with energy metabolism, digestion and metabolism of carbohydrates and other host and gut metabolites.

Conclusions Overall, urinary metabolite profiles in the FMS patients suggest: (1) energy utilization is a central aspect of this pain disorder, (2) dysbiosis seems to prevail in FMS patients, indicated by disrupted microbiota metabolites, 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 is an avenue to explore for adding non-invasive clinical information for diagnosis and treatment of FMS.

Keywords

Fibromyalgia syndrome (FMS), Gas chromatography- mass spectrometry (GC-MS), Dysbiosis, Carbohydrate, Pain, Biomarkers.

1. Introduction

Fibromyalgia syndrome (FMS) is currently viewed as part of the Functional Pain Syndromes (FPS) (Egloff et al. 2015): 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 (Yunnus et al. 1992; Russell et al. 1994; Legangneux et al. 2001; Meyer 2002). Peripheral pain generators may contribute to the pathophysiology of some FMS patients (Shah et al. 2005) 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 over-growth (SIBO), interstitial cystitis and mood disorders (Pimentel et al. 2001; Levine and Burakoff 2011; Clauw 2015).

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 (Nicholson et al. 2005). A recent untargeted NMR metabolomics study of FMS (Malatji et al. 2017) 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 (Li et al. 2008). In health, host-gut microbiota metabolic (Nicholson et al. 2012) and brain-gut interactions are crucial in the maintenance of homeostasis (Mayer and Tillisch 2011). 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 (Cryan and O'Mahony 2011). The frequent comorbidity of fibromyalgia with stress-related disorders, such as chronic fatigue (Cryan and Dinan 2012) and IBS (Kennedy et al. 2014) and some CNS-related abnormalities (Foster and Neufeld 2013), suggests that gut–brain axis regulation may at least be a partial common denominator for these disorders (Carabotti et al. 2015).

The presence of the exogenous markers of gut origin observed in our NMR metabolomics study (Malatji et al. 2017) 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 derived from partial digestion of complex dietary plant polysaccharides, known to be intermediates in metabolic pathways specific for gut microbiota (Nicholson et al. 2012). Anaerobic colonic bacteria are required for the final metabolism of carbohydrate intermediates towards lactic acid and short-chain fatty acids — part of the human energy resources. Dysbiosis, defined as a disruption of gut bacterial and fungal species (McFarland 2014), is a common clinical symptom seen in exposure to exogenous substances, like antibiotics, or in endogenous conditions like IBS, Alteration of the gut microbiota can lead to significant changes in the colonic microenvironment which becomes reflected in perturbed excretion of organic compounds, such as carbohydrates (Hogenauer et al. 1998), as seen here in the GC-MS data obtained from the present FMS cohort. Notably, a link was proposed between FMS and symptoms of gastrointestinal dysfunction, as some FMS cases present concurrently with SIBO (Wallace & Hallegua 2004), and complaints related to IBS are common (Yunus et al. 1992). We thus propose from our observations that metabolomics information holds promise as an avenue for understanding the dynamic basis of host–microbiome perturbations in FMS, contributing to clinical information that distinguish FMS patients from related comorbidities and direct the development of a functional approach towards its treatment.

2. Materials and methods

2.1 The study population and sampling

We applied a sample collection and analysis pipeline (Fig. 1) for exploratory metabolic profiling of urine samples (Jackson et al. 2016) 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.

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 (Malatji et al. 2017). The diagnosis for FMS in our study group was based on a comprehensive clinical assessment using the American College of Rheumatology (ACR) criteria, first published for FMS in 1990 (Wolfe et al. 1990). All FMS 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) (Bennett et al. 2009) and an in-house clinical questionnaire (IHCQ) (Malatji et al. 2017). Further details are presented in the SI. 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 flagged by statistical analysis and confirmed clinically. 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) with ethical approval given by Pharma Ethics Pty, Ltd (Reference number 11064365), as further specified under Declarations.

2.2 Analytical procedures and quality control

Organic acid profiles of urine collected from our sample groups were analysed by a standard GC-MS method (see SI for detailed descriptions), which was standardized to comply with required levels of qualitatively and quantitatively repeatable and reproducible (Mason et al. 2014). 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 N,O-bis-(trimethylsilyl)trifluoraceteamine (BSTFA), trimethylchlorosilane (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.

2.3 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 (Boutra et al. 2013).

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 (Wishart et al. 2012), 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 after clinical confirmation. Univariate statistics, specifically the Mann-Whitney (MW) test (p values and effect sizes (ES)) 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/).

