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The Metabonomic Signature of Celiac Disease

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Celiac disease (CD) is a multifactorial disorder involving genetic and environmental factors, thus, having great potential impact on metabolism. This study aims at defining the metabolic signature of CD through Nuclear Magnetic Resonance (NMR) of urine and serum samples of CD patients. Thirty-four CD patients at diagnosis and 34 healthy controls were examined by ^{1}H NMR of their serum and urine. A CD patients' subgroup was also examined after a gluten-free diet (GFD). Projection to Latent Structures provided data reduction and clustering, and Support Vector Machines provided pattern recognition and classification. The classification accuracy of CD and healthy control groups was 79.7−83.4% for serum and 69.3% for urine. Sera of CD patients were characterized by lower levels ($P < 0.01$) of several metabolites such as amino acids, lipids, pyruvate and choline, and by higher levels of glucose and 3-hydroxybutyric acid, while urines showed altered levels ($P < 0.05$) of, among others, indoxyl sulfate, meta-[hydroxyphenyl]propionic acid and phenylacetylglycine. After 12 months of GFD, all but one of the patients were classified as healthy by the same statistical analysis. NMR thus reveals a characteristic metabolic signature of celiac disease. Altered serum levels of glucose and ketonic bodies suggest alterations of energy metabolism, while the urine data point to alterations of gut microbiota. Metabolomics may thus provide further hints on the biochemistry of the disease.

Keywords: Celiac disease • energy metabolism • gut microflora • NMR spectroscopy • support vector machines

Introduction

Celiac disease (CD, OMIM# 212750), also known as celiac sprue, is a multifactorial disorder, and both genetic and environmental factors play a crucial role in its pathogenesis.[1] CD is an immune-mediated enteropathy triggered by the ingestion of wheat gluten or related rye and barley proteins in genetically susceptible individuals.[2,3] Although untreated symptomatic CD is associated with substantial morbidity and mortality,[4] clinical and histological improvement is usually observed on a strict gluten-free diet (GFD).[2] Until fairly recently, CD was regarded as rare, but serological screening studies have shown that it is relatively common, affecting approximately 1% of the population in both Europe[5,6] and North America.[7] Genetic susceptibility to CD is strongly associated with specific class II MHC alleles: more than 90% of CD patients possess the HLA-DQ2 allele (*DQA1*05/DQB1*02*), with DQ8 (*DQA1*03/DQB1*0302*) being the most common allele in the remaining patients;[8] non-HLA genes, however, have been also found to contribute to the disease.[9,10]

The main environmental factor responsible for CD is gluten, a protein complex formed by glutenin and gliadin proteins. Gliadin (with α-, β-, γ-, and ω-gliadin fractions) is the active disease component.[11] Being rich of proline and glutamine, gliadin is partially resistant to enzymatic proteolytic degradation.[12] Several immunogenic peptides have been identified, especially in α-gliadin: these peptides are deamidated by the intestinal brush border enzyme tissue transglutaminase (tTG), thus, enhancing their capacity to bind to the pocket of the human leukocyte antigen HLA-DQ2 or HLA-DQ8 and stimulat-

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ing both the humoral and cell-mediated arms of the adaptive immune response.[13]

Several conditions have been described in association with CD, like T-cell lymphoma,[14,15] osteoporosis[16] and neurologic diseases.[17,18] Furthermore, like many autoimmune diseases, CD is often associated with other autoimmune disorders[19] such as type 1 diabetes,[20] autoimmune thyroiditis[21] and Addison's disease.[22]

To date, no metabolomic studies have been reported on CD, although it is a pathology that is known to have a direct impact on metabolism.

Metabolomics has established itself as a useful complement to the characterization of pathologies. The metabolome can be considered the downstream of genome, transcriptome and proteome, and it is the best representation of a healthy or diseased phenotype of an organism. The metabolome, in fact, amplifies changes[23−25] caused by a biological perturbation. As opposed to metabolomics, which places a greater emphasis on comprehensive metabolic profiling, *metabonomics* is more often used to describe multiple (but not necessarily comprehensive) metabolic changes caused by a biological perturbation. Metabonomics has been defined as "the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification".[26]

Nuclear Magnetic Resonance (NMR)-based metabonomics offers evident advantages in contrast with knowledge-guided search of metabolites in pathological samples. NMR-based metabonomics makes no assumptions on the identity of the metabolites that are relevant for the selected pathology. Information on the metabolite pattern alterations that can be significantly associated to the pathology is directly obtained through statistical analysis of the NMR profiles. Usually, metabonomics does not rely on the measurement of a single metabolite-associated peak(s) but analyze spectra as whole: metabonomic profiles are essentially the superposition of the ^{1}H NMR (in the most popular version of NMR-based metabonomics) spectra of tens to thousands different small molecules (up to 2500 in the case of urine) present in the sample at >1 μM concentration.[27] In principle, an NMR profile contains qualitative and quantitative information on all of them. Small changes in enzyme concentrations can reflect in considerable alterations in intermediate products and in the fact that metabolic networks are connected by few high concentrated nodes, that can be investigated by NMR-based metabonomic analysis of biological fluids such as serum, plasma, and urine.

