Metabolomic Identification of Novel Biomarkers of Myocardial Ischemia

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Background—Recognition of myocardial ischemia is critical both for the diagnosis of coronary artery disease and the selection and evaluation of therapy. Recent advances in proteomic and metabolic profiling technologies may offer the possibility of identifying novel biomarkers and pathways activated in myocardial ischemia.

Methods and Results—Blood samples were obtained before and after exercise stress testing from 36 patients, 18 of whom demonstrated inducible ischemia (cases) and 18 of whom did not (controls). Plasma was fractionated by liquid chromatography, and profiling of analytes was performed with a high-sensitivity electrospray triple-quadrupole mass spectrometer under selected reaction monitoring conditions. Lactic acid and metabolites involved in skeletal muscle AMP catabolism increased after exercise in both cases and controls. In contrast, there was significant discordant regulation of multiple metabolites that either increased or decreased in cases but remained unchanged in controls. Functional pathway trend analysis with the use of novel software revealed that 6 members of the citric acid pathway were among the 23 most changed metabolites in cases (adjusted $P=0.04$). Furthermore, changes in 6 metabolites, including citric acid, differentiated cases from controls with a high degree of accuracy ($P<0.0001$; cross-validated c-statistic$=0.83$).

Conclusions—We report the novel application of metabolomics to acute myocardial ischemia, in which we identified novel biomarkers of ischemia, and from pathway trend analysis, coordinate changes in groups of functionally related metabolites. (***Circulation***. 2005;112:3868-3875.)

Key Words: exercise ■ ischemia ■ metabolism

Coronary artery disease is a leading cause of morbidity and mortality worldwide.[1] Recognition of myocardial ischemia is critical both for diagnosing coronary heart disease and for selecting and evaluating the response to therapeutic interventions. Currently, myocardial ischemia is diagnosed through a combination of a history consistent with typical angina pectoris and labile ECG ST-segment and T-wave changes, occurring either spontaneously or on provocation with exercise testing.[2,3] This approach, however, is often unsatisfactory because of the transient nature of ECG changes as well as the subjective nature of history taking. Exercise testing with myocardial perfusion imaging is relatively accurate but adds substantially to the cost[4] and is difficult to implement rapidly in settings such as the emergency department. Although several biomarkers accurately diagnose patients with irreversible injury secondary to myocardial infarc-

tion, none are suitable for detecting the more subtle insult of myocardial ischemia.[5]

Recent advances in proteomic and metabolic profiling technologies have enhanced the feasibility of high-throughput patient screening for the diagnosis of disease states.[6] The profiling of low-molecular-weight metabolites is particularly relevant to exercise physiology and myocardial ischemia. Small biochemicals are the end result of the entire chain of regulatory changes that occur in response to physiological stressors, disease processes, or drug therapy. In addition to serving as biomarkers, circulating metabolites may themselves participate as regulatory signals, such as in the control of blood pressure.[7] Three technologies for measuring large sets of biochemical metabolites appear to have the most promise for wide application in diagnostic biochemical profiling: Nuclear magnetic resonance (NMR) spectroscopy,[8,9]

Circulation is available at http://www.circulationaha.org   **DOI: 10.1161/CIRCULATIONAHA.105.569137**

mass spectrometry,[10,11] and liquid chromatography.[12] Coupling bioinformatics and biostatistics with these technology platforms permits the identification and quantification of molecules to characterize the whole organism's response to a given intervention or disease.

Nonetheless, an important limitation to this approach is the profound degree of interindividual variability and, for a disease state such as myocardial ischemia, the inherent unpredictability of the onset of an acute coronary syndrome. To circumvent these problems, we applied metabolic profiling technologies with the use of liquid chromatography coupled with high-sensitivity electrospray mass spectrometry to blood samples obtained from patients undergoing exercise stress testing. This approach is particularly powerful because serial sampling can be performed in patients before and after a controlled ischemic insult, thereby allowing each patient to serve as his or her own biological control. Our goals were threefold. First, we wanted to demonstrate a proof of concept that current technologies are sufficiently robust to identify perturbations in circulating metabolites. Second, we hoped to identify and characterize specific metabolic pathways and circulating metabolites that change depending on the presence of myocardial ischemia. Such metabolites and their pathways might ultimately serve as targets for therapeutic intervention or as substrates for molecular imaging. Third, we wanted to investigate whether metabolic profiling could be used to help identify patients with acute myocardial ischemia and thus potentially add to our diagnostic armamentarium.

Methods

Patients

Patients who underwent stress testing with myocardial perfusion imaging at Brigham and Women's Hospital and Massachusetts General Hospital were enrolled in a prospective biomarker cohort study. The Human Research Committee approved the study protocol, and all patients provided written, informed consent. All patients who were referred for stress testing for the evaluation of possible myocardial ischemia were eligible for participation. Patients who underwent pharmacological testing were excluded. For these analyses, blood samples from a total of 36 patients, 18 with clear-cut inducible ischemia (cases) and 18 without (controls), were selected for the metabolic profiling described next.

