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Profiling Amino Acids of Jordanian Scalp Hair as a Tool for Diabetes Mellitus Diagnosis: A Pilot Study

Ayat H. B. Rashaid,[†] Peter de B. Harrington,*[,‡] and Glen P. Jackson[§,∥]

[†]Department of Applied Biological Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan

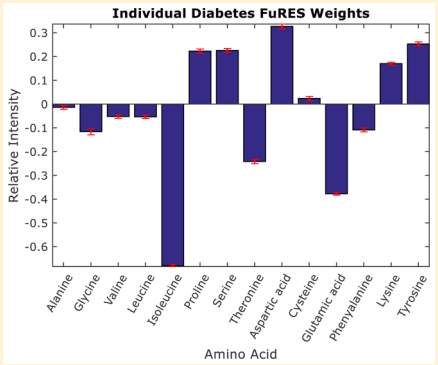
[‡]Center for Intelligent Chemical Instrumentation, Clippinger Laboratories, Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio 45701, United States

[§]Department of Forensic & Investigative Science, West Virginia University, Morgantown, West Virginia 26506, United States

[∥]C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, West Virginia 26506, United States

S *Supporting Information*

ABSTRACT: Hair analysis is an area of increasing interest in the fields of medical and forensic sciences. Human scalp hair has attractive features in clinical studies because hair can be sampled easily and noninvasively from human subjects, and unlike blood and urine samples, it contains a chronological record of medication use. Keratin protein is the major component of scalp hair shaft material and it is composed of 21 amino acids. The method used herein for the amino acid determination in hair included keratin protein acid hydrolysis using 6 M hydrochloric acid (HCl), followed by amino acids derivatization using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and the determination of derivatized amino acids by gas chromatography/mass spectrometry (GC/MS). Amino acid profiles of scalp hair of 27 Jordanian subjects (15 diabetes mellitus (DM) type 2 patients and 12 control subjects) were analyzed. A fuzzy rule-building expert system (FuRES) classified the amino acid profiles into diabetic and control groups based on multivariate analyses of the abundance of 14 amino acids. The sensitivity and specificity were 100% for diabetes detection using leave-one-individual-out cross-validation. The areas under the receiver operative characteristics (ROC) curves were 1.0, which represents a highly sensitive and specific diabetes test. The nonessential amino acids Gly and Glu, and the essential amino acid Ile were more abundant in the scalp hair of diabetic patients compared to the hair of control subjects. The associations between the abundance of amino acids of human hair and health status may have clinical applications in providing diagnostic indicator or predicting other chronic or acute diseases.



Diabetes mellitus (DM) is a major public health problem: there were an estimated 150 million persons with diabetes in the developing world in 2000, and this number is predicted to increase 300 million in 2025.[1] DM is a chronic illness that is diagnosed by a fasting blood glucose greater than or equal to 126 mg/dL.[2] Diabetes mellitus results when pancreatic β-cells are unable to maintain adequate insulin secretion to prevent hyperglycemia or because cells become resistant to insulin.[2,3] There are two main types of DM; type 1 and type 2. Type 1 is characterized by insulin deficiency because pancreatic beta cells fail to produce insulin. Type 2 DM is characterized with insulin resistance, wherein target cells fail to use or respond to insulin properly. In the case of gestational diabetes, pregnant women without a previous diagnosis of diabetes develop high blood glucose levels and the symptoms are similar to type 2 DM.[2−4]

The major practical advantage of hair analysis is that the detection window, days to years, depending on the length of the shaft hair, is considerably longer than those of urine and blood (i.e., hours to 2−4 days).[5] Moreover, hair collection is noninvasive, can be witnessed first-hand, and can be stored for long times at room temperature, and there is no need for special apparatus or training for sampling human hair.[5,6] Hair samples contain longer-term histories of diseases and medications compared with other biological samples. Therefore, hair could be a potential sample for clinical testing and convenient diagnostic marker of diseases. Considering that the collection of blood specimens from elderly patients, acutely ill or injured, and newborn babies may be difficult, invasive, or painful, the development of analytical methods using hair samples has many advantages.

There are several studies concerned with the relationship between the amino acid content of blood and diabetes. Anuradha[4a] reviewed the relationships between insulin secretion and sensitivity with respect to amino acid content (e.g., via exogenous sources) of plasma. In general, insulin decreases the breakdown of protein; thus a lack of insulin increases the circulating amino acids and as a consequence

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putatively increases the concentration of some amino acids in hair.

