

Multiple Myeloma Patients Have a Specific Serum Metabolomic Profile That Changes after Achieving Complete Remission

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Abstract

Purpose: Multiple myeloma remains an incurable disease. New approaches to develop better tools for improving patient prognostication and monitoring treatment efficacy are very much needed. In this study, we aimed to evaluate the potential of metabolomics by ¹H-NMR to provide information on metabolic profiles that could be useful in the management of multiple myeloma.

Experimental Design: Serum samples were collected from multiple myeloma patients at the time of diagnosis and after achieving complete remission. A matched control set of samples was also included in the study. The ¹H-NMR measurements used to obtain the metabolic profile for each patient were followed by the application of univariate and multivariate statistical analyses to determine significant differences.

Results: Metabolic profiles of multiple myeloma patients at diagnosis exhibited higher levels of isoleucine, arginine, acetate, phenylalanine, and tyrosine, and decreased levels of 3-hydroxybutyrate, lysine, glutamine, and some lipids compared with the control set. A similar analysis conducted in multiple myeloma patients after achieving complete remission indicated that some of the metabolic changes (i.e., glutamine, cholesterol, lysine) observed at diagnosis displayed a variation in the opposite direction upon responding to treatment, thus contributing to multiple myeloma patients having a closer metabolic profile to those of healthy individuals after the disappearance of major disease manifestations.

Conclusions: The results highlight the potential of metabolic profiles obtained by ¹H-NMR in identifying multiple myeloma biomarkers that may be useful to objectively discriminate individuals with and without multiple myeloma, and monitor response to treatment. *Clin Cancer Res*; 19(17); 4770–9. ©2013 AACR.

Introduction

Multiple myeloma is a malignant disorder of postgerminal center B cells, characterized by the clonal proliferation of malignant plasma cells within the bone marrow. It is the second most frequent hematologic malignancy, after non-Hodgkin lymphoma with an incidence of 4 to 5 new patients per 100,000 people and year. In the past few years, many significant advances have occurred in the field of

multiple myeloma, about all aspects of research, from basic knowledge to supportive care, novel diagnostic procedures, and prognostic classifications, as well as novel therapeutic approaches. The administration of high-dose therapy followed by stem cell support (HDT-ASCT) and the introduction of novel agents, such as immunomodulatory drugs (thalidomide and lenalidomide) and proteasome inhibitors (bortezomib) have contributed to improve the overall survival of multiple myeloma patients. Nevertheless, multiple myeloma remains an incurable disease and new efforts are necessary not only to improve survival but also to develop better tools to evaluate the prognosis and to monitor treatment efficacy.

Metabolomics, a new and powerful tool based on the combination of metabolic profiling techniques with multivariate statistical approaches, has been proved to be extremely useful in the identification of early biomarkers related with inborn errors of metabolism, cardiovascular disease, metabolic disorders, or cancer (1). This approach involves the systematic study of small molecules that characterize the metabolic pathways of biological systems. The most commonly employed analytical

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Multiple myeloma is a hematological neoplasm difficult to diagnose in its early stages. Despite the introduction of novel agents that have contributed to improve the overall survival of multiple myeloma patients, the disease is still incurable. This scenario explains why new efforts are necessary not only to improve survival but also to develop better tools to evaluate the prognosis and to monitor treatment efficacy. In this context, we assessed the potential of metabolomics to provide information that could be useful in the clinical management of multiple myeloma patients. Although validation in a prospective fashion using a larger sample set is required, our results provide evidence that metabolomics could be a promising tool for a better understanding of the molecular mechanisms of multiple myeloma with implications as a diagnostic tool, as well as for elucidating the biochemical pathways involved in the response to chemotherapy.

techniques used for metabolic profiling include nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy and mass spectrometry (MS; ref. 2). High-resolution $^1\text{H-NMR}$ spectroscopy provides quantitative analysis of metabolite concentrations and reproducible information with minimal sample handling. Particularly in the cancer setting, metabolomics has been applied to develop novel early diagnostic biomarkers in renal cancer (3, 4), colorectal cancer (5), pancreatic cancer (6), leukemia (7), ovarian cancer (8, 9) and oral cancer (10). More recently, this approach has also been used to provide predictive biomarkers associated with prognosis, response to treatment and toxicity (11, 12).

In this study, we assessed the potential role of metabolomics by $^1\text{H-NMR}$ spectroscopy to characterize the specific metabolic profile of multiple myeloma patients by conducting a comparative analysis of healthy subjects and patients with multiple myeloma. Furthermore, to extend the clinical applications of metabolomics, a com-

parison between the metabolic profiles of multiple myeloma patients at the time of diagnosis and after achievement of complete remission was conducted. Overall, we found that this approach provides a promising tool for a better understanding of the molecular mechanisms of multiple myeloma with implications as a diagnostic tool, as well as for elucidating the biochemical pathways involved in the response to chemotherapy, an information that could have a relevant impact in treatment design and monitoring.