Study Protocol

Data were obtained on each patient's age, sex, race, weight, cardiac risk factors, prior cardiac disease, and cardiac medications. Patients underwent exercise testing with the standard Bruce protocol.[13] Symptoms, heart rate, blood pressure, and a 12-lead ECG were recorded before the test, midway through each stage, and during recovery. The stress test was terminated if there was physical exhaustion, severe angina, a >2-mm horizontal or downsloping ST-segment depression, a ≥20-mm Hg fall in systolic blood pressure, or sustained ventricular arrhythmia. Duration of the stress test, metabolic equivalents achieved, peak heart rate, and peak blood pressure were recorded. If the patient developed angina during the test, the timing, quality (typical versus atypical), and effect on the test (limiting or nonlimiting) were noted. The maximal horizontal or downsloping ST-segment changes were recorded in each ECG lead.

Single-Photon Emission Computed Tomography Myocardial Perfusion Imaging

A stress-rest imaging protocol was used. ^{99}Tc-tetrofosmin was administered at peak stress, and imaging was performed soon thereafter. Four hours later, a second injection was administered, and repeated imaging was performed. Quantitative analysis of perfusion was performed with the CEqual method to calculate the percent reversible and fixed perfusion defects.[14] Patients with a >5% reversible perfusion defect were selected as cases, and those without any perfusion defect were selected as controls. Left ventricular ejection fraction was calculated with the use of commercially available software.[15]

High-Performance Liquid Chromatography and Mass Spectrometry Analysis

Blood samples were obtained immediately before, immediately after, and 4 hours after stress testing. Blood samples were placed on ice and processed within 60 minutes. Plasma was stored at $-80°C$, and aliquots were thawed for these analyses. Amino acids and amines were separated on a Luna phenyl-hexyl column (Phenomenex) by reverse-phase chromatography with an acetonitrile/water/0.1% acetic acid mixture at pH 3.5 to 4.0 for a run time of 1.5 minutes. Sugars and ribonucleotides were separated on a Luna amino column (Phenomenex) by normal-phase chromatography with an acetonitrile/water/0.25% ammonium hydroxide/10 mmol/L ammonium acetate mixture at pH 11 for a run time of 3.5 minutes. Organic acids were separated on a Synergi Polar-RP column (Phenomenex) by reverse-phase chromatography with an acetonitrile/water/5 mmol/L ammonium acetate mixture at pH 5.6 to 6.0 for a run time of 3.5 minutes. Columns were connected in parallel with an automated switching valve on a robotic sample loader (Leap Technologies). A triple-quadrupole mass spectrometer (API4000, Applied Biosystem/Sciex) was operated in an automated switching polarity mode with a turbo ion spray liquid chromatography/mass spectrometry interface under selected reaction monitoring conditions. A total of 477 parent/daughter ion pairs were monitored through 6 selected reaction monitoring experiments for each sample.

Peak areas for each parent/daughter ion pair were integrated, and analytes with areas below the limit of detection of the liquid chromatography–tandem mass spectrometry were excluded from further analysis. Peak area ratios to an internal standard were computed to normalize variation in injection volume. The peak area ratios were then logarithmically transformed, and the logarithms of peak area ratios per sample were normalized by subtracting the median of all analytes to account for sample-to-sample variation in blood concentration.

One hundred seventy-three of the analytes assayed were known, having been evaluated by high-accuracy mass spectrometry in studies of purified compounds spiked into plasma across a range of concentrations. In prior studies, the coefficient of variation at typical circulating plasma concentrations was <10% in 25% of the analytes, 10% to 20% in 35% of the analytes, 20% to 30% in 20% of the analytes, and >30% in the remainder. Some of the low-molecular-weight peaks seen reproducibly in human plasma have not yet been unambiguously identified and are designated as such by the prefix MET. A list of the known metabolites analyzed is included in the Data Supplement Table I.

Statistical Analysis

For baseline characteristics of cases and controls, continuous variables were compared with Student's *t* test, and categorical variables were compared with Fisher's exact test. Metabolites for which the distribution of the logarithmically transformed levels in the study population had absolute values of skewness and kurtosis <1 and a nonsignificant Wilkes-Shapiro test were deemed to have a normal distribution and analyzed with parametric tests; metabolites for which the distribution failed to meet these criteria were analyzed with nonparametric tests. The significance of the change in logarithmically transformed metabolite levels from pretest to posttest values was assessed by paired Student's *t* tests or Wilcoxon signed-rank tests, as appropriate. Changes in metabolites are expressed as percentage increases or decreases from the untransformed baseline levels, and for the sake of consistence, medians and interquartile ranges (IQRs) are used for all metabolites. To compare the correla-

tion between logarithmically transformed metabolite levels and degree of exertion or extent of ischemic myocardium, correlation coefficients were calculated.