3. Results

3.1 Data generation and case selection

Using the original variable data, case reduction was first applied to all four experimental groups (Fig. S1). 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 (n=17); and for the controls CF (n=11), CO (n=10) and CN (n=41).

3.2 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.

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 validity of the PLS-DA model was assessed using predictive accuracy (i.e. the ability to explain variation in the dependent or grouping variable Y) on all data ($R^2(Y)$) as well on a leave-one-out approach ($Q^2(Y)$). These metrics, reported in the legend to Fig. 3, indicating a good model fit, partially confirmed the complete separation between the FMS and CN young control groups, with $R^2(Y) = 97\%$ and $Q^2(Y) = 88\%$. More fit statistics between all experimental groups are reported in the SI.

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.

3.3 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 ($p < 0.05$ and smaller than the B-F 5% values), ES >0.8 and FC >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 (Malatji et al. 2017), 2-hydroxyisobutyric acid was significantly increased, but did not contribute to group separation (p = 0.00012; FC = + 2.13, and VIP = 0.32).

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. 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. 2009. 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. S2a), the 36 energy and intermediary metabolites (Fig. S2b) and the 30 carbohydrates and related metabolites (Fig. S2c). Incomplete cluster separation was observed from FMS relative to the CO for the gut-related metabolites (Fig. S2a), as well as for the CF and CN controls. Complete case separations occurred by application of the energy (Fig. S2b for FMS and CO) and carbohydrate (Fig. S2c 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 and glutamic acids and 4-hydroxybutyric acid) showed very good differentiation between the FMS patients and CO controls (boxed areas 1 and 2 in Fig. S2b). 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. S2b). Area 2 again includes an additional metabolite (galactonic acid-lactone) which did not comply with the selection criteria for Table 1, 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. S2) and the quantitative metabolite concentrations (Table 1) points to the effectiveness of the monosaccharide metabolite signature of gut-host metabolites.

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. 4. 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 (Medina et al. 2013).

4. Discussion

Although FMS is presently better understood than ever before (Clauw 2014), 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 (Jones et al. 2016). Untargeted metabolomics studies on FMS have revealed that tryptophan (Hackshaw et al. 2013), lysophosphocholine (Caboni et al. 2014) and gut metabolism (Malatji et al. 2017) 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. 5).

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) (Willats et al. 2001). 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 (Boutra et al. 2013) but exceed normal reference ranges in dysbiosis, the imbalance in the gut microbiome (Henström and D'Amato 2016). Metabolic profiling of urine provides a strategy to characterize metabolites from microbial origin and to define dysbiosis (Nicholson et al. 2012). An example was the increase in arabinose observed in our FMS patients relative to the CO controls (arabinose: Ref. value: 0.8–19.4 µmol/mmol creatinine (HMDB); FMS 19.60 and CO 0.44 µmol/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). The symbiosis between the host and its gut microbiota results in the coproduction of a number of small molecules from dietary and other sources, which play critical roles in shuttling information between the host's microbial symbionts, and within the host (Nicholson et al. 2012). This chemical dialogue predictably becomes disturbed in dysbiosis, which implies a molecular link between host metabolism and gut microbiota genes, rather than just species identity (Li et al. 2008), which could enable a more profound understanding of metabolic basis of host health and its disruption in disease. 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 (Baumgart and Carding 2007; Hansen et al. 2010). 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) (Postma and Lengeler 1985). Since its discovery in 1964 (Kundig et al. 1964), 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 (Saier 2015). The PTS has tight substrate specificity for the translocation and subsequent phosphorylation (formation of the monophosphate monosaccharide) process, shown schematically in Fig. 5b for tagatose (van der Heiden et al. 2013). 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 (Murphey and Rosenblum 1964), while mutants of *E. faecium* displayed a colonization defect in antibiotic-treated mice (van Schaik et al. 2010). These observations clearly linked an aspect of

dysbiosis to potential genetic aberrations in the PTS 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 (Miquel et al. 2015), 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 (Brown et al. 1987)) or to microbiome-host co-metabolism (sorbose; see Fig. 5c and 5d). Sorbose is a structural product of fruit polysaccharides (Ge et al. 2009). Two metabolic sorbose pathways reside in bacteria: (1) The PTS mediated pathway, of L-sorbose → L-sorbose-1-P → D-gluticol-6-P →D-fructose-6-P → energy release (Kelker et al. 1972); (2) The 2-keto-L-gulonic acid pathway (Fig. 5d), observed in a large number of bacterial strains, including the gut-linked *Eschericia coli* (Isono et al. 1968). Next, sorbitol, an abundant osmolite with a key role in regulating human cell volume homeostasis and cytoprotection, is produced from L-sorbose (sorbose 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-guconic, 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 sorbose 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.

