An objective means of diagnosing anorexia nervosa and bulimia nervosa using $^{15}$N/$^{14}$N and $^{13}$C/$^{12}$C ratios in hair

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An objective means based on the carbon and nitrogen stable isotope analysis of five hairs per individual is presented for distinguishing between individuals with anorexia nervosa and/or bulimia nervosa from non-clinical individuals (i.e. clinically normal controls). Using discriminant analysis, an algorithm has been developed that provides both sensitivity and specificity of 80% in making diagnoses of individuals with these eating disorders. With further refinements, the results suggest that it may be also possible to distinguish between individuals with anorexia or bulimia. Finally, the study shows the value of conducting blind tests and using larger sample sizes of both control and treatment groups. Both groups are needed to validate the diagnostic value of a method and to provide measures of sensitivity and specificity of any diagnostic test. Copyright © 2006 John Wiley & Sons, Ltd.

Conservative estimates suggest that as many as 1–5% of all high school and college-age women in the United States, as well as increasing numbers throughout the developed world, are affected in some way by pathological dietary practices.1,2 The two most prevalent forms of disordered eating are anorexia nervosa, characterized by an extreme fear of weight gain and a severe concomitant restriction of calories, and bulimia nervosa, manifested in excessive binging and purging cycles.3–5

Common to both anorexia and bulimia is the element of denial. Those who suffer from these illnesses are often unwilling or unable to recognize and admit to the psychopathology of their behaviors.3–5 This problem raises specific concerns and challenges for researchers and medical professionals seeking to obtain accurate dietary information for diagnostic and evaluative purposes when working with eating disorder patients. In most cases, doctors and researchers must rely on potentially unreliable self-reported data.3–5 This problem underscores the need for and the usefulness of a clinical test to determine diet and condition independent of subjective criteria and self-reporting, both to accurately diagnose and to effectively treat eating disorder patients.

The measurement of stable isotope ratios in hair has the potential to provide a unique method for objectively diagnosing individuals with eating disorders.8 When the body is in an anabolic state, it incorporates dietary proteins into growing hair. Although the mechanism is unclear, it appears that $^{14}$N is excreted by the organism at a slightly higher rate than $^{15}$N. As a result, $^{15}$N concentrates in body tissues9 (but see also Ref. 10). Thus, as one moves up the food chain, $\delta^{15}$N increases on average at 3% with each step or trophic level11 (but see also Ref. 12). Because of these trophic level effects,11–15 the $^{15}$N/$^{14}$N ratios of herbivores (or vegetarians and vegans) are less than those of predators (or humans who eat more meat). Likewise, when the body becomes catabolic, the $^{15}$N/$^{14}$N ratio of the individual’s tissues increases further due to something functionally similar to an increase in trophic level.12,16,17 As the individual loses weight, the individual’s body consumes its own energy and protein stores. The remaining proteins are $^{15}$N enriched and their amino acids are recycled. Some of these amino acids are incorporated into new hair growth, providing a signal of the change in diet.8,12,13,18,19 Research has long suggested that the progression of anorexic pathology is accompanied by changing patterns in dietary habits.20–22 These patterns include periods of low or no carbohydrate intake and an avoidance of dietary fats.20 They can also include patterns in which the primary foods consumed are fruits and vegetables. During this period, meat is often avoided.21 In one study of anorexic women, for example, 47% of the participants reported they had become vegetarians.23
Changes in relative amounts of heavy to light isotopes in the hair indicate changes in the body’s metabolic state and dietary intake. Since hair is produced sequentially and is inert after production by the body, the serial sections of hair record these changes over time. The stable isotope ratios should change over time as the eating disorder progresses and the individual eliminates animal products from the diet, thereby reducing the amount of protein in the diet. The older segments of hair would have higher δ¹⁵N values than newer segments. Finally, the newest segments of hair would show an increase in δ¹⁵N values in individuals who have become highly catabolic, because they are eating a high fiber diet providing little protein, they are losing weight rapidly, and they are metabolizing their own proteins for energy. Since the stable isotope ratios of hairs of normal individuals should follow no such pattern, we predicted that it would be possible to distinguish individuals with anorexia or bulimia (or both) from normal individuals (non-clinical controls) based on stable isotopic analysis of the hairs alone.

