

## Malabsorption syndrome : Studies on diagnosis and pathogenesis using $^1\text{H}$ NMR metabonomics approach

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**Abstract :** High resolution NMR spectroscopy for the qualitative and quantitative estimation of metabolites in biofluids has become an important tool in biomedicine as it is non-destructive, doesn't require any chemical pre-treatment step, is highly selective and allows study of cellular biochemistry and metabolism. This article reviews such studies in urine and upper gut aspirate specimens of patients with Malabsorption Syndrome (MS) for the purpose of diagnosis and investigating pathogenesis of the disease. It has been demonstrated that  $^1\text{H}$  NMR method for the diagnosis of patients with MS is more specific, highly accurate, devoid of interferences from other similar metabolites, if any, as compared to the conventional colorimetric method.

The metabolic profile of upper gut aspirate provided by  $^1\text{H}$  NMR spectroscopy demonstrates that MS patients associated with Small Intestinal Bacterial Overgrowth (SIBO) have significantly higher concentrations of unconjugated bile acids, acetate, lactate and formate as compared to MS patients without SIBO and controls. These biochemicals may damage intestinal mucosa and impair small intestinal functions in MS patients.

**Keywords :** Malabsorption syndrome,  $^1\text{H}$  NMR, pathogenesis.

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### Introduction

Although NMR spectroscopy in bulk material was observed in 1946 and has been in use in various areas of science since then<sup>1,2</sup>, its application in biomedicine has kindled interest in medical community in a spectacular way in the last few years, especially after the award of four Nobel Prizes in the recent past<sup>3</sup>. A new term "metabonomics" (systemic metabolic profiling and regulation of function in whole organisms via analysis of biofluids) has been coined and it is presently being used to diagnose many disease conditions<sup>4-6</sup>, monitoring treatment<sup>7</sup>, for the prognosis of diseases<sup>8</sup>, toxicological screening in drug development and for better understanding of pathogenesis of various diseases<sup>9-12</sup>. The growth in this area may completely revolutionize health care in next few years<sup>13</sup>. In this direction, attempts have been made to explore the utility of NMR spectroscopy in urine and

upper gut aspirate specimens from patients with Malabsorption Syndrome (MS) for the diagnosis<sup>14</sup> as well as in understanding pathophysiology of the disease<sup>15</sup> and the results are critically evaluated in this review.

Malabsorption syndrome results from a group of diseases<sup>16</sup> affecting small intestine from where absorption of metabolites occur<sup>17</sup>. It is a common cause of chronic diarrhoea all over the world<sup>18,19</sup>. The problem becomes more severe in children and elderly subjects<sup>20-22</sup>. Different geographical regions may have different etiology of MS. In developed countries, non-infectious causes like celiac sprue (persons having wheat sensitivity) and Crohn's disease are common while in developing countries like India, infectious diseases e.g. tropical sprue (TS), parasitic infections and Small Intestinal Bacterial Overgrowth (SIBO) are likely to be more common<sup>23,24</sup>. Patients exhibit the symptoms of diarrhea, steatorrhea (fatty stool),

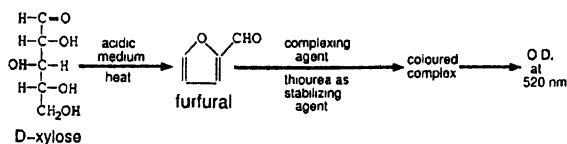
flatulence, fatigue, anemia and weight loss<sup>16</sup>.

### Diagnosis of MS

Many methods are available to diagnose malabsorption. Such methods are intake – output balance test involving fat (Van de Kamar method i.e. 72 h fat excretion)<sup>25</sup> and radioactive tracers<sup>16</sup>. These tests are difficult to conduct and therefore are not very popular. The most commonly used screening method for malabsorption in the laboratory is the D-xylose absorption test<sup>26</sup>.

