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Combined Metabolomic and Proteomic Analysis of Human Atrial Fibrillation

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Objectives We sought to decipher metabolic processes servicing the increased energy demand during persistent atrial fibrillation (AF) and to ascertain whether metabolic derangements might instigate this arrhythmia.

Background Whereas electrical, structural, and contractile remodeling processes are well-recognized contributors to the self-perpetuating nature of AF, the impact of cardiac metabolism upon the persistence/initiation of this resilient arrhythmia has not been explored in detail.

Methods Human atrial appendage tissues from matched cohorts in sinus rhythm (SR), from those who developed AF post-operatively, and from patients in persistent AF undergoing cardiac surgery were analyzed using a combined metabolomic and proteomic approach.

Results High-resolution proton nuclear magnetic resonance (NMR) spectroscopy of cardiac tissue from patients in persistent AF revealed a rise in beta-hydroxybutyrate, the major substrate in ketone body metabolism, along with an increase in ketogenic amino acids and glycine. These metabolomic findings were substantiated by proteomic experiments demonstrating differential expression of 3-oxoacid transferase, the key enzyme for ketolytic energy production. Notably, compared with the SR cohort, the group susceptible to post-operative AF showed a discordant regulation of energy metabolites. Combined principal component and linear discriminant analyses of metabolic profiles from proton NMR spectroscopy correctly classified more than 80% of patients at risk of AF at the time of coronary artery bypass grafting.

Conclusions The present study characterized the metabolic adaptation to persistent AF, unraveling a potential role for ketone bodies, and demonstrated that discordant metabolic alterations are evident in individuals susceptible to post-operative AF. (J Am Coll Cardiol 2008;51:585–94) © 2008 by the American College of Cardiology Foundation

Atrial fibrillation (AF), the most common arrhythmia encountered in clinical practice, results in significant morbidity and mortality. A significant proportion of patients with paroxysmal AF develop persistent or even permanent AF over time, but the exact reasons for this remain unknown (1).

Animal models of pacing-induced AF have demonstrated that AF is a self-perpetuating arrhythmia (i.e., the longer it is present, the more persistent it becomes), leading to the notion that "AF begets AF" (2). Atrial fibrillation leads to several different forms of remodeling within the atria—electrical, contractile, and structural (3). Electrical and contractile remodeling appear to be closely related through a down-regulation of the L-type Ca^{2+} current and are fully reversed within a few days of sinus rhythm (SR) having been restored (4). Structural remodeling, on the other hand, encompasses a plethora of potentially irreversible intracellular and extracellular processes (5). Whereas electrical, structural, and contractile remodeling processes are well-recognized contributors to the self-perpetuating nature of AF, the impact of cardiac metabolism upon the initiation/persistence of this resilient arrhythmia has not been explored in detail.

A recent microarray analysis in patients with permanent AF revealed ventricularization of gene expression

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Abbreviations and Acronyms

AF = atrial fibrillation
CABG = coronary artery bypass grafting
CRP = C-reactive protein
MALDI = matrix-assisted laser-desorption ionization
NMR = nuclear magnetic resonance
PCA = principal component analysis
SR = sinus rhythm

and prominent up-regulation of transcripts involved in metabolic activities (6), including several glycolytic enzymes, suggesting a switch to glucose metabolism in AF. However, deduction of metabolic changes based on messenger ribonucleic acid transcript analysis requires confirmation at the protein and metabolite levels. We previously demonstrated that the combined application of new technologies, such as proteomics and metabolomics, allows novel insights into the pathophysiology of various cardiovascular diseases (7). To further clarify metabolic processes at work during human persistent AF and to ascertain whether metabolic derangements might play a role in instigating this resilient arrhythmia, we analyzed human atrial tissues from matched cohorts in SR, from those who developed AF post-operatively (SR-AF group), and from patients in persistent AF (AF group) using a combined metabolomic and proteomic approach.

Materials and Methods

Patients. Atrial appendages were obtained as surgical specimens from patients undergoing cardiac surgery by use of established procedures approved by our local Regional Ethics Committee Board. Informed consent was obtained from all patients recruited into the study. All subjects in the AF cohort had severe nonrheumatic valvular disease as the principal underlying pathology for surgery. Patients with rheumatic valvular disease, mixed valvular pathophysiology, impaired glucose tolerance, and diabetes mellitus were excluded from the study. Recruited subjects with AF had had the documented arrhythmia for at least 1 year (geometric mean 4.4 years). Patients in the SR-AF cohort were undergoing either coronary artery bypass grafting (CABG) or valve surgery. For comparison, matched patients in SR were screened to ensure that they had never experienced AF by direct questioning about symptoms suggestive of such and by the retrospective analysis of all 12-lead electrocardiograms during their entire preoperattive review periods. No patients in the SR cohort had ever been or were on any antiarrhythmic drugs other than beta-blockers or calcium channel blockers. Cohorts were matched for those patient characteristics that had been highlighted to be relevant by the largest prospective and longitudinal multicenter analysis of post-cardiac surgery AF (8). Atrioventricular nodal blocking agents in the AF cohort included beta-blockers, calcium channel blockers, and digoxin; no subjects in AF were on other antiarrhythmic drugs at the time of surgery. Tissue was harvested during cannulation of the right atrium for bypass, and specimens were immediately snap-frozen and stored in liquid nitrogen.

Proton nuclear magnetic resonance (NMR) spectroscopy. Snap-frozen hearts were extracted in 6% perchloric acid (9). Neutralized extracts were freeze-dried and reconstituted in deuterium oxide. One-half milliliter of each extract was placed in 5-mm NMR tubes. ^{1}H-NMR spectra were obtained using a Bruker 600-MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The water resonance was suppressed by using gated irradiation centered on the water frequency. Fifty microliters of 5 mM sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate in deuterium oxide was added to the samples for chemical shift calibration and quantification. Immediately before the NMR analysis, the pH was readjusted to 7 with perchloric acid or potassium hydroxide.

Proteomic analysis. A detailed methodology for proteomic analysis and mass spectrometry is available in the Online Appendix and on our web site at http://www.vascular-proteomics.com.