Insulin also helps cells to absorb the circulating amino acids from the blood. Therefore, a lack of insulin or resistance to insulin inhibits amino acid absorption.[4a] For example, significant increases in threonine (Thr), alanine (Ala), and isoleucine (Ile) in plasma have been reported in cases of type 2 DM. For one study, a significant increase in threonine (Thr), alanine (Ala), and isoleucine (Ile) were reported in type 2 DM.[4a] In other studies, exogenous amino acids are able to enhance insulin secretion of insulin from pancreatic cells. For example, Van loon et al.[7] reported that plasma insulin concentrations increase significantly for type 2 DM patients and controls after oral intake of a mixture of leucine (Leu) and phenylalanine (Phe) amino acids. Intravenous administration of a mixture of 10 essential amino acids: arginine (Arg), lysine (Lys), Phe, Leu, methionine (Met), valine (Val), histidine (His), Ile, Thr, and tryptophan (Trp) increased the insulin concentration in plasma.[8] Also, key amino acids such as Ala, glutamine (Gln), Leu, Ile, and Arg were reported to stimulate β-cell insulin secretion.[9] This mechanism of stimulation is complex and involves mitochondrial metabolism.

Amino acid profiling could provide a significant indicator for high-risk individuals who have type 2 DM. For example, Wang et al. concluded that raised levels of five branched-chain and aromatic amino acids: Ile, Leu, Val, Phe, and tyrosine (Tyr) amino acids in the blood could be an indicator for the development of diabetes later in life.[10]

Hair may contain possible biomarkers for DM and an alternative sample to blood and urine, which are the routine samples of choice for the analysis of biological specimens. Hair consists of two main parts: the hair root, which is embedded in the cavity known as the hair follicle, and hair shaft, which is the visible part of hair projecting from the skin surface.[11] Human scalp hair grows at a rate of approximately 0.4 mm/day, or 1 cm/month.[11,12] Human hair comprises approximately 80% keratin protein[13] and the remaining constituents are water (6%), lipids(1%−9%), pigment (less than 3%), and trace elements (less than 1%).[11] Amino acids are the building blocks of keratin of hair, which is composed of 21 amino acids.[11,14] Because the hair follicle uses circulating blood as the source of amino acids, hair growth can be influenced by the concentrations or isotope ratios of amino acids in blood.[12,14]

There are relatively few studies associating diabetes and the amino acid composition of human scalp hair. For example, Oimomi et al. found fructose-lysine (which is formed by binding glucose to lysine) is significantly higher in the hair of diabetic patients than in control subjects.[15] Mogos et al. compared scalp hair samples of control subjects to scalp hair samples of diabetic patients based on the content of 12 amino acids, that is, cysteine (Cys), cystine (Cyt), lysine (Lys), Thr, serine (Ser), Ala, Tyr, Val, Leu, Ile, aspartic acid (Asp), and glutamic acid (Glu).[16] They found higher levels of all amino acids in the diabetic hair than the hair of control subjects except for Cyt.

Fourteen amino acids, Ala, Gly, Val, Leu, Ile, Pro, Ser, Thr, Asp, Cys, Glu, Phe, Lys, and Tyr, were profiled in this preliminary study. The method used here was previously developed.[17] Other amino acids were not studied because they are either destroyed during the acid hydrolysis step (Trp, Cyt, and His) or undergo oxidation (Met). Gln and Asn are typically deamidated to Asp and Glu, respectively, under acid hydrolysis conditions. Also, Arg decomposes to ornithine during silylation, which can yield inaccurate values in the analysis of free amino acids in extracts of biological fluids, as well as cell and tissue extracts.[18] These known issues were taken into account in the present study.

This case-control study used a method of analysis for classifying diabetic patients from control subjects based on measuring the concentrations of 14 amino acids of the scalp hair shaft. The strengths of this pilot study stem from the case-control design, which matches the average age and sex for both groups. That three separate digestions and derivatization steps were completed on different days for each subject also provides confidence in the robustness of the method and data analysis.