NMR-based metabonomic studies have provided significant information on a wide range of pathologies, from hypertension[28,29] to cancer,[30,31] ischemia-reperfusion,[32] meningitis,[33] neurological disorders,[34] diabetes (especially type 2)[35] and diabetic nephropathy,[36] coronary-heart disease,[37] aging,[38] as well as a variety of gastrointestinal diseases. The latter are, not surprisingly, more likely to be associated with metabolic alterations. Inflammatory bowel disease,[39] fatty liver disease,[40] liver fibrosis and cirrhosis,[41] and *Helicobacter pylori* infection[42] have all been shown to present characteristic metabolic alterations, that is, they seem to possess a characteristic metabonome.

In this study, we have examined CD patients, before and after GFD, and healthy controls, by ^{1}H NMR profiling of their serum and urine samples; through multivariate analysis, we were able to identify a number of serum and urine metabolites whose levels were significantly different in CD at diagnosis as compared to controls, and most of which reverted to normal within 12 months of a strict GFD. These metabolites can provide hints both to define new biomarkers and to better understand the biochemistry involved in this pathology.

Material and Methods

Recruitment of Patients and Controls and Sample Collection. Thirty-four adult CD patients (7 males, 27 females, mean age 38.7 ± 13.7 years), consecutively diagnosed at the Tuscany Referral Center for Adult CD participated in the study. The diagnosis was based on positive serology and confirmed by histological examination of small bowel biopsies taken from the second part of the duodenum. Small intestinal mucosal damage was graduated according to the classification of Marsh modified by Oberhuber.[43] In three patients, only minor small bowel mucosal changes were documented by histology (i.e., Marsh I−II), and so they were maintained on a gluten-containing diet. They all developed an overt CD with small intestinal mucosal atrophy during the follow-up and were therefore retrospectively diagnosed as latent CD. The clinical characteristics of CD patients are summarized in Supporting Information Table S1. Thirty-four healthy subjects (HS) (13 males, 21 females, mean age 37.6 ± 14.8 years) from the medical staff of the Gastrointestinal Unit served as a control group: all had negative anti-endomysium (EMA) and anti-tissue transglutaminase antibodies (tTGA), no history of gastrointestinal disease, and for ethical reasons, did not undergo intestinal biopsy. Blood and urine samples were obtained from healthy controls and CD patients at diagnosis. In a subgroup of patients with CD, blood and urine samples were also collected after 3 (3 males, 14 females, age = 40.5 ± 14.1), 6 (1 male, 9 females, age = 38.7 ± 13.8) and 12 (2 males, 11 females, age = 41.3 ± 11.5) months of treatment with strict GFD.

Each subject had fasted overnight, and urine and blood were collected in the morning preprandial. Venous blood samples were collected into plastic serum tube (6 mL), with increased silica act clot activator, silicone-coated interior (Becton Dickinson, Plymouth, U.K.). Samples were allowed to clot by standing tubes vertically at room temperature (22 °C) for 60 min. Tubes were centrifuged at 1800 RCF for 10 min at room temperature. Within 15 min of centrifugation, the supernatant (serum) was transferred in 500 μL aliquots to prelabeled 1 mL cryovials (Bruker BioSpin, Milan, Italy). Three aliquots per patient were immediately frozen and stored at − 80 °C until used.

First morning preprandial urine void was used for the collection period. Patients were supplied with appropriate collection instructions and information on fasting, diet and medication restrictions when necessary. The urine samples were collected into prelabeled sterile collections cups. One milliliter of urine sample was transferred into prelabeled 1 mL sterile cryovials (Nalgene, Rochester, NY). Three aliquots per patient were immediately frozen and stored at − 80 °C until used.

Additionally, CD patients and healthy controls were asked to record their dietary intake and the use of any medication (either prescribed or self-administered) on the day before each visit and to fast from midnight until blood samples and urine were collected the following morning.

Antibody Testing. Anti-tissue transglutaminase antibodies were measured by a commercially available enzyme-linked immunosorbent assay kit (EutTG, Eurospital, Trieste, Italy) that employs human recombinant tTG as antigen, as previously reported.[44] EMA were determined by indirect immunofluorescence, using tissue section of monkey esophagus (Eurospital).