Finally, 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). Although larger patients and control groups are advocated, from our experience with metabolomics studies, groups of selected cases of no less than twenty would suffice. (2) Dysbiosis emerged in this study as a post-hoc clinical aspect of FMS. Although 88% of our patient group indicated discomforts with their gastrointestinal functions (defined as IBS in our clinical questionnaire – see SI Table S1, B. Clinical Questionnaire, §13), a follow-up study should include some molecular phylogenetic analysis to define in the diversity of fecal bacteria in patients diagnosed for a study on a link between FMS and dysbiosis. (3) 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. (4) 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)]. Regardless of these limitations encountered in metabolomics studies, metabolomics retains a promise well beyond the scope of standard clinical chemistry techniques, for affording detailed characterization of metabolic phenotypes. It is generally believed that metabolomics studies would contribute to insights, leading to so-called precision medicine in which knowledge of their unique metabolic derangements explains the disease state of individual patients (Clish 2015).

5. Conclusions

The semi-targeted GC-MS metabolomics study on urine samples from FMS patients and controls confirmed and extended the metabolite information reported in a previous metabolomics study, using NMR technology (Malatji et al. 2017). Here, the overall urinary metabolite profile observed in the FMS patients suggests that (1) energy utilization is a central aspect of this pain disorder, and (2) dysbiosis seems to prevail in FMS patients, supporting the model that microbiota may alter brain function through the gut-brain axis (Mayer et al. 2015), with the gut being a gateway to generalized pain. All-in-all, 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. Screening of urine from FMS is an avenue to explore for adding non-invasive clinical information for diagnosis and treatment of FMS, supporting a present view that effective treatment for fibromyalgia becomes achievable (Clauw 2014). 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 (Mayer et al. 2015). This might provide the key to future management of FMS.

List of abbreviations

2-D-3,5-DHPL: 2-deoxy-3,5-dihydroxypentanoic lactone.
2,3,4-trihydroxy-butyl-L: 2,3,4-trihydroxybutyl-lactone
4-HBA: 4-hydroxybutyric acid
ACR: American College of Rheumatology
ADP: adenosine-diphosphate
ATP: adenosine-triphosphate
B-F: Bonferroni-Holm test
BSTFA: N,O-bis-(trimethylsilyl)trifluoraceteamine
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
EI and HP: a general and a histidine-containing cytoplasmic phosphor-carrier bacterial protein system
EII-AT, EII-BT and EII-CT: tagatose-specific B. licheniformis multi-domain membrane proteins
EC: Enzyme Commission number
ES: effect size
FC: fold change
FIQR: Fibromyalgia Impact Questionnaire
FM: fibromyalgia
FMS: fibromyalgia syndrome
FPS: functional pain syndromes
G: galactonic acid-lactone
GC-MS: gas chromatographic-mass spectrometric
H: 2-hydroxy-glutaric acid.
HMDB:
IBS: irritable bowel syndrome
IHCQ: In-house clinical questionnaire
M: malic acid
MW: Mann–Whitney test
NIST: National Institute of Standards and Technology
1H-NMR: proton nuclear magnetic resonance
NTeMBI: Nuclear Technologies in Medicine and Biosciences Initiative
NWU: North-West University
~P: high-energy phosphate
PC: principal component
PCA: principal components analysis
PLS-DA: partial least squares discriminant analysis
PTS: phosphoenolpyruvate:carbohydrate phosphotransferase system
Q2: predictive ability parameter
R2: goodness-of-fit parameter
SIBO: small intestinal bacterial over-growth
Tag-1P: tagatose-1-phosphate
Tag-6P: tagatose-6-phosphate
TagK: tagatose-1-phosphate kinase
TMCS: trimethylchlorosilane
TPs: tender-points
VIP: variable important in projection

Declarations

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). 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." Informed consent was obtained from all the participants in this study by means of a voluntarily completed consent form, included in the SI.

Consent to publish

All authors have given their approval of the version of the manuscript as submitted, their consent for publication and agreed to the accountability requirements.

Availability of data and materials

The clinical details on the patients, as well as the full complement of NMR metabolomics data, are available in the Supplementary Information of (Malatji et al. 2017).