Materials and Methods

Selection and description of participants

Serum samples were obtained from multiple myeloma patients (Table 1) included in 2 Spanish Myeloma Group clinical trials: the phase 3 GEM2005<65y trial, in which young patients were randomized to receive induction with VBMCP (vincristine, carmustine, melphalan, cyclophosphamide, and prednisone)/VBAD (vincristine, carmustine, doxorubicin, and dexamethasone) plus bortezomib in the last 2 cycles or thalidomide/dexamethasone or bortezomib/thalidomide/dexamethasone followed by HDT-ASCT, and the phase 3 GEM 2005>65y for elderly patients, consisting on bortezomib plus melphalan/prednisone or thalidomide/prednisone (13). Both trials included patients with newly diagnosed, untreated, symptomatic, measurable multiple myeloma (defined as serum monoclonal protein of more than 10 g/L or urine monoclonal protein of 0.2 g or more per day). For most patients included in this study, 2 serum samples were analyzed; the first one was collected at the time of diagnosis and the second after achieving complete remission, defined as absence of the original M component by immunofixation and <5% plasma cells in bone marrow. A matched control set of 31 serum samples (age, sex) from healthy subjects was also included. Samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki and with approval from the Ethics Committees of all participating institutions. Both clinical trials were registered with ClinicalTrials.gov, number NCT 00461747 for GEM2005<65y and NCT00443235 for GEM2005>65y.

Table 1. Samples included in the metabolomic study

Group	n	Gender	Age \pm SEM ^a	M protein
Control	31	M = 16 F = 15	67.1 \pm 1.6	–
Diagnosis	27	M = 14 F = 13	63.6 \pm 2.2	IgG = 13 IgA = 9 Bence-Jones = 5
Remission	23	M = 12 F = 11	63.7 \pm 2.3	–

n = sample size.

^aMean years at time of sample collection (for the control group) and diagnosis (for diagnosis and remission groups) \pm SEM.

Sample preparation and $^1\text{H-NMR}$ acquisition

Serum was collected and immediately stored at -80°C . At the time of analysis, the serum samples were thawed on ice. Five hundred microliters of 10% D_2O buffer (5 mmol/L TSP, 140 mmol/L Na_2HPO_4 , 0.04% NaN_3 , pH 7.4) were added to 500 μL of serum sample. After this, 550 μL of the mixture were transferred to a 5-mm NMR tube for analysis. $^1\text{H-NMR}$ spectra were acquired using a Bruker Avance II 600 MHz spectrometer equipped with triple resonance cryo-probe with a cooled ^{13}C preamplifier (TCI). $^1\text{H-NMR}$ experiments were acquired at 310 K (37°C) for every sample. Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (14), which generates spectra edited by T2 relaxation times with reduced signals from high molecular weight species and giving improved resolution of low molecular weight metabolite resonances, was collected for each sample with a total of 16 accumulations and 72 K data points over a spectral width of 16 ppm. A 4-second relaxation delay was included between free induction decays (FIDs). The total spin-spin relaxation delay was 40 milliseconds. A one-dimensional (1D) NOESY pulse sequence that generates an unedited spectrum with improved solvent peak suppression (15) was collected using the same parameters as the CPMG experiment, with a 4-second relaxation delay and 10 milliseconds of mixing time. For both experiments, a water presaturation pulse of 25 Hz was applied throughout the relaxation delays to improve solvent suppression. In addition, for assignment purposes, 2D J -resolved spectra, homonuclear 2D $^1\text{H-}^1\text{H}$ total correlation spectroscopy and 2D ^1H , ^{13}C heteronuclear single quantum correlation were acquired for selected samples. All spectra were multiplied by a line-broadening factor of 1 Hz and Fourier transformed. Spectra were automatically phased and baseline corrected, chemical shift referenced internally to the methyl group signal of alanine at 1.47 ppm using TOPSPIN 3.0 (Bruker Biospin) and analyzed with Analysis of MIXtures (AMIX; Bruker) for metabolites assignment. Metabolites of interest were identified using Amix v 3.9.7 in combination with the Bruker NMR Metabolic Profiling Database BBIORFCODE 2.0.0 database (Bruker Biospin), as well as other existing public databases and literature reports (7, 15, 16).

Multivariate statistical analysis

All samples, but one having high-intensity signals derived from a contamination during the sample collection/preparation process, were considered valid samples for the statistical analysis. CPMG spectra were used for the multivariate statistical analysis because of improved baseline and metabolite detection. Furthermore, any reduction in signal intensity induced by relaxation effects would be consistent across samples. $^1\text{H-NMR}$ spectra were binned using Amix 3.9.7 (Bruker Biospin) into 0.04-ppm-wide rectangular buckets over the region δ 10.5 to 0.26 ppm. The water (δ 5.12–4.35 ppm) and urea signal (δ 5.85–5.65 ppm) regions were excluded from the analysis to avoid interference arising from dif-

ferences in water suppression and variability from urea signal, respectively. Bucket tables generated were imported into SIMCA-P 12.0 software (Umetrics AB). Because statistical analysis data were scaled to unit variance by dividing each variable by $1/(\text{SD})$, where SD represents the standard deviation value of each variable, so that all the 234 variables were given equal weight regardless of their absolute value.

Then, principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted. PCA, an unsupervised pattern recognition method that does not use prior knowledge of a system, was used to examine the intrinsic variability within the dataset, to observe clustering or separation trends and for the identification of outliers. Next, OPLS, a supervised approach used to produce models of "best fit" was applied to minimize the possible contribution of intergroup variability and to further improve separation between the groups of samples. The default method of seven-fold internal cross validation of the software was applied, from which Q^2Y (predictive ability parameter, estimated by cross-validation) and R^2Y (goodness of fit parameter) values were extracted. Those parameters were used for the evaluation of the quality of the OPLS-DA models obtained. To identify the variations responsible for the separation between groups of samples, the corresponding loading plots and the variable importance in projection (VIP) list of each OPLS-DA model were carefully inspected, and used to identify which variables were important for discrimination between groups.