HLA Genotyping. Genomic DNA was extracted from peripheral venous blood using a salting-out procedure. All CD patients and healthy controls were genotyped for HLA-DRB1, -DQA1, and -DQB1 genes by Polymerase Chain Reaction-Sequence Specific Primers (PCR-SSP) using commercial kits (Innogenetics, Gent, Belgium).

NMR Samples Preparation. Frozen serum samples were thawed at room temperature and shaken before use. A total of 300 μL of a phosphate sodium buffer (70 mM Na_2HPO_4; 20% (v/v) ^2H_2O; 0.025% (v/v) NaN_3; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3-$^2H_4$]propionate (TSP); pH 7.4) was added to 300 μL of each serum sample, and the mixture was homogenized by vortexing for 30 s. A total of 550 μL of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl) for analysis.

Frozen urine samples were thawed at room temperature and shaken before use. Seventy microliters of sodium phosphate buffer (0.2 M Na_2HPO_4; 0.2 M NaH_2PO_4 in 100% ^2H_2O; pH 7.0) also containing 10 mM (TSP) and 30 mM NaN_3 was added to 630 μL of urine. Samples were centrifuged at $1.4 \times 10^4 g$ for 5 min and 600 μL aliquots of the supernatant were pipetted into 4.25 mm NMR tubes (Bruker BioSpin srl).

NMR Analysis. ^{1}H NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm TXI ^{1}H−^{13}C/^{15}N−^{2}H probe including a *z* axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.01 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead, for temperature equilibration (298 K).

For each urine sample, a one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (NOESYpresat; Bruker), using 64 free induction decays (FIDs), 64k data points, a spectral width of 12 019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 100 ms.

One-dimensional spectra of serum samples were acquired using a Carr−Purcell−Meiboom−Gill (CPMG; Bruker) spin−echo sequence to suppress signals arising from high molecular weight molecules and a standard pulse sequence (NOESYpresat, same acquisition parameters as in the case of urine samples).

Spectral Processing. Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were manually corrected for phase and baseline distortions and calibrated (TMSP peak at 0.00 ppm) using TopSpin (Version 2.1, Bruker). Each 1-D spectrum in the range between 0.02 and 10.00 ppm was segmented into 0.02-ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (Bruker BioSpin). Regions between 6.0 and 4.5 ppm containing residual water and urea signals were removed. The total spectral area was calculated on the remaining bins and normalization was carried out on the data prior to pattern recognition.

Statistical Analysis. Several methods for data reduction and classification and combination thereof were applied on the serum and urine spectra data sets: PLS-SVM, PLS-RCC-SVM, K-OPLS, K-OPLS-SVM. Projection to Latent Structure (PLS) on the mean-centered data without scaling was performed using the classical SIMPLS algorithm[45] as implemented in the R-library "plsgenomics". Support Vector Machines (SVM) method[46] was used for data classification using the "libsvm" module[47] of the R-library "e1071". Kernel-Ortogonal Projection to Latent Structures (K-OPLS) on the mean-centered data without scaling was performed using the R "kopls" package.[48] Regularized Canonical Correlation Analysis[49] (RCC) was performed using the "CCA"[50] R-library.

To assess the prediction ability of the model, the original data set (34 + 34) was split according to a 2:8, that is, removing 20% of samples prior to any step of statistical analysis, including PLS component selection. Parameter selection (best number of predictive and orthogonal components and kernel type for K-OPLS, λ_1, and λ_2 for RCC, kernel type, cost of constraints violation for SVM) was carried out by means of 6-fold cross validation on the remaining 80%. The whole procedure was repeated 250 times, and each time the same data set was fed to the four statistical methods.

Data classification using SVM was performed by applying the classifier on RCC, PLS, or K-OPLS scores. For K-OPLS models, classification was also performed using the built-in classification function based on the maximum class belonging.

Accuracy, sensitivity and specificity were estimated using standard definitions.[51]

To assess which buckets (that is, resonance peaks) were significantly different between controls and patients (before and after GFD), a one-way analysis of variance was used. Normality of data distributions was assessed using the Jarque-Brera normality test.[52] The statistical significance of the means over the two groups was assessed using ANOVA or the nonparametric analogue Kruskal−Wallis test. A P value ≤ 0.05 was considered statistically significant. The buckets whose values appeared to be significantly different from the above statistical analysis were manually checked to identify the associated resonance peak(s). All resonances of interest were then manually checked and signals were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2-0-0; Bruker BioSpin) reference database and published literature when available.

All calculations were made using R[53] and MATLAB (The MathWorks, Natick, MA) scripts developed in-house.