Western blotting. Twenty-five micrograms of protein was separated on 8% or 12% Tris-glycine gels (Invitrogen Limited, Paisley, United Kingdom) and blotted to nitrocellulose. Membranes were blocked in 5% phosphate-buffered saline and milk. Consequently, they were probed with primary antibodies to peroxiredoxin 1 (LF-PA0001, 1:100; Lab Frontier, Seoul, Korea), superoxide dismutase (FL-154, 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, California), alpha/B crystallin (SPA-223, 1:100; Stressgen, Ann Arbor, Michigan), and desmin (ab8470, 1:200; Abcam, Cambridge, Massachusetts). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (GE Healthcare, Buckinghamshire, United Kingdom) was applied and proteins were detected using X-ray films.

Cytokine measurements. Levels of C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor-alpha (TNF-alpha) were measured using commercially available assays (Euro/DPC Limited, Gwynedd, United Kingdom) performed on an Immulite automated analyzer (Euro/DPC Limited).

Statistical analysis. Statistical analysis of the sample populations was performed using the Student *t* test for continuous variables and Fisher exact test for discrete data. Student *t* tests and the nonparametric Mann-Whitney *U* test were used to assess protein and metabolite differences in SR and AF cohorts, respectively. Results are given as mean ± SD or SE. A p value <0.05 was considered significant. Statistical analysis of metabolite changes among more than 2 groups was performed using analysis of variance. This test, however, does not control for multiple comparisons. The correlation between metabolite ratios and onset of post-operative AF was estimated by a Pearson correlation coefficient. For the calculation of the probability of metabolite changes being associated with protein changes

by chance, simulations were run on Matlab (Mathworks Limited, Cambridge, United Kingdom).

Pattern recognition. Proton NMR spectroscopy metabolic profiles from 1.0 parts per million (ppm) to 4.0 ppm with positive intensities scaled to total intensity in the spectrum were used for generating bucket tables, producing signals of 600 data points. Principal component analysis (PCA) reduced the data dimensionality to 10 variables, enough to accurately represent more than 96% of the original data. Linear discriminant analysis (LDA), a classification method, was then applied on the new representation of the data. Linear discriminant analysis constructs a separating hyperplane from an optimal projection, which maximizes the distances between groups while minimizing the distances within groups (10). The classification results obtained were validated through a leave-one-out scheme, in which the classifier was trained and tested n times, respectively, on n − 1 and 1 sample to reduce selection bias (11).

Results

Patient characteristics. The clinical characteristics of the cohorts are summarized in the Online Appendix Table 1. No significant differences were present among the groups except for atrial dilatation and the use of digoxin in patients with AF, which are impossible to overcome because persistent AF itself leads to and is favored by left atrial dilatation and digoxin is predominantly used in AF.

Table 1 Differences in Metabolites Between Hearts in SR and Permanent AF

	SR Valve	AF Valve	p Value
Leucine	0.070 (±0.013)	0.108 (±0.020)	0.104
Isoleucine	0.030 (±0.011)	0.042 (±0.013)	0.318
Valine	0.084 (±0.020)	0.128 (±0.028)	0.189
Beta-hydroxybutyrate	0.058 (±0.021)	0.193 (±0.029)	0.006*
Glucose	0.371 (±0.074)	0.428 (±0.066)	0.462
Lactate	5.198 (±0.521)	6.452 (±0.766)	0.208
Alanine	1.085 (±0.189)	1.296 (±0.203)	0.462
Acetate	0.518 (±0.140)	0.444 (±0.066)	0.916
Glutamate	2.098 (±0.395)	2.267 (±0.311)	0.834
Succinate	0.740 (±0.147)	0.751 (±0.134)	0.916
Glutamine	0.929 (±0.156)	1.241 (±0.240)	0.529
Aspartate	0.135 (±0.090)	0.236 (±0.072)	0.270
Choline	0.066 (±0.015)	0.062 (±0.006)	0.753
Phosphocholine	0.099 (±0.017)	0.111 (±0.011)	0.753
Glycocholic acid	0.591 (±0.086)	0.644 (±0.147)	0.999
Carnitine	0.365 (±0.045)	0.363 (±0.050)	0.599
Myoinositol	1.195 (±0.273)	1.482 (±0.191)	0.401
Taurine	5.576 (±0.765)	5.810 (±0.848)	0.916
Fumarate	0.003 (±0.001)	0.017 (±0.009)	0.401
Tyrosine	0.011 (±0.004)	0.049 (±0.009)	0.002*
Glycine	0.232 (±0.052)	0.336 (±0.039)	0.016*
Formate	0.617 (±0.157)	0.319 (±0.041)	0.296
Creatine	1.603 (±0.216)	1.482 (±0.273)	0.600
Adenosine pool	0.899 (±0.100)	1.076 (±0.158)	0.401

Data presented are given in μmol/g wet weight (mean ± SE; N = 16, n = 8 for patients in SR or AF undergoing valve surgery, respectively). p values were derived from nonparametric Mann-Whitney *U* tests. *Statistically significant.

AF = atrial fibrillation; SR = sinus rhythm.

Metabolite changes in persistent AF. To analyze metabolic alterations in AF, we measured cardiac metabolites with high-resolution NMR spectroscopy. A representative spectrum is shown in Figure 1. Quantitative data for 24 metabolites are presented in Table 1. Concentrations of beta-hydroxybutyrate were elevated in AF paired with a rise in ketogenic amino acids (tyrosine and leucine) and increase in glycine. The latter, along with succinyl-coenzyme A (CoA), contributes all the carbon and nitrogen atoms required for the nonhepatic tissue utilization of ketone bodies. Notably, fumarate, a metabolic intermediate in the tricarboxylic acid (TCA) cycle, became detectable in AF, resulting in an increase of the fumarate/succinate ratio ($p = 0.010$), similar to what had previously been observed in the working rat heart on administration of glucose plus ketone bodies (12,13).

Protein changes in persistent AF. To provide insight into the consequences of persistent AF on protein expression, we compared the proteome of atrial appendages from patients in SR with those in AF. Protein extracts were separated by 2-dimensional gel electrophoresis using a broad pH gradient (pH 3 to 10 nonlinear) (Fig. 2). Two-dimensional gels comprised approximately 1,500 protein features. Differentially expressed spots were numbered, excised, and subjected to in-gel tryptic digestion. Enlarged silver-stained images are shown in Figure 3A. Protein identifications as obtained by matrix-assisted laser-desorption ionization-mass spectrometry (MALDI-MS) are listed in Table 2. A representative MALDI-MS spectrum and identifications obtained by tandem MS are included in the online data supplement (Online Appendix Fig. 1 and Table 2).