**D-xylose absorption test :** It is an oral carbohydrate tolerance test for screening patients with MS with reasonable sensitivity and specificity (95% and 98%, respectively)<sup>27–29</sup>. D-xylose, a pentose sugar, is preferred over any other sugar e.g. D-glucose since the former is normally neither present nor significantly metabolized in the body<sup>26</sup>. The test involves administration of an oral dose of 5 g or 25 g D-xylose to an overnight fasting individual with ample amount of water, collection of urine for the next 5 h and estimation of D-xylose therein. Normally, in healthy subjects, over 20% of the administered dose appears in the urine while it is less in patients with MS. The excreted amount closely correlates with the amount of D-xylose absorbed in the gastrointestinal tract. It is absorbed from proximal part of the small intestine (duodenum and jejunum)<sup>30</sup>; its successful absorption is a reflection of the integrity of the surface area of the small intestine. In patients with MS, the surface area is drastically reduced due to partial or sub-total atrophy of the intestinal villi (cross section of small intestine in Fig. 5)<sup>16</sup>. 5 g D-xylose instead of 25 g is usually preferred due to the cost reasons, reduced side effects like nausea and diarrhea and is more acceptable for children<sup>27–29,31</sup>.

The results may be misleading in the case of improper urine collection, impaired renal function, ascites, delayed gastric emptying, diabetes mellitus, presence of bacterial overgrowth of upper intestine (the substrate D-xylose may be destroyed), and high-dose aspirin therapy<sup>32,33</sup>. Though D-xylose test can be performed in urine as well as in plasma, urinary estimation is routinely preferred over plasma<sup>34</sup> as the former is non-invasive. However, at times such as in infants and in patients with impaired renal functions, estimation in plasma is preferable<sup>29,35</sup>. D-xylose can be estimated by several methods such as gas chromatography (GC)<sup>35,36</sup>, thin layer chromatography (TLC)<sup>37</sup> and colorimetric methods<sup>38–40</sup>. Of these, colorimetric method is conventionally used (since 1950's) in the laboratory for urinary estimations of D-xylose<sup>38</sup>.



**Conventional colorimetric method for D-xylose test :** Estimation of D-xylose using colorimetric method is based on the conversion of xylose to furfural in an acidic medium and thereafter addition of complexing agent (*p*-bromoaniline)<sup>14,38</sup>. Final colour is obtained by involving steps of heating, cooling and incubation of the reaction mixture for fixed duration of time whose optical density (OD) is measured at a certain wavelength<sup>14,38,40</sup>.

Post-xylose urine specimens are diluted to 10 to 80 times with saturated solution of benzoic acid depending on the volume collected in 5 h<sup>26</sup>. The concentrations of the xylose are then calculated using the standard protocol<sup>26,38</sup>.

**Limitations of the colorimetric method :** Despite being routinely used technique, the colorimetric method suffers from many disadvantages which are well documented in the literature<sup>38,40</sup>. The stability of the coloured complex depends upon the strict conditions of light, temperature and incubation time. In addition, it is tedious, time consuming and requires a number of reagents. Further errors are compounded when some other interfering sugar is present in urine e.g. glucose in the patients with uncontrolled diabetic mellitus as this may also form colored complex under the conditions used for xylose estimation<sup>14,38,40</sup>.

**Need for an alternative method :** The need for a highly accurate (highly sensitive so that treatable patients are not missed and fairly specific to avoid over estimation of non-disease patients), reliable, simple method, which is devoid of interference by other substances such as glucose while estimating D-xylose, has been long felt. NMR method has thus been proposed and used<sup>14</sup>.

The advantages of NMR spectroscopy are manifold over other conventional methods. <sup>1</sup>H NMR spectroscopy is non-invasive, non-destructive, rapid, requires no sample preparation other than buffering and addition of D<sub>2</sub>O to provide a field frequency lock signal. The large interfering signal that arises from water in all biofluids is easily eliminated using appropriate solvent suppression methods<sup>11</sup>. The metabolites can be estimated qualitatively and quantitatively in a single measurement as they have unique <sup>1</sup>H NMR signatures<sup>11,12</sup>. Though, other important tools of metabonomics like mass spectroscopy (MS)<sup>41</sup>,

gas chromatography (GC) and liquid chromatography (LC)<sup>42</sup> are inherently quite sensitive but these suffer from limitation of being rather destructive in nature and pre-selective for different classes of substances and require a chemical pre-treatment or a separation step<sup>43</sup>.

