Metabolic Profiling with Magnetic Resonance Mass Spectrometry and a Human Urine Bank: Profiles for Aging, Sex, Heart Disease, Breast Cancer and Prostate Cancer

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ABSTRACT

A sample bank has been established at the Oregon Institute of Science and Medicine to which 5,000 volunteers are periodically contributing urine specimens and medical histories. Samples are stored at -80 ºC (degrees Celsius). As groups of samples accumulate from persons with similar subsequent medical events, samples are quantitatively analyzed by magnetic resonance mass spectrometry (MRMS). MRMS permits the simultaneous quantitative measurement of more than 800 molecular urinary constituents of human metabolic origin. Profiles for aging, sex, heart disease, breast cancer, and prostate cancer have been found and analyzed for diagnostic usefulness. There is a 99.99% probability that a profile predictive of a subsequent cardiac event has been identified, and a 94% and 97% chance, respectively, that profiles predictive of breast or prostate cancer have been identified. Such profiles could be made available at very low cost and have great potential for preventive, diagnostic, and therapeutic medicine. In our set of patients with diagnosed cardiac disease, a diagnostic coefficient greater than a specified threshold was present in 19 of 21 subjects who experienced a cardiac event 4 to 30 months after contributing a urine specimen, but present in only 2 of 21 age and sex-matched controls. Sixteen of these 21 had experienced no cardiac event prior to providing the urine sample. In a randomly selected set of 200 undiagnosed healthy subjects (100 men and 100 women), the cardiac event diagnostic coefficient was above the threshold in 28%. About 27% of the U.S. population in this age group is actuarially expected to die from heart disease.

Introduction

In this paper, we introduce the concept and utility of quantitatively measuring metabolic profiles of samples from a human urine bank. The approach described here holds remarkable promise as a means of providing early indication of disease, making it ideally compatible with precision or personalized medicine. To facilitate this approach, we also introduce the concept of magnetic resonance mass spectrometry (MRMS) for rapid metabolic profiling. MRMS combines the ultra-high performance of Fourier transform ion cyclotron resonance (FTICR) mass spectrometry with the advantages of matrix assisted laser desorption ionization (MALDI) for rapid, straightforward profiling analysis.

The MRMS method utilizes the extremely high resolution and high mass measurement accuracy of FTICR mass spectrometry.[1] This high resolution allows thousands of independent chemical substances to be detected and quantified simultaneously for a single sample without the requirement for prior separation. This provides the ability to discern the extensive metabolite information generated from the ionization of urine samples, while providing a unique speed advantage. Moreover, the molecular formulae for most of these signals can be confirmed by accurate mass measurement, providing great specificity.

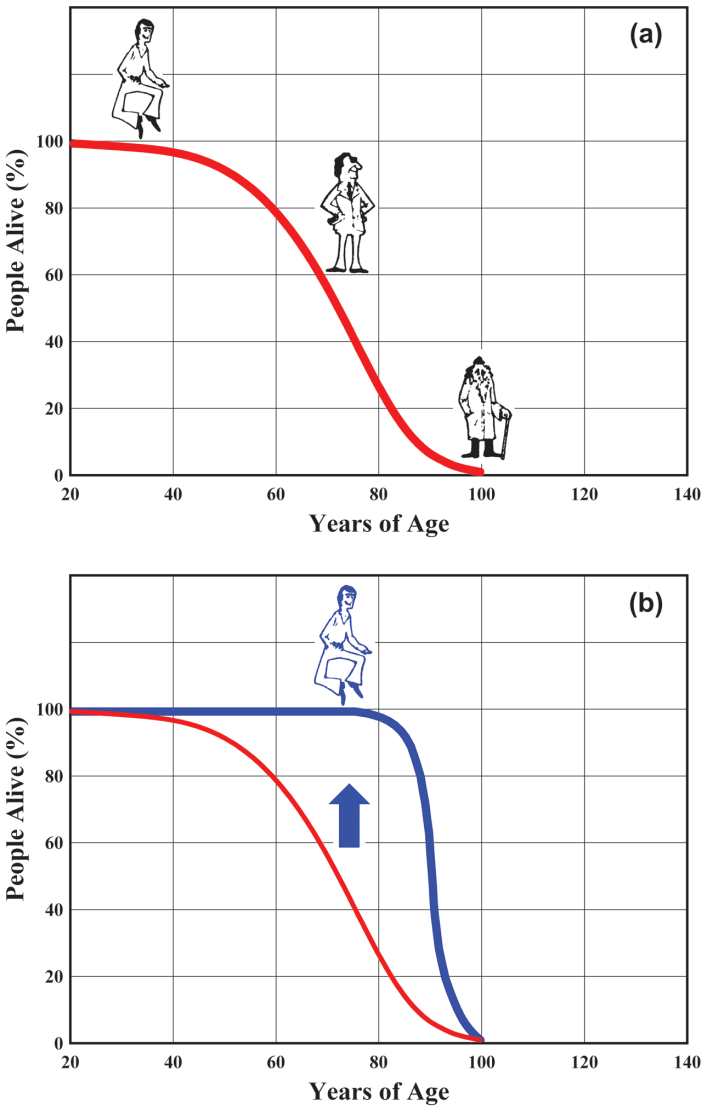

Figure 1. Actual and Potentially Enhanced U.S. Survival. The red line in (a) and (b) is the U.S. survival curve. Physiologically calibrated MRMS of urine could prevent much of this suffering and early death as illustrated in (b) by enabling early diagnosis and preventive treatment.

MRMS can be configured with any of the various and routine ionization methods such as electrospray (ESI)[2] and MALDI.[3,4] MALDI was chosen as the ionization method in this work for its simplicity of operation and nonsusceptibility to sample carryover, making it attractive as a potential diagnostic tool. Additionally, MALDI is fast and less vulnerable to the deleterious effects of salt concentration on ionization efficiency.

These characteristics of the MALDI-MRMS combination permit metabolic profiling with greater numbers of substances than other methods with a single rapid analysis rather than analyses combined with chromatographic separations or other preparative procedures, which require more time and added expense.

In the work presented here, a single 7-minute MALDI-MRMS run reproducibly resolves more than 100,000 different chemical constituents from a 5 µl human urine sample in positive ion mode. Negative ion mode will further increase this inventory of measurable substances. Also, as the inventory of metabolic profiles grows, this method can be refined for analytical turnaround in much less than 7 minutes.

With sufficient research and very high quality human calibration samples, a true cornucopia of information about virtually all important health conditions that affect human metabolism should be detectable by means of a single analysis with this one analytical technique.

We also carried out substantial experimentation with ESI as an alternative to MALDI for this work. In our experience, ESI provided more complete ionization; however, it introduces unacceptable sample-introduction contamination and also adsorptive sample substance losses that make it less ideal for this particular application.