Structural damage inflicted by prolonged AF was evident on 2-dimensional gels as actin, tubulin, and desmin fragmentation and compensatory changes in small heat shock proteins, including heat shock protein beta 7 and alpha/B crystallin. However, no difference in net expression was observed for alpha/B crystallin on 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 3B) because post-translational modifications as well as changes in net expression can be visualized on 2-dimensional gels. The structural changes were accompanied by a depletion of the antioxidant protein peroxiredoxin 1 (spot 14) (Figs. 3A and 3B), which is thought to play an important role in eliminating peroxides generated during metabolism (14), and a decrease in the levels of the atrial natriuretic peptide (ANP) precursor, which correlates with previous reports that AF alters plasma ANP (15). Consistent with our metabolomic findings, the majority of changes observed in fibrillating atria were related to energy metabolism. Among the differentially expressed enzymes were triose phosphate isomerase (spot 7) (Fig. 3A) and glyceraldehyde-3-phosphate dehydrogenase (spot 8 to 10) (Fig. 3A), 2 glycolytic enzymes known to be up-regulated as part of the reprogramming of the human atrial transcriptome in per-

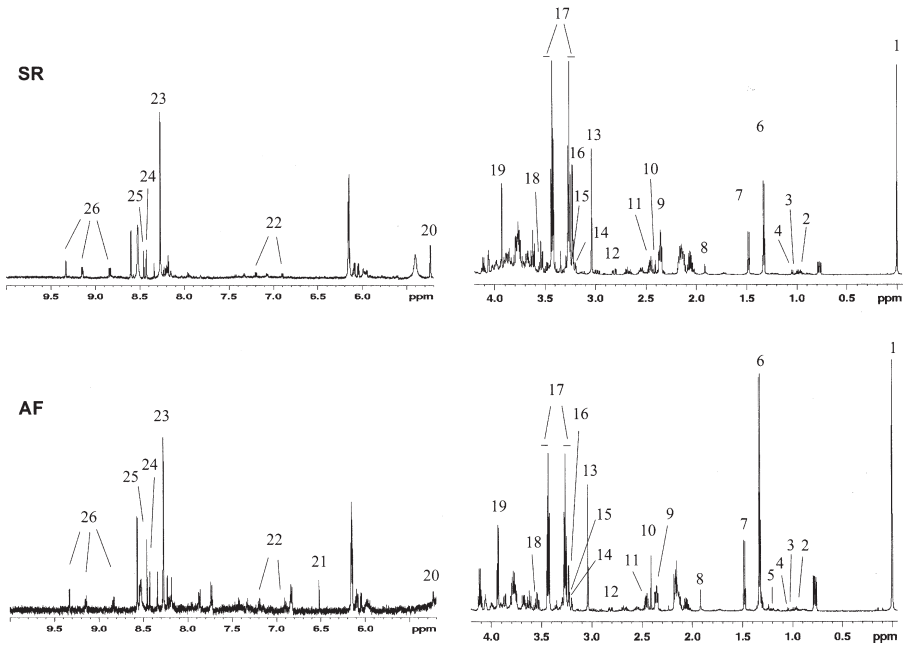

Figure 1 NMR Spectra of Atrial Appendages

Within the aliphatic and aromatic regions (−0.05–9.5 parts per million [ppm]) of the nuclear magnetic resonance (NMR) spectra, resonances were assigned as: (1) 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate; (2) leucine; (3) isoleucine; (4) valine; (5) beta-hydroxybutyrate; (6) lactate; (7) alanine; (8) acetate; (9) glutamate; (10) succinate; (11) glutamine; (12) aspartate; (13) creatine and phosphocreatine; (14) choline; (15) phosphocholine; (16) carnitine; (17) taurine; (18) glycine; (19) creatine; (20) glucose; (21) fumarate; (22) tyrosine; (23) AMP, ADP, and ATP; (24) NAD and NADH; (25) formate; and (26) NAD and NADH. The presence of glycerophosphocholine within the carnitine peak was ruled out by NMR analysis at low pH. ADP = adenosine diphosphate; AF = atrial fibrillation; AMP = adenosine monophosphate; ATP = adenosine triphosphate; NAD = nicotinamide adenine dinucleotide; NADH = reduced form of nicotinamide adenine dinucleotide; SR = sinus rhythm.

manent atrial fibrillation (6); 3-oxoacid transferase, the key enzyme in the extrahepatic utilization of ketone bodies; and NADH dehydrogenase and ubiquinol cytochrome C reductase, complex I and III of the respiratory chain, both of which are linked via the redox span of the coenzyme Q couple. A schematic model of enzyme and metabolite changes is shown in Figure 4.

Metabolite changes preceding AF. Metabolic changes in persistent AF do not allow any conclusions to be made on their proarrhythmogenic or antiarrhythmogenic potential. To determine whether alterations in cardiac metabolism might precede the onset of the arrhythmia, we analyzed atrial appendages from patients undergoing CABG who developed AF post-operatively (onset of arrhythmia geometric mean, 2.1 days). Online Appendix Table 3 summarizes the clinical characteristics of these patients. Previous research has suggested that the risk of AF post-operatively correlates to the amount of inflammation in surgically treated heart tissues (16). In our cohort, however, post-operative CRP and cytokine levels (IL-6, TNF-alpha) at day 1 after surgery were not significantly different than those of patients who maintained SR throughout. In contrast, their cardiac tissue samples had significantly lower concentrations of glucose, beta-hydroxybutyrate, and acetate, an end product of lipid metabolism (Online Appendix Table 4). Cross-validation was performed using PCA to investigate the global variation of the 22 patient samples in the 24-dimensional metabolite space (Fig. 5). Principal component analysis reduces the dimensionality of a multivariate data set by identifying linear combinations of features (metabolites) that provide the most information about global sample variance. A direct comparison with SR controls allowed the occurrence of post-operative AF to be correctly classified in 8 of 11 and 9 of 11 patients by PC1 versus PC5 (Fig. 5B) and PC3 versus PC6 (Fig. 5C), respectively, with 1 of 11 and 2 of 11 patients maintaining SR being misclassified. Overall, leave-one-out cross-validation on both scores correctly classified 81.8% of patients undergoing CABG surgery.