### <sup>1</sup>H NMR method for D-xylose test

(i) *In vitro studies* : The feasibility of <sup>1</sup>H NMR spectroscopy for D-xylose test was first ascertained with *in-vitro* experiments employing standard D-xylose as follows :

(a) One dimensional NMR experiments using single pulse sequence with presaturation were first performed with a known quantity of D-xylose dissolved in measured volume of urine specimen from controls containing a measured amount of reference TSP (trimethyl silyl propionic acid in D<sub>2</sub>O, kept in a closed reusable capillary). It was observed that the marker signals of anomeric protons of D-xylose (in solution under equilibrium conditions, D-xylose exists in two structural anomers : α-D-xylose and β-D-xylose) were well separated from the crowded regions of the spectra. Some of the signals of other protons from each of α-D-xylose and β-D-xylose were also found non-overlapping with metabolites of urine. Quantitative estimation of D-xylose was made by calculating the sum of areas of at least one proton from each isomer which is non-overlapping to the signals of metabolites of urine, relative to the internal reference. Concentration of xylose excreted in 5 h in urine was calculated by using following equation which is inbuilt in a computer programme - which requires integrated area (*A*), molecular weight (*M*), number of protons contributing to the signal (*N*) of TSP (*r*) and xylose (*x*). *V<sub>c</sub>* is the total volume of urine in 5 h, *V<sub>x</sub>* is the volume of urine taken in NMR tube and *W<sub>r</sub>* is the weight of TSP in capillary. Thus *W<sub>x</sub>*, the weight of xylose excreted in urine in 5 h can be calculated from :

$$W_x = \frac{A_x M_x N_r W_r V_c}{A_r M_r N_x V_x} \text{ mg}$$

(b) Similar experiments when performed on specimens containing measured amount of D-glucose in addition to xylose clearly showed the well separated marker signals of anomeric protons of D-glucose from those of D-xylose leading to the separate quantitation of both sugars.

(c) Each set of experiment was simultaneously performed by colorimetric method as well.

(d) Xylose concentrations estimated by colorimetry in standard samples showed a maximum error of 20% as

against 7% by NMR. While in presence of glucose the errors went upto 30% by colorimetry as against 8% by NMR<sup>14</sup>.

(ii) *Studies on patients* : The <sup>1</sup>H NMR method for D-xylose test for diagnosis of MS was exploited in a pilot

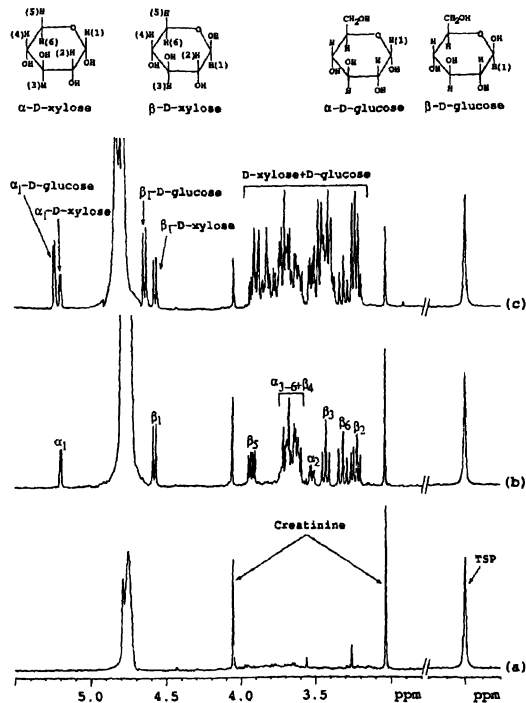


Fig. 1. One dimensional 400 MHz <sup>1</sup>H NMR spectra of urine specimen from a patient with suspected MS : (a) pre-test, (b) post-xylose test, (c) post-xylose test of another patient with uncontrolled diabetes. The assignments of D-xylose signals of all the non-labile protons from both the isomers (α and β) along with structures are given. The structure of D-glucose is also included.

study in patients with suspected MS (Fig. 1). The patients were classified as MS and No MS by gold standard tests of fecal fat<sup>25</sup> and/or duodenal/jejunal biopsy<sup>14</sup>. It was observed that colorimetry significantly underestimates quantity of D-xylose excreted as compared with NMR in the same urine specimens, particularly in those without MS.