Competing interests

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

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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. 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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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).

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.

Fig. 3 Supervised analysis for the FMS patients relative to the controls, based on all metabolites. Upper panels: PLS-DA (A supervised multivariate analysis), 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: Volcano plots (A supervised univariate analysis). Red lines in the volcano plot (d to f) indicate the univariate boundaries of $p < 0.05$ and $|FC| > 2$, respectively.

Fig. 4 Correlation matrix for the 14 significant metabolites causing discrimination between FMS patients and controls. Abbreviations used as in Table 1. Red: High up-regulation; blue: high down-regulation.

Fig. 6 Conceptual representations of microbiome-host metabolic interactions proposed to be elemental in dysbiosis in FMS patients. (a) A model of the pectin (adapted from Caboni et al. 2014) 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 van der Heiden et al. 2013) 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.

Figure 1

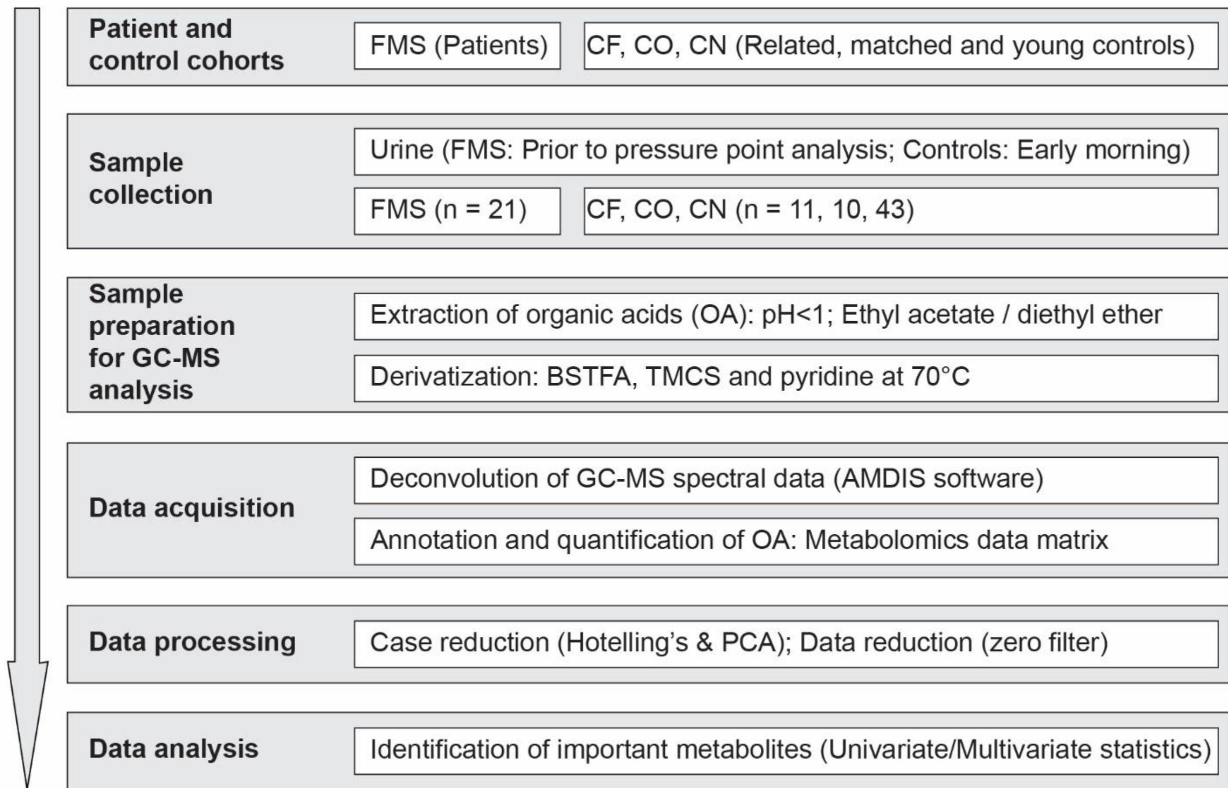

Figure 2

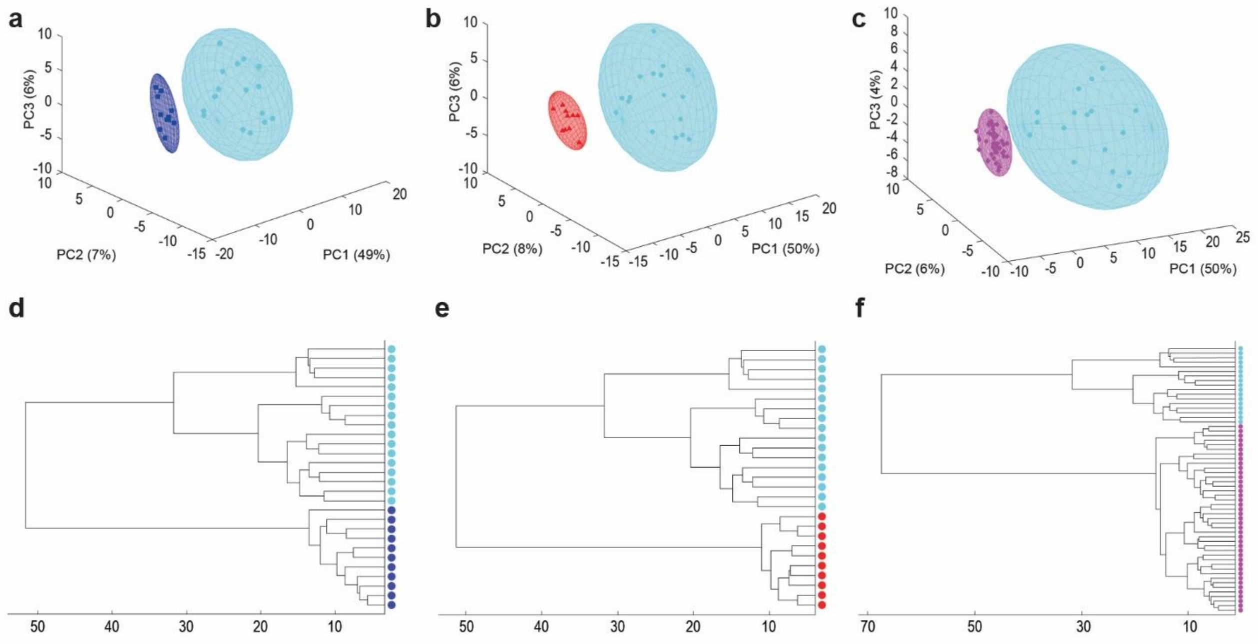

Figure 3

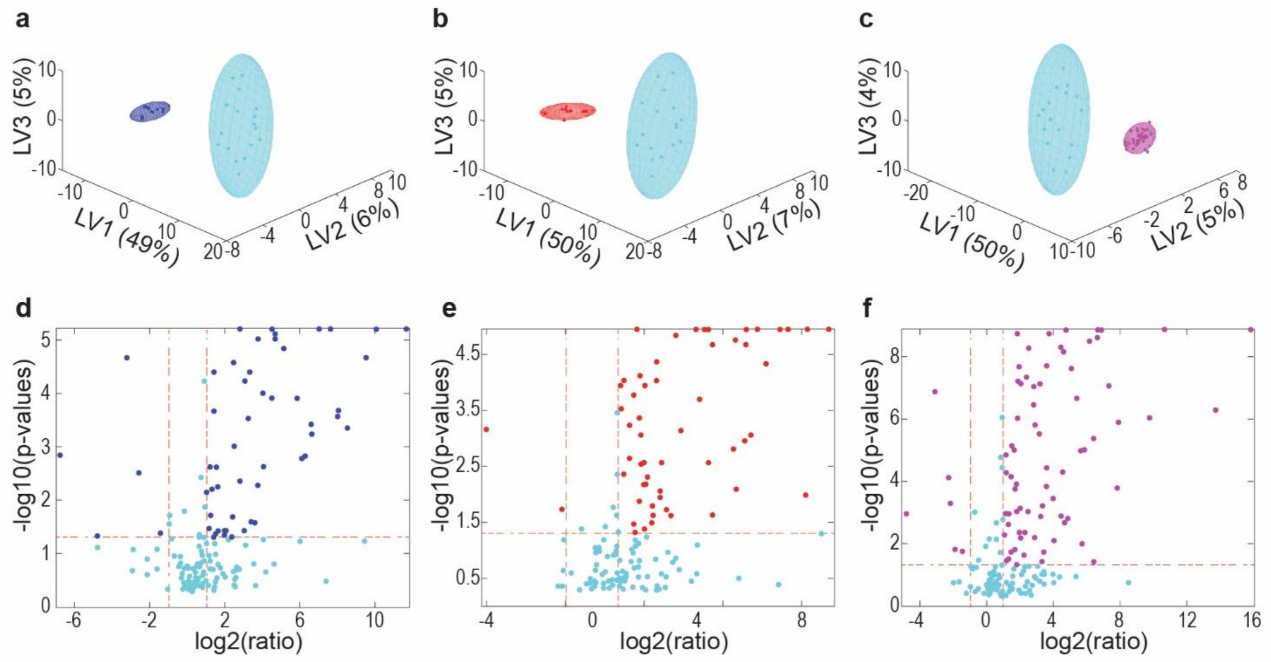

Figure 4

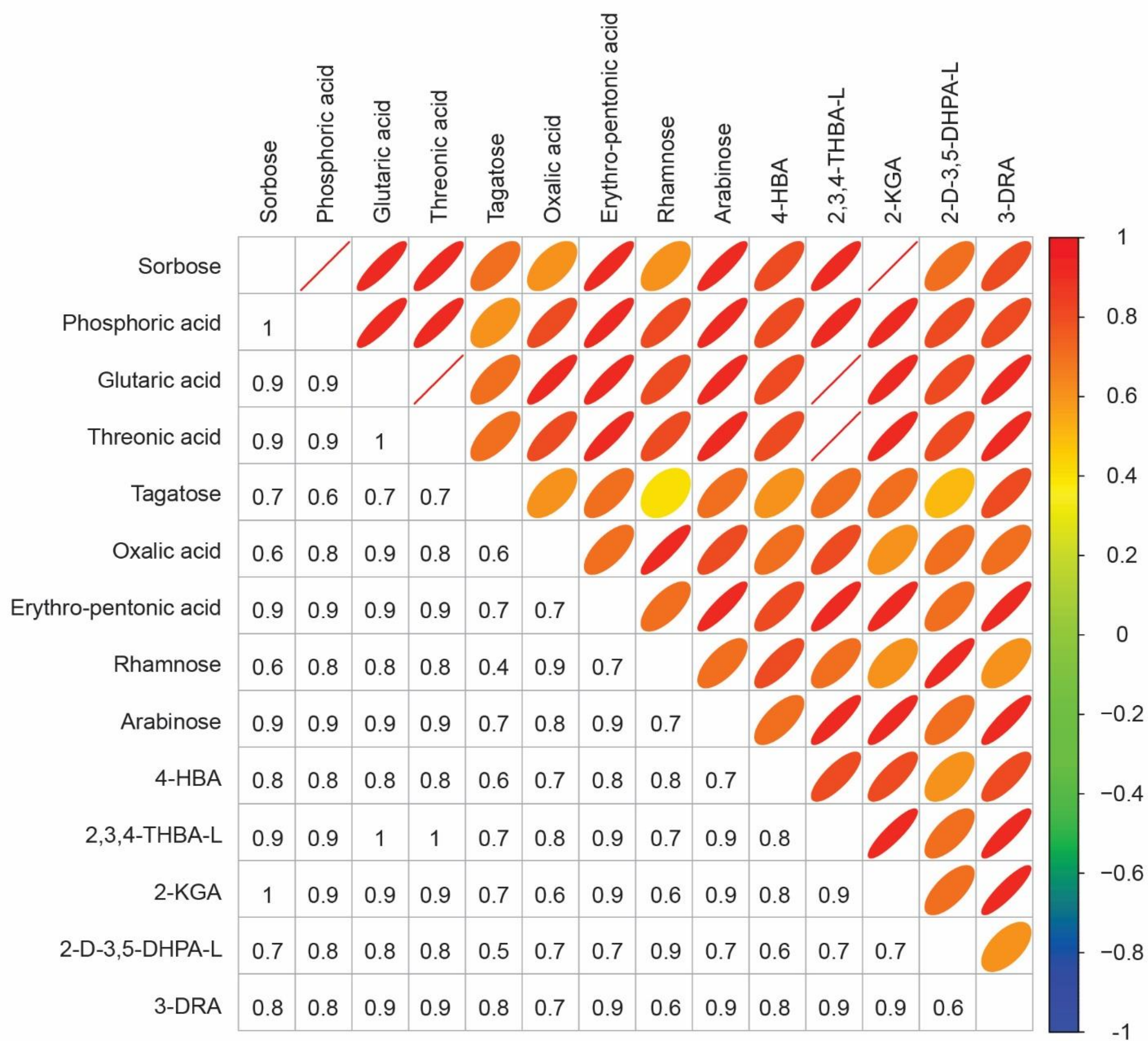

Figure 5