To perform functional trend analysis, we developed software based on FuncAssociate (for details, see Berriz et al[16]; also http://llama.med.harvard.edu/cgi/func/funcassociate). We characterized metabolites with attributes from the KEGG database (www.genome.jp/kegg). These attributes are of the form "participates in reaction *R*," "participates in pathway *P*," or "is associated with human disease *D*." (Because we were particularly interested in identifying pathways involving multiple metabolites, we used only attributes that were associated with at least 3 metabolites. The total number of attributes examined was 96.) We then ranked metabolites as follows. For every metabolite, we applied a Wilcoxon rank-sum test; for controls, we used a 1-sample test against the null hypothesis of zero exercise-related change in the metabolite (to determine which metabolites increased in controls simply in response to exercise); for cases, we used a 2-sample test against the null hypothesis that ischemic patients and control patients had the same exercise-related response (to determine which metabolites increased in cases in response to exercise but did not rise in controls in response to exercise). The metabolites were then sorted by the signed significance of each respective test. Signed significance is defined as the negative of the logarithm (base 10) of the test probability value, multiplied by the sign of the median (in the case of the list of metabolites obtained from controls) or the difference in medians between the 2 samples (in the case of the list of metabolites obtained from cases). For the subsequent analysis, we discarded the unknown metabolites. With this procedure, we generated 2 ranked lists of metabolites, 1 for cases and 1 for controls. Then, for each of these 2 ranked lists of metabolites, together with the same lists in reverse order (4 ranked lists in total), we used a cumulative hypergeometric test (Fisher's exact test) at each possible rank threshold to score attributes of these metabolites according to their degree of overrepresentation among metabolites above the rank threshold. Specifically, for each metabolite attribute *A* and each "initial *k*-sublist" of metabolites (which is the ranked list of metabolites consisting of the first *k* metabolites in the original ranked list), we computed the Fisher's exact test probability value for the categorical variables "belongs to initial *k*-sublist" and "has attribute *A*." To each attribute, we assigned the *k*-sublist with the smallest probability value and ranked the attributes in ascending order by this probability value. Then, for each ranked list of metabolites, we repeated this analysis 1000 times with random permutations of the original ranked metabolite list as input. The null hypothesis for each ranked list was that no metabolite attribute is more enriched among the top-ranked metabolites than would be expected from a randomly ranked list of metabolites. To limit type I errors, the multiple-hypothesis-corrected (adjusted) probability value for a given metabolite attribute is the fraction of random control runs with an unadjusted probability value (for any metabolite attribute) less than or equal to the observed unadjusted probability value for the metabolite attribute of interest. (For example, if the unadjusted probability value was 0.002 and the adjusted probability value was 0.01, this means that after generating 1000 random permutations of the data, the fraction of permutations in which the unadjusted probability value was ≤0.002 was 0.01.) This procedure has been described elsewhere in detail.[16]

Differences between the change (before versus after exercise testing) in a metabolite in cases versus controls were compared by Wilcoxon rank-sum tests. For metabolites that displayed significantly discordant regulation in cases versus controls ($P<0.01$), cutpoints were selected according to receiver-operator characteristic curve analysis to maximize accuracy. A metabolic risk score was computed by assigning patients 1 point for each metabolite for which the change exceeded the cutpoint for ischemia. To estimate the degree of optimism in the discriminatory ability of our score, 6-fold cross-validation was performed.[17,18] The dataset was randomly divided into 6 subsets, each containing 3 cases and 3 controls. With the methodology described earlier, a metabolic score was developed in a training set containing 5 subsets. This score was then validated in a testing set consisting of the remaining withheld subset. This

TABLE 1. Patient Characteristics

	Controls (n=18)	Cases (n=18)	P
Demographics			
Age, y	64±10	65±11	0.66
Male	9 (50)	15 (83)	0.08
White	12 (67)	16 (89)	0.23
Cardiac risk factors			
Hypertension	14 (78)	13 (72)	>0.99
Diabetes			0.13
Insulin dependent	0	4 (22)	
Non–insulin dependent	5 (28)	5 (28)	
None	13 (72)	9 (50)	
Smoking			0.04
Current	0	1(6	
Former	7 (39)	13 (72)	
Never	11 (61)	4 (22)	
Hyperlipidemia	11 (61)	14 (78)	0.47
No. of cardiac risk factors	2.1±0.9	3.0±0.9	0.02
Prior cardiovascular disease			
Coronary artery disease	5 (28)	15 (83)	0.002
Myocardial infarction	4 (22)	13 (72)	0.007
Coronary revascularization	5 (28)	12 (67)	0.04
Congestive heart failure	0 (0)	5 (28)	0.05
Peripheral arterial disease	0 (0)	3 (17)	0.23
Long-term cardiovascular medications			
Aspirin	13 (72)	16 (89)	0.40
β-Blockers	14 (78)	16 (89)	0.66
Calcium channel blockers	4 (22)	8 (44)	0.29
Nitrates	2 (11)	8 (44)	0.06

Values are presented as mean±SD or n (%).

process was repeated so that each subject in the dataset was used in 1 testing set. The *c*-statistics in each testing set were then averaged to provide a cross-validated *c*-statistic.

Results

A total of 36 patients undergoing exercise stress testing with myocardial perfusion imaging served as the study population: 18 with no evidence of ischemia (controls) and 18 with evidence of inducible ischemia (cases). The baseline characteristics and stress test performance parameters for these patients are listed in Table 1. The mean ages of the 2 groups were comparable, although as expected, patients with inducible ischemia had slightly more cardiac risk factors (3.0±0.9 versus 2.1±0.9) and were more likely to have a documented history of coronary disease.