Future research and development will make this analytical methodology even more quantitative and practical. The research reported herein, however, demonstrates that MALDI-MRMS already offers the potential for extraordinary diagnostic advances in its present form.

Quantitative metabolic profiling originated in a 10-year project between 1968 and 1978 to test the hypothesis that a single analysis of the amounts of large numbers of metabolites in human body fluids and tissues, followed by computerized pattern recognition, would be a useful means for the simultaneous quantitative measurement of many aspects of human health.[5]

Using mostly chromatographic measurement of between 50 and 150 substances, in primarily human urine with a few experiments on breath and tissues, this project verified this hypothesis by discovering unique profiles for multiple sclerosis, Duchenne dystrophy, Huntington's disease, breast cancer, diet, fasting, sex, diurnal variation, and chemical birth control. With diet control, profiles sufficient to fingerprint single person biochemical individuality were observed, and it was discovered that urinary substances are monomodally, bimodally, and even trimodally distributed in the human population at birth. In addition, profiles characteristic of physiological age were found in fruit flies, mice, and men.[5-8]

With the objective of very low cost mass screening of people to increase their quality and length of life as illustrated in Figure 1, this 1970s research had three problematic limitations. First, the analytical procedures were slow and expensive. Second, the disease work was carried out on people who were already overtly ill, which introduces systematic variables other than the disease itself. Third, it involved primarily single samples from individuals. Multiple samples taken over an extended period of time would have allowed individuals to serve as their own controls and markedly enhanced the precision of the metabolic profiles.

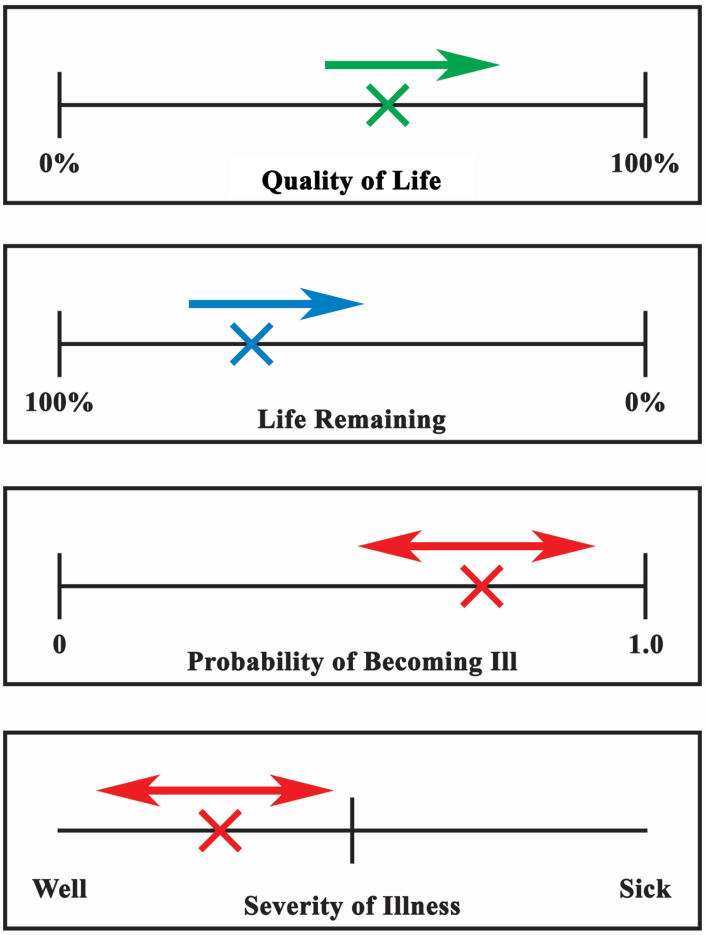

Figure 2. Potential Use of Diagnostic Coefficients. The empirical determination of the positions, designated X, of single individuals on these illustrative linear axes by means of metabolic profiling available to everyone at low cost would facilitate lifestyle and medical intervention on their behalf. Research with such profiles on groups of individuals would help to guide those interventions.

Advances in mass spectrometry, especially MRMS as utilized herein, now make possible very fast and low-cost simultaneous measurement of thousands of substances, which solves the first problem. So, a sample bank has been created at the Oregon Institute of Science and Medicine to solve the second and third problems, with urine samples and medical data being collected periodically from 5,000 volunteers and the urine stored at -80 ºC. The initial results from this project are reported herein.

This research is conceptually different from the vast

worldwide effort begun by biochemists a century ago to ultimately and thoroughly understand human metabolism and, along the way, to identify biochemical markers or, now, groups of markers that carry information useful for specific medical purposes.[9,10]

We seek instead to measure large numbers of metabolites in a single analysis, gaining usually small amounts of empirical correlating information from each individual metabolite—the summations of these correlations being used to simultaneously detect and measure many different health profiles that are diagnostically useful. This technique depends upon the subtle biochemical interactions through which metabolites throughout the metabolism pick up information about one another.

This permits one simple empirical analytical procedure, optimized for those substances that are easy and inexpensive to measure, to gather a wide variety of useful quantitative information characteristic of various aspects of human health.

In each profile, the information from the measured substances in a urine sample is combined mathematically into one number, designated the "diagnostic coefficient," for each condition of interest, since most uses of this information are one-dimensional as shown in Figure 2.

Placing an individual quantitatively on a life-remaining, physiological aging axis would allow him to watch and manipulate his progress along that axis as a function of diet and other adjustable lifestyles, and placing groups of individuals on this axis would allow objective research on such parameters.

Placing individuals on a probability-of-illness axis would be useful in efforts to combat the probability of specific illnesses rather than the illnesses themselves, and, if illness is present, placing an individual on a severity-of-illness axis would be useful in monitoring and optimizing therapy.

In general, placing an individual on a "quality of life" axis can include any parameter of importance to the individual—even athletic performance, sleep pattern, or just sense of well-being.

To be sure, these are not new goals. The point here is that a single inexpensive quantitative metabolic profiling tool now has the potential to do all four without the need for solving the underlying biochemistry of the condition of interest or using targeted procedures, the expense of which often limits their use.

Optimization of human nutrition motivated the origin of this concept. In 1968, Linus Pauling and Arthur Robinson were searching for ways in which to determine optimum nutritional intakes of essential nutrients in individuals and populations. They needed to make graphs of health as a function of intake of vitamins and other nutritional substances, but lacked a quantitative means of measuring biochemical health. Quantitative metabolic profiling was devised as a possible solution.

While biochemistry is expected to ultimately provide learned answers to these questions, the goal was then and is now to provide empirical information at very low cost to improve the lives of people living now and prior to the ultimate maturation of biochemical knowledge.