Results

Four different statistical analyses have been applied on the experimental data set. A total of 250 different training and test sets were built by random splitting, with an average classification accuracy for the test set ranging from 79.7% to 83.4% for CPMG spectra. A detailed comparison of the methods, carried out on the CMPG spectra data set, is given in Table 1. The average classification accuracy for each individual in the test set is reported in Supporting Information Table 2.

The clustering of HS and untreated CD subjects for the three sets of samples/experiments is shown in Figure 1. The best clustering was provided by the serum CPMG spectra. The clustering was achieved by using the best performing methods for sample discrimination, that is PLS-RCC-SVM. In this case, the best model parameters were chosen by minimizing the 6-fold cross validation error on the whole data set. Confusion matrices for this model are given in Table 2. The best model for the CPMG serum spectra was built using 13 PLS components, values of 0.1 for both the regularization parameters λ_1 and λ_2, and a radial kernel with a γ of 0.06 and a constant C of 100. The best model for the NOESY serum spectra was built

Table 1. Summary of the Comparison of the Four Different Methods Applied on the Serum CPMG Spectra Data Sets[a]

serum CPMG spectra 2:8 split	no. comp	train accuracy (%)	test accuracy (%)	no. comp	train accuracy (%)	test accuracy (%)
		K-OPLS			K-OPLS SVM	
Median	16.0	83.9	83.3	14.0	88.7	83.3
Mean	17.9	84.4	79.7	15.0	89.4	81.2
S.D.	9.2	2.7	17.0	5.5	2.1	16.4
Max	40.0	91.9	100.0	40.0	95.2	100.0
Min	2.0	79.0	16.7	6.0	83.9	33.3
		PLS SVM			PLS RCC SVM	
Mean	16.5	89.4	80.6	16.1	95.6	83.4
Median	14.0	88.7	83.3	15.0	95.2	83.3
S.D.	7.26	1.8	17.4	5.7	1.6	17.0
Max	40.0	93.5	100.0	39.0	96.8	100.0
Min	6.00	85.5	16.7	6.0	88.7	33.3

[a] Average values for components, train and test accuracy have been computed over 250 different data sets. Calculations have been carried out using a 2:8 data splitting as described in Materials and Methods.

using 15 PLS components, values of 0.001 and 0.1 for the regularization parameters λ_1 and λ_2, respectively, and a linear kernel with a constant C of 10. The best model for NOESY urine spectra was built using 7 PLS components, values of 0.1 and 0.01 for the regularization parameters λ_1 and λ_2, respectively, and a linear kernel with constant C of 1.

By processing the follow-up serum samples with the same statistical model built for the discrimination, we found that all but one CPMG serum samples of the follow-up after 12 months were classified as belonging to the HS group (Figure 2), while after 3−6 months, several samples were still classified as CD (Figure 3).

When comparing spectra of serum samples of untreated CD patients and controls, we find that CD patients are characterized by lower levels ($P < 0.01$) of asparagine, isoleucine, methionine, proline, valine, methylamine, pyruvate, creatinine, choline, methylglutarate, lactate, lipids (from their R-**CH_2**-CH_2-CO- and -C=C-**CH_2**-C=C- signals) and glycoproteins (from their N-acetyl signals), and higher levels ($P < 0.01$) of glucose and 3-hydroxybutyric acid. In the case of urine, CD patients are characterized by lower levels ($P < 0.05$) of mannitol, glutamate, glutamine and pyrimidines, and higher levels of indoxyl sulfate (IS), choline, glycine, acetoacetate, uracil, meta-[hydroxyphenyl]propionic acid (mHPPA), and phenylacetylglycine (PAG). These data are summarized in Tables 3 and 4.

Analysis of serum spectra of CD patients after 12 months of a gluten-free diet showed decreased levels of glucose and an increase of lipoprotein associated peaks. We also noticed a decrease of 3-hydroxybutyric acid and an increase of amino acids such as methionine and valine, as well as of lactate and creatinine levels. No significant variations (at a $P \leq 0.05$ level) in levels of other resonances were found between patients and controls. The distributions of all metabolite levels that are significantly different between CD patients and HS, as well as their changes in the 12 months follow-up, are summarized in Table 5 and Supporting Information Figures S1−S5.

Discussion

We have demonstrated here a novel metabonomic approach to study and understand celiac disease mechanism from a holistic point of view. As the metabonome reflects changes both of genome and proteome, an NMR-based metabonomics profiling provides, when paired with an *ad hoc* statistical analysis, a comprehensive metabonomic picture of such a complex and multifactorial pathology. This picture trespasses the classical CD diagnosis, based on serological assay and endoscopy, highlighting new molecular mechanisms that can help to elucidate CD-associated symptoms that do not currently find explanation in the known CD etiology.