The focus of much stable isotope research, to date, has been to reconstruct dietary histories. Much of this work has been done by anthropologists, paleontologists, and archaeologists who initially focused on bone collagen. Nitrogen was shown to correlate well with diet, but to be approximately 3‰ more positive than the diet. Carbon in well-preserved hairs was also found to correlate well with diet. Stable isotope ratios in hair were found to closely reflect the diet of living humans. Since the δ¹⁵N values of hairs of normal individuals should follow no such pattern, we predicted that it would be possible to distinguish individuals with anorexia or bulimia (or both) from normal individuals (non-clinical controls) based on stable isotopic analysis of the hairs alone.

Surprisingly, it was not until the late 1990s that researchers provided experimental evidence suggesting hair as a superior material for establishing the dietary history of archeological humans. Meanwhile, because of its accessibility and because its sampling was not invasive, the usefulness of hair in understanding the dietary habits of living humans was also explored. Stable isotope ratios in hair were found to closely reflect the diet of living humans, and to be able to indicate a diet change in 6–12 days. Shampooing and graying were shown not to affect the stable isotopic ratios in hair, but dying and bleaching may affect them. Studies of living humans also provided solid evidence that increasing the proportion of meat in a diet increases the δ¹⁵N of the hairs of the individual.

Several studies demonstrated the potential that stable isotopic analysis has for providing information on an individual’s dietary history as well as her/his body condition over time. By using stable nitrogen isotopic ratios, researchers have been able to distinguish people eating diets containing animal protein (such as meat, eggs, and milk) from people eating vegan diets. Those individuals eating diets higher in animal proteins are naturally labeled with elevated ratios of ¹⁵N/¹⁴N. Animals that go through periods of fasting have also been shown to have tissues enriched in ¹⁵N as a result. Other studies indicated that the stable isotopic ratio analysis of hair serial sections can determine anabolic and catabolic states in humans. Nevertheless, few attempts have been made to apply stable isotopic analysis of hairs to the diagnosis and treatment of medical conditions in humans. The use of stable isotopic analysis of hairs has been explored to detect the use of illegal drugs, to determine adequate zinc intake in infants, to determine the diet of elderly people with senile dementia, and to understand the effects of pregnancy. A study of Ross’s geese first suggested that stable isotopic analyses might be used to indicate nutritional stress (but see also Ref. 49). Fuller et al. were the first to show that nutritional stress, by affecting the nitrogen balance in pregnant women, can change the δ¹⁵N ratios in hairs, although not the δ¹³C ratios. Most recently, Meek et al. presented evidence that the dietary changes of women with eating disorders are recorded in the δ¹⁵N and δ¹³C of the hairs of these women.

While several studies have demonstrated that nitrogen isotope ratios of hairs in humans are influenced by diet and nutritional stress, they were not designed to demonstrate how to apply stable isotopic analysis of hairs to the diagnosis or treatment of these conditions.

The goal of our study was to determine if stable isotopic analysis of hairs from a sample of young adult women could provide an objective measure of whether or not they have an eating disorder. The δ¹³C and δ¹⁵N values were measured of the hairs of non-clinical volunteers (i.e. clinically normal controls) and individuals diagnosed with anorexia nervosa, bulimia, or both anorexia and bulimia. Individuals diagnosed with eating disorders had had these eating disorders for an average of 4.6 years (standard deviation (SD) = 3.1 years) before the study and first sought treatment after, on average, 35.5 months (SD = 29.2 months). A discriminant analysis of the δ¹³C and δ¹⁵N values of the hairs was then conducted to determine how successfully these data could be used to separate individuals with eating disorders from those without. Simple variables were used that avoided any need for a subjective evaluation of the hairs. Our results strongly suggest that stable isotopic analysis of hairs can provide an effective, objective diagnosis of whether an individual has either or both anorexia and bulimia or whether that individual is clinically normal.