It is unacceptable that the human survival curve includes, as shown in Figure 1, a large percentage of people who experience suffering and death at ages far shorter than the intrinsic human life span. Analytical technology now provides the ability to significantly improve this. Technological advance in mass spectrometry makes possible not only eventual detailed understanding of human metabolism, but also empirical methods to markedly and significantly reduce this early suffering and death. We describe, herein, progress in research on such a method.

Methods and Resources

Urine Bank

A total of 8,500 interested volunteers in southern and central Oregon were located by direct mail. After expected initial losses, 5,000 volunteers now actively participate in this project, with an attrition and necessary replacement rate of about 5% per year.

Periodic urine samples and current self-reported medical information are collected from the volunteers. Each sampling

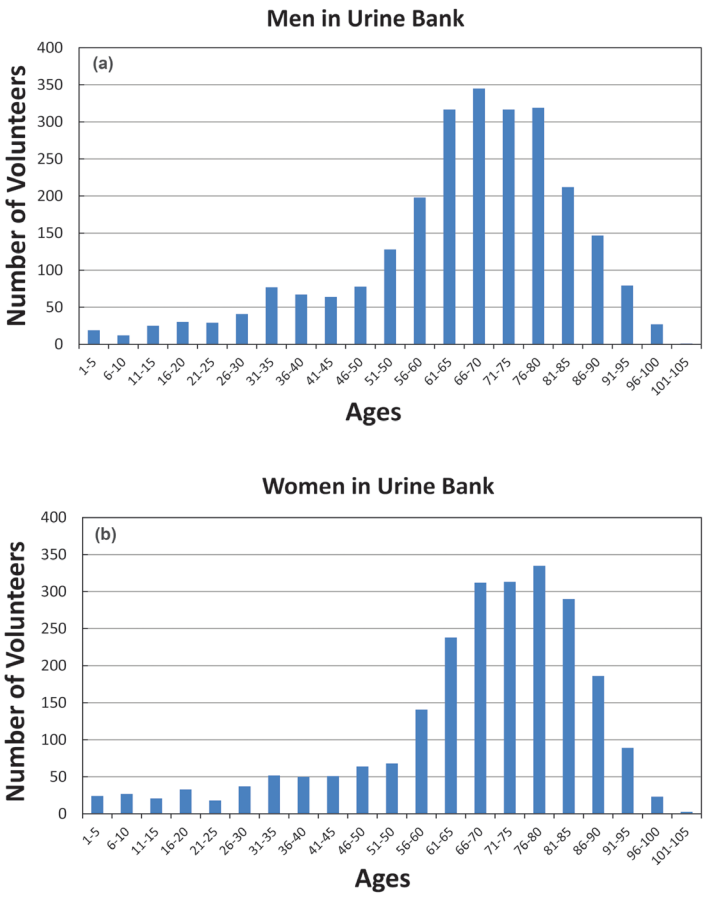

Figure 3. Age Distribution of 5,000 Contributors to OISM Urine Sample Bank. Men (a) and women (b) from southern and central Oregon, U.S., volunteer to contribute urine samples and medical information to the urine bank.

consists of two approximately 1.5 ml (1,500 µl) samples of urine placed in 1.8 ml Nunc Cryotube vials.

In the initial stages of the project, these samples were mailed in ambient temperature USPS approved mailers to the urine bank, where they were cataloged and stored at -80 ºC. Currently, we are collecting both mailed samples and door-to-door collected samples that are frozen immediately.

There are partial losses of some substances during the ambient temperature mailing, but, with thousands of substances from which to choose, these losses are moderate. Door-to-door collection is about twice as expensive. Mailing is also more compatible with our goal of very low cost, enabling as many people as possible to afford and benefit from this technology.

Self-selection has led to an older age distribution of our volunteers as shown in Figure 3, so they have expected disease incidences about three times greater than a linear age distribution of ordinary Americans.

The urine bank storage is in military grade -80 ºC freezers with three-fold power backup. The two samples permit storage in two locations.

Mass Spectrometry

Mass spectral analysis was performed in an unmodified Bruker 7T-SolariX XR ICR FTMS tuned to the 100 to 1,000 m/z mass range. The MALDI (matrix assisted laser desorption ionization) source was operated at 50% laser power. The MALDI plate was a Protea Biosciences Redichip, pin type with no chemical matrix.

Urine sample dilutions were determined by spectrophotometry over 350-360 nanometers in a Molecular Devices SpectraMax M2 spectrophotometer, with the concentrations then normalized by adding between 0 and 50 µl of VWR Aristar Ultra pure water to a 5 µl urine sample. This adjusted the urine concentrations approximately with one another. A total of 4 µl of each diluted sample was carefully applied to the MALDI plate in order to completely cover one circular pin array, and dried before analysis.

A total of 200 FTICR transients were averaged together, with each 1.0-second transient generated by a 500-shot pulsed laser directed onto a unique position on the plate as selected by means of Bruker automation. The sample cycle time was seven minutes.

Ions from the 500 laser shots accumulate in the MALDI source and enter the ICR cell as one group, the measurement of which produces one transient analytical image. The average of 200 such transients is converted into the mass spectrum by Fourier transform.

Sample introduction for these analyses has unique requirements. With 200 transients and an estimated 1 million molecules accommodated by the ICR cell, an estimated 200 million molecules varying in amounts over three orders of magnitude can be measured. These are composed of more than 100,000 molecular components, with about 30,000 making up most of the total. So, most of the individual chemical species are present in very small amounts.

Therefore, the analytical system must be very clean. The use of laser desorption ionization overcomes contamination of the sample during introduction, but the MALDI chemical matrices commercially available to us were unacceptably contaminated with impurities. So, we used the pin type plates, which were sufficiently clean.

Similarly, ordinary desalting procedures are sources of sample contamination and sample loss at these low concentrations of urinary constituents, so desalting was omitted, which also simplifies the procedure.

MRMS technology is a preferred choice for this application because of its evident superior capabilities as illustrated herein and because of marked improvements in MRMS technology that can be expected in the future.

The quantitative noise in the measurements reported herein is manageable. The high sample quality and especially the large number of experimental parameters utilized have partially overcome noise in these experiments. Suppression of noise by future improvements in MRMS technology and the maturation of the OISM urine sample bank over time will provide even more remarkable profiling capability.

Subjects Used in the Analyses

For the age and sex analyses, 100 men and 100 women spanning the age range and distribution shown in Figure 3 were drawn from volunteers who reported good health.

For the volunteers who reported a diagnosis of breast or prostate cancer, we analyzed samples given ***before*** the cancer was otherwise diagnosed—all compared with individually age and sex-matched controls.

The cardiac event testing was conducted twice. The first trial was with 11 cardiac-event subjects and 11 age and sex-matched controls, with five of the subjects having experienced heart problems prior to providing the urine sample and six having not experienced a prior heart problem. After this was done, we received reports from 10 additional volunteers (or their survivors) that they had experienced their first known cardiac event. We then analyzed the samples provided by the 16 volunteers who had not reported cardiac symptoms prior to providing a urine sample.