In this preliminary study, the scalp hair samples from 27 subjects were collected from the mountain region of Jordan. The analysis included keratin protein acid hydrolysis and the determination of derivatized amino acids by gas chromatography/mass spectrometry (GC/MS). The proposed method of analysis could be quite useful for screening biomarkers of other diseases that influence metabolism and amino acid concentrations in blood.

EXPERIMENTAL SECTION

Sampling and Data Collection. This study was approved by Ohio University institution review board (IRB no. 12X029) and King Abdullah University Hospital institution review board (IRB no. 10/215/2444). The 27 participating subjects were selected randomly from the mountain area in Jordan. The subjects were identified from the database of the General Civil Status Department. This Department is a national bureau that stores the personal information from all Jordanians. A case-control study was conducted by collecting scalp hair samples from 15 type 2 diabetic patients and a control group of 12 subjects who reported no lifetime history of type 2 DM. The subjects of both groups (case and control) were matched by age and sex.

During the scheduled home visits, written informed consent was obtained from all cases and controls before participating in the study. The subjects completed extensive questionnaires about their demographic information (age, sex, and nationality), medical history, and dietary habits. For long hair, a 1 cm diameter lock of long hair adjacent to the scalp was cut from the posterior vertex region using surgical scissors. For subjects with shorter hair, a 2−3 cm diameter lock of short hair was cut adjacent to the scalp using clippers. Each hair sample was placed in a sealed plastic baggie labeled with the subject's identification number. Hair samples used in this study were reported to have no dye treatments, bleaching, or any other chemical treatment besides conditioners. Also, none of the subjects consumed alcoholic beverages or smoked tobacco.

Reagents and Supplies. The use of an internal standard is required to correct any variation during hydrolysis, derivatization, and analysis. Norvaline (Nor) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as an internal standard because it is an exogenous amino acid that is not synthesized by the human body. The derivatizing reagent was *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco Analytical, Bellefonte, PA, USA). Hydrochloric acid (HCl) was used as a hydrolysis agent to liberate free amino acids from human hair keratin. Acetonitrile, methanol, acetone, and chloroform were purchased from GPS Chemicals (Columbus, OH, USA).

This study used 4 mL glass vials with a phenolic rubber lined cap (Qorpak, Bridgeville, PA, USA). Solutions were filtered

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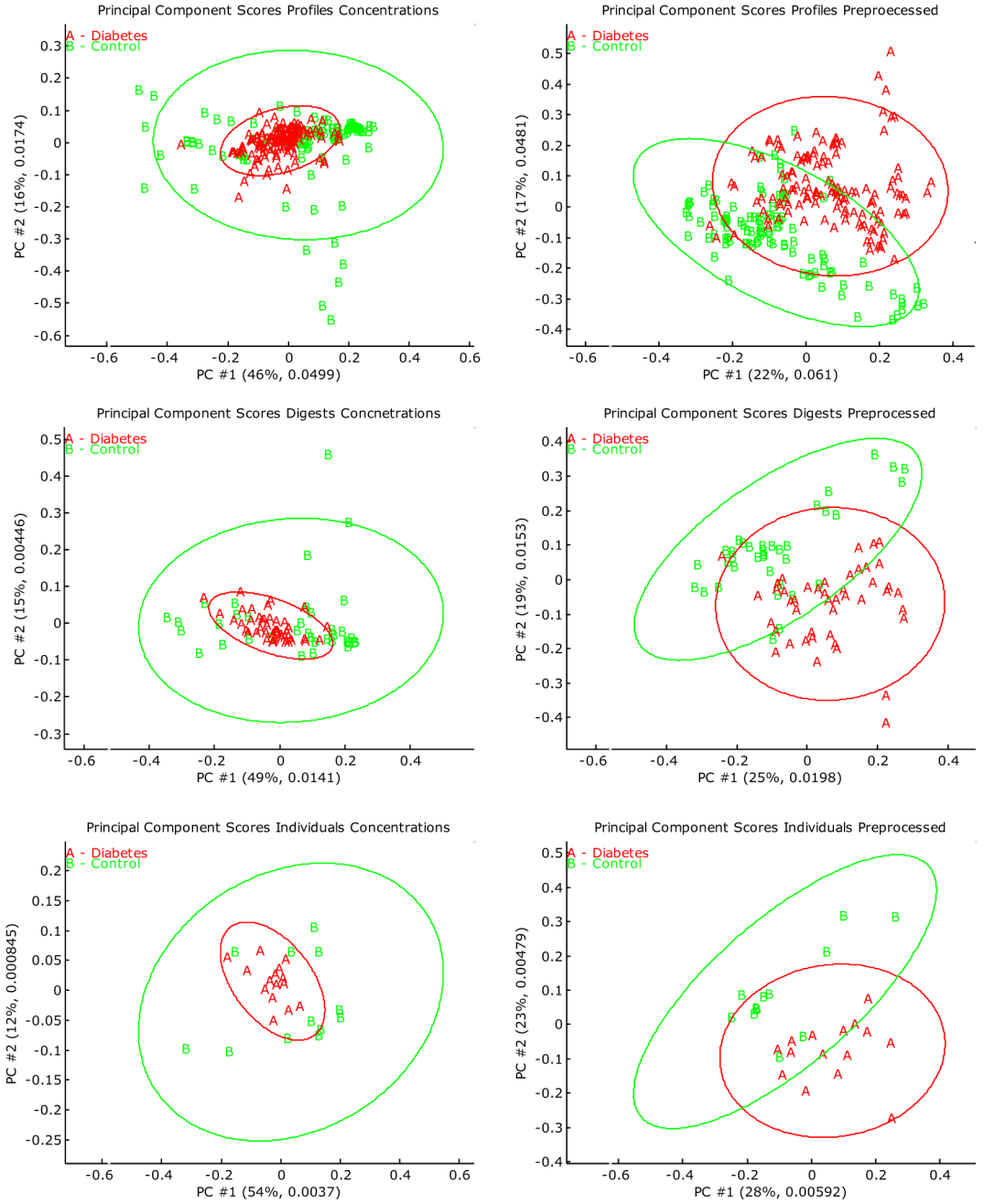