The exercise stress test results of cases and controls are shown in Table 2. By design, all 18 cases had reversible perfusion defects, with the mean percentage of myocardium with a reversible perfusion defect being 17±8%, whereas no controls had any degree of a reversible perfusion defect. Although coronary angiography was not mandated by the protocol of this study, 14 of the 18 cases did undergo coronary angiography, and all 14 had angiographic confirma-
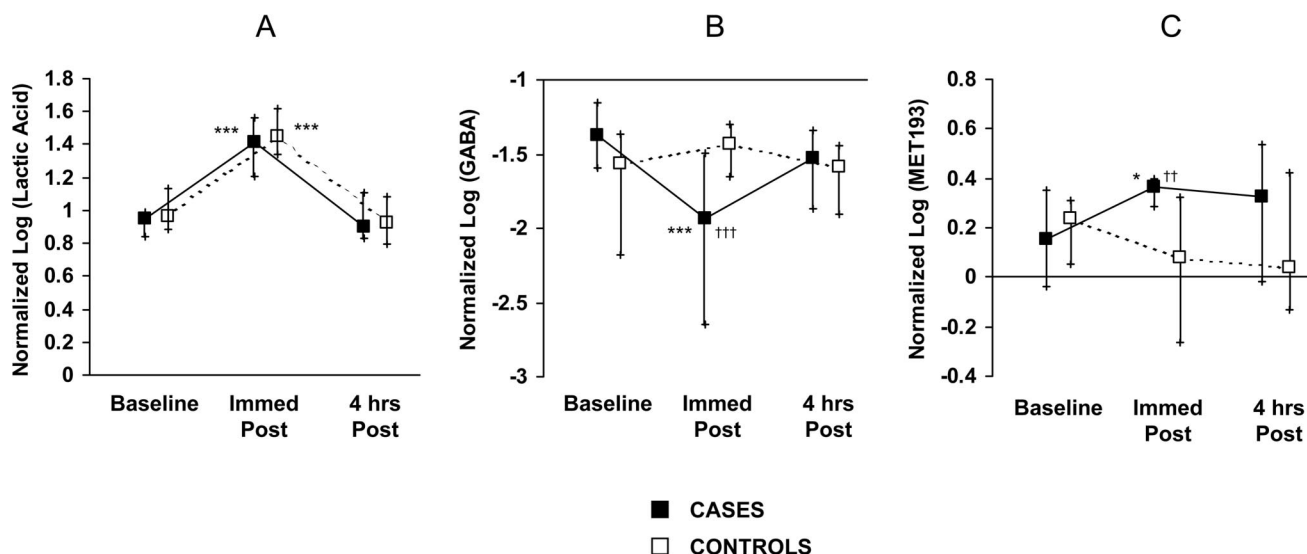
TABLE 2. Stress Test Performance Results

Parameters	Controls (n=18)	Cases (n=18)	P
Duration, min	8.8±2.3	6.8±2.2	0.01
Metabolic equivalents	10.0±2.6	7.9±2.7	0.03
Ejection fraction <50%	2 (11)	5 (28)	0.40
Chest pain	7 (39)	9 (50)	0.74
ST-segment deviation ≥1 mm	2 (12)	10 (56)	0.01
Percentage of myocardium with any perfusion defect, mean±SD	3±4	28±12	...
Percentage of myocardium with reversible perfusion defect, mean±SD	0±0	17±8	...

Data are presented as mean±SD or n (%) of patients. Because cases and controls were selected on the basis of their reversible perfusion defects during stress testing, no statistical comparisons were performed.

tion of multivessel or severe, complex, single-vessel coronary artery disease.

Metabolic Profiling

For each metabolite, the statistical significance of the change in the circulating level from immediately before exercise to immediately after exercise was calculated separately in cases and controls. The results are plotted in Figure 1, in which the position on the *x* axis represents the statistical significance of the change in controls, and the position on the *y* axis represents the statistical significance of the change in cases. Metabolites on the right half of the scatterplot increased in controls after stress testing, whereas metabolites on the left half decreased. Similarly, metabolites on the top half of the scatterplot increased in cases, whereas metabolites on the bottom half decreased.

The majority of metabolites displayed concordant changes in cases and controls (ie, increased in both or decreased in both). The upper right quadrant of Figure 1 contains metabolites that increased in both cases and controls. For example, immediately after exercise, median levels of lactic acid, an end product of glycolysis when the amount of oxygen is limiting, increased by 177% (IQR, 105% to 257%; $P<0.0001$]. The changes observed after exercise were similar in cases and controls (Figure 2A) and had resolved by 4 hours after exercise. Similarly, median levels of metabolites involved in skeletal muscle AMP catabolism increased after exercise in both cases and controls (upper right quadrant of Figure 1). These included hypoxanthine (46%; IQR, −8% to 106%; $P=0.0004$) and inosine (67%; IQR, −18% to 175%; $P=0.003$). In addition, median levels of alanine, a nitrogen transporter exported by skeletal muscle, also increased after exercise in cases and controls (19%; IQR, 2% to 35%; $P<0.0001$).

We then examined metabolites that demonstrated discordant regulation between cases and controls. As shown at the bottom center of Figure 1, plasma levels of γ-aminobutyric acid and MET 288 decreased strikingly in cases (−77%; IQR, −37% to −94%; $P=0.0004$; and −65%; IQR, −23% to −85%, $P=0.001$, respectively) but remained unchanged in controls. The levels of γ-aminobutyric acid in cases and controls are shown over time in Figure 2B, which illustrates how levels returned to baseline in cases by 4 hours. We also observed significant decreases in the levels of oxaloacetate (−25%; IQR, 5% to −39%; $P=0.023$), citrulline (−25%; IQR, 2% to −36%; $P=0.009$), and argininosuccinate (−73%; IQR, 25% to −84%; $P=0.012$) in cases only. Both oxaloacetate ($r=−0.65$, $P=0.0035$) and citrulline ($r=0.46$, $P=0.054$) exhibited moderately strong trends toward correlating with the extent of the perfusion defect during stress testing. As shown in the lower right quadrant of Figure 1, 3 metabolites were significantly differentially regulated in