Quantitative analysis of variations of selected metabolites

The main metabolites contributing to group discrimination in each model (healthy individuals vs. multiple myeloma patients at the time of diagnosis, multiple myeloma patients at the time of diagnosis vs. multiple myeloma patients after complete remission, and healthy individuals vs. multiple myeloma patients after complete remission) were integrated to enable comparison between samples. To this end, the variable size bucketing module in Amix 3.9.7 was used to obtain the exact integral value corresponding to the NMR signal of each selected metabolite. The statistical significance of the differences between the means of the 2 groups was assessed using the Student t test. A P -value <0.05 (confidence level 95%) was considered statistically significant.

Results

Thirty-one serum samples from healthy donors, 27 of multiple myeloma patients at the time of diagnosis and 23 paired samples from these same multiple myeloma patients after achieving complete remission were included in the analysis. The characteristics of the subjects included in this study are summarized in Table 1. A typical $^1\text{H-NMR}$ CPMG spectrum of a sample corresponding to a multiple myeloma patient collected at the time of diagnosis is illustrated in Fig. 1. Spectra of similar quality were obtained

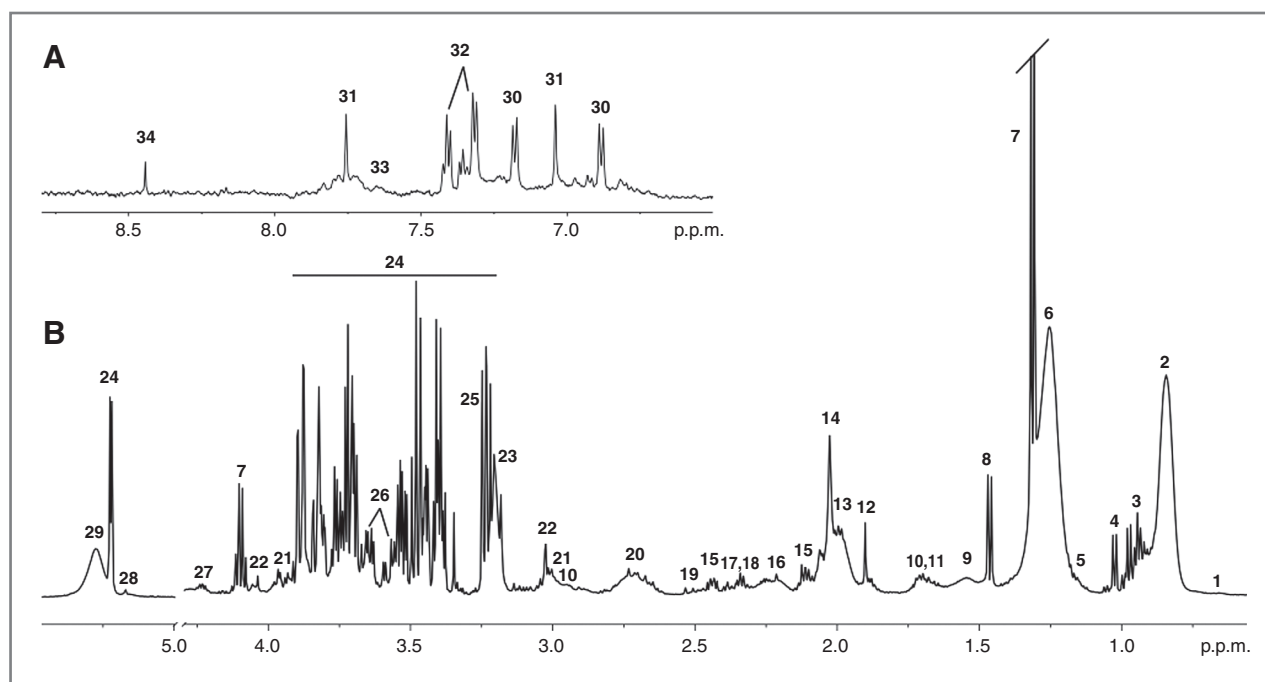


Figure 1. Assignment of the $^1\text{H-NMR}$ signals of a representative 600 MHz one-dimensional Carr–Purcell–Meiboom–Gill (1D-CPMG) $^1\text{H-NMR}$ spectra of a multiple myeloma patient serum sample measured at 310K. A, full spectrum ($\delta = 5.50\text{--}0.5$ ppm) and B, magnification of aromatic region ($\delta = 9.00\text{--}6.50$ ppm). Peak assignments: 1, cholesterol (C18-CH_3); 2, lipids ($-\text{CH}_3$) (mainly LDL/VLDL); 3, leucine/isoleucine; 4, valine; 5, 3-hydroxybutyrate; 6, lipids (CH_2) (mainly LDL/VLDL); 7, lactate; 8, alanine; 9, adipic acid; 10, lysine; 11, arginine; 12, acetate; 13, lipids ($\text{CH}_2\text{-C}=\text{C}$); 14, NAC (acetyl signals from glycoproteins); 15, glutamine; 16, lipids ($\text{CH}_2\text{-CO}$); 17, glutamate; 18, pyruvate; 19, citrate; 20, lipids ($\text{CH}=\text{CH-CH}_2\text{-CH}=\text{CH-}$); 21, creatine; 22, creatinine; 23, choline; 24, glucose; 25, trimethylamine N-oxide; 26, glycerol; 27, threonine; 28, glycerol of lipids; 29, lipids ($-\text{CH}=\text{CH-}$); 30, tyrosine; 31, histidine; 32, phenylalanine; 33, 3-methylhistidine; 34, formate.

for multiple myeloma patients after achieving complete remission and control individuals. Spectra were dominated by signals of low-molecular weight metabolites. Assigned signals represent a typical range of metabolites normally found in serum, including several amino acids (e.g., lysine, arginine, glutamine, phenylalanine, histidine, tyrosine, alanine), glucose and sugars, creatine, creatinine, organic acids (e.g., acetate, lactate, citrate, formate). Higher molecular weight metabolites were also observable in the CPMG spectrum: fatty acyl chain protons from lipoproteins, unsaturated acids, and glycoproteins.