Figure 1. Left column presents principal component scores of the unprocessed amino acid concentrations, while the right column present the scores after scaling by the pooled replicate standard deviation and normalization of the objects to unit vector length. The top corresponds to the 243 amino acid profiles, the middle to the 81 digests, and the bottom to the 27 individual subjects.

through a 13 mm × 0.45 μm, polyvinylidene difluouride (PVDF) filter (Bonna-Agela Technologies, Wilmington, DE, USA). The nitrogen generator was purchased from Parker Hannifin Corporation (Haverhill, MA, USA). Hair samples were washed, prepared, and analyzed in a similar procedure to other reports.[17,19]

Hair Sample Preparation. Each hair specimen was rinsed two times in deionized water and was vortexed in a 1:1:1 (by volume) solution of chloroform, methanol, and acetone for 1 min to remove surface contamination of oils and lipids.[19] The hair was then dried under a constant stream of 4 L/min nitrogen to remove excess solvent. The clean, dry long hair was then cut into 1 cm lengths from the proximal ends (i.e., close to the scalp) and distal ends and physically pulverized and homogenized by placing ∼20 mg of hair into a capped plastic vial with 8 stainless steel beads for 1 min using a mini bead beater (BioSpec Products, Inc., Bartlesville, OK, USA) at 4800 rpm. A 3 mg portion of fine powder was transferred into a 4 mL glass vial for hydrolysis.

Hair Sample Analysis. The hair powder of each sample was separated into three precisely weighed subsamples (3.00 ± 0.03 mg [SD]), and placed in 4 mL glass vials with phenolic-rubber-lined caps. Thereafter, 0.30 mg of Nor was added. Each subsample was hydrolyzed by adding 0.3 mL of 6 M HCl to the vial and tightly capping them. To heat the samples, an aluminum block with holes to accommodate the glass vials was mounted on a hot-plate for 24 h at 110 °C.[18b,c,20] After the solution was cooled to room temperature for 5 min, the

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solutions were filtered using 0.45 μm PVDF filters to remove undigested melanins and dried under a constant stream of 4 L/min nitrogen. Hair samples from an individual underwent three replicate digestions on different days.

N,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is a silylating reagent that replaces acidic protons of the amino acids (e.g., SH, OH, NH, and COOH) with a nonpolar trimethylsilane group. By replacing the acidic protons of the amino acids, the polarity is reduced and the volatility is increased.[21] The dried amino acid residues were dissolved in a 100-μL aliquot of acetonitrile and derivatized with the addition of a 100-μL aliquot of BSTFA. The vials were tightly capped, ultrasonicated for 1 min, and mounted on a hot-plate for 30 min at 100 °C. After cooling the solution at room temperature for 10 min, a 100-μL aliquot of the solution was transferred into a new vial for GC/MS analysis.