The estimation of D-xylose by <sup>1</sup>H NMR method on urine specimens of a suspected MS patient with uncontrolled Diabetes Mellitus clearly demonstrated the separate quantitation of xylose without interference of glucose (Fig. 1c) while on the contrary, the values obtained by colorimetric method were much higher and might have misinterpreted this patient as normal.

## Understanding pathogenesis of MS

Metabonomics is especially important in giving not only qualitative and quantitative measurement of endogenous metabolites but also biochemicals that arise as a result of external influences, perturbations with time and those which are produced as a consequence of dietary or environmental interactions, such as gut bacterial populations. MS, irrespective of its diverse etiology have been reported to be frequently associated with SIBO<sup>44-46</sup>. The gut-microbial flora in patients with MS may perpetuate malabsorption of nutrients and cause refractoriness to specific treatment directed against the primary cause<sup>47,48</sup>. However, the mechanism by which SIBO may cause malabsorption of nutrients is not clearly understood. Conjugated bile salts, synthesized in liver are excreted in bile and are involved in the absorption of fat via formation of micelles in upper gut. It has been reported that bacteria may be involved in deconjugation of conjugated bile salts and may play a role in causing MS in some patients<sup>49</sup>. Bacteria produce several metabolites<sup>50</sup>, which may induce mucosal injury<sup>51</sup>.

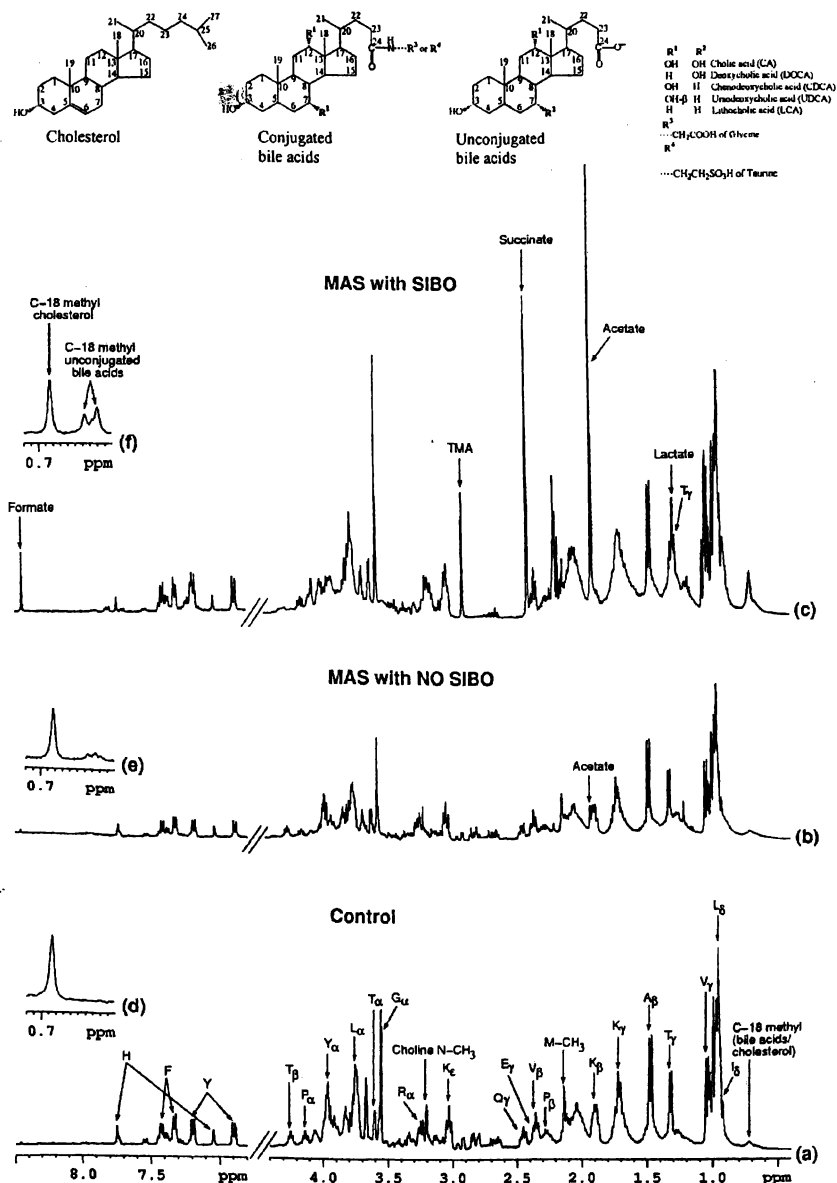
Patients with MS and disease-free controls were included in the study. Diagnosis of MS was based on abnormal D-xylose absorption, 72 h fecal fat excretion using Van de Kamer's technique<sup>15,25</sup> (normal < 7 g/24 h), endoscopic duodenal or jejunal biopsy. Upper gut aspirate specimens from each subject were obtained as described<sup>45</sup>. Briefly, a sterilized double walled catheter assembly was used to avoid contamination by oropharyngeal flora and it was passed through a biopsy channel while performing endoscopy, and contents of upper gut were aspirated with the help of a cannula. Each aspirate was divided into two parts (i) one was used for microbiological assay as described<sup>45,52</sup> to evaluate SIBO by quantitative culture of the upper gut aspirate. SIBO was diagnosed if upper gut aspirate grew bacteria  $\geq 10^5$  colony forming unit (CFU/mL) and (ii) the other part for NMR spectroscopic studies. Median colony counts of bacteria in upper gut aspirate in patients with MS with bacterial colonization were significantly higher than in controls with bacteria. SIBO was not present in any of the controls studied. The supernatant and residues were separated from gut aspirate and subjected for NMR measurements.

