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Atrial Fibrillation Rhythm is Associated with Marked Changes in Metabolic and Myofibrillar Protein Expression in Left Atrial Appendage

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Abstract

Atrial fibrillation (AF) is strongly associated with risk of stroke and heart failure. AF promotes atrial remodeling that increases risk of stroke due to left atrial thrombogenesis, and increases energy demand to support high rate electrical activity and muscle contraction. While many transcriptomic studies have assessed AF-related changes in mRNA abundance, fewer studies have

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assessed proteomic changes. We performed a proteomic analysis on left atrial appendage (LAA) tissues from 12 patients with a history of AF undergoing elective surgery; atrial rhythm was documented at time of surgery. Proteomic analysis was performed using liquid chromatography with mass spectrometry (LC/MS-MS). Data-dependent analysis identified 3090 unique proteins, with 408 differentially expressed between sinus rhythm and AF. Ingenuity Pathway Analysis of differentially expressed proteins identified mitochondrial dysfunction, oxidative phosphorylation, and sirtuin signaling among the most affected pathways. Increased abundance of electron transport chain (ETC) proteins in AF was accompanied by decreased expression of ETC complex assembly factors, tricarboxylic acid cycle proteins, and other key metabolic modulators. Discordant changes were also evident in the contractile unit with both up- and down-regulation of key components. Similar pathways were affected in a comparison of patients with a history of persistent vs. paroxysmal AF, presenting for surgery in sinus rhythm. Together, these data suggest that while the LAA attempts to meet the energetic demands of AF, an uncoordinated response may reduce ATP availability, contribute to tissue contractile and electrophysiologic heterogeneity, and promote a progression of AF from paroxysmal episodes to development of a substrate amenable to persistent arrhythmia.

Keywords

Atrial fibrillation; metabolism; mitochondria; proteomics; calcium signaling; left atrium

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia. The lifetime risk of AF increases with advancing age and other risk factors that include obesity, smoking, hypertension, and diabetes mellitus[36]. AF is often a progressive condition, frequently beginning with brief episodes that start and stop spontaneously (paroxysmal AF), progressing to longer episodes that do not terminate without either drug- or electrical shock induced cardioversion (persistent AF). AF episodes of increasing persistence are associated with increased risk of stroke, cognitive decline, heart failure, and death [36]. The cost and health burden of AF are expected to rise as the American population ages and the obesity rates increase, thus highlighting the need for interventions and therapies that can slow the progression and reduce the burden of AF. Risk of AF is in part heritable, and genome wide association studies have sought to identify the genetic loci and the genes associated with AF risk [34,38]. More than 140 loci have been identified, many of which are associated with the expression of a nearby gene (or genes) (*cis*-expression quantitative trait loci, *cis*-eQTL). Deciphering the role of AF risk genes in AF progression is not straightforward. Efforts to characterize AF at varying states of progression (paroxysmal vs. persistent or long-standing persistent) may be facilitated by analyzing relevant atrial tissues obtained during cardiac surgery from patients with AF of varied duration and persistence. In principal, these studies could include assessment of mRNA abundance, atrial histology, atrial metabolites, or atrial protein composition. Several studies have assessed the abundance of mRNA in atrial tissues from individuals with AF [3,25,52,12,49,19] and in animal models of induced or spontaneous AF [6,30,39].

In longitudinal cohort studies, proteomic studies have been performed on blood samples using multiplexed antibody approaches [42] and protein-specific aptamer assays [21]. Blood based assays are indirect, and the source of differentially expressed proteins in blood cannot be readily determined.

The first AF-targeted atrial tissue proteomic analysis was performed using two dimensional gel electrophoresis coupled with mass spectrometry [31]. This study simultaneously evaluated metabolite profiles. AF-associated changes in protein expression [11,39] and kinomic profile [32] have also been reported in animal models of AF, but have not been reported in patients in sinus rhythm compared to AF at time of tissue acquisition.

Methods

Cardiac Surgery/Tissue acquisition

Human left atrial appendage (LAA) tissues were obtained from patients undergoing elective mitral valve repair and Maze surgery to treat AF (n=12). All patients had a documented history of either paroxysmal or persistent AF, but were in sinus rhythm (SR, n=8) or AF at the time of surgery (AF, n=4). Atrial rhythm status was confirmed by review of pre-surgical electrocardiograms. All surgical patients provided informed consent for research use of discarded atrial tissues. Prior to 2008 verbal consent was obtained and documented in the patient medical records in a process approved by the Cleveland Clinic Institutional Review Board (IRB). Specimens were snap frozen in liquid nitrogen and stored at −80°C.

Patient Demographics

Samples in each group were matched by race, sex, age, body mass index (BMI) and AF duration (Table 1). All patients had a history of AF with mitral disease. None had a history of diabetes, aortic valve disease, or heart failure.

Proteomics

Tandem mass spectrometry (MS/MS) to identify proteins was performed by the Cleveland Clinic Proteomics Core. Frozen LAA tissue samples (50–75 mg) were used from each patient. Frozen tissues were thawed on ice. Each tissue sample was transferred to a lysing Matrix D (MP Biomedicals, Solon, OH) in 200 µl 8M urea 0.1M Tris-HCl buffer pH 8.0 with freshly added complete™ Mini Protease Inhibitor Cocktail (ROCHE, Basel, Switzerland). Samples were processed on a FastPrep 24 homogenizer (MP Biomedicals, Solon, OH) using a 30s × 4 program at 6 m/s speed with 5 minutes rest between runs. Sample homogenates were centrifuged at 17000× g for 10 minutes at room temperature. Supernatants were transferred to new Eppendorf tubes, and a small aliquot from each supernatant was diluted 5x with water and used for a bicinchoninic acid assay (BCA). One hundred µg of protein from each sample based on the BCA result was transferred to a new Eppendorf tube, reduced by dithiothreitol and alkylated by iodoacetamide before digestion by adding 2.5 µg sequencing grade trypsin (Promega, Madison, WI). Digested samples were desalted using Waters Sep-Pak C18 cartridges (Waters, Milford, MA). Each desalted sample was fractionated using a Waters XBridge C18 2.1×150 mm column (Waters, Milford, MA) on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) under basic pH.