The statistical models applied proved to be quite powerful in discriminating between patients and healthy subjects, as shown in Tables 1 and 2. This clear discrimination, achieved by metabolic fingerprinting, undoubtedly demonstrates the existence of a metabonomic signature for CD both in serum and urine. Notably, the best discrimination is obtained from CPMG spectra, that is, from spectra in which signals arising from large macromolecules such as lipids have been suppressed. This observation provides the surprising result that, although it is known that CD patients usually appear to be hypocholesterolemic,[54−56] lipids do not contribute significantly to the metabonomic signature of CD. The lowest discrimination is obtained from urine NOESY spectra, as expected from the higher day-to-day variability of the metabolic NMR profile of urine samples.[57,58] With reference to Figure 1A, it is interesting to note that the two incorrectly clustered CD patients (patients 23 and 26 in Supporting Information Table S1) were both completely asymptomatic, and were detected during familial screening. The only incorrectly clustered control subject has a past history of thyroid carcinoma and persistent low levels of ferritin and folic acid, and was on substitutive therapy with levothyroxine. Additionally, one of the control subjects, correctly clustered in Figure 1, was also misclassified; anecdotically, this subject has a familial history of lymphoma and myeloid leukemia.

Untreated CD patients often (up to 87%[59]) report symptoms of (chronic) fatigue[60,61] and this condition—that in patients' descriptions overlaps with feelings of tiredness, muscle weakness, depression, fatigability and irritability—is sometimes the only symptom of undiagnosed celiac disease.[62,63] The origin of this syndrome, generically attributed to malabsorption, is still unclear.

The NMR screening of CD patients and healthy controls sera revealed higher levels of glucose and reduced levels of pyruvate. Higher glucose levels in plasma of untreated CD patients have been reported,[64] together with a higher carbohydrate consumption than controls,[65] but to the best of our knowledge, decreased pyruvate and lactate levels have never been associated with CD. Furthermore, we also found higher levels of 3-hydroxybutyric acid in blood and of acetoacetate in urine of CD patients.

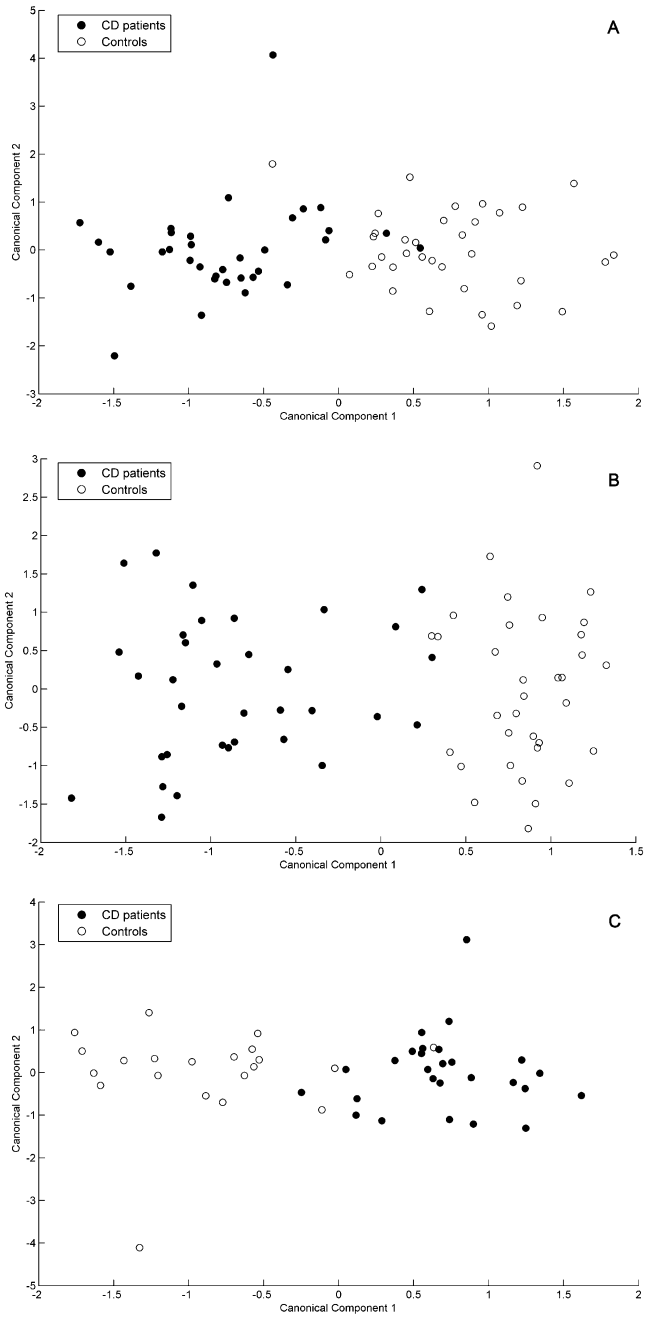

Figure 1. Descriptive clustering obtained by use of PLS-RCC method on CPMG (A) and NOESY (B) serum spectra, and (C) NOESY urine spectra.