Figure 1. *X-y* scatterplot of the statistical significance (see Methods) of the changes in metabolite levels from baseline to immediately after exercise testing. The position on the *x* axis represents the statistical significance of the change in controls, and the position on the *y* axis represents the statistical significance of the change in cases. Metabolites on the right half of the scatterplot increased in controls after stress testing, whereas metabolites on the left half decreased. Similarly, metabolites on the top half of the scatterplot increased in cases after exercise, whereas metabolites on the bottom half decreased. Metabolites whose concentrations changed significantly ($P<0.05$) after stress testing in either cases or controls are shown as colored circles, the rest as black dots. Red indicates that the concentration of the metabolite increased; green, that it decreased. The color of the rim of the circle indicates the direction of change in controls, whereas the center indicates the direction of change in cases. Some of the low-molecular-weight peaks seen reproducibly in human plasma have not yet been unambiguously identified and are designated as such by the prefix MET.

Figure 2. Median and IQRs of normalized metabolite levels (logarithmically transformed values) in patients with ischemia (closed squares) and in those without (open squares) at all 3 time points (baseline, immediately after stress testing, and 4 hours after stress testing). The degree of statistical significance for the change compared with baseline levels is indicated by *$P<0.05$, **$P<0.01$, or ***$P<0.001$. The degree of statistical significance for the comparison between the change in cases versus controls is indicated by †$P<0.05$, ††$P<0.01$, or †††$P<0.001$. GABA indicates γ-aminobutyric acid; other abbreviations are as defined in text.

cases (decreased) and controls (increased), including uric acid ($P=0.0006$), citric acid ($P=0.008$), and MET 200 ($P=0.008$). Conversely, MET 193 ($P=0.0068$) and MET 221 ($P=0.01$) increased in cases (with the changes in MET 193 persisting through 4 hours after the ischemic insult, Figure 2C) but decreased in controls (upper left of Figure 1). Of note, in this small clinical cohort, there was no evidence of significant heterogeneity in the magnitude of the changes in metabolites in cases with and without diabetes, hyperlipidemia, heart failure, or peripheral arterial disease or in those taking or not taking aspirin, β-blockers, calcium channel blockers, or nitrates, consistent with the notion that the changes are due to myocardial ischemia rather than cardiac risk factors.

Functional Pathway Analysis

To determine whether our observations of changes in individual metabolites in the setting of myocardial ischemia in fact reflected coordinate changes in defined metabolic pathways, we developed software to identify functional or pathway trends. This software was based on FuncAssociate, originally designed to reveal pathway trends in high-throughput mRNA expression data.[16] We used a priori annotation of metabolites according to their associated reactions, pathways, and diseases from the KEGG database (www.genome.jp/kegg). Analysis of all known metabolites in our dataset revealed that members of the citric acid pathway were significantly overrepresented in the list of metabolites that changed specifically in the setting of myocardial ischemia, with 6 members of the citric acid cycle pathway falling within the top 23 most-changed metabolites ($P=0.00031$, $P=0.04$ after adjusting for multiple testing; see Methods).

Ischemia Risk Score

On the basis of these observations, we then investigated whether metabolic profiling could be used to accurately

distinguish patients with ischemia from those without. With receiver-operator characteristic curve analysis, cutpoints were selected for the change in the 6 most discordantly regulated metabolites (Figure 3). A metabolic ischemia risk score was created by assigning patients 1 point for each metabolite for which the change with exercise exceeded the cutpoint for ischemia (Figure 4). The score yielded a highly statistically significant relation to the probability of ischemia ($P<0.0001$). Six-fold cross-validation was performed in which a metabolic score was developed in a training subset and then validated in a distinct testing subset. This process was repeated 6 times, and the c-statistic was averaged over the iterations. This revealed excellent discriminatory ability, with a c-statistic of 0.83.