PCA analyses were applied independently to the full set of control samples, patients samples collected at the time of diagnosis and after complete remission to detect potential intrinsic clustering and possible outliers. No age or gender-related differences were observed to influence the homogeneity of the groups of samples included in the study (Supplementary Fig. S1). Only one sample from the control set and one from the group of multiple myeloma patients at the time of diagnosis were identified as strong outliers on their group, outside the Hotellings 95% confidence limit. They displayed a metabolic profile that has been previously reported to be related to hypertensive patients (17, 18), characterized by changes in signals attributed to lipids and lipoproteins, as well as the choline-containing region. Furthermore, the detailed revision of the clinical history of the multiple myeloma patient at the time of diagnosis revealed that the patient was diabetic and hypertensive.

These findings justified the exclusion of these 2 samples from the study.

Multiple myeloma patients at diagnosis versus healthy donors

To better examine differences between the groups of samples an OPLS-DA model was used. The score plot (Fig. 2A) shows a clear separation between healthy individuals and multiple myeloma patients at the time of diagnosis ($R^2 = 0.613$; $Q^2 = 0.462$). Examination of the corresponding loading plot and VIP list indicated the buckets that were mainly contributing to the discrimination between healthy and multiple myeloma individuals. The metabolites corresponding to those regions of the spectra were identified through a combination of their ^1H chemical shift in the 1D-CPMG experiment and the spin system patterns obtained from the 2D spectra acquired for representative samples of each group. Metabolic profiles of multiple myeloma patients exhibited higher levels of isoleucine, arginine, acetate, phenylalanine, and tyrosine and decreased levels of 3-hydroxybutyrate, lysine, glutamine, as well as some lipids. Supplementary Table S1 summarizes the mean and SEM values obtained from the integration of the defined regions in each group of samples and the percentage of variation for the selected metabolite between these 2 groups. The percentage of variation found for the above-mentioned metabolites between the 2 groups is