Table 2. The 6-fold Classification Results for Sensitivity, Specificity, Accuracy and Cross Validation (CV) Accuracy Obtained Using CPMG and NOESY Serum Spectra and NOESY Urine Spectra[a]

	CD patients	HS	sensitivity	specificity	accuracy
Serum CPMG Spectra					
CD patients	32	2	94.1%		94.1%
HS	2	32		94.1%	
Serum NOESY Spectra					
CD patients	30	4	88.2%		92.6%
HS	1	33		97.2%	
Urine NOESY Spectra					
CD patients	24	3	88.9%		83.3%
HS	5	16		76.2%	

[a] Classification was performed by means of parameter-optimized Support Vector Machines.

The analysis of this metabolic profile points to pyruvate as the key to elucidate the origin of this peculiar metabonomic signature of CD. Pyruvate is the product of glycolysis, the anaerobic metabolism of glucose. While higher levels of glucose can be related to a variety of causes (such as upregulation of glucose intake at the level of microvillous membrane caused by alteration of the lipid-to-protein ratio of the microvillous membrane), a decrease of pyruvate sera levels is consistent with the fact that in untreated CD patients glycolysis is somehow impaired by reduction of glucose intake at cellular level or by impairment of one or more steps in the glycolysis process itself. Impairment of glycolysis explains both a lowering of pyruvate and lactate levels and an increase of glucose levels in blood. If glycolysis is reduced, lipid β-oxidation should be increased, as this is the second major metabolic pathway for energy conversion, but under conditions of malabsorption, caused in CD patients by villous atrophy, intake of lipids is reduced. Both facts, enhanced lipids β-oxidation and malabsorption, can then explain lower levels of lipids in sera. Because of the fact that also lipid catabolism is reduced, the use of ketonic bodies becomes a more important source of energy in CD patients, explaining higher levels of 3-hydroxybutyric acid in blood and acetoacetate in urine. Energy conversion from lipids and ketonic bodies catabolism is far less efficient than that from glucids, and this can explain the well-known chronic fatigue in CD patients. We found that in patients on a GFD (Table 4), levels of glucose and 3-hydroxybutyric acids revert to normality (that is, these levels were no longer statistically discriminating), and this can be an indication of a renormalization of the main energy metabolic pathway together with a recovery of villous functionality. The latter is indicated also by the increased level of lipids observed after 12 months on a GFD; this result is consistent with another recent study.[66] In patients on a GFD, fatigue tends to be reduced, and in fact, it has been proposed that this condition is gluten-related.[63] Furthermore, we observed a strong correlation between glucose and 3-hydroxybutyric acid levels ($r = 0.93$) over a 12 months period (Supporting Information Figure S6).

While glucose and 3-hydroxybutyric acid are the only metabolites with higher serum levels in untreated CD patients, the level of several other metabolites such as amino acids and choline are found to be lower. This is likely to be a direct consequence of intestinal malabsorption caused by villi atrophy.

Apart from gluten assumption, the composition and the metabolic activity of the gastrointestinal microflora have been indicated as a probable major environmental factor involved in the pathogenesis of celiac disease.[67] Microbial consortia are acquired soon after birth and the balance between host, gut microflora and environment is essential for well being. Microbiota contribute to several mammalian processes like defense against pathogens, immunity, development of intestinal microvilli and metabolic energy recovery.[68] Evidence of microbial involvement in the pathogenesis of CD has recently been found and a study of the metabolic activity in the gut suggests aberrant bacterial flora in the small bowel of CD patients with respect to healthy controls.[69] Here, we have found that the metabonome of CD patients is characterized by higher levels ($P < 0.05$) of m-HPPA, IS and PAG. m-HPPA mostly originates from gut microflora, being one of the several products of the