Discussion

Investigators have begun to incorporate a number of emerging technologies as part of a systems biology approach to the identification of disease states. One specific focus of recent investigation has tested the concept that perturbations that arise either as a cause or a consequence of disease may be detected as particular patterns of metabolites or proteins in the blood. To that end, we now demonstrate the application of metabolomics to myocardial ischemia in a carefully characterized cohort of 36 patients undergoing exercise stress testing. Our study has 3 major findings. First, using state-of-the-art metabolic profiling, we were able to demonstrate significant changes after exercise stress testing in circulating levels of multiple metabolites. To our knowledge, this is the first example of metabolomics applied to acute myocardial ischemia in human subjects. Second, we were able to identify distinct clusters of related metabolites that demonstrated coordinate responses to either exercise in some cases or to ischemia in others. Third, we were able to use metabolic profiling to differentiate patients who developed inducible
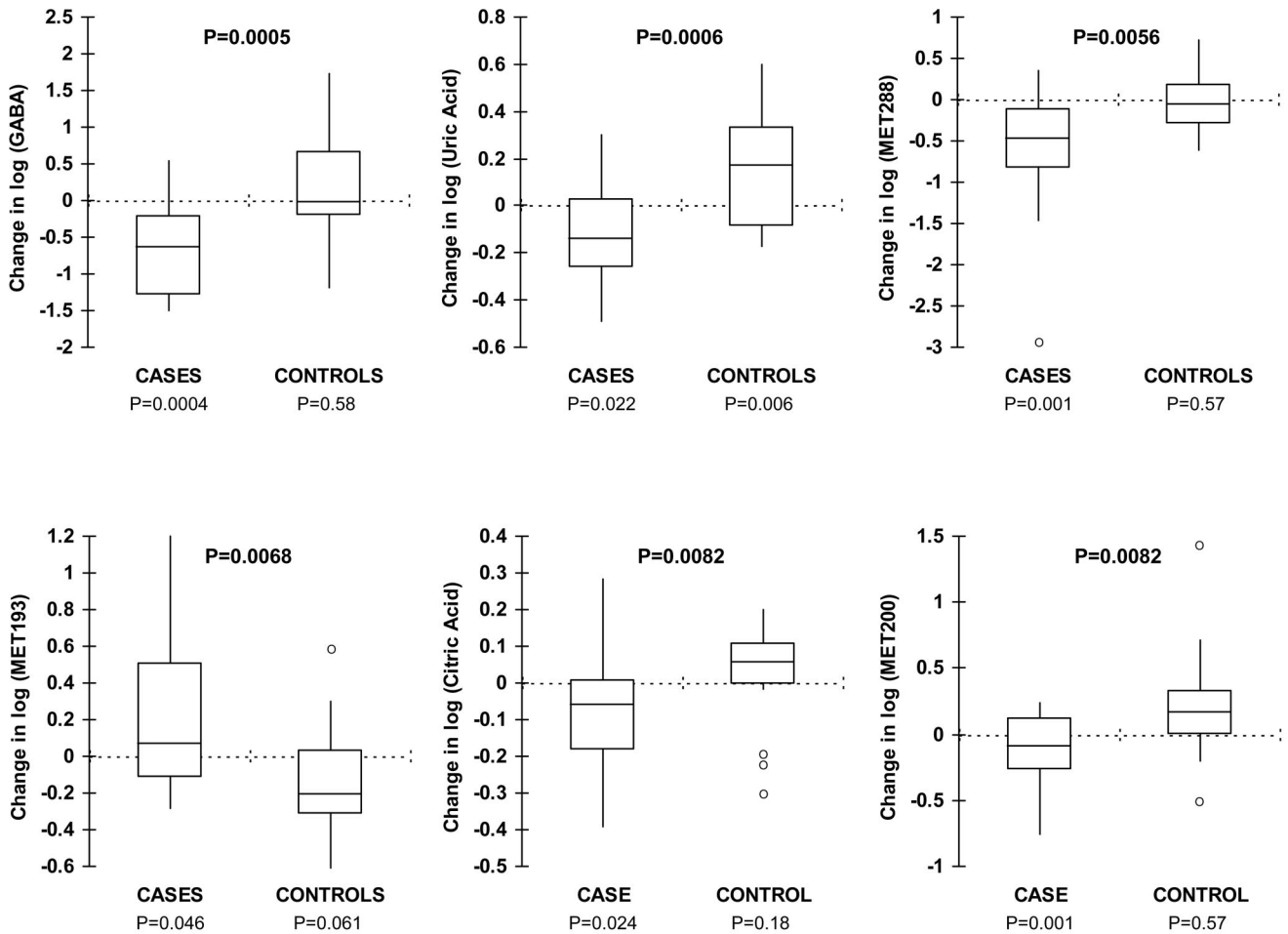
Figure 3. Box-and-whisker plots of the changes seen immediately after stress testing in 6 metabolites in cases and controls. The line in the box represents the median change in the normalized logarithm value; the lower and upper boundaries of the box represent the 25th and 75th percentiles, respectively; the lower and upper whiskers represent the 5th and 95th percentiles, respectively; and open circles represent the outliers. For each metabolite, P values shown below cases and controls indicate the significance of the change from baseline. P values shown at the top measure the significance in the difference in the change between cases and controls. GABA indicates γ-aminobutyric acid; other abbreviations are as defined in text.

ischemia from those who did not with a high degree of accuracy.

Metabolic Profiling Approaches

NMR has been used successfully for "metabolic footprinting" of lower organisms such as the yeast *Saccharomyces cerevisiae*.[8,19] In those studies, metabolic profiling of conditioned medium was used to "diagnose" otherwise-silent mutant phenotypes. In terms of cardiovascular biology, 1 report also applied pattern-recognition techniques to proton NMR spectra of human sera to aid in the noninvasive diagnosis of chronic coronary artery disease, although no studies to date have explored biomarkers of acute myocardial ischemia.[9] In the previously published studies, however, the metabolites that underlay the spectroscopy peaks were not unambiguously identified. Furthermore, NMR is much less sensitive than the mass spectrometry–based approach used for the present studies.[10,20] An important rationale for unequivocally identifying analytes or surveying known analytes is to gain insight into the functionally relevant cellular mechanisms contributing to disease pathways. Having hundreds of named

metabolites allowed us to identify multiple participants in particular biological pathways moving in tandem, which enhanced confidence that individual participants in that pathway were truly correlated with the perturbation. In principle, incorporating knowledge of pathways into candidate marker triage increases the likelihood that selected biomarkers will be validated in subsequent prospective studies. The use of pathway analysis should also prove advantageous in our ongoing efforts to identify novel peaks according to recently developed techniques, such as Fourier transform mass spectrometry. Thus, a key goal of future investigation is to develop a more comprehensive survey of metabolites to better inform underlying biological processes, both by identifying the novel peaks and by incorporating more known compounds into the platform.