Of the 21 subjects with cardiac events in the two trials, "heart attacks" were reported for 14 subjects, "congestive heart failure" for 4, and "heart failure" for 3.

Calculations

Statistical tests of the discovery and diagnostic reliability of the metabolic profiles reported here were computed in two different and complementary ways. Both of these ways use the Wilcoxon method of nonparametric statistics.[11]

Much analytical data, by custom and culture, is tested by methods that assume the measurements to be distributed as Gaussian. If the measured values are determined by underlying phenomena that depend upon a significant number of similarly sized, largely independent variables, then the distribution function (range and relative magnitude of the values within the range) of the measurements tends to be Gaussian. For example, human intelligence is found to be Gaussian distributed. If, however, the data is not

distributed as a Gaussian or another defined functional form or if it is not known to be so distributed, then rigor requires that "nonparametric" statistics, which do not depend on the distribution function shape, be used. Medical research, including that reported herein, often involves too few measurements to determine the distribution function shapes, so it must be evaluated nonparametrically. Also, in the case of urinary metabolic profiling, research has shown that the measured values are often not distributed as Gaussian.[5]

For the first test (of profile presence), the raw mass spectrometric data herein were tested for the existence of metabolic profiles for sex, age, prostate cancer, breast cancer, and heart disease, with no data manipulation at all other than normalization to remove systematic variation caused by variable in vivo dilution, primarily from variable water intake by the subjects. Thus, the peak areas of all substances in each urine sample were divided by a sample-dependent dilution constant derived by four iterations of normalizing,[12] using the values of a large number of the peaks in the sample.

The probabilities of non-correlation (1.0 minus the probabilities of correlation) were then nonparametrically calculated for each mass spectral peak found in 80% of the spectra of the test subjects and matched controls. Control matching was primarily for sex and age, as appropriate. These probabilities were arranged in order of increasing magnitude and plotted as cumulative distribution functions as shown in Figures 4 and 6.

So, for example, if the non-correlation probabilities for peaks in two compared groups for 20 peaks are equal to or lower than P=.001, that point is plotted; if 35 peak areas lie at or below P=.002, that point is plotted; and so on for all P value divisions in the comparison. These are the blue lines on the graphs. For 5,000 peaks, if there were no correlation and the data were random, 5 peaks would be expected at or below P=0.001; 10 at or below P=.002; and so on, leading to a linear plot as shown in red on the graphs. In this example, therefore, 5 peaks are expected at P=.001 and 20 are found, an excess of 15. This does not reveal which are the random 5 and which are the 15 from a systematic profile, but the excess reveals a profile.

The red lines shown are theoretically straight, but deviate from linearity with finite data sets and experimental noise. So, we calculated 20 red lines from the measured spectra for 40 control subjects arranged randomly in 20 different paired groups of 20 subjects each. The Gaussian standard deviation, σ, at P = 0.1 for this series of experiments (Gaussian statistics being appropriate for this purpose), was computed. Deviation of the blue lines from the red lines was thus measured in units of σ, providing values of 5.5 σ, 2.2 σ, and 0.4 σ for the cardiac event, breast cancer, and prostate cancer measurements, respectively. So, there is an estimated greater than 99.99% probability that a predictive cardiac event profile is present, a greater than 95% probability that a predictive breast cancer profile is present (as was discovered for overt breast cancer in the 1970s), and no detected predictive prostate profile at low probabilities of non-correlation, although the overall graph reveals an apparent weaker profile for prostate cancer.

For the second test (of profile usefulness), the experimental values were used in a simple diagnostic procedure to test the diagnostic potential of these profiles. This procedure does not depend on the cumulative distribution functions, but it is to be expected that the relative strength of these cumulative distributions would correspond to relative diagnostic power, as it does.

Using the protocol we developed for these experiments, a great many chemical species with unique masses are detectable in these urine samples, with more than 100,000 appearing in most of the samples and about 30,000 appearing as unique, reliably quantifiable peaks. On average, each unique substance in these spectra appears at eight specific different masses due to combinations during analysis with other urinary substances and isotope effects, wherein various elemental isotopic variations appear frequently enough to be detected.

A recent review of urine composition[13] lists 2,700 unique chemical substances that have been detected in human urine in the mass range of our experiments, with 917 of those listed believed to be endogenous products of human metabolism. These could include both human and bacterial products and byproducts. We tentatively identified 2,300 of these in our spectra based upon their masses being within 2.5 parts per million of the exact theoretical masses in the 2,700. We verified 837 of the 917 by means of observed masses of multiple adduct forms and isotopes, and found that about 700 met the criteria of appearing in 80% of the samples in each profiling experiment. We added all detected amounts of different mass forms of each of the 837 together to obtain the total amount of each unique substance used in the diagnostic calculations.

The molecular identities of these 837 substances have been tentatively determined by exact mass and are listed with the Internet version of this publication. This mass measurement provides elemental formulas, not structural formulas. The molecular identities have been enhanced by structural information regarding urine composition compiled from other sources,[13] but it is to be expected that some of these assigned molecular identities may be incorrect.

We found many substances that correlate with the human conditions we measured in these experiments that are not reported[13] to be products of human metabolism. These are widely abundant in food, air, water, and other sources, and metabolic information is apparently impressed on them while they are inside human tissues. We decided to limit these diagnostic calculations to those designated as human products,[13] so about 700 human metabolites that passed our 80% criteria in each experiment are used herein in each diagnostic evaluation. On average, the amounts of about eight adducts and isotopes in the spectra were added together to obtain the value for each of the 700.

We have found that the logarithmic ratios of amounts of metabolites contain better diagnostic information than the absolute values, which is in accord with the general behavior of chemical systems. So, we calculated about 500,000 parameters by dividing all of the 700 amounts of metabolites with each other and then computing the logarithms. These 500,000 parameters

were ordered by nonparametric correlation probability, and the most correlating unique 500 parameters were used for diagnosis. These 500 (1,000 including the inverses) included between 120 and 150 different metabolites, depending upon individual disease, and no single metabolite was present in more than 5% of the 500 selected. Although there were far more than 500 parameters with high correlation probabilities, we found that inclusion of more than 500 had little marginal diagnostic value herein. Use of ratios in this computation also removes any remaining concentration-normalizing insufficiencies.

In the case of the predictive cardiac event profile, the 500 parameters included 147 human metabolic urinary substances in the first trial, 146 in the second, and 148 in the combined diagnostic power evaluation shown in Figure 9.

While sophisticated pattern recognition techniques are available, we have used a simplified procedure herein, in which diagnostic coefficients[5] are calculated.