To explore whether a common signature for post-operative AF could be identified in patients with different underlying pathophysiologies, we collected additional samples from patients who developed arrhythmia after valve surgery (Online Appendix Table 5). Although tissue levels of individual metabolites were not significantly altered in the valve cohort (Online Appendix Table 6), similarities in metabolite changes, such as reductions in glucose and creatine levels, were observed to the initial cohort with coronary artery disease and found to be significant in a combined analysis including all SR (n = 19) and SR-AF (n = 18) patients (Table 3). Furthermore, when ratios rather than absolute concentrations were analyzed to com-

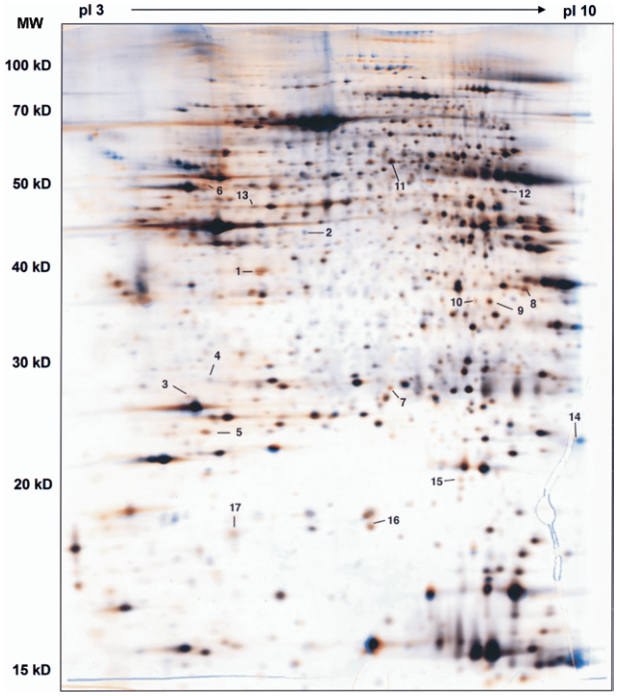

Figure 2 **2-Dimensional Gel Electrophoresis Map of Atrial Appendages**

Protein extracts were separated on a pH 3 to 10 nonlinear immobilized pH gradient strip, followed by separation on a 12% sodium dodecyl sulfate polyacrylamide gel. Spots were detected by silver staining. The figure represents a direct overlay of average gels from hearts in sinus rhythm (SR) and atrial fibrillation (AF) (**blue** and **orange** for SR and AF, respectively) created by using the Proteomweaver software (Definiens AG, Munich, Germany). Differentially expressed spots are numbered. Proteins were identified by mass spectrometry and are listed in Table 2.

pensate for the observed differences between patients undergoing CABG or valve surgery, the ratio of glucose to acetate was reduced to a similar extent in all patients who developed post-operative AF, irrespective of the underlying cardiac pathophysiology (Fig. 6A), and the analysis of all SR to SR-AF patients was found to be highly significant (1.16 ± 0.32 vs. 0.33 ± 0.13; p = 0.006; n = 37). Moreover, the ratio of glycolytic end products (alanine, lactate) to end products of lipid metabolism (acetate) correlated positively to the time of onset of post-operative AF in the CABG group (Fig. 6B), which is consistent with previous findings in an animal model that glycolytic inhibition predisposes to AF (17). Overall, these findings provide additional support for a discordant regulation of cardiac metabolites before the onset of the arrhythmia and strongly suggest that metabolite ratios may serve as a better classifier for post-operative AF than absolute metabolite concentrations.

Discussion

Atrial fibrillation, as confirmed by recent studies (6), denotes a hypermetabolic state, but how its increased energy requirement is serviced in the long term and whether metabolism influences the onset or persistence of AF has, to our knowledge, not previously been explored. This study in a carefully chosen, well-characterized cohort of patients undergoing cardiac surgery used 2 emerging techniques, proteomics and metabolomics, to provide novel insights into the metabolic processes at work during AF. Unlike previous plasma-based metabolomic analyses of myocardial ischemia (18,19), our study applied metabolomic techniques directly to human cardiac tissue and demonstrated that the combined proteomic and metabolomic platform provides an exiting armamentarium for investigating cardiovascular pathology (7).

Cardiac metabolism in AF. To date, animal studies have shown that atrial oxygen consumption and coronary flow increase almost 3-fold after the induction of AF; as a result, the atrial coronary flow reserve decreases markedly (20,21). Furthermore, high atrial oxygen and energy

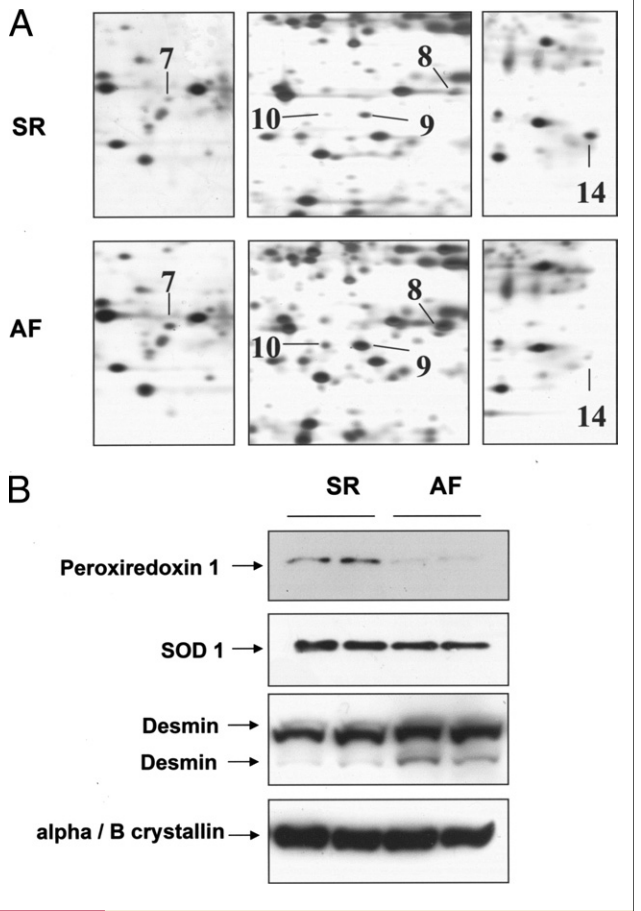

Figure 3 **Enlargements of Silver-Stained Gels**

(A) Representative areas of 2-dimensional gels from patients in SR and AF highlight quantitative differences in images. **Numbers** correspond to proteins listed in Table 2. **(B)** Quantification of protein abundance using 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Note the decline of peroxiredoxin 1 levels in persistent AF. SOD = superoxide dismutase; other abbreviations as in Figure 2.