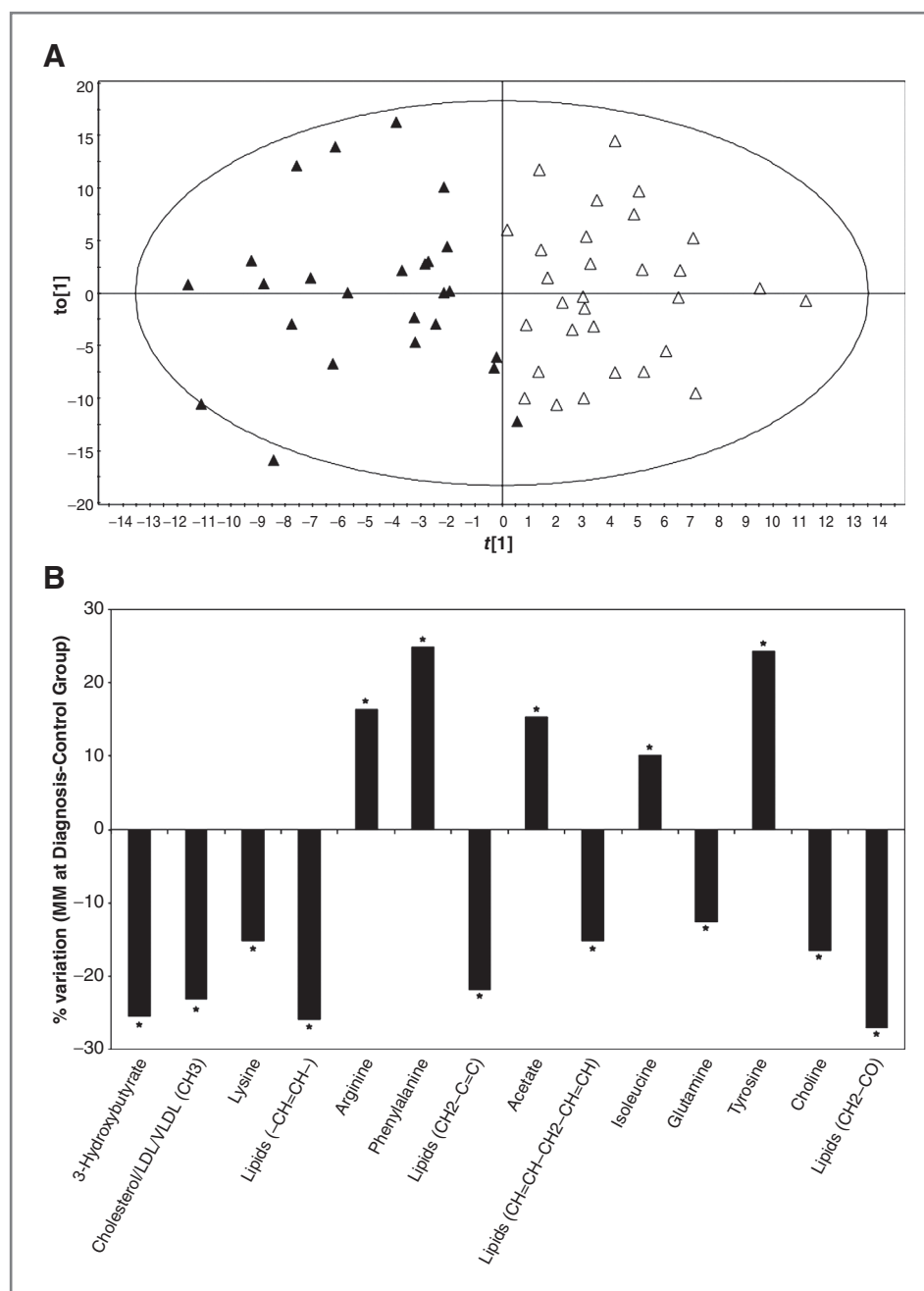


Figure 2. Multivariate modeling resulting from the analysis of serum ¹H-NMR spectra of control individuals (open symbols) vs. multiple myeloma patients at the time of diagnosis (full symbols). A, OPLS-DA score plot showing the separation between the 2 groups based on their metabolic profile. B, mean signal intensity variation (%) of the metabolites that contributed most to the separation between groups. Positive bars in the graphic correspond to signals that are increased in multiple myeloma patients at the time of diagnosis. *, Statistically significant differences compared to control ($P < 0.05$).

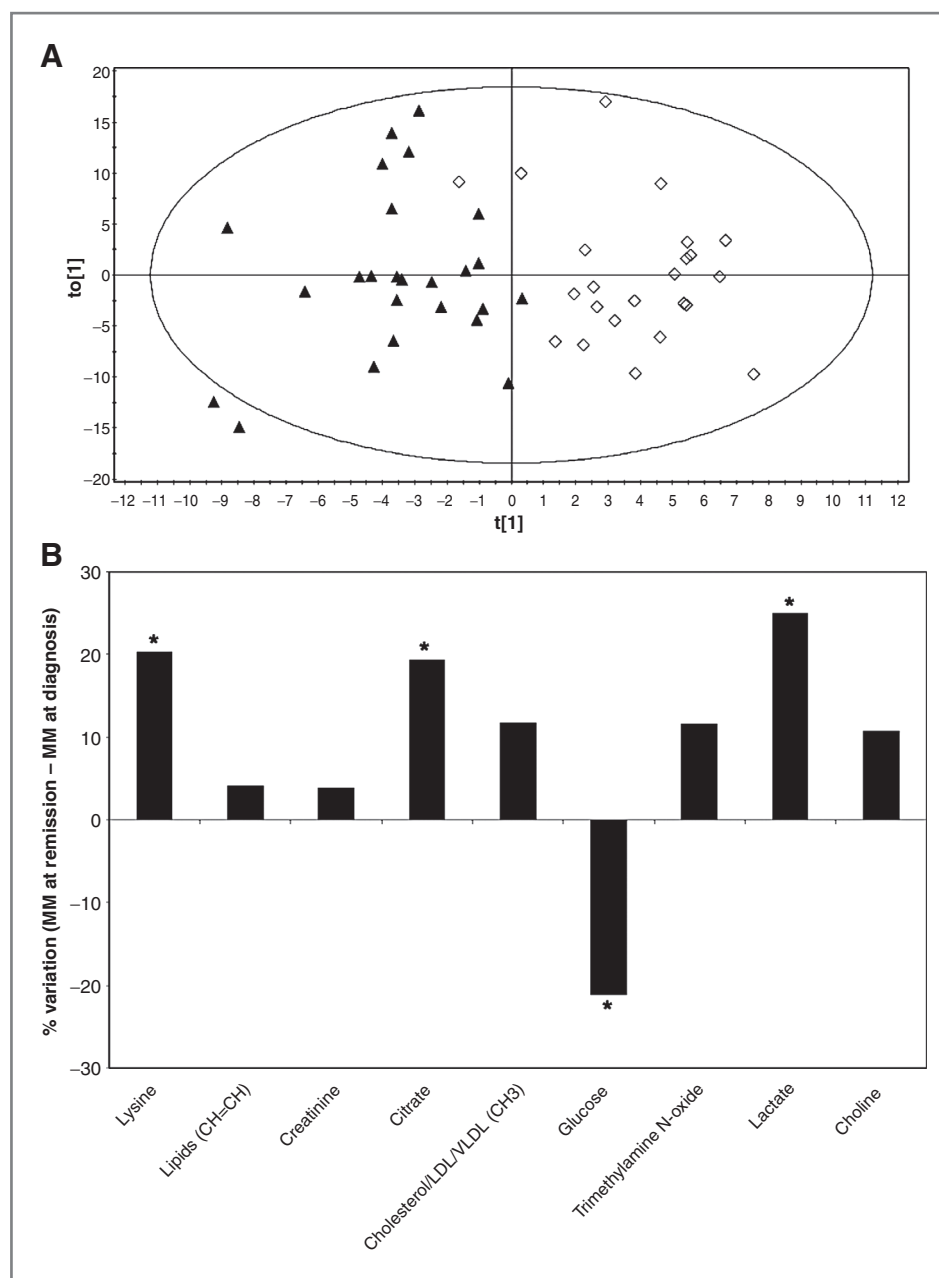
graphically represented in Fig. 2B. The results obtained are consistent with the OPLS-DA model built for the discrimination of both groups of samples.