GC/MS Analysis. Amino acid derivatives were analyzed with a Shimadzu GC/MS instrument (QP-2010SE, Scientific Instrument, Inc. Columbia, MA, USA) using a previously validated method. Amino acids were separated on a 5% diphenyl-dimethylpolysiloxane capillary column (SHRX1−5MS, 30 m × 0.25 mm × 0.25 μm). Ultrahigh-purity helium was used as a carrier gas at a constant flow rate of 1.0 mL/min. The temperature of the column was programmed to rise from 70 to 170 °C at a rate of 10 °C/min, and then was ramped to 280 °C at a rate of 30 °C/min, at which it was held for 3 min. The total run time was 16.6 min.

Sample injection was performed in split injection mode (1:20 ratio) at 280 °C using an injection volume of 1 μL. Triplicate injections of triplicate samples (n = 9) were made with a random block design using acetonitrile as a solvent blank before each injection. The random block design was used to collect triplicate measurements of each hair digestion with each block of replicates separated by 2 days. Three solvent vials of acetonitrile, methanol, and acetone were used sequentially as cleaning solvents for the autosampler injection syringe.

The mass spectrometer was operated in fast automated scan and selected-ion-monitoring type (FASST) mode, which switches back and forth between full scan mode and selected ion monitoring (SIM) at a rate of 2 Hz during a single analysis. In full scan mode, the mass analyzer scanned from m/z of 50 to m/z of 500 with a scan time of 0.3 s. For SIM, the analyzer scanned three selected ions with a scan time of 0.2 s. The target ions for quantitation were established previously, and were typically the fragment ions of the highest intensity (base peak) for each amino acid. As is typical, two additional fragments of the next greatest intensities were used to support the qualitative assignment for each amino acid.[22] The target and reference ions used for the integration of peak areas were reported in previous work.[17a,22]

Statistical Data Analysis. Statistical analyses were performed using MATLAB R2012b or R2014a (The MathWorks Inc., Natick, MA). All statistical tests were conducted at the 95% confidence level. Amino acids in the hair protein were determined using the relative peak areas (i.e., referenced to the Nor internal standard). Concentrations (w/w) were obtained from external calibration curves obtained from liquid injections of standard amino acid solutions.

The fuzzy rule-building expert system (FuRES) constructs classification models of amino acid profiles that are amenable to interpretation. This system builds a classification tree, which is a sequence of multivariate rules used to separate the profiles into their appropriate classes. The branches of the classification tree comprise rules in the form of linear discriminants. The rules are obtained by minimizing the fuzzy entropy H of classification.[23]

The receiver operating characteristic curve (ROC) displays the true positive detection rate (i.e., sensitivity) with respect to the false positive detection rate (1 − specificity) with respect to the change in classification threshold. The area under the curve (AUC) is a measure of the classifiers performance with an AUC of 1 as an ideal result and AUC below 0.5 a random result.[24]

One-way analysis of variance (ANOVA) was used to compare the mean concentrations of each amino acid in the diseased and the nondiseased groups of subjects, and to find which amino acids have the largest ratio between between-individual and within-individual variation.[25] These results are reported in the Supporting Information.

RESULTS AND DISCUSSION

Details regarding the participant characteristics, the detected amino acid profile of scalp hair, the relative precision of the amino acid profiles, and the analysis of variance of the amino acid concentrations can all be found in the Supporting Information.

There is inherent variance in the digestion and GC/MS analysis of the hair samples, so the concentration profiles were subjected to principal component analysis to characterize this variation. The pooled standard deviations of the amino acids with respect to each individual (i.e., 9 profiles) were calculated to scale the amino acids by their variability. The amino acid concentrations were scaled by the pooled standard deviations. Then each profile was normalized to unit vector length.

Figure 1 gives the principal component scores for three levels of the data, 243 amino acid profiles, 81 digestions, and 27 individuals before and after preprocessing. Three evaluations were run classifying the profiles, the average of the three profiles for each digest, and the average of nine profiles for each individual.

FuRES Classification. FuRES was used to classify the subjects according to their diabetes status based on multivariate analyses of the preprocessed 14 amino acids. The same preprocessing that was used for the PCA was also used for the classification studies. Averaged profiles were calculated from the scaled profiles and then were normalized.