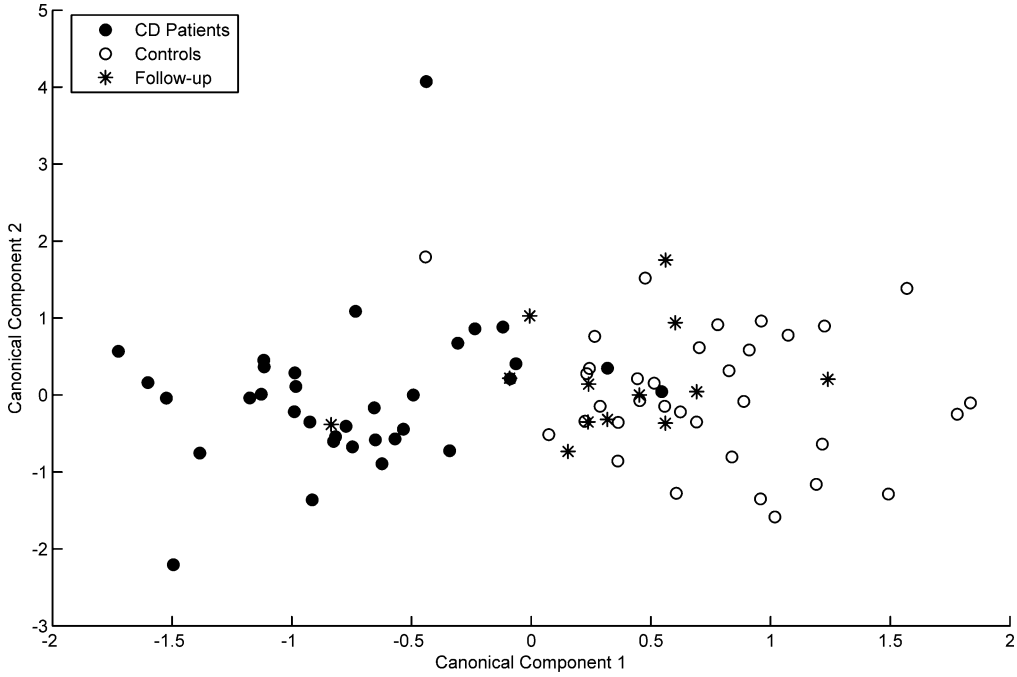

Figure 2. Predictive clustering of CMPG serum spectra of patients after 12 months on a gluten-free diet. Clustering is obtained by projection of follow-up patients on a model built on the 34 + 34 untreated patients and controls spectra.

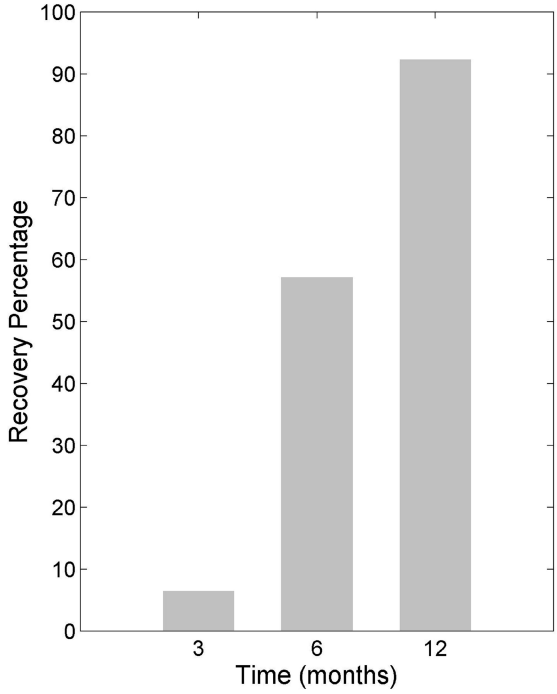

Figure 3. Time progression of the percentage of individuals that are classified as healthy by the statistical model after 3, 6 and 12 months on a gluten-free diet. Percentages refer to the number of individuals that provided samples at each time step, as detailed in Material and Methods.

Table 3. Metabolites Found To Be Statistically Different ($P < 0.01$) in Sera of Untreated Celiac Disease Patients with Respect to the Control Group[a]

	CD patients		HS	
	mean	CI 95%	mean	CI 95%
3-OH-Butyrate	0.7570	0.5926−0.9215	0.4512	0.3348−0.5675
Asparagine	0.0628	0.0563−0.0693	0.0782	0.0709−0.0855
Choline	1.3822	1.2231−1.5413	1.5543	1.3724−1.7361
Creatinine	0.1815	0.1658−0.1973	0.2164	0.1976−0.2352
Glucose	2.0659	1.6415−2.4903	1.3127	1.0249−1.6006
Glycoproteins	0.0081	0.0079−0.0082	0.0082	0.0082−0.0083
Isoleucine	0.4563	0.4159−0.4966	0.5029	0.4579−0.5479
Lactate	3.7368	3.3743−4.0992	4.1618	3.8688−4.4548
Leucine	0.7779	0.7223−0.8335	0.8474	0.7945−0.9003
Lipids	0.025	0.025−0.026	0.027	0.026−0.027
Methionine	0.0699	0.0645−0.0754	0.0863	0.0782−0.0945
Methylamine	0.0660	0.0601−0.0719	0.0823	0.0750−0.0895
Methylglutarate	0.0754	0.0660−0.0848	0.0897	0.0795−0.0999
Pyruvate	0.1203	0.1065−0.1341	0.1477	0.1282−0.1671
Proline	0.1300	0.1188−0.1412	0.1389	0.1309−0.1469
Valine	0.1241	0.0979−0.1504	0.1613	0.1370−0.1856