**<sup>1</sup>H NMR analysis of supernatant and residue of upper gut aspirate :** <sup>1</sup>H NMR experiments using single pulse sequence with pre-saturation were performed on measured amount of supernatant (D<sub>2</sub>O is used, wherever necessary) and residue dissolved in DMSO-*d*<sub>6</sub> (deuterated

dimethylsulfoxide) (Fig. 2). In the supernatant parts of upper gut aspirate, signals from total bile acids (conjugated and unconjugated) and cholesterol overlap and hence the combined C-18 methyl proton signal appears (in the range 0.61–0.64 ppm). On the other hand, the spectra of the residue in DMSO solvent showed separate C-18 methyl proton signals for cholesterol and bile acids (Fig. 2f) and hence DMSO was used for the residue analysis. Conjugated bile acids are freely soluble under the physiological pH which is in the range 6 to 7<sup>15,53</sup> of upper gut aspirate whereas the unconjugated analogues are not. The various biochemicals identified were free amino acids, glucose (in some cases) in general in controls as well as in patients whereas signals coming from acetate, lactate, formate and succinate (in some cases) were present in significant quantities in MS patients with SIBO and they were either weak or not seen in controls. Various amino acids were assigned unambiguously by performing the combined use of two-dimensional Double Quantum Filtered Correlated Spectroscopy (DQF-COSY) (Fig. 3) and Total Correlated Spectroscopy (TOCSY). For distinguishing between signals of odd and even multiplicity, Hahn Echo sequence and to suppress broad signals, experiments using Carr-Purcell-Meiboom-Gill (CPMG) sequence were also employed<sup>15</sup>, thus assignments of overlapping signals of acetate and lysine at  $\delta$  1.91 and doublets of CH<sub>3</sub> of lactate and threonine were reinstated (Fig. 2c). Quantity of total bile acids/cholesterol was calculated from the integral of C-18 methyl proton signals relative to that of TSP signal.