Three models of FuRES were used: the profiles without averaging (n = 9 for each individual), the average of the three profiles obtained from the same digestion (average across digestions, n = 3 for each individual), and the average of all 9 profiles for each individual (average across individuals, n = 1 for each individual). The classification rates of diabetes after leave-one-profile-out, leave-one-digestion-out, and leave-one-individual-out cross-validations are reported in Table 1.

The performance of a diagnostic test is evaluated by sensitivity, the number of proportion of correct detections,

Table 1. FuRES Classification Rates of Diabetes, Sensitivity, and Specificity of the Three Modes of Evaluation

	validation		
factor	profiles (n = 243)	digestions (n = 81)	individuals (n = 27)
classification accuracy	96%	94%	100%
sensitivity	0.97	0.96	1.00
specificity	0.94	0.92	1.00

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and specificity, the proportion of correct nondetections. Sensitivity and specificity can be displayed graphically using a technique known as the receiver operating characteristic (ROC) curve.[24] They are given in Figures 3 and 5.

A FuRES classification tree is the collection of rules used to separate all the samples. First, the classification was modeled using the 243 preprocessed amino acid profiles. The diabetic group is classified from the control group with a 7-rule tree (Figure 2). The classification rate for the model was 96% after the leave-one-profile-out validation. Note that profiles from the same digestion were measured several days apart.

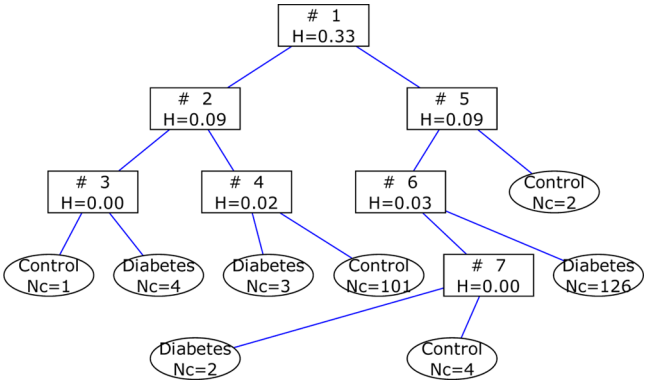

Figure 2. FuRES diabetes classification tree from the 243 preprocessed amino acid profiles. Nc is the number of subjects and the class is given in each leaf node. *H* is the entropy of the classification rule and the number of rules required to build the tree are given in the rectangle. There is no splitting of the subjects among the circular leaf nodes.

Sensitivity, or the true-positive rate, is plotted on the ordinate as a function of 1 − specificity (the false-positive rate) on the abscissa. Performance is measured by the area under the ROC curve. Figure 3 is the ROC curve for identifying type 2 DM using the 243 amino acid profiles. The sensitivity of the evaluation is 0.97, while the specificity is 0.94 (Table 1). The areas under the two curves are both 0.99, which represents a highly sensitive and specific classification. The ROC curves are consistent with the FuRES diabetes prediction rate of 96%. FuRES assigns the class to the profile with the largest response or output, while the ROC curve displays the relationships of the true positive rate with respect to the false positive rate with respect to the classification threshold. Therefore, if the classification threshold is carefully adjusted for each evaluation then the prediction rates may improve to the values given by the ROC curves.

Using an alternative approach, the average of the three GC/MS profiles of each subsample (digestion) was used as a basis for classification. Figure 4 gives the resulting three-rule classification tree built from the 81 preprocessed profiles. The 81 FuRES models were built from the profiles of the 14 amino acids and the 80 digestions using a leave-one-digestion-out validation. The prediction rate for this evaluation was 94%.

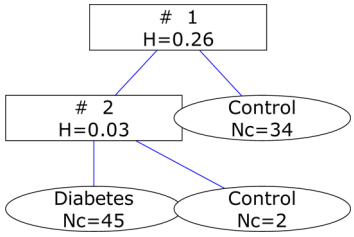

Figure 4. FuRES diabetes classification tree for type 2 diabetes from the average amino acid profiles of hair for 81 digestion (n = 3 for each digestion). Nc is the number of subjects, and the name of each group is given in each leaf node. *H* is the entropy of the classification rule, and the number of rules required to build the tree are given in the rectangle.