[a] Values are given in arbitrary units together with confidence intervals at 95%.

microbially mediated breakdown of larger plant phenolic compounds such as caffeic acid and its conjugate chlorogenic acids.[70,71] IS is a harmful uremic toxin produced in the liver from indole through indoxyl. Indole is one of the subproducts of tryptophan metabolism by intestinal bacteria.[42,72] Modulation of PAG excretion in urine has been recently attributed to gut microflora, though increases of PAG have been reported in case of drug-induced phospholipidosis and the contribution to PAG excretion from mammalian and microbial source have not been yet fully characterized.[68]

These findings are consistent with the hypothesis that in CD patients gut microflora of the small bowel is altered, or presents peculiar species with their own microbial metabonome. To our knowledge, m-HPPA, IS and PAG have never been associated with celiac disease.

The presence of these metabolites in urine can also be caused by an altered intestinal permeability: it is commonly accepted that CD onset is initiated by the leakage of proline/glutamine-rich gliadin fragments through the intestinal epithelium that activates, in susceptible subjects, the response of immune system.[73] The cause−effect relationship between

Table 4. Metabolites Found To Be Statistically Different ($P < 0.05$) in Urine of Untreated Celiac Disease Patients with Respect to the Control Group[a]

	CD patients		HS	
	mean	CI 95%	mean	CI 95%
Acetoacetate	0.5734	0.5100−0.6369	0.4543	0.4027−0.5059
Choline	0.2682	0.2468−0.2895	0.2474	0.2284−0.2664
Glutamate/ Glutamine	0.3109	0.2764−0.3454	0.3235	0.2852−0.3618
Gylicine	1.2308	1.0778−1.3839	1.0321	0.8970−1.1672
Indoxyl Sulfate	0.2438	0.1990−0.2887	0.1897	0.1565−0.2228
Mannitol	1.2370	1.1230−1.3510	1.7713	1.3372−2.2053
mHHPA	0.1753	0.1455−0.2052	0.1438	0.1318−0.1558
PAG	0.4764	0.4082−0.5445	0.3544	0.3076−0.4012
Pyrimidines	0.0294	0.0207−0.0381	0.0544	0.0271−0.0837
Uracile	0.1261	0.1097−0.1426	0.0929	0.0725−0.1132

[a] Values are given in arbitrary units together with confidence intervals at 95%.

Table 5. Metabolites That Showed a Statistically Significant Variation between Untreated Celiac Disease Patients and Corresponding Follow-up after a 12 month GFD[a]

metabolite	12 month follow-up
3-Hydroxybutyric acid	↓
Asparagine	↑
Choline	↑
Glucose	↓
Isoleucine	↑
Lactate	↑
Lipids	↑
Lipoproteins	↑
Leucine	↑
Methionine	↑
Valine	↑

[a] The arrows (↑ and ↓) indicate increase and decrease of levels after diet, respectively.

abnormal permeability and the appearance of the inflammatory process is still debated, and it has been proposed that increased permeability is a precondition for CD.

In conclusion, we have shown that a metabonome for celiac disease can be defined. This metabonome has three components, one directly related to malabsorption, one related to energy metabolism, and the third related to alterations of gut microflora and/or of intestinal permeability. The pattern of several metabolites related to energy metabolism sheds new light on the origin of the chronic fatigue syndrome common to many CD patients.

A study aiming at defining the metabonomic signatures of other diseases such as Crohn's disease, common variable immune deficiency, small intestinal bacterial overgrowth, and short bowel syndrome is ongoing in our laboratory in order to confirm the ability of this NMR-based metabolomic approach to distinguish CD from other causes of malabsorption.

Abbreviations: CD, Celiac Disease; GFD, Gluten-free diet; NMR, Nuclear Magnetic Resonance.

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Supporting Information Available: Tables of clinical characteristics of CD patients and the average classification accuracy for each individual in the test set; figures of distributions of all metabolite levels that are significantly different between celiac and HS. This material is available free of charge via the Internet at http://pubs.acs.org.

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