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Table 2 Differences in Protein Profiles Between Hearts in SR and AF

Spot No.	Protein Identity	Δ*	p Value	NCBI Entry	Function	Calculated pI/MW (Da × 10^3)	Observed pI/MW (Da × 10^3)	Sequence Coverage/ Mascot Score
Myofilaments								
1	Tubulin alpha chain (*fragment*)	+3.3	0.026	55977864	Intermediate filament	4.9/50.6	5.5/39.7	24%/87 MS/MS‡
2	Actin alpha cardiac	−2.1	0.031	54036697	Cytoskeleton	5.2/42.3	5.8/41.2	MS/MS‡
3	Actin alpha cardiac (*fragment*)	+2.6	0.023	54036697	Cytoskeleton	5.2/42.3	5.0/32.1	19%/72
4	Actin alpha cardiac (*fragment*)	+2.0	0.030	54036697	Cytoskeleton	5.2/42.3	5.2/33.0	18%/76
5	Cardiac myosin light chain 1	+2.3	0.007	127149	Contraction	5.0/22.1	5.1/24.7	36%/94 MS/MS‡
6	Desmin (*fragment*)	+5.3	0.010	23506465	Intermediate filament	5.2/53.4	5.0/58.4	35%/120 MS/MS‡
Glucose metabolism								
7	Triosephosphate isomerase	+4.8	0.001	4507645	Glycolysis	6.5/26.8	6.5/31.0	42%/137
8	Glyceraldehyde-3-phosphate dehydrogenase	+2.4	0.002	32891805	Glycolysis	8.2/36.2	7.9/33.0	MS/MS‡
9	Glyceraldehyde-3-phosphate dehydrogenase	+2.6	0.000	32891805	Glycolysis	8.2/36.2	7.2/38.0	28%/113
10	Glyceraldehyde-3-phosphate dehydrogenase	+1.8	0.001	32891805	Glycolysis	8.2/36.2	7.0/38.0	17%/64
Lipid metabolism								
11	3-Oxoacid transferase	+2.0	0.004	4557817	Utilization of ketone bodies	7.1/56.6	6.7/59.5	16%/89
Respiratory chain								
12	NADH dehydrogenase (ubiquinone) flavoprotein 1	+2.1	0.032	15990511	Energy production, Fe-S–containing protein	8.5/50.5	7.5/52.6	43%/248
13	Ubiquinol cytochrome C reductase core protein I	+2.3	0.012	46593007	Energy production, Fe-S–containing protein	5.9/53.3	5.5/50.2	25%/156
Other								
14	Peroxiredoxin 1	−3.0	0.023	32455266	Thioredoxin-dependent peroxide reductase 2	8.3/22.3	8.1/28.0	37%/105
15	Crystallin alpha beta	AF†	0.020	30582379	Actin polymerization	6.8/20.1	6.9/22.3	25%/64
16	Heat-shock protein beta-7	+2.3	0.024	12643877	Small heat shock protein	6.0/18.6	6.4/19.1	MS/MS‡
17	Atrial natriuretic peptide precursor	−2.2	0.017	178638	Vasoactive peptide	5.1/16.5	5.3/19.4	34%/87

*Fold increase/decrease in AF samples (n = 8) compared with SR (n = 8). †Protein species were detectable in AF samples only. ‡Identification by tandem mass spectrometry. MW = molecular weight; NCBI = National Center for Biotechnology Information; pI = isoelectric point; other abbreviations as in Table 1.

demand during AF result in a transient drop in phosphocreatine levels during early stages of AF (22), but phosphocreatine levels are replenished after a few days of the sustained arrhythmia, a time scale remarkably comparable to the reversibility of contractile dysfunction. How energy needs of the persistent arrhythmia are subsequently met remains unknown. Human studies measuring coronary arteriovenous differences have reported increased lactate production during AF (23), and others increased oxidative stress and impaired myofibrillar energetics, which may lead to atrial contractile dysfunction during AF (24). High energy demand might explain many of the permanent changes in the atria resulting from persistent AF such as cellular hypertrophy, myolysis, changes in mitochondrial shape, and perinuclear accumulation of glycogen (25).

Glucose metabolism in AF. Recent microarray analysis in patients with permanent AF (6) revealed a prominent up-regulation of transcripts involved in metabolic activities, including several glycolytic enzymes. Consistent with these microarray data, enzymatic changes constituted the majority of differences in the protein profiles of human samples of persistent AF, and glycolytic enzymes were among the differentially expressed proteins; however, a corresponding build-up of glucose metabolites (i.e., lactate or alanine, a transamination product of pyruvate) failed to reach statistical significance. The intracellular calcium concentration, a known activator of glycogenolytic pathways, rises in early

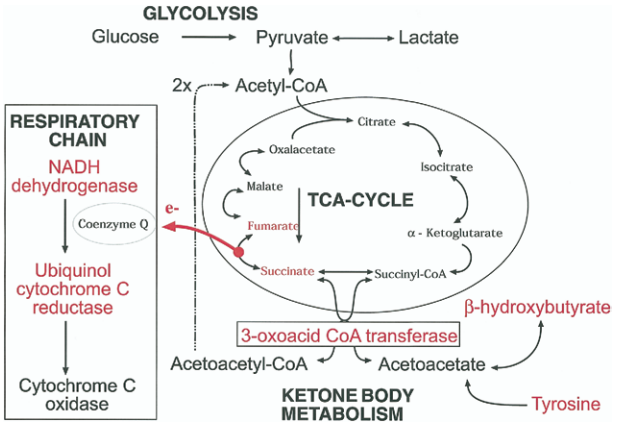

Figure 4 **Representation of Enzyme and Metabolic Changes in Persistent AF**

The illustration shows the TCA cycle, oxidative phosphorylation, and protein and metabolite changes observed in patients with persistent AF **(red)**. Notably, the probability of 3 metabolites being associated with 3 enzymes out of the 17 differentially expressed proteins by chance was estimated at <5% after running 1 million simulations on Matlab (Mathworks Limited). Each simulation consisted of randomly selecting 3 metabolites out of the 24 measured, generating 1,500 proteins, randomly associating 300 of them with pairs of metabolites (based on the fact that approximately 20% of spots on 2-dimensional gel maps from human hearts are metabolic enzymes), and randomly selecting 17 of the 1,500 proteins. A success was defined as the 3 selected metabolites being associated with at least 3 proteins among the 17 selected. CoA = coenzyme A; e = electron; TCA = tricarboxylic acid; other abbreviations as in Figures 1 and 2.