Multiple myeloma patients at diagnosis versus multiple myeloma patients after complete remission

A different OPLS-DA model was built to determine if it was possible to distinguish when a multiple myeloma patient has achieved complete remission of the disease based on its ¹H-NMR metabolic profile. Figure 3A and B shows the score plot for this model, which illustrates the clear separation obtained between the 2 groups ($R^2 =$

0.553; $Q^2 = 0.358$), and a graphic representation of the main metabolites contributing to this separation, based on the results obtained from the loading plot and the most representative buckets defined in the VIP list. Based on these results, the ¹H-NMR serum metabolic profile of multiple myeloma patients after achieving complete remission differed from the profile at the time of diagnosis mainly in the levels of lactate, lysine, TMAO, choline, and cholesterol, which were significantly higher in patients after complete remission. To validate the statistical modeling, the metabolites identified were quantified using variable size bucketing for the integration of the corresponding ¹H-NMR

Figure 3. Multivariate modeling resulting from the analysis of serum $^1\text{H-NMR}$ spectra of multiple myeloma (MM) patients at the time of diagnosis (full symbols) vs. MM patients after complete remission (open symbols). A, OPLS-DA score plot showing the separation between the 2 groups based on their metabolic profile. B, mean signal intensity variation (%) of the metabolites that contributed most to the separation between groups. Positive bars in the graphic correspond to signals that are increased in MM patients after the complete remission of the disease. *, Statistically significant differences compared to diagnosis ($P < 0.05$).



signals in the spectra. The summary of the metabolites that were quantified for this model is shown in Supplementary Table S2, including the percentage of variation found in the group of multiple myeloma patients after complete remission compared to that of multiple myeloma patients at the time of diagnosis. A graphical representation of the percentage of variation found in the level of these metabolites between the 2 groups is shown in Fig. 3B.

The reliability of the statistical OPLS-DA models was evaluated based on the parameters obtained from the internal cross validation, summarized in Table 2, in terms of Q^2 and R^2 values (representing, respectively, the predictive capability and the explained variance),

sensitivity (percentage of true positive results), specificity (percentage of true negative results), and accuracy (total percentage of samples correctly classified) values. This information was extracted from the misclassification table obtained when the class labels of all samples were predicted using the corresponding model.

Multiple myeloma patients after complete remission versus healthy donors

Finally, an OPLS-DA analysis conducted to compare the metabolic profiles of multiple myeloma patients after complete remission and healthy individuals showed that the statistical discrimination between these 2 groups was

Table 2. Prediction results obtained for OPLS-DA models built for control vs. diagnosis samples and diagnosis vs. remission samples

	<i>n</i>	<i>R</i> ²	<i>Q</i> ²	Sensitivity ^a (%)	Specificity ^a (%)	Accuracy ^a (%)	Fishers prob. ^b
Control vs. diagnosis	55	0.613	0.462	88	100	94.5	1.8E-012
Diagnosis vs. remission	48	0.553	0.358	95.7	96	95.8	1.9E-011

n = sample size.

^aClassification results were used to define true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) and therefore calculate as percent sensitivity [TP/(TP + FN) × 100], specificity [TN/(TN + FP) × 100], and accuracy [TP + TN/(TP + TN + FP + FN) × 100].

^bProbability of a particular model occurring by chance and is satisfied when *P* < 0.05 for 95% confidence.

smaller ($Q^2 < 0.25$) to that obtained in the previous comparisons (i.e., healthy controls vs. multiple myeloma patients at the time of diagnosis, and multiple myeloma patients at the time of diagnosis vs. multiple myeloma patients after complete remission). Table 3 summarizes the mean and SEM values obtained from the integration of the metabolites that showed the most significant variations between the 3 groups of samples.

Discussion

Efforts to identify multiple myeloma early biomarkers that could help to better understand disease pathogenesis

and to evaluate response to treatment remain a fundamental goal in this area (19–21). In that sense, to the best of our knowledge, this study represents the first application of high field ¹H-NMR metabolomics to the characterization of serum samples from multiple myeloma patients. The multivariate statistical approach used in this study takes into account numerous variables (metabolites) across multiple samples, facilitating a global description of the serum metabolic profile of multiple myeloma patients. Overall, the results suggest that the serum metabolic profile of multiple myeloma patients could provide useful information about the molecular

Table 3. Mean intensities of most significant variations found in the metabolites for the 3 groups of samples included in the study