**The biochemicals, their relation to bacteria and pathological significance :** Quantities of amino acids and glucose in upper gut aspirate of patients with MS and controls were comparable. However, total bile acids/cholesterol, acetate, lactate and formate were significantly higher in patients with MS than in controls (Fig. 2). Similarly, unconjugated bile acids, acetate, lactate and formate were higher in upper gut aspirate in patients with MS with SIBO than those without SIBO (Fig. 4). Unconjugated bile acids were absent in controls (Figs. 2 and 4). Succinate and trimethylamine (TMA) were also present in few patients with MS with SIBO (Fig. 2c). In MS patients, a positive correlation was obtained between concentration of acetate with total colony counts of bacteria and unconjugated bile acids with quantity of fecal fat excretion<sup>15</sup>

Acetate, formate and lactate are short chain fatty acids (SCFA)<sup>51</sup> which arise as a result of bacterial fermentation<sup>50</sup>. However, a significant correlation between quan-



**Fig. 2.** Typical one-dimensional  $^1\text{H}$  NMR spectra of supernatant of upper gut aspirate from controls (a) and MS patients without SIBO (b) and those with SIBO (c). Assignments of important signals of various amino acids are marked using one letter codes. Bacterial overgrowth in upper gut aspirate is clearly seen from the presence of intense signals of bacterial metabolites (acetate, lactate and formate) in patients with SIBO (c), which are either not seen or are weak in MS patients without SIBO (b) and controls (a). Succinate and trimethyl amine were seen in a few cases with SIBO (c). Parts of the spectra of residue of upper gut aspirate in  $\text{DMSO}-d_6$  in controls (d), in patients without SIBO (e) and in patients with SIBO (f). The structure of cholesterol, conjugated bile acids and unconjugated bile acids are also shown. The subscripts  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  refer to the corresponding protons. The abbreviations used are - I : Isoleucine; L : Leucine; V : Valine; T : Threonine; A : Alanine; K : Lysine; M-CH<sub>3</sub> : Methionine; P : Proline; E : Glutamic acid; Q : Glutamine; G : Glycine; Y : Tyrosine; F : Phenylalanine; H : Histidine.

tity of acetate (and not formate and lactate) with the degree of bacterial overgrowth suggests that lactate and formate are intermediate products of bacterial metabolism

that are further metabolized to acetate<sup>50</sup>. Earlier studies involving analysis of small intestine contents for SCFA by GC in patients with small-bowel bacterial overgrowth