AF (26,27), suggesting that short-term energy demand might be met by increased glycolysis. However, intracellular calcium levels normalize in prolonged AF, and perinuclear storage of glycogen in fibrillating atria continues (27). Two explanations have been suggested: cardiac accumulation of glycogen may be either the result of a metabolic excess of glucose or the consequence of impaired catabolism of glycogen (5). Our findings make a metabolic excess of glucose less likely because tissue concentrations of glucose were unchanged in patients with persistent AF.

Ketone bodies in AF. Extrahepatic tissues utilize ketone bodies by converting beta-hydroxybutyrate successively to acetoacetate and acetoacetyl-CoA, which is finally cleaved to yield 2 molecules of acetyl-CoA for oxidation in the TCA cycle. Among the differentially expressed proteins in AF was 3-oxoacid transferase, a mitochondrial matrix enzyme required to activate ketones by transferring CoA from succinyl-CoA to acetoacetate, the key reaction in ketolytic energy production (Fig. 4). Consistent with the proteomic findings, metabolomic analysis revealed a rise in the levels of beta-hydroxybutyrate and ketogenic amino acids, notably tyrosine, which forms acetoacetate and fumarate during catabolism. Fumarate levels were markedly elevated in persistent AF, and consequently the fumarate/succinate ratio increased. The latter is often used as an indicator for the redox state of the coenzyme Q couple (13), which is the cofactor for the succinate dehydrogenase reaction and links complex I and complex III of the respiratory chain via its redox span (Fig. 4). Both NADH dehydrogenase (complex I) and ubiquinol cytochrome C reductase (complex III) were among the differentially expressed proteins in the proteomic screen. Moreover, animal studies have shown that administration of glucose plus ketone bodies resulted in an increase in the fumarate/succinate ratio similar to our observation in humans (13). Thus, there are several lines of evidence to suggest that ketone bodies might be an important fuel during human persistent AF.

Energy metabolism in AF. As beta-hydroxybutyrate is more reduced, it provides 30% more calories per carbon unit than pyruvate, the normal mitochondrial fuel produced by glycolysis (13). The higher inherent energy present in ketone bodies exerts a glucose-sparing effect in cardiac muscle mediated by increased acetyl-CoA, which competes with CoA for binding to the pyruvate dehydrogenase complex (28). The pyruvate dehydrogenase complex is responsible for converting pyruvate into acetyl-CoA, representing the irreversible step from glycolysis to the TCA cycle. A previous transcript analysis of human persistent AF (6) revealed that, along with several glycolytic enzymes, pyruvate dehydrogenase kinase 1, which inactivates the pyruvate dehydrogenase complex, was up-regulated, whereas pyruvate dehydrogenase phosphatase, which reverses this inhibition, was down-regulated in AF. Phosphorylation of the pyruvate dehydrogenase complex would impair the coupling of glycolysis to the mitochondrial TCA cycle, and as a result, raising myocardial levels of glucose 6-phosphate would reroute glucose toward glycogen synthesis (28). Indeed, previous animal experiments confirmed that addition of ketones to glucose perfusion results in a switch from constant glycogen breakdown to glycogen synthesis in working hearts (12), and increased expression of glycogen synthase was observed in human AF, accompanied by a corresponding down-regulation of glycogen phosphorylase, the enzyme responsible for glycogen breakdown (6). Taken together, the proteomic, metabolomic, and transcriptomic data on human AF would be consistent with a negative feedback of ketone bodies on glucose metabolism.

Instead, the observed compensatory up-regulation of glycolytic enzymes during persistent AF (6) might be required to fill up TCA cycle intermediates. Under normoxic conditions, pyruvate is not only decarboxylated but also carboxylated to oxaloacetate and malate, allowing the replenishment of TCA cycle intermediates and recycling of carbon dioxide (28). Such anaplerotic mechanisms appear to be particularly important during prolonged oxidation of ketone bodies, which can "unspan" the Krebs cycle by sequestration of CoA, thereby impairing the energy production required to sustain contractile performance of the heart (29). Thus, metabolic changes during persistent AF may contribute to the self-perpetuating nature of this resilient arrhythmia.

Post-operative AF. Post-operative AF is a frequent complication of most types of cardiac surgery, occurring in up to

Table 3 Metabolite Concentrations in Atria of SR Patients Undergoing CABG or Valve Surgery