**EXPERIMENTAL**

**Sample collection**

Hair samples were collected from two groups of female subjects: inpatients diagnosed as having anorexia, anorexia and bulimia, or bulimia (20 individuals), and non-clinical volunteers (23 individuals). The treatment group consisted of inpatients at an eating disorder treatment facility (The Center for Change, Orem, UT, USA). All patients were diagnosed by therapists at the facility as having either anorexia nervosa, bulimia nervosa, or both anorexia and bulimia, based on the criteria established in the Diagnostic and Statistical Manual for Mental Disorders, fourth edition (DSM-IV). While the diagnoses were made based on the factors listed in the description of anorexia and bulimia in the DSM-IV, we were not privy to these data, including the weight and height of the individuals. The non-clinical volunteers were recruited from communication classes at Brigham Young University. Each subject filled out a survey indicating dietary habits and eating disorder history, and then pulled five hairs from her own head. The hairs were wrapped in aluminum foil for later stable isotopic analysis. The patients were asked to pull rather than to cut hairs because cutting hairs can cause loss of
Diagnosing eating disorders using stable isotopes in hair

might be useful in distinguishing between anorexic, bulimic, and non-clinical individuals. The variables considered were as follows:

1. The average $\delta^{15}N$ values of the whole length of hair;
2. The average $\delta^{15}N$ values of the length of hair grown before the individual entered treatment (subjects had had the eating disorder for an average of 35.5 months, with a standard deviation of 29 months, before seeking treatment);
3. The average $\delta^{15}N$ values of the length of hair grown by the individual after entering treatment. (Since all the subjects were inpatients at The Center for Change, staff in the center were able to monitor them and to ensure they were getting adequate nutrition.);
4. The $\delta^{15}N$ values of the oldest (most distal) section of hair;
5. The $\delta^{15}N$ values of the youngest (most proximal) section of hair;
6. The difference in $\delta^{15}N$ values between the oldest and youngest sections of hair (i.e. $\Delta^{15}N$);
7. The maximum and minimum $\delta^{15}N$ values found along the length of the hair;
8. The slopes of the $\delta^{15}N$ values of hair sampled before and after the subjects entered treatment; and
9. The standard deviation of the $\delta^{15}N$ values along an individual’s hair.

The same variables were considered for carbon. These values were regressed against the following factors: months since eating disorder was diagnosed, months in treatment, months working with a nutritionist, age, how frequently the individual ate meat, and how frequently the individual ate less than 1200 calories per day. The F-to-enter was 4.00 and the F-to-remove was 3.99. The variables that were predictive of any one of these factors were included in the stepwise discriminant analysis.

The ability of the equations developed by discriminant analysis to correctly categorize individuals as either having an eating disorder or as being clinically normal was tested. The binomial distribution of the normal approximation of the lower 95% confidence intervals (CIs) for both sensitivity and specificity of these results was calculated. Only the lower 95% CI was used, since we were primarily concerned with the degree to which a test might perform worse than, not better than, the predicted outcome.

ANOVA (type III sums of squares) was used to determine which of the above variables appeared to be useful in distinguishing between individuals with anorexia, bulimia, both anorexia and bulimia, and non-clinical volunteers. Post hoc analyses were carried out using Fisher’s probable least-squares difference (PLSD, $p < 0.05$). Variables that appeared useful in this regard were also included in the stepwise discriminant analysis.

RESULTS

ANOVA showed that several factors could be useful in distinguishing between non-clinical individuals, individuals with eating disorders and between different eating disorders. When the $\delta^{15}N$ values of the oldest sections of hair were compared with those of the youngest sections of hairs for all

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four groups (anorexia, bulimia, both anorexia and bulimia, and normal volunteers), the differences were significant ($F_{3,39} = 5.688, p = 0.0025$). Patients who described themselves as anorexic showed the greatest difference from the controls. Patients with both anorexia and bulimia showed less difference. There was no significant difference between bulimic patients and controls or between anorexic patients and patients with both anorexia and bulimia. However, anorexic patients were significantly different from bulimic patients (Fig. 1).