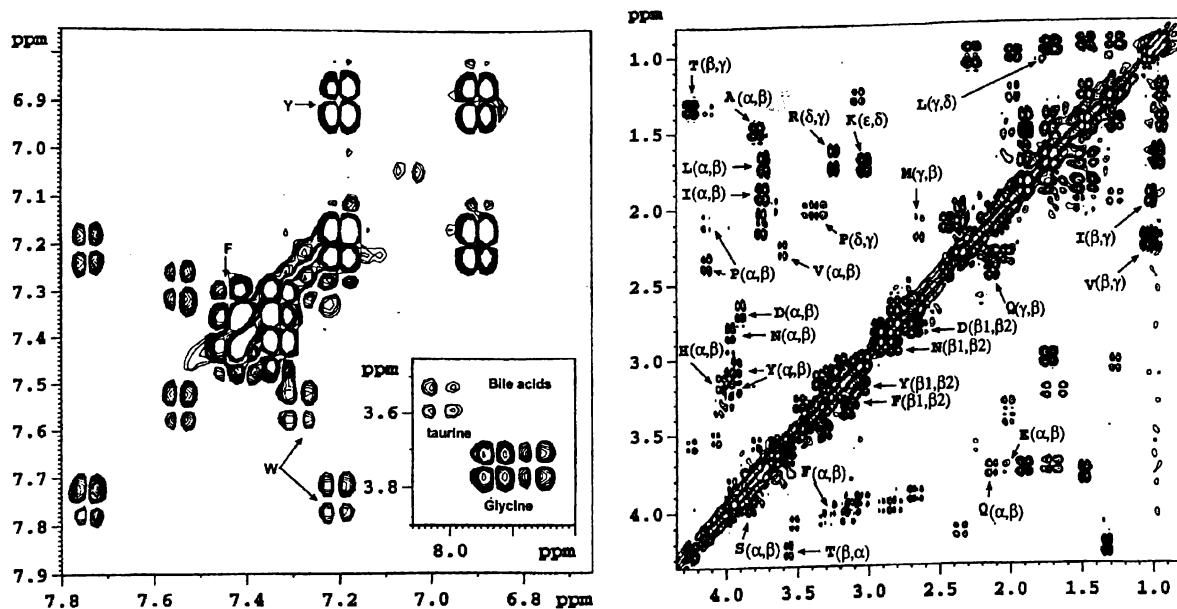


Fig. 3. Typical two-dimensional DQF-COSY (double quantum filtered correlated spectroscopy) spectrum of upper gut aspirate of a patient. W : tryptophan; taurine : amide proton of taurine conjugated bile acids; glycine : amide proton of glycine conjugated bile acids. All other cross peaks between protons, within each amino acid, are marked using single letter codes as is given in Fig. 2. The subscripts  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  refer to the corresponding protons.

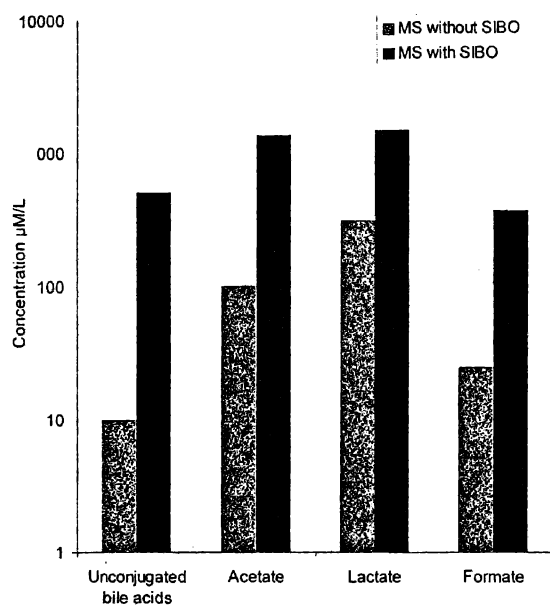


Fig. 4. Concentration ( $\mu\text{M/L}$ ) of some important biochemicals in MS patients with and without SIBO.

syndrome<sup>54</sup> and in rat with blind loops<sup>55</sup> have reported presence of significant amount of acetate. Acetate along with other SCFA(s) has been implicated in causing co-

lonic mucosal injury in rats<sup>51</sup>. The acetate produced by bacteria may increase the osmolarity of small bowel contents and decrease the intraluminal pH, which may cause damage to enterocytes<sup>51,56</sup>.

The presence of significant amount of unconjugated bile acids in MS patients with SIBO, and its significant correlation with amount of fecal fat excretion (and not with colony counts of bacteria) suggests that although all bacteria may not have deconjugating ability<sup>57</sup> but unconjugated bile acids may have pathological significance. The unconjugated bile acids may form impaired micelles and could interfere in fat absorption<sup>58</sup>. The low solubility of unconjugated bile acids at physiological pH i.e. 6–7 of small intestine (jejunum)<sup>15,16,23</sup>, results in their precipitation and may be detrimental to intestinal mucosa<sup>59</sup>. Bacterial metabolites and free unconjugated bile acids may stimulate secretion of water and electrolyte into the bowel lumen and cause diarrhea. They may cause motility disturbances. Studies involving SIBO in rats have shown small bowel motility disturbances, which are reversible with antibiotics<sup>60</sup>. Further, how bacterial metabolites and unconjugated bile acids could cause damage to intestinal mucosa has not been substantiated and requires further investigations.