Diagnostic coefficients R_A are defined as:

$$R_A = \frac{100}{\sum_{i=1}^{n} r_i}\left[\sum_{i=1}^{n}\frac{|A_i - Y_i|r_i}{A_i + Y_i} - \sum_{i=1}^{n}\frac{|A_i - O_i|r_i}{A_i + O_i}\right]$$

where A_i is the normalized value of the ith parameter in the mass spectrum, A, that is being classified. Y_i and O_i are the average values of the corresponding parameters in the two groups being compared, n is the number of parameters in the calculation, and r_i is a weight constant that was set equal to 1 for all parameters in the calculations herein for simplicity in evaluating these results.

By this procedure, each parameter (logarithmic metabolite ratio) is averaged for the test group and an appropriate control group. Each subject in the disease analyses was paired with an age and sex-matched control, and diagnostic coefficients for each of the pair were computed. The pair is excluded from determination of the averages of the parameters to which it is compared, the averages being thus recomputed for each comparison. This exclusion prevents the pair from biasing the averages in its own favor. The average coefficient for the two is computed and the quantitative diagnostic coefficient deviation toward the experimental or control group averages for each determined. These deviations are plotted on a diagnostic coefficient graph as shown in Figures 7 and 8.

To simplify comparisons in Figures 7 and 8, these values were normalized to a range between -50 for the most extreme average of the control subjects and +50 for the most extreme average of the subjects manifesting the condition of interest.

Figure 7 shows bar graphs, which illustrate the diagnostic separations achieved. From the combined diagnostic coefficient order of the trial and control groups shown in these graphs, the nonparametric probability that a separation into two groups by metabolic profiling has been achieved is computed. For the two cardiac event analyses, the breast cancer analysis, and the prostate cancer analysis, these probabilities are 99.5%, 99.8%, 94%, and 97%, respectively. While the breast cancer separation appears better than the prostate cancer pattern, it has a lower probability. The reason for this is that fewer pairs of diseased and non-diseased subjects were used in the breast cancer analysis.

These diagnostic coefficients can then be ordered and plotted in diagnostic power graphs as illustrated for cardiac event prediction in Figure 9. The coefficients are placed in numerical order for the subjects being evaluated, and this linear distribution is divided at all possible division points to create the diagnostic power graph. This graphing method was developed[5] to account for the fact that diagnostic profiles do not contain within themselves essential information about how they will be used, such as the tolerances for false positives and false negatives, which depend on anticipated medical or other actions.

Since cardiac events very often lead to unexpected and immediate death (9 of 21 or 43% of the urine bank volunteers suffering cardiac events in these two analyses died from the event), more false positives would be tolerable for this disease than, for example, prostate cancer. Figure 9 shows that 19 out of 21 cardiac event-prone subjects were identified with only two apparent false positives among the normal controls. If fewer false negatives are desired the increased number of false positives is evident from the graph. For random data and no diagnostic power, the data would follow the theoretical red line on the graph. The "diagnostic power" of 82% in Figure 9 represents the percentage area between the random red line and a perfect correlation of a point in the origin.

Results and Discussion

Sex and Age

The cumulative distribution function of nonparametric probabilities of non-correlation with sex (Figure 4a) shows a very strong profile, affecting more than 30% of the peaks. There are 1,000 peaks strongly correlating and 3,000 reasonably correlating, reflecting the pervasive metabolic differences between men and women. There were 100 men and 100 women with no known health problems in this evaluation. When the individual correlation probabilities of a large number of substances are calculated, these probabilities are linearly distributed between 0 and 1 if there is no overall correlation. For example, if there are 1,000 peaks, the sum of the probabilities of non-correlation at or below 0.01 will be about 10, below 0.1 about 100, below 0.2 about 200, and so on.

If, however, some of the peaks are correlated, the low probabilities are raised in number, which raises the low probability part of the line. So, for example, the sex probability distribution here is composed of an approximately linear distribution of about 5,000 non-correlated peaks and about 3,000 correlated peaks.

Statistical detection of correlation increases with the number of measurements of each substance, so there may well be far more than 3,000 peaks actually correlated, but the additional weaker correlations will not be evident unless more individual urines are analyzed.

The cumulative distribution function for aging was calculated for these same men and women. The diagnostic

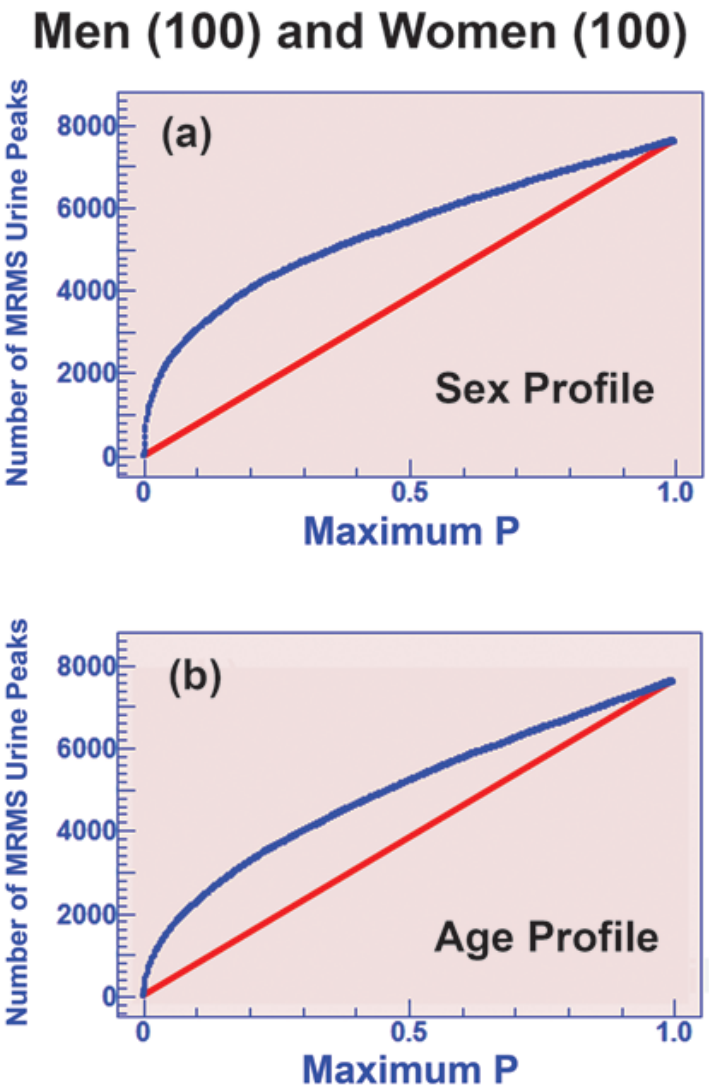

Figure 4. Cumulative Distribution Function of Nonparametric Probability of Non-correlation, P, of MRMS-measured Urinary Peaks with Sex and Age. The peaks for sex used age-matched controls, and those for age used sex-matched controls. The red line is the theoretical plot for non-correlated measurements.

coefficients for aging computed for this profile were calculated using half of the male subjects to establish a profile for aging (group 1) and the other half used to evaluate the profile (group 2). This revealed a diagnostic power for group 2 of 76% shown in Figure 5.