Metabolite	$\delta^1\text{H}$ (ppm) ^a	Mean spectral intensity \pm SEM (arbitrary units)		
		Control group	Multiple myeloma patients at diagnosis	Multiple myeloma patients at remission
3-Hydroxybutyrate	1.15–1.19	1.95E07 \pm 6.59E05	1.45E07 \pm 6.97E05	1.44E07 \pm 6.45E05
Arginine	1.63–1.77	1.18E07 \pm 3.38E05	1.37E07 \pm 5.64E05	1.42E07 \pm 5.10E05
Glutamine	2.41–2.46	6.76E06 \pm 1.31E05	5.90E06 \pm 3.57E05	6.13E06 \pm 2.52E05
Phenylalanine	7.29–7.33	1.06E06 \pm 4.65E04	1.33E06 \pm 7.62E04	1.33E06 \pm 8.38E04
Lipids (CH = CH–CH ₂ –CH = CH)	2.60–2.84	3.22E07 \pm 1.21E06	2.73E07 \pm 1.52E06	2.90E07 \pm 1.04E06
Acetate	1.88–1.91	4.03E06 \pm 1.62E05	4.65E06 \pm 2.78E05	5.28E06 \pm 5.14E05
Lipids (CH = CH)	5.24–5.37	4.09E07 \pm 2.74E06	3.03E07 \pm 2.59E06	3.15E07 \pm 2.03E06
Glucose	5.21–5.24	1.22E07 \pm 8.00E05	1.41E07 \pm 1.25E06	1.11E07 \pm 9.67E05
Lipids (CH ₂ –CO)	2.17–2.25	1.81E07 \pm 2.11E06	1.32E07 \pm 1.38E06	1.18E07 \pm 9.77E05
Lactate	4.13–4.18	1.84E06 \pm 1.87E05	2.01E06 \pm 2.00E05	2.51E06 \pm 2.04E05
Lipids (CH ₂ –C = C)	1.93–2.01	4.69E07 \pm 2.51E06	3.67E07 \pm 2.20E06	3.92E07 \pm 1.67E06
Citrate	2.50–2.55	1.46E06 \pm 9.17E04	1.51E06 \pm 6.65E04	1.80E06 \pm 1.00E05
Cholesterol/LDL/VLDL (CH ₃)	0.70–0.90	1.62E08 \pm 7.42E06	1.24E08 \pm 7.53E06	1.39E08 \pm 4.91E06
Lysine	2.87–2.93	3.70E06 \pm 5.93E04	3.14E06 \pm 1.11E05	3.77E06 \pm 8.45E04
Tyrosine	7.16–7.20	5.64E05 \pm 3.46E04	7.02E05 \pm 7.02E05	6.30E05 \pm 4.80E04
Choline	3.19–3.21	3.31E07 \pm 1.60E06	2.77E07 \pm 1.70E06	3.06E07 \pm 1.60E06
Glutamate	2.32–2.35	3.40E06 \pm 2.35E05	4.35E06 \pm 3.09E05	4.71E06 \pm 2.98E05
Threonine	4.21–4.29	7.94E06 \pm 5.55E05	6.41E06 \pm 5.41E05	6.44E06 \pm 3.66E05

^aChemical shift of region used for quantification.

mechanisms behind this disease and could have a potential role in the identification of new biomarkers indicative of response to treatment.

As a proof-of-principle, serum metabolic profiles of healthy individuals and multiple myeloma patients at the time of diagnosis were examined. Initial analysis using unsupervised PCA did not indicate any obvious disease-related variation in the metabolic profile. However, a supervised multivariate OPLS-DA modeling enabled good separation of healthy individuals and multiple myeloma patients revealing clear differences in their metabolic profiles. The major metabolic differences detected in multiple myeloma patients at the time of diagnosis included increased levels of amino acids such as isoleucine, phenylalanine, arginine and tyrosine, and acetate, as well as decreased levels of 3-hydroxybutyrate, cholesterol, lysine, lipids, choline, and glutamine. Alterations in the fatty acid profiles of plasma lipids are quite common in cancer and have been shown in a variety of neoplastic processes (22). All saturated, monounsaturated, and essential fatty acids and their polyunsaturated derivatives are decreased, apparently as a consequence of the disease itself (23). Decreased cholesterol levels in multiple myeloma patients have been previously described, probably representing increased LDL clearance and utilization of lipids and cholesterol by myeloma cells (24). In fact, it has been shown that NS0 myeloma cells are naturally cholesterol dependent; probably due to a biochemical deficiency in the demethylation of lanosterol to C-29 sterols (25, 26). Previous studies (27, 28) have also shown that lysine and glutamine are absolutely required for myeloma cells growth. Glutamine is not only rapidly used by myeloma cells but also depleted by spontaneous conversion to pyrrolidone carboxylic acid and by enzymatic modification to glutamic acid (29). Differences in the observed amino acid serum levels of multiple myeloma patients could be associated with the specific features of the proteins that are produced in the course of this disease. Thus, it has been reported (30) that serum proteins isolated from multiple myeloma patients display a preferential utilization of aspartic acid, glutamic acid, lysine, proline, and serine, and comparatively show a low utilization of arginine, isoleucine, phenylalanine, tryptophan, and tyrosine. The comparison of the metabolic profiles of multiple myeloma patients at the time of diagnosis and healthy controls did not reveal statistically significant differences in the glucose levels of the 2 groups, a result that is in agreement with PET/CT observations obtained during evaluation of multiple myeloma patients. Thus, it has been reported that, despite its sensitivity and usefulness in multiple myeloma diagnosis, the uptake of fluorodeoxyglucose (^{18}F -FDG) in multiple myeloma assessed with the maximum standardized uptake value (SUV_{max}) can be very low, sometimes even comparable to the SUV_{max} of a benign lesion, making sometimes very difficult to distinguish between a benign lesion and a low-metabolic multiple myeloma lesion (31). However, ^{11}C -Choline PET/CT seems to be more sensitive than ^{18}F -FDG PET/CT for the detection of bony myelomatous lesions. Cho-

line, a small molecule precursor of phospholipids whose uptake is increased in proliferating cells as it is involved in membrane metabolism and growth, shows a statistically significant decrease when the metabolic profile of multiple myeloma patients and healthy donors were compared, thus correlating with the PET/CT data. Interestingly, choline serum levels show an opposite tendency after complete remission has been achieved, perhaps reflecting the disappearance of myeloma lesions. Taken together, these results provide evidence that serum ^1H -NMR profiling harbors the capacity to identify, using a rapid noninvasive assay, disease-related changes in the serum of multiple myeloma patients.