Functional Pathway Analysis

Through application of novel functional trend software, we have demonstrated coordinate, highly statistically significant changes in circulating levels of metabolites belonging to the citric acid pathway. Moreover, though not a direct participant
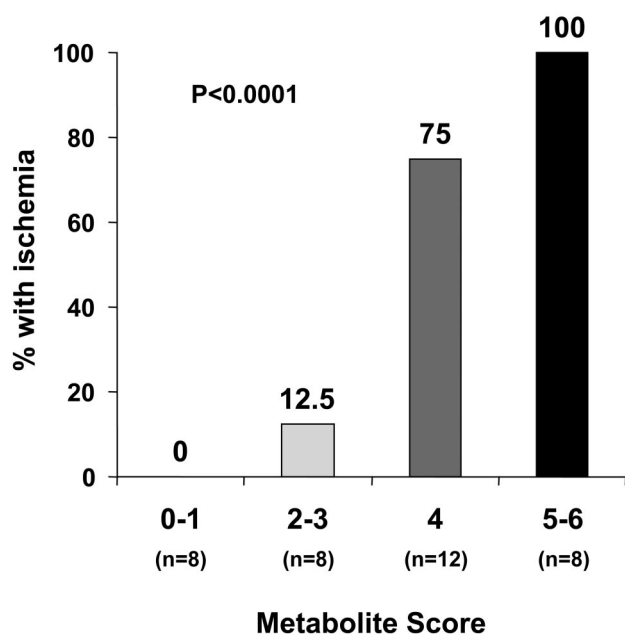
Figure 4. Bar graph, showing the proportion of patients with inducible ischemia among patients categorized by the metabolic risk score.

in the citric acid cycle, γ-aminobutyric acid is an end product of the metabolism of 1 of the members of the citric acid cycle (α-ketoglutarate), and citrulline and argininosuccinate are members of the urea cycle, which feeds into the citric acid cycle. All 3 of these metabolites also decreased in cases but not in controls.

The citric acid cycle plays a central role in oxidative phosphorylation in the myocardium. Cardiomyocyte levels of citric acid cycle intermediates are tightly regulated to ensure adequate throughput of substrates derived from glycolysis and β-oxidation of fatty acids. In normal cardiomyocytes, there is a constant rate of mitochondrial efflux of citric acid cycle intermediates (cataplerosis). In the setting of acute ischemia, preservation of citric acid cycle intermediates becomes of paramount importance to defend ATP production. Coronary occlusion in an in vivo porcine model resulted in an abrupt 60% to 70% increase in myocardial citric acid content.[21] Furthermore, by cannulating the anterior interventricular vein in an in vivo porcine model, Panchal et al[22] demonstrated that an abrupt decrease in left anterior descending coronary artery flow resulted in an 80% decrease in citric acid efflux from the myocardium. These data support the notion that the metabolic changes that we observed are a direct consequence of myocardial ischemia and extend prior studies by their direct application to humans.

Limitations

Because high-throughput metabolomics methodologies are still under development, our overall approach to enhance biomarker and pathway discovery emphasized the in-depth analysis of a small, extremely well-phenotyped patient cohort. However, our study has several potential limitations that should be considered. First, although serial sampling in patients who served as their own biological controls helped

diminish interindividual variability and signal-to-noise problems, our study population was nevertheless small. Thus, it is important to note that changes in metabolites that failed to reach nominal significance in our study still may be scientifically important and bear further investigation. For this reason, biological pathway trend analysis offers increased power to detect subtle but significant differences. Further testing in larger cohorts will provide the opportunity for both confirmation and exploration of subgroups of interest, including those based on sex, race, and comorbidities, which our study was underpowered to do. Moreover, larger datasets will provide sufficient precision in the estimates of the utility of each marker to allow for appropriate relative weighting of each component. Second, we selected cases and controls on the basis of clear-cut evidence on perfusion stress testing imaging for or against myocardial ischemia, respectively. This approach may have influenced which metabolites were altered, the magnitude of the perturbations, and the utility of our diagnostic score. Future evaluation of patients with more modest degrees of ischemia will be important. On the other hand, the ischemic insult in our experimental model was brief. This is an unavoidable consequence of our model, in which prolonged ischemia is not permitted during exercise stress testing. In contrast, spontaneous ischemia tends to be more severe and more prolonged than that induced during stress testing and thus bodes well for the utility of these biomarkers in the setting of acute coronary syndromes. However, whether ischemia due to coronary plaque rupture and decreased supply generates the same metabolic profile as ischemia due to increased myocardial demand remains to be seen. Third, even metabolites whose levels changed exclusively in cases and not in controls may have derived from a noncardiac source. Data from animal studies support a cardiac source for several of our metabolites, but future studies of coronary sinus samples will be necessary to confirm this supposition. Fourth, although our metabolic profile score had excellent discriminatory power in 6-fold cross-validation, these findings must be further tested in a separate, large validation cohort, which will also permit comparison with and adjustment for traditional cardiovascular risk factors and other exercise test performance parameters.

Conclusions

Taken together, our findings provide important evidence that current technologies can be used to identify clinically relevant perturbations in circulating metabolites. Furthermore, consideration of metabolites as pathway members rather than stand-alone entities begins to shed insight into exercise performance and myocardial ischemia. We expect that metabolomic studies will increasingly add to our diagnostic armamentarium and ultimately identify new targets for therapeutic intervention.