This diagnostic power is below 100% partly because the separations are by chronological age, while the measurements are of physiological age. As more data on medical histories and lifespan accumulates over time, the metabolic profile will give an increasingly accurate estimate of a subject's position on an axis of physiological aging and thus of years remaining in the individual's lifespan.

Also, since the statistical years of life remaining to these younger and older men overlap, a complete separation and diagnostic power of 100% is not possible. This has been discussed more completely elsewhere.[8]

Thus, the urine bank and profiling analysis will eventually reveal the statistically estimated years of life remaining for these volunteers. Moreover, since all samples are stored at -80 °C and analytical technology will continue to improve, more accurate measurements of more substances will become available to refine this profile.

The aging profile herein shows about 30% of the peaks correlate with age. This is consistent with the approximately 30% of substances found to be age correlated in the 1970s[5,8] with far fewer substances.

During the original 1970s research wherein age-dependent metabolic profiles were first observed, most metabolites were not identifiable by the chromatographic techniques utilized. Among 20 that were identified were aspartic acid, glutathione, cystine, alpha-amino butyric acid, and glutamic acid, which increased with age, and histidine, asparagine+glutamine, serine, glycine, threonine, alpha-amino adipic acid, alanine, lysine, valine, ethanolamine, and taurine, which decreased with age.[8] All 16 of these deviated with age in the same directions in the MRMS analyses reported herein as they did in the 1970s research. The other 4 substances identified in the 1970s did not deviate in the same directions, but these were present in very small amounts and therefore subject to high experimental error.

These results illustrate a characteristic of quantitative metabolic profiling in that the urinary amounts of thousands of compounds are useful for profiling, even though they would not necessarily be expected to be especially biochemically relevant. Biochemical interconnectedness in human metabolism induces weak correlations into thousands of molecular species, and these can be statistically summed to provide practical diagnostic value.

For example, it was found in the 1970s[12] that urinary amines

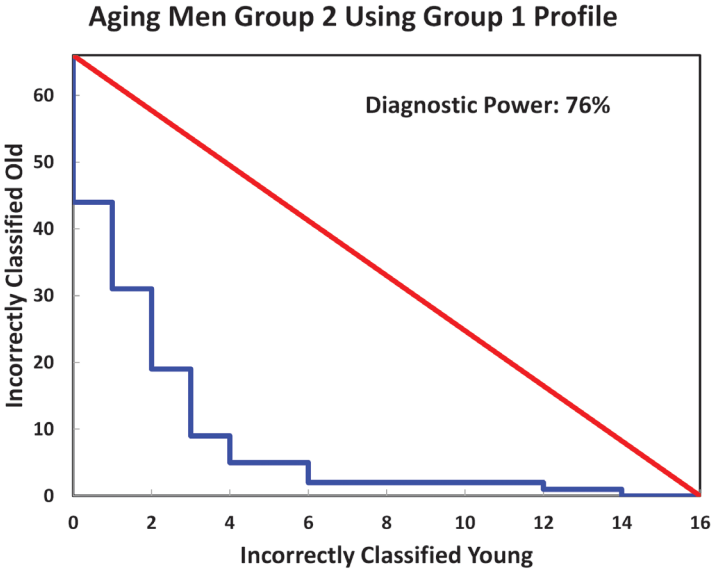

Figure 5. Diagnostic Power Graph. This shows the accuracy of classifying men as "older" (above chronological age 50) or "younger" (below age 50) by metabolic profiling. Note that the measurement is of physiological age.

and amino acids were highly correlated with sex, and we have replicated this finding here, even though specific biochemical links of these substances to human sex are generally unknown.

The physiological age profile should reflect the probable years of life remaining as a result of physiological deterioration and increased susceptibility to disease, especially to life-threatening illnesses. Quantitative metabolic profiling of physiological age should eventually permit useful experiments

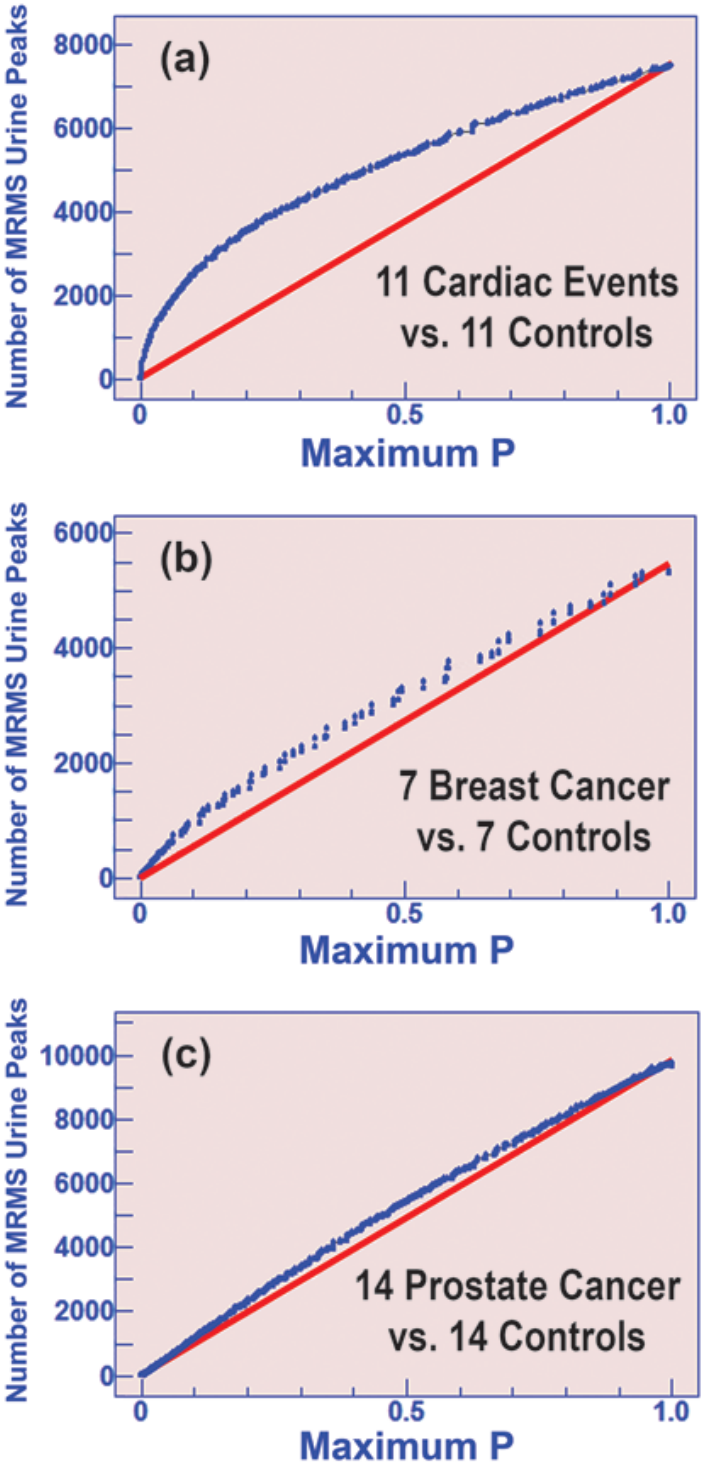

Figure 6. Cumulative Distribution Functions of Nonparametric Probability of Non-correlation of MRMS-measured Substances in Urine Provided Pre-diagnosis: (a) the first analysis of cardiac events; (b) breast cancer; (c) prostate cancer