Having determined that multiple myeloma patients present a serum metabolic signature distinguishable from healthy controls, the metabolic profiles of multiple myeloma patients at the time of diagnosis and after complete remission were examined using the same methodology. Here again, an OPLS-DA multivariate model provided a clear separation between these 2 groups of samples. In this case, the levels of glucose, citrate, lactate, and lysine were found to be significantly different. The decreased levels of glucose and concomitant increased lactate levels observed in the multiple myeloma group after complete remission, when compared to the multiple myeloma patients at diagnosis, could be related to the chemotherapy treatment. A number of reports have suggested that increased levels of serum lactate dehydrogenase (LDH), the enzyme that catalyzes the interconversion of pyruvate into lactate, could be associated to treatment with bortezomib, a drug common to most of the therapeutic regimes used in this study, and not by progressive disease (32). Moreover, the increased levels of citrate observed for the multiple myeloma group after complete remission could be explained by the inhibitory effects of antimyeloma drugs, bortezomib in particular (33), on hypoxia-inducible factors (HIFs), a family of transcription factors that promote anaerobic respiration (34) through different mechanisms. One of them involves the upregulation of pyruvate dehydrogenase kinase (PDK), an enzyme that inactivates the pyruvate dehydrogenase (E1) by ATP-mediated phosphorylation. E1 is the first component of pyruvate dehydrogenase complex that catalyzes the transformation of pyruvate into acetyl-CoA, a key molecule in the citric acid cycle. In this context, HIF inhibition promoted by bortezomib could play an important role in shifting from anaerobic to aerobic respiration, thus explaining the increased levels of citrate observed after chemotherapy treatment.

In contrast to findings obtained when comparing the metabolic profiles of multiple myeloma patients at the time of diagnosis with those of controls or multiple myeloma patients after complete remission of the disease, the statistical model obtained from the comparison of the metabolic profiles of multiple myeloma patients after complete remission and healthy individuals showed, as it would be expected, a lower discriminatory capability. This result tend to suggest that the metabolic profile of a multiple myeloma patient gets closer to the metabolic

profile of a healthy subject after having achieved complete remission.

The analysis of the variations in the mean intensities of the metabolites that were identified to play an important role in the discrimination between groups revealed 3 different behaviors (Table 3 and Supplementary Fig. S2). Some of the metabolites that differed between the healthy individuals and multiple myeloma patients at the time of diagnosis showed the same tendency when comparing the variation between multiple myeloma patients at diagnosis and after complete remission (e.g., 3-hydroxybutyrate, arginine, acetate, etc.). This behavior could reflect metabolic changes caused by the disease that are not reversed after achieving complete remission. However, there are other metabolites (e.g., citrate, lactate, etc.) that showed a statistically significant variation when comparing multiple myeloma patients at the time of diagnosis and after achieving complete remission, but not between healthy donors and multiple myeloma patients at the time of diagnosis, perhaps indicating metabolic changes, as suggested earlier, caused by the treatment but not necessarily linked to the response to treatment. Finally, metabolites, such as glutamine, cholesterol, lipids, lysine, etc., displayed a variation in the opposite direction after treatment has been initiated. This suggests that these metabolites could be reflecting the changes in the metabolic profiles of the multiple myeloma patients in response to treatment. Interestingly, these results highlight the potential value of serum metabolic profiling in the identification of specific metabolites that could be used to objectively monitoring treatment efficacies.

Although further validation of the results, using additional patient samples, will be necessary to increase the robustness of this analysis, our data support the idea that multivariate statistical analysis of ¹H-NMR serum metabolic profiles obtained from multiple myeloma patients comprises both subtle and more obvious metabolic differences that could be used for objectively discriminating individuals with and without multiple myeloma, and identifying patients who achieved complete remission. The characterization of a specific metabolic profile associated to multiple myeloma holds great promise in the identification and development of new serum biomarkers for the diagnosis and treatment of this disease. Furthermore, the results associated with the response to treatment are particularly relevant because they could represent a starting point in the individualization of the therapeutic regimes for different patient groups. The fact that we found only few false negatives (88% and 95.7% sensitivity for the 2 sets of comparisons) and almost no false positives (100% and 96%

specificity for both comparisons) suggest that the measurement of serum metabolic profiles could become, if validated in prospective studies based on a much larger patient cohort, an effective screening tool in conjunction with other clinical and molecular parameters. The use of these biomarkers would help to avoid both under- and overtreatment, particularly when evaluating consolidation or maintenance therapies. Finally, a better understanding of the multiple myeloma metabolic profiles could be useful in identifying those cases that will be resistant to specific agents, would avoid repetitive errors and cumulative toxicity, and allow to explore new experimental strategies in these cohorts (35).

From a pharmaceutical point of view, one of the major problems in the therapeutic management of multiple myeloma is the development of resistance to drugs and the occurrence of adverse events. Future studies would likely focus on exploring the identification of biomarkers that could be informative about the prediction of drug toxicity, which is often measured by the presence of aberrant drug-metabolizing enzymes. Such biomarkers could be used to select drug doses and dose regimens, identify the best duration of treatments, as well as potential toxicities and efficacies of several drug candidates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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