The youngest hair growth of patients with either anorexia or both anorexia and bulimia had significantly lower $\delta^{15}N$ values than non-clinical controls while there was no difference between individuals with bulimia and the non-clinical controls ($F_{3,39} = 4.916, p = 0.005$; Fig. 2). However, there was no statistical difference between the mean $\delta^{15}N$ values of the oldest segments of hair (the 1 cm nearest the tip of the hair) of any of the groups. Finally, the degree of variance in $\delta^{15}N$ along the length of an individual’s hair was examined and compared with the hair variance between the groups. Here there was weak evidence ($F_{3,38} = 2.647, p = 0.0624$) that the hairs of individuals with anorexia were more variable in $\delta^{15}N$ along their length than were those either from individuals with bulimia or from the normal volunteers and that individuals with both anorexia and bulimia showed a similar trend (Fig. 3).

Stepwise (forward) multiple regression analyses showed that the $\delta^{15}N$ values of the newest sections of hair were correlated with an individual’s age ($t_{40} = 2.124, p < 0.04$) and with the amount of meat that an individual consumed each day ($t_{40} = 4.341, p < 0.0001$; $R^2 = 0.371$). The standard deviation of the $\delta^{15}N$ values along the length of the hair was found to correlate with how frequently an individual ate less than 1200 calories per day ($t_{41} = 2.803, p = 0.008; R^2 = 0.161$). The difference between the $\delta^{15}N$ values of the oldest and the youngest sections of hair (distal and proximal ends of the hair) was found to correlate both with the frequency of eating less than 1200 calories per day ($t_{40} = 2.185, p = 0.035$) and with the frequency of eating meat ($t_{40} = 1.872, p = 0.07; R^2 = 0.56$).

The $\delta^{13}C$ values of the hairs appeared to be less useful in distinguishing among individuals with eating disorders and the non-clinical controls. Nevertheless, the $\delta^{13}C$ of the oldest—the $\delta^{13}C$ of the youngest 1 cm sections of hair differed significantly between individuals with and without eating disorders ($F_{1,40} = 11.237, p = 0.002$; Fig. 4). Forward stepwise
multiple regression found that the $\delta^{15}C$ of the youngest 1 cm of hair was correlated with the number of months since the eating disorder began ($p = 0.04$; $R^2 = 0.10$). The average $\delta^{15}C$ value of an individual's hair after entering treatment was significantly correlated with the frequency of eating meat ($t_{\text{sig}} = 3.161$, $p = 0.003$; $R^2 = 0.22$).

Thus, through the use of ANOVA and stepwise multiple regression, the list of variables to include in a stepwise discriminant analysis was narrowed down to the following:

1. Standard deviation in $\delta^{15}N$ values along the length of an individual's hair;
2. The $\delta^{15}N$ values of the youngest (most proximal) section of hair;
3. The average $\delta^{15}N$ value of an individual's hair after entering treatment;
4. The difference in $\delta^{13}C$ values between the oldest and youngest sections of hair; and
5. The $\delta^{13}C$ values of the oldest sections of hair.

Other comparisons were not significant and therefore were not included in the above list.

The forward stepwise discriminant analysis further reduced the list of useful variables. Variables were added if $p < 0.15$ and dropped if their $p$ values increased above 0.15 after other variables were added. The difference between the $\delta^{15}N$ values of the oldest and youngest sections of hair was added first, followed by the standard deviation in $\delta^{15}N$ values along the length of the hair. When, however, the difference in the $\delta^{13}C$ values of the oldest and the youngest sections was added, the difference in $\delta^{15}N$ values between them was dropped. Finally, the $\delta^{15}N$ values of the youngest section of hair were added to the model.