	SR	SR-AF	SR	SR-AF	p Value		
	CABG (n = 11)	CABG (n = 11)	Valve (n = 8)	Valve (n = 7)	CABG vs. Valve	SR vs. SR-AF	ANOVA
Leucine	0.056 (±0.012)	0.041 (±0.016)	0.070 (±0.013)	0.060 (±0.024)	0.301	0.411	0.641
Isoleucine	0.018 (±0.006)	0.010 (±0.004)	0.030 (±0.011)	0.026 (±0.012)	0.080	0.415	0.303
Valine	0.045 (±0.012)	0.029 (±0.011)	0.084 (±0.020)	0.084 (±0.027)	0.007*	0.561	0.055
Beta-hydroxybutyrate	0.051 (±0.016)	0.002 (±0.002)	0.058 (±0.021)	0.045 (±0.022)	0.135	0.041	0.058
Glucose	0.256 (±0.065)	0.079 (±0.020)	0.371 (±0.074)	0.242 (±0.079)	0.035*	0.016*	0.014*
Lactate	5.266 (±1.650)	1.334 (±0.380)	5.198 (±0.521)	5.224 (±1.856)	0.158	0.084	0.064
Alanine	1.220 (±0.317)	0.609 (±0.193)	1.085 (±0.189)	1.198 (±0.299)	0.407	0.246	0.282
Acetate	0.413 (±0.074)	0.202 (±0.028)	0.518 (±0.140)	0.917 (±0.245)	0.005*	0.807	0.004*
Glutamate	1.060 (±0.275)	0.428 (±0.122)	2.098 (±0.395)	1.950 (±0.663)	0.001*	0.269	0.008*
Succinate	0.883 (±0.119)	0.566 (±0.093)	0.740 (±0.147)	0.975 (±0.168)	0.266	0.673	0.174
Glutamine	0.576 (±0.146)	0.229 (±0.080)	0.929 (±0.156)	0.828 (±0.254)	0.005*	0.146	0.015*
Aspartate	0.134 (±0.043)	0.044 (±0.020)	0.135 (±0.032)	0.174 (±0.082)	0.169	0.432	0.221
Choline	0.041 (±0.013)	0.030 (±0.010)	0.066 (±0.015)	0.065 (±0.020)	0.038*	0.589	0.212
Phosphocholine	0.067 (±0.016)	0.031 (±0.009)	0.099 (±0.017)	0.084 (±0.021)	0.011*	0.100	0.022*
Glycocholic acid	0.634 (±0.066)	0.611 (±0.066)	0.591 (±0.086)	0.852 (±0.203)	0.400	0.370	0.343
Carnitine	0.222 (±0.051)	0.098 (±0.022)	0.365 (±0.045)	0.329 (±0.094)	0.002*	0.140	0.005*
Myoinositol	1.168 (±0.296)	0.502 (±0.151)	1.195 (±0.273)	1.082 (±0.294)	0.257	0.098	0.170
Taurine	3.282 (±0.949)	2.013 (±0.693)	5.576 (±0.765)	5.255 (±1.566)	0.007*	0.376	0.047*
Fumarate	0.003 (±0.002)	0.001 (±0.001)	0.003 (±0.001)	0.004 (±0.002)	0.411	0.437	0.561
Tyrosine	0.015 (±0.004)	0.012 (±0.009)	0.011 (±0.004)	0.017 (±0.007)	0.979	0.842	0.918
Glycine	0.326 (±0.086)	0.301 (±0.061)	0.232 (±0.052)	0.233 (±0.047)	0.231	0.869	0.690
Formate	0.694 (±0.086)	0.576 (±0.074)	0.617 (±0.157)	1.022 (±0.313)	0.298	0.540	0.249
Creatine	0.985 (±0.212)	0.600 (±0.145)	1.603 (±0.216)	0.966 (±0.257)	0.021*	0.029*	0.015*
Adenosine pool	0.773 (±0.212)	0.422 (±0.129)	0.899 (±0.100)	0.896 (±0.267)	0.115	0.266	0.215

Data presented are given in μmol/g wet weight (mean ± SE). p values for differences between SR patients undergoing surgery for CABG (n = 22) and valve disease (n = 15) and combined SR (n = 19) and SR-AF (n =18) cohorts were derived from unpaired *t* tests; differences among all groups were derived from ANOVA tables. *Statistically significant.
ANOVA = analysis of variance; CABG = coronary artery bypass grafting; SR-AF = post-operative atrial fibrillation; other abbreviations as in Table 1.

30% of patients. PCA and LDA of cardiac metabolite profiles from proton NMR spectroscopy, as highlighted in this study, were able to correctly classify more than 80% of the patients susceptible to post-operative AF undergoing CABG. A tendency toward lower glucose and creatine concentrations was also observed in patients who developed post-operative AF with underlying valve disease. This approach at validating our findings in a valve cohort was adopted to ensure that the differences observed were not ischemia driven. However, valve surgery in itself is associated with a much higher risk of post-operative AF, and the different underlying pathophysiologies were associated with variable metabolite concentrations (Table 3). Therefore, metabolite ratios were assessed in all groups, and these, by diminishing interindividual variability in cohorts, were found to provide a better classifier for post-operative AF, irrespective of the underlying pathology.

Although persistent AF is a distinct clinical entity, our additional analysis of atrial appendages of patients who were in SR at the time of surgery but developed AF post-operatively provides evidence that metabolic alterations might facilitate the onset of the arrhythmia. This assumption is supported by the recent demonstration in an animal model that glycolytic inhibition predisposes to AF (17). Similarly, we found that the lower the ratio of glycolytic end products to end products of lipid metabolism in human atrial tissue, the earlier the onset of the arrhythmia in CABG patients. The higher incidence of post-operative AF in the valve cohort excluded it from the time of onset analysis. To date, CRP levels have been shown to correlate not only with the risk of post-operative AF but also with its peak incidence (16). To our knowledge, this is the first evidence that alterations in tissue metabolites might serve as classifiers for post-operative AF. Although a link between tissue metabolism and inflammation has been established in skeletal muscle (30), it has yet to be proven in cardiac tissue, especially with respect to AF.

Study limitations. During the course of this study, to enhance pathway discovery, we performed an in-depth analysis of extremely well-matched but admittedly small cohorts of patients undergoing cardiac surgery. The heterogeneous nature of AF means that despite the sample size of this study (n = 45) exceeding any previous metabolomic studies using human cardiac tissue samples, the assessment of the impact of different underlying mechanisms on metabolism will require future exploration in larger independent data sets. Furthermore, NMR currently only monitors approximately 20 to 30 of the most abundant metabolites, and the predictive value of absolute metabolite concentrations is hindered by the different pathophysiologies themselves expressing variable metabolic profiles. More sensitive systems, however, are rapidly evolving, and the usefulness of

these techniques as an additional risk assessment remains to be determined (7).

Clinical implications. A considerable lag in recovery from contractile dysfunction prevails following restoration of SR from prolonged AF, and accumulation of lactate and increased oxidative stress have both been implicated in myocardial contractile dysfunction (24). Although the combined use of glucose and ketone bodies increases cardiac efficiency (13), oxidation of ketones as a sole substrate can also lead to cardiac contractile failure (28,31) and increased risk of arrhythmogenesis through accumulation of glycogen (32). Thus, metabolic alterations occurring during AF may be closely associated with contractile dysfunction and the genesis of re-entrant circuits providing possible new explanations to understanding the self-perpetuating nature of this arrhythmia.