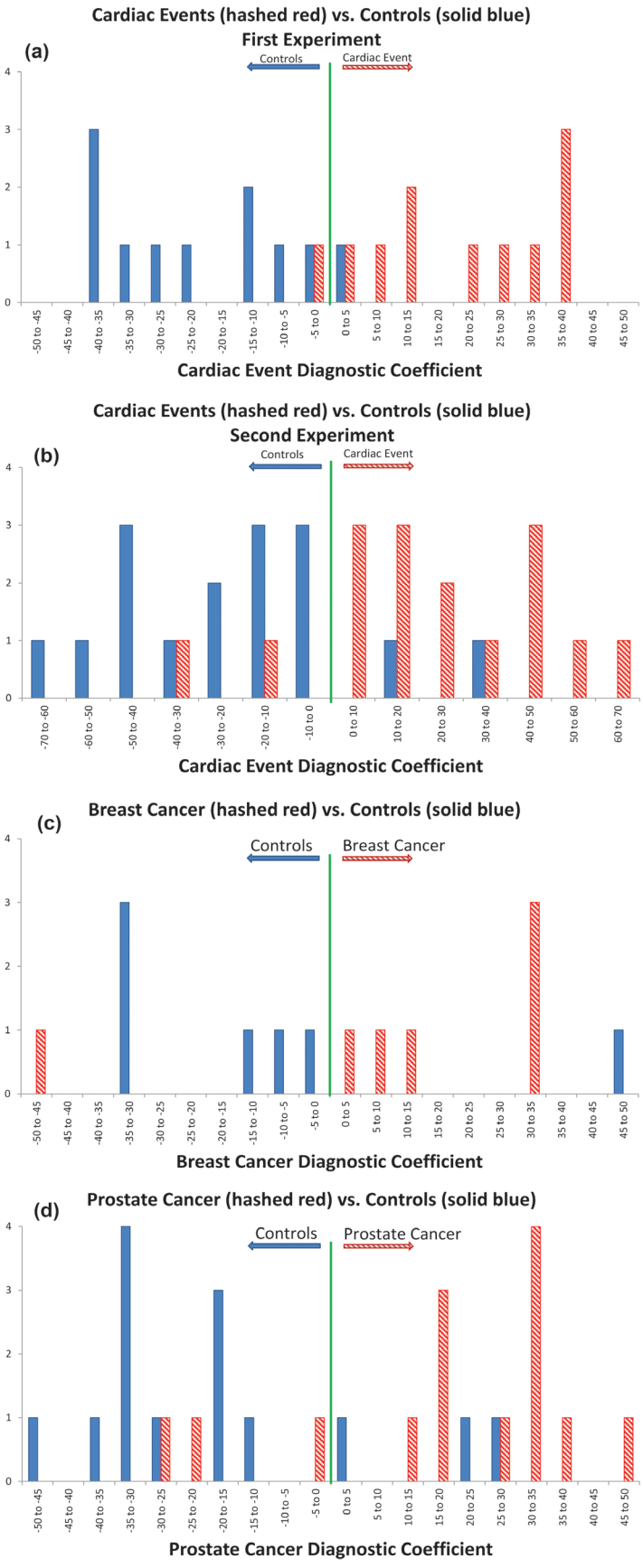

Figure 7. Diagnostic Separations of Subjects vs. Age and Sex-matched Controls: (a) cardiac events in first analysis; (b) cardiac events in second analysis; (c) breast cancer; (d) prostate cancer. Probability of correlation is 99.5%, 99.8%, 94%, and 97%, respectively.

to be performed on populations and on individuals with respect to the effects of diet, exercise, chemical supplements, and other lifestyle-adjustable parameters.

Cardiac Events and Breast and Prostate Cancer Analyses

The cumulative distribution functions of nonparametric non-correlation probabilities for cardiac events, breast cancer, and prostate cancer were determined as shown in Figure 6. The urine samples were provided by the volunteers and stored in the urine bank 4 to 30 months ***before*** these illnesses were symptomatically experienced by the volunteers and medically diagnosed, with the exception that 5 volunteers of the 11 in the first cardiac-event group had also experienced earlier heart health problems.

At P = 0.1 and based on our experimentally determined σ for the red line, the blue lines obtained from our analyses differ from the red lines by 5.5 σ for the first cardiac event profile, 2.2 σ for the breast cancer profile, and 0.4 σ for the prostate cancer profile.

There is, therefore, a greater than 99.99% probability that a cardiac event profile has been detected and a greater than 95% probability that a breast cancer profile has been detected.

There are fewer unique cumulative probabilities plotted for breast cancer as a result of the smaller number of subjects diagnosed with breast cancer, and therefore it exhibits a more broken blue line.

The relatively strong cardiac event profile might be anticipated because a deteriorating heart would be expected to have especially widespread consequences in metabolic processes.

In order to confirm the first cardiac event profile, we performed a second analysis with 16 volunteer subjects, none of whom were known to have ever experienced a heart problem prior to providing the analyzed urine sample, but all of whom suffered a cardiac event in the 4 to 30-month period following deposit of the sample. The result is shown in Figure 7b.

In this analysis, an improved version of the Bruker FTICR-MS with greater sensitivity was utilized; the mass range was 75 to 1,000; 1.5 second transients were collected; and 300 transients were averaged.

Cardiac Event Prediction

The cardiac event samples analyzed herein were given by the volunteers ***before*** they suffered symptoms and were diagnosed with cardiac disease (with the exception of 5 in the first cardiac event analysis).

A correlation was qualitatively observed wherein the cardiac event diagnostic coefficient apparently became larger as the time of the cardiac event approached, but the small size of these sample sets prevents corroboration of this observation with statistical reliability.

Of the 21 cardiac event subjects in Figure 7, the time between the analyzed sample and the cardiac event was between 4 and 11 months for 11 subjects, 14 and 22 months for 9 subjects, and 30 for one subject.

Are these profiles strong enough for predictive and possibly preventive use?