The discriminant analysis procedure used three of the above variables to produce two equations: one for individuals with normal eating habits (Eqn. (1)) and one for individuals with eating disorders (Eqn. (2)):

\[
\text{Control} = -143.7 + 14.1S + 35.0Y - 5.57O \tag{1}
\]

\[
\text{Eating disorder} = -125.6 + 20.1S + 32.5Y - 3.42O \tag{2}
\]

where $S$ is the standard deviation of the $\delta^{15}N$ values along the length of the hairs, $Y$ is the $\delta^{15}N$ value of the youngest hair section, and $O$ is the difference in the $\delta^{13}C$ value of the oldest—the youngest hair section. The values for the individual are entered into each equation. If the resulting value is larger for Eqn. (1), it is inferred that the individual is clinically normal. If, however, the larger of the two results belongs to Eqn. (2), it is then inferred that the individual has an eating disorder.

Based on this algorithm, the computer assigned each individual to either the control group or the eating disorder group. The algorithm was correct approximately 80% of the time in both cases (see Table 1).

**DISCUSSION**

Our study suggests that a statistically sound and objective measure can be triangulated with the current objective and subjective measures to improve the diagnosis of eating disorders. While previous studies have shown that eating habits,8,15,18,19,35,41–43,45 weight loss or gain,8,16,45 or eating disorders8 can affect the ratios of stable isotopes in hair, this study is the first to establish an objective and statistically sound means, based on stable isotope ratios of hairs, for diagnosing eating disorders. The diagnosis of an eating disorder is a triangulation of multiple facts, including both objective measures (e.g. weight, amenorrhea, body mass index, change in weight over time) and subjective assessments based on qualitative interviews and self-reporting by the patient. The accuracy of the subjective assessments is based on the honesty of the information reported by the patient, and it can be difficult for a clinician seeing a patient for the first time to get a clear idea of the true history of the patient’s disorder. Researchers, clinicians and patients would therefore greatly benefit from an objective, biological measure that could aid in diagnosing eating disorders.

While Mekota et al.8 were the first to attempt to analyze isotope ratios of hair as a means of diagnosing eating disorders, their study was not designed to establish the validity of using stable isotope analysis to diagnose eating disorders. To establish the validity of a method or test to diagnose a condition, three points need to be considered. First, the study should be a blind and independent comparison with a reference standard. Secondly, the study should include an appropriate spectrum of individuals, including both those with and those without the condition so that the sensitivity and specificity of the method can be evaluated. Thirdly, the results should not influence the outcome.53,54

Similarly to previous authors, we initially used a visual examination of the data to evaluate the individual's eating disorder. However, this new approach differed in that the tests were blind so that previous knowledge of the individuals could not influence the outcome. In addition, we included non-clinical individuals in our study. This had not been done previously when studying the effects of eating disorders8 or nutritional stress18,45 on the carbon and nitrogen isotope ratios in human hairs.

A blind pilot test was carried out in which one of the authors (KAH) was given the $\delta^{15}N$ and $\delta^{13}C$ values from the serial analyses of the hairs of six individuals. KAH was told only that three individuals were normal and three had eating disorders. By visually examining graphs of the stable isotope ratios of the hairs down their lengths, KAH was able to correctly assign all six individuals to their correct group.

This procedure was repeated with all the individuals in the study. However, this time not only did KAH not know who had an eating disorder and who did not, but he did not know how many had an eating disorder and how many did not. On this occasion, the overall success was only 70%. This represents only a fair ability to discriminate.55

Table 1. The correct and incorrect diagnosis of individuals as either having an eating disorder or as being clinically normal by the discriminant analysis algorithm

<table>
<thead>
<tr>
<th>Subject group (n)</th>
<th>Subjects assigned to control group (% of total)</th>
<th>Subjects assigned to eating disorder group (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (22)</td>
<td>18 (81.8)</td>
<td>4 (18.2)</td>
</tr>
<tr>
<td>Eating disorder (20)</td>
<td>4 (20)</td>
<td>16 (80)</td>
</tr>
</tbody>
</table>

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Typically, when establishing the diagnostic performance of a test, one should establish the sensitivity and specificity of the test. The sensitivity is the probability that the test is positive for an individual who actually has the disease, while the specificity is the probability that the test is negative for an individual who does not have the disease. The sensitivity of the subjective, visual method was 75% with a 95% lower confidence interval (CI) of 59%. The specificity was 65% with a 95% lower CI of 49%.