Acknowledgments

References

1. Gaziano JM. Global burden of cardiovascular disease. In: Braunwald E, Zipes DP, Libby P, eds. *Heart Disease: A Textbook of Cardiovascular Medicine.* 6th ed. Philadelphia: W.B. Saunders Company;2001:1–18.

2. Gibbons R, Abrams J, Chatterjee K, Daley J, Deedwania P, Douglas J, Ferguson TJ, Fihn S, Fraker TJ, Gardin J, O'Rourke R, Pasternak R, Williams S. ACC/AHA 2002 guideline update for the management of patients with chronic stable angina: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1999 Guidelines for the Management of Patients with Chronic Stable Angina). Available at: www.acc.org/clinical/guidelines/stable/stable.pdf. Accessed March 30, 2005.

3. Braunwald E, Antman EM, Beasley JW, Califf RM, Cheitlin MD, Hochman JS, Jones RH, Kereiakes D, Kupersmith J, Levin TN, Pepine CJ, Schaeffer JW, Smith EE 3rd, Steward DE, Theroux P. ACC/AHA guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on the Management of Patients With Unstable Angina). 2002. Available at: http://www.acc.org/clinical/guidelines/unstable/unstable.pdf. Accessed March 30, 2005.

4. Ritchie JL, Bateman TM, Bonow RO, Crawford MH, Gibbons RJ, Hall RJ, O'Rourke RA, Parisi AF, Verani MS. Guidelines for clinical use of cardiac radionuclide imaging: a report of the American College of Cardiology/American Heart Association Task Force on assessment of diagnostic and therapeutic cardiovascular procedures (Committee on Radionuclide Imaging)–developed in collaboration with the American Society of Nuclear Cardiology. *J Nucl Cardiol.* 1995;2:172–192.

5. Morrow DA, de Lemos JA, Sabatine MS, Antman EM. The search for a biomarker of cardiac ischemia. *Clin Chem.* 2003;49:537–539.

6. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov.* 2003;2:668–676.

7. He W, Miao FJ, Lin DC, Schwandner RT, Wang Z, Gao J, Chen JL, Tian H, Ling L. Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature.* 2004;429:188–193.

8. Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ, Westerhoff HV, van Dam K, Oliver SG. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Nat Biotechnol.* 2001; 19:45–50.

9. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligin E, Mosedale DE, Grainger DJ. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ¹H-NMR-based metabonomics. *Nat Med.* 2002;8: 1439–1444.

10. Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L. Metabolite profiling for plant functional genomics. *Nat Biotechnol.* 2000; 18:1157–1161.

11. Rashed MS. Clinical applications of tandem mass spectrometry: ten years of diagnosis and screening for inherited metabolic diseases. *J Chromatogr B Biomed Sci Appl.* 2001;758:27–48.

12. Vigneau-Callahan KE, Shestopalov AI, Milbury PE, Matson WR, Kristal BS. Characterization of diet-dependent metabolic serotypes: analytical and biological variability issues in rats. *J Nutr.* 2001;131:924S–932S.

13. Fletcher GF, Balady GJ, Amsterdam EA, Chaitman B, Eckel R, Fleg J, Froelicher VF, Leon AS, Pina IL, Rodney R, Simons-Morton DA, Williams MA, Bazzarre T. Exercise standards for testing and training: a statement for healthcare professionals from the American Heart Association. *Circulation.* 2001;104:1694–1740.

14. Garcia EV, Cooke CD, Van Train KF, Folks R, Peifer J, DePuey EG, Maddahi J, Alazraki N, Galt J, Ezquerra N, Ziffer J, Areedab J, Berman DS. Technical aspects of myocardial SPECT imaging with technetium-99m sestamibi. *Am J Cardiol.* 1990;66:23E–31E.

15. DePuey EG, Nichols K, Dobrinsky C. Left ventricular ejection fraction assessed from gated technetium-99m-sestamibi SPECT. *J Nucl Med.* 1993;34:1871–1876.

16. Berriz GF, King OD, Bryant B, Sander C, Roth FP. Characterizing gene sets with FuncAssociate. *Bioinformatics.* 2003;19:2502–2504.

17. Stone M. Cross-validatory choice and assessment of statistical predictors. *J R Stat Soc.* 1974;(series B, 36):111–147.

18. Efron B, Gong G. A leisurely look at the bootstrap, the jackknife, and the cross-validation. *Am Statistician.* 1983;37:36–48.

19. Allen J, Davey HM, Broadhurst D, Heald JK, Rowland JJ, Oliver SG, Kell DB. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. *Nat Biotechnol.* 2003;21: 692–696.

20. Fiehn O. Metabolomics—the link between genotypes and phenotypes. *Plant Mol Biol.* 2002;48:155–171.

21. Hassel B, Ilebekk A, Tonnessen T. Cardiac accumulation of citrate during brief myocardial ischaemia and reperfusion in the pig in vivo. *Acta Physiol Scand.* 1998;164:53–59.

22. Panchal AR, Comte B, Huang H, Dudar B, Roth B, Chandler M, Des Rosiers C, Brunengraber H, Stanley WC. Acute hibernation decreases myocardial pyruvate carboxylation and citrate release. *Am J Physiol Heart Circ Physiol.* 2001;281:H1613–H1620.