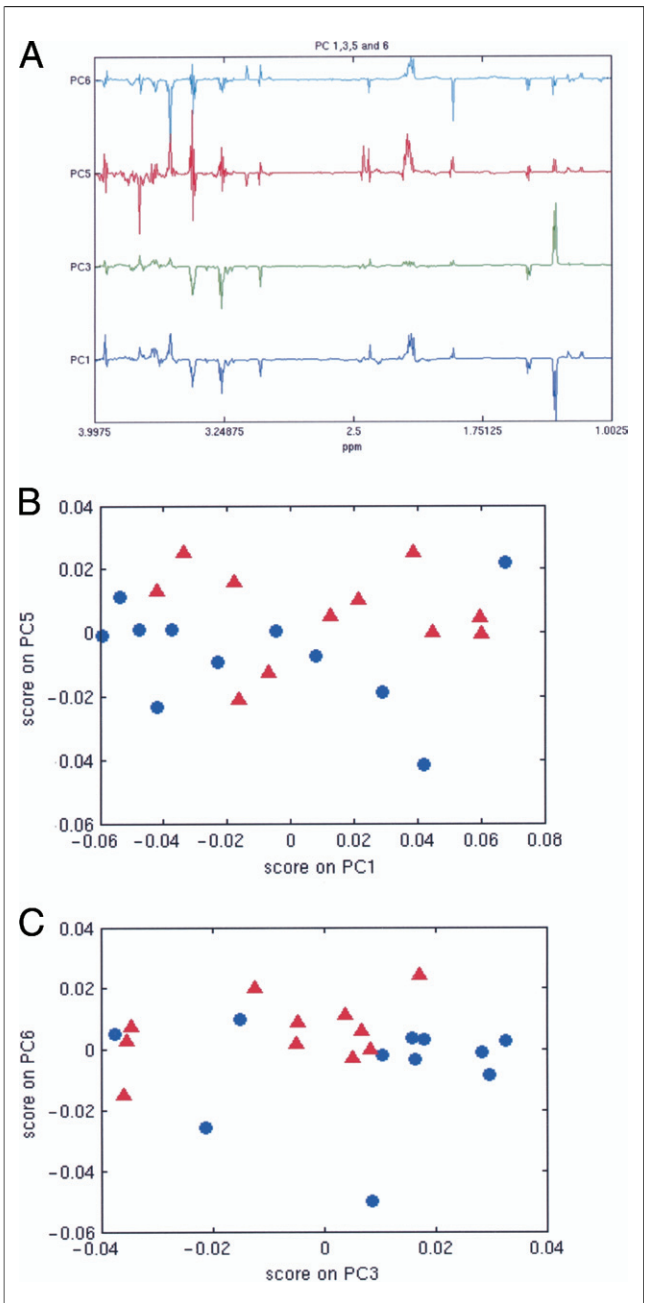

Figure 5 Pattern Recognition Analysis

^{1}H-NMR metabolite profiles underwent principal component analysis as described in "Materials and Methods." **(A)** Plots of the 4 principal components (PC 1, 3, 5, and 6) were used for discrimination. **Red triangles** and **blue circles** represent patients who developed post-operative AF and patients, who maintained SR, respectively. Note that more than 80% of patients were correctly classified using either PC1 versus PC5 **(B)** or PC3 versus PC6 **(C)**. Abbreviations as in Figure 2.

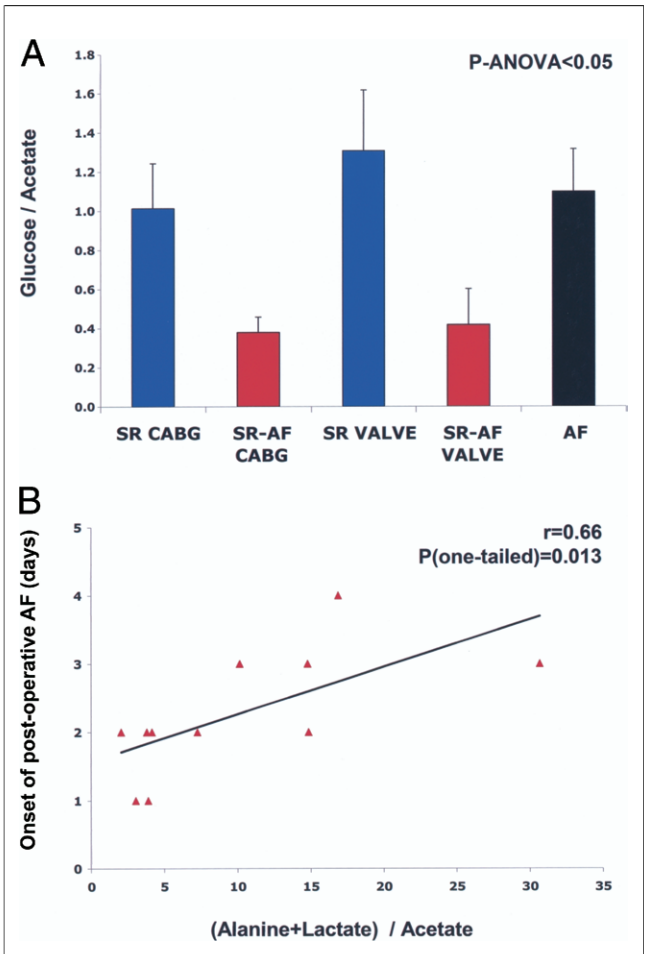

Figure 6 Metabolite Ratios

(A) Reduction of the glucose to acetate ratio in patients susceptible to post-operative AF irrespective of the underlying cardiac pathology. **(B)** Positive correlation between the ratio of glycolytic end products (alanine, lactate) to end products of lipid metabolism (acetate) and the onset of post-operative AF in patients with coronary artery disease. ANOVA = analysis of variance; CABG = coronary artery bypass grafting; other abbreviations as in Figure 2.

Summary. This study is, to our knowledge, the first metabolomic and proteomic analysis into human AF characterizing the metabolic adaptation processes at work in persistent AF. It unravels a potential role for ketone bodies and demonstrates that a discordant regulation of energy metabolites precedes the onset of the arrhythmia after surgery. As such, metabolic profiles might help to

classify patients who are at risk of developing postoperative AF.

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APPENDIX

For the supplemental methods, please see the online version of this article.