Discriminant analysis, however, offered a means by which one could determine which measures were most important and predictive. It is also completely objective, since the algorithms on which the diagnoses are based cannot be influenced by previous knowledge or biases. Both the sensitivity and the specificity of this method were better than that of subjective, visual analysis. The sensitivity using the algorithms was 80% (95% lower CI = 65%) as was the specificity. Thus the overall ability to discriminate was 80% (95% lower CI = 66%), which indicates good discrimination.

While these results are promising, there are several other potential problems that should be considered. First, it is important to recognize that the above equations were established using a North American population. North Americans differ in their diets from other populations primarily in that they eat more corn. Corn is a C stalk plant that has a different carbon isotope ratio from the C plants (e.g., wheat and rice) typical of other industrial societies. Thus, the above algorithms may not be appropriate for diagnosing individuals with eating disorders who are residents of Europe or Japan, for example. However, this study suggests that if the above algorithms are not universal in their applicability, algorithms can be developed specific to the eating habits of other societies.

Secondly, variation in the stable isotope ratios of hairs can be caused by other factors such as seasonal variations in diets, travel between locations with differing diets, or other conditions that affect metabolism. Incorporating this kind of information into the above algorithms or evaluating the results of the algorithms may improve their accuracy.

Thirdly, the small number of hairs sampled may have introduced some error. Only five hairs per individual were sampled, and these hairs occasionally may not have been representative, particularly if several of the hairs sampled were in the quiescent stage. Previous authors have suggested using 25 or more hairs to avoid this. However, as the results were 80% correct with regard to both sensitivity and specificity, this suggests that the small sample size of hairs was not a major problem. While cutting 25 hairs does not cause a patient great stress, the most recent information is lost when hair is cut and it is difficult to properly align the cut hairs for analysis. If one wants to avoid this problem by pulling hairs, the use of a small sample size reduces the stress experienced by the patient.

Fourthly, it may also be possible to gain greater accuracy by sequentially analyzing segments of hair shorter than the 1 cm length used in this study and by avoiding combining consecutive sections of hair for analysis. Occasionally, 1 cm segments (for a total of 2 cm) had to be combined in order to obtain sufficient mass of hair for analysis. This may also have introduced some error. However, the study by Schwert et al. suggests that this is unlikely to cause significant errors because of the buffering effect that the body’s amino acid pool has. In addition, the algorithms did not include the sequence of hairs as a factor, except for comparing the first and the last sequences. Rather, it included the variance of the signal along the length of the hair. Order is not important here nor is the occasional combining of two 1 cm segments likely to greatly affect the variance along the length of the hair.

Despite these potential problems, Figs. 1–3 suggest that it may be possible to develop an algorithm that distinguishes, not just between individuals with eating disorders and non-clinical individuals, but also among four different groups: individuals with anorexia, individuals with bulimia, individuals with both anorexia and bulimia, and non-clinical controls. In the above-mentioned figures, individuals with either anorexia or both anorexia and bulimia were consistently similar, while individuals with only bulimia were consistently similar to non-clinical volunteers. By definition, individuals with anorexia or anorexia and bulimia are losing weight and do not get adequate nutrition. These individuals either get their nitrogen largely from plants, and/or do not get sufficient nitrogen in their diet and are in nitrogen imbalance. Consequently, the individuals in our study with either anorexia or anorexia and bulimia differed from non-clinical volunteers. By contrast, individuals diagnosed with only bulimia are maintaining their weight and therefore get adequate nutrition, and are likely not to be in nitrogen imbalance. These individuals may get much of their nitrogen from meat or milk products in their diet and were therefore similar to normal volunteers. These results do suggest that further distinctions may be possible.

In conclusion, the fact that students from across the USA were being compared with patients, that the hairs of these students included seasonal variation and variation from travel, that only five hairs from each individual were used, and that it was still possible to obtain 80% or better accuracy suggest that we were able to capture the majority of the information needed for diagnosis and that the above problems are not overwhelming. Accounting for problems may refine the above algorithms further, thereby improving the accuracy of the resulting diagnoses, but our study suggests that the method is already quite robust.

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