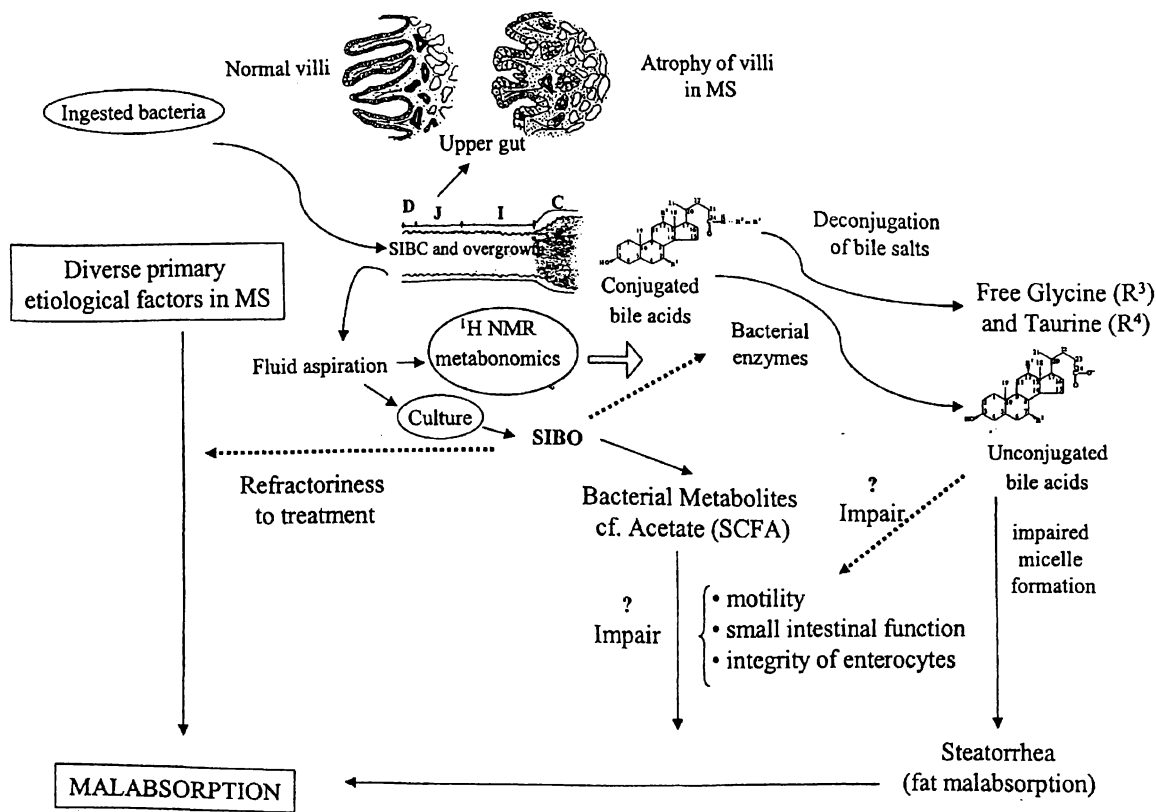


Fig. 5. A model giving an overview of pathogenesis, in patients with MS. The abbreviations used are SIBC : Small Intestinal Bacterial Colonization; SIBO : Small Intestinal Bacterial Overgrowth; D : duodenum; J : jejunum; I : ileum; C : colon; R<sup>3</sup> : glycine; R<sup>4</sup> : taurine; SCFA : short chain fatty acids.

A model for overview of pathogenesis of MS is presented in Fig. 5. In the normals, absorption takes place within the small intestine. The cross section of proximal small intestine clearly shows partial or subtotal atrophy of villi in MS leading to reduced surface area for absorption. In the normals, the bacterial colonization is greatest ( $> 10^{11}$  cfu/mL) in distal ileum and colon but in MS, the bacteria colonizes proximally (primarily due to ingested bacteria), allowing deconjugation of bile salts and production of toxic bacterial metabolites in upper gut leading to further perpetuation of the disease.

#### Conclusion :

It is clear that  $^1\text{H}$  NMR method for D-xylose test has the unique potential of being highly specific, is devoid of interference from other similar metabolites and is simple. It overcomes the limitations of colorimetric method which has been exploited in the diagnosis of malabsorption syndrome in clinical situations. Furthermore,  $^1\text{H}$  NMR spectroscopy of upper gut aspirate clearly shows significant

quantities of acetate, formate, lactate and unconjugated bile acids in MS patients with SIBO as compared to no SIBO. It has been demonstrated how the external factors (bacteria) change the metabolic profile of upper gut aspirate in MS patients leading to the severity of the disease. Such studies provide an insight in further understanding of pathogenesis of MS. Further application of this method to disease models of MS and intestinal epithelial cell lines would extract information in identifying true biomarkers of MS with SIBO that exert toxic effect on intestinal mucosa and may help in solving intriguing problem of pathophysiology of the disease.

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