To evaluate this, diagnostic coefficients were calculated for the disease victims and their individual sex and age-matched controls as shown in Figures 7a and 7b. The cross-hatched red bars are those who suffered cardiac events after providing the samples and the blue bars are controls.

The numerical distributions in these disease diagnostic coefficient values shown in Figure 7 provide nonparametric probabilities that these measured profiles are actually diagnostic of the diseases ***prior*** to the later symptoms and medical diagnoses. These probabilities are 99.5% and 99.8% for the two separate cardiac event profile analyses.

The cardiac event prediction profile, discovered in the first set of subjects and confirmed in the second, is especially remarkable.

Diagnostic coefficients were also calculated for the cardiac event profile of the 100 men and 100 women whose samples were used in the age and sex analyses, as shown in Figure 8.

Those among the 200 with a positive heart disease diagnostic coefficient comprise 28% of the group, while CDC (Centers for Disease Control and Prevention) statistics indicate that about 27% of individuals in this age distribution are expected to eventually die from heart disease.

The reliability of this percentage-of-population finding of 28% is enhanced as compared with individual diagnosis, since analytical profiling experimental noise is averaged over 200 analyses in the result.

These results demonstrate that there is a metabolic profile present in the urine of people who have not yet experienced cardiac events, which is likely to be of value in warning such people of this vulnerability.

The diagnostic power graph in Figure 9 created from the ordered diagnostic coefficients of the 21 cardiac event victims and 21 age and sex-matched controls in the two cardiac event analyses combined (with averaged coefficients from these two analyses used for the 11 people in both analyses) has a

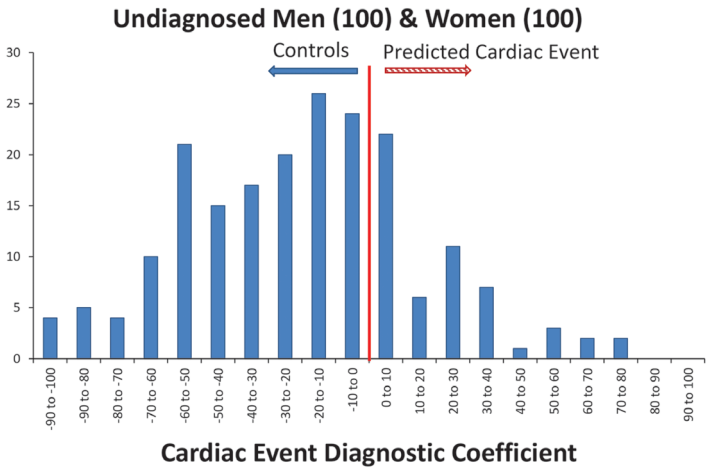

Figure 8. Cardiac Event Diagnostic Coefficients for 200 Men and Women with No Known Health Problems. It is shown that 28% of these people have positive diagnostic coefficients. About 27% of the U.S. population in the age distribution of the 200 are actuarially expected to eventually die from heart disease.

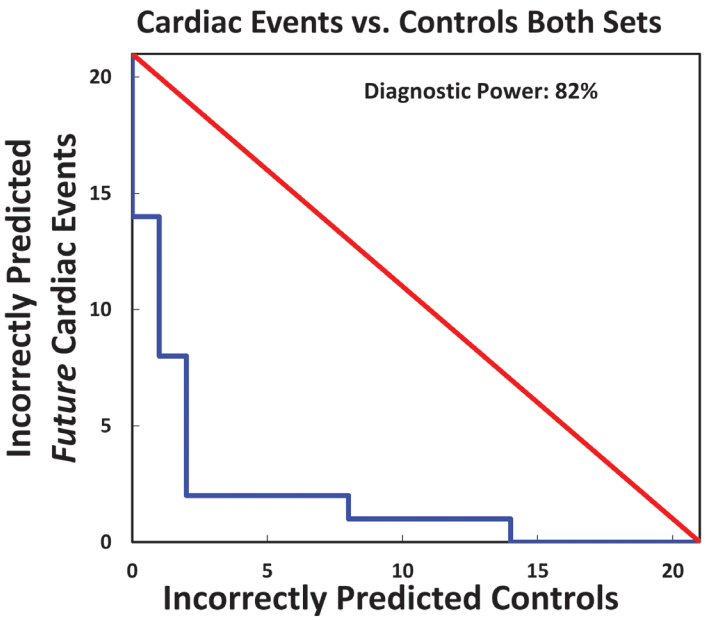

Figure 9. Diagnostic Power Graph of Cardiac Event Prediction by MRMS of Urine for 21 Subjects

diagnostic power of 82%.

If warned by MRMS profiling, these people could have sought medical help, made changes in their lives in hopes of diminishing their cardiac event probability, and taken precautions, such as equipping themselves or their associates with portable defibrillators.

Thus, we see in Figure 9 that, if this profile had been used to inform those whose urine was analyzed, 19 of the 21 who were at risk could have been warned if the cutoff criteria had been set to a level that warned only 2 who had not suffered an event. Given the prevalence of heart disease, it is probable that several of the "control" subjects in this study will also eventually experience cardiac events, so the diagnostic power may actually be higher than 82%. This diagnostic power will improve when constructed with many more samples and subjects.

Figure 9 demonstrates the value of the graphical diagnostic power evaluation[5] because the results of a quantitative profiling study do not contain information about how the profile will be used. Since cardiac events very often lead to unexpected and immediate death, more false positives would be tolerable for this disease than, for example, prostate cancer.

The metabolic profiles for these three conditions (cardiac event, breast cancer, and prostate cancer) appeared before symptoms and medical diagnosis and are unique. Each of the three profiles, when applied to the profiles of subjects with the other two diagnoses, showed no diagnostic value whatever.

Conclusions

It has been shown that magnetic resonance mass spectrometry (MRMS), when combined with the OISM human urine bank for calibration, has substantial potential as a method for the empirical quantitative metabolic profiling of human health. Instrumental improvements and the maturation, extension, and expansion of the urine bank, both of which will occur over time, can further improve this capability.

Advances in mass spectrometry have revolutionized biochemistry, which is gradually leading to increasingly extensive biochemical models and reasoned medical advances.

In the meantime, the empirical use of high-resolution mass spectrometry and careful sampling as illustrated here can make important contributions to the quality and length of human life.

Imagine, for example, a sample kit containing a suitable disposable laser desorption target on which the user places a drop of urine, allows it to dry, and then mails the target USPS First Class in an ordinary envelope to a central mass spectrometry laboratory. The user could receive by Internet a coded confidential report with valuable health information for a total cost of perhaps $5, including kit, postage, and automated analysis, within a few days. Also, receiving the analysis itself, the user could submit his analysis to a statistical evaluation Internet provider of his choice.

In this way, mass spectrometric technology could make valuable information for preventive, diagnostic, and therapeutic medicine immediately available to all people, regardless of their social and economic circumstances.

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