The ROC curve was used to display these prediction results. The sensitivity of the model was 0.96, while the specificity was 0.92 (Table 1). The area under the curve is 0.98 (Figure 5) that typifies an effective classification model that discriminates diabetic patients from the control subjects and is consistent with the leave-one-digestion-out prediction rate of 94%.

Finally, the average of all nine measurements for each individual was calculated and used as the basis for classification

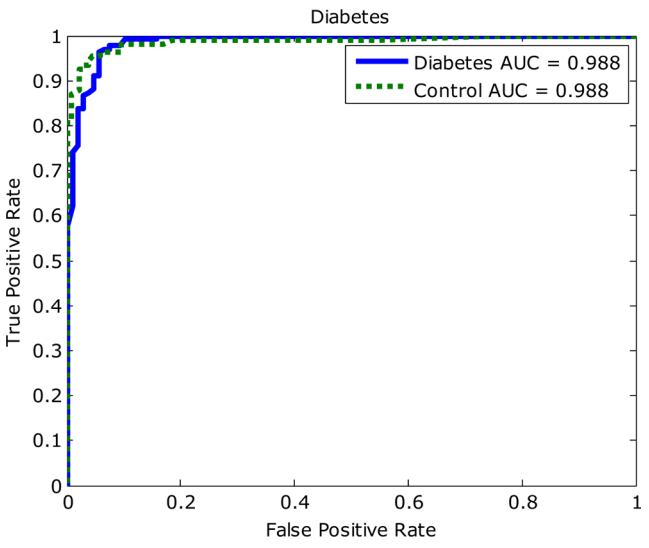

Figure 3. ROC curves for the leave-one-profile-out cross-validation for the FuRES type 2 DM and control classes. AUC is the area under the curve.

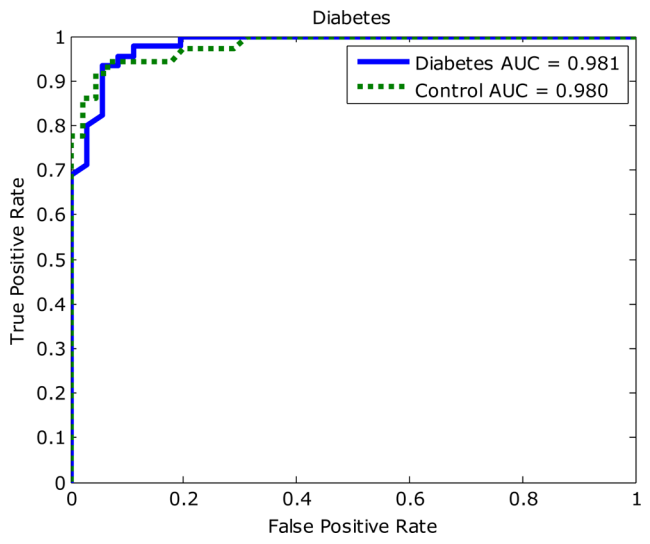

Figure 5. Receiver operating characteristic curves digestions (n = 3 profiles) obtained from the FuRES classes from the leave-one-digestion-out validation.

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of the two groups using FuRES. The results show that the diabetic group can be classified from the control group using a tree comprising a single rule. The leave-one-individual-out cross-validation rate is 100% (Figure 6).

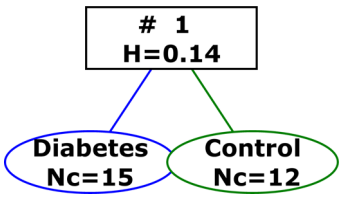

Figure 6. FuRES diabetes classification tree from the preprocessed 14 amino acids of the hair of 27 subjects (n = 1 for each individual). Nc is the number of subjects, and the name of each group is given in each leaf node. H is the entropy of the classification rule, and the number of rules required to build the tree are given in the rectangle.

The corresponding ROC curve gave sensitivities and the specificities that were each 1.00 (Table 1), so the area under this ROC curve also was 1.00. Thus, when the amino profiles were averaged for each individual subject a highly sensitive and specific test was achieved for type 2 DM, although the population in the study was relatively small compared to large-scale clinical studies. Note that during the validation the same individual's profiles were never used for model building and prediction, so this model generalized across the individuals.

On the basis of the prediction results of FuRES from the three data sets, we could properly classify the diabetic patients from the control subjects with greater than 94% prediction rate using the average of one, three, or nine replicate GC/MS analyses for each subject. In addition to classification, the FuRES loadings of the amino acid profiles provide further insight into the relationship between amino acid profile and disease state.

FuRES Variable Loadings. The average variable loadings obtained from 27 FuRES models for the 14 amino acids of diabetes classification using the leave-one-individual-out cross-validation is presented in Figure 7. Average variable loadings of the 14 amino acids of diabetes classification with 95% confidence intervals. The positive peaks correspond to amino acids that are loaded more strongly for the control group relative to the diabetic group, and they include the amino acids Pro, Ser, Asp, Lys, and Tyr. In contrast, the negatively weighted peaks Ile, Thr, and Glu, in Figure 7 correspond to amino acids that are more heavily loaded for the diabetic subjects relative to the control subjects. The 95% confidence intervals provide a measure of the precision of the amino acid profile.

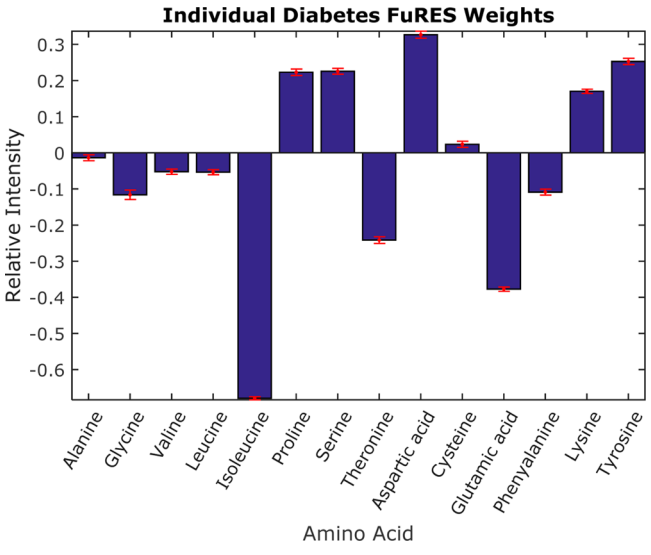

Figure 7. Average variable loadings of the 14 amino acids of diabetes classification with 95% confidence intervals.

CONCLUSIONS

The FuRES classification rates using leave-one-out validation were 96%, 94%, and 100% using the 243 amino acid profiles, the 81 averaged profiles for each digestion, and the average of the nine profiles for the 27 individual subjects, respectively. Note that the amino acid profiles from the same individual and digestion were collected several days apart. The hair samples from an individual were digested 3 times and were digested 3 days or further apart, so this study shows that the robustness for diagnosing diabetes using amino acid profiles in hair. In addition to FuRES prediction results, the area under the ROC curve exceeded 0.95 for each of the three validations, which further demonstrated the detection of diabetes from hair samples. These results suggest that GC/MS combined with FuRES provides a powerful method for the classification of type 2 DM. These preliminary results suggest that amino acid profiles in hair could assist in diabetes risk assessment. A large scale clinical study would help validate these results. The key issue is to improve the digestion of the hair proteins to improve the reproducibility of the amino acid profiles. Moreover, intraindividual biological variability of amino acids composition should be studied by collecting different hair samples of same subject from different proximal head sites.

The outcomes, as well as other previous studies, provide good evidence for the feasibility of the abundance of amino acids to distinguish between the hairs of control individuals and type 2 diabetic patients. Further studies have to be carried out first to decrease the hydrolysis time and second to develop a method that could enable analysis of more than 14 amino acids or include more orthogonal variables to assist the classification. Other studies of large number of subjects from different geographical origins are in need to evaluate the ability of amino acid profiles to predict type 2 DM of different stages for a global population. In addition, fasting blood of the same subjects could be collected to investigate the correlation between the amino acid concentrations in blood and hair with the diagnosis history (i.e., onset of diabetes).

ASSOCIATED CONTENT

Supporting Information

Participant characteristics, amino acid profile of the scalp hair, precision of GC/MS analysis of amino acids of the same subject, and analysis of variance (ANOVA). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00460.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 740 994 0265. Fax: +1 740 593 0148. Email: peter.harrington@ohio.edu.

Notes

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The authors declare no competing financial interest.

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