A total of 16 collections were made for each sample and every 4th collection was combined resulting in 4 fractions per sample. Combined samples were dried in a SpeedVac and re-suspended in 1% acetic acid for LC-MS/MS analysis.

Each fraction was analyzed on a ThermoFisher Scientific UltiMate 3000 UHPLC system (ThermoFisher Scientific, Bremen, Germany) interfaced with a ThermoFisher Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Bremen, Germany). Liquid chromatography was performed prior to MS/MS analysis for peptide separation. The HPLC column used is a Thermo Scientific™ Acclaim™ PepMap™ 100 C18 reversed-phase capillary chromatography column (Thermo Fisher Scientific, Waltham, MA) 75 μm × 15 cm, 2 μm, 100 Å. Five μL peptide sample was injected and peptides eluted from the column by a 100-minute acetonitrile/0.1% formic acid gradient at a flow rate of 0.30 μL/min and introduced to the source of the mass spectrometer on-line. Nano electrospray ion source was operated at 2.3 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra using a Fourier Transform (FT) orbitrap analyzer to determine peptide molecular weights and higher energy collision dissociation (HCD) MS/MS product ion spectra with the Orbitrap analyzer at 30% collision energy (CE) to determine the amino acid sequence in successive instrument scans. The MS method used in this study was a data-dependent acquisition (DDA) with 3 second duty cycle. It includes one full scan at a resolution of 120,000 followed by as many MS/MS scans as possible on the most abundant ions in that full scan in the 3S cycle time. Dynamic exclusion was enabled with a repeat count of 1 and ions within 10 ppm of the fragmented mass were excluded for 60 seconds.

Data were analyzed using MaxQuant V1.5.2.8 with the search engine Andromeda (integrated in MaxQuant software) and the parameters used were default settings for an Orbitrap instrument. The MS/MS spectra were searched using the Uniprot human protein database containing 85,299 entries, with an automatically generated decoy database (reversed sequences). The search was performed looking for fully tryptic peptides with a maximum of two missed cleavages. Oxidation of methionine and acetylation of protein N-termini were set as dynamic modifications and carbamidomethylation of cysteines was set as a static modification. The precursor mass tolerance for these searches was set to 10 ppm and the fragment ion mass tolerance was set to 0.5 Da. The search was performed including the common contaminant database available in MaxQuant and these proteins were excluded in the data analysis. A false discovery rate (FDR) was set to 1% for both peptide and protein identification and calculated using the number of identified peptides/proteins from decoy database divided by the total number of identified peptides/proteins. Two peptides were required for positive protein identification to decrease the chance of false discovery by a random match.

Normalized protein quantities were expressed as label free quantification intensities (LFQI) by MaxQuant. LFQI were the output of the MaxLFQ algorithm[10]. These are based on the (raw) intensities that are normalized on multiple levels to ensure that profiles of LFQI across samples accurately reflect the relative amounts of the proteins.

Statistical Analysis

Proteins with more than 50% missing LFQI in any of the groups were removed from further analyses. LFQIs of remaining proteins were between-sample normalized using variance stabilized normalization[40,46] as implemented in the justvsn function of the R package vsn. Missing normalized LFQIs were imputed using a hybrid imputation approach as implemented in the impute.MAR.NMAR function of the R package imputeLCMD[26]. Multidimensional scaling plots of the normalized and imputed LFQIs were used to assess sample outliers. Linear regression models were fit to assess associations of AF groups with normalized and imputed LFQIs. Regressions were fit using robust linear regression followed by empirical Bayes shrinkage of variance parameters across proteins using the limma R package[37]. Linear models included additive adjustment for sex and two inferred surrogate variables, estimated using the smartsva R package[9]. Adjusted contrasts of interest were estimated from the linear model fits. The Benjamini and Hochberg method [4] was used to control the false discovery rate and provide adjusted p-values per contrast of interest. All analyses were performed using R version 3 and described packages.

Pathway Analysis

Canonical pathways, upstream regulators, and downstream target molecules were identified using the Ingenuity Pathway Analysis (IPA®) software package (Qiagen). IPA uses protein expression data to predict pathways that are activated by comparing known proteins within a set of pathways to expression patterns in the imported dataset. UniProtKB, p-value, and log fold change values for identified proteins were imported into IPA. Differentially expressed proteins with a p-value ≤ 0.05 were used for analysis. Significance of association between the data and the canonical pathway was determined by the ratio of the number of overlapping proteins from our dataset to the total number of IPA dataset proteins in a particular pathway. Benjamini-Hochberg adjustment was used on the IPA p-values of association between proteins and the IPA canonical pathway and upstream regulators, where adjusted $p<0.05$ was considered significant. Localization of identified proteins to the mitochondria was determined using Human MitoCarta2.0 [5]. Nuclear-encoded mitochondrial-associated proteins were determined using a published list [24].

Results

Protein Identification

Our analysis identified 3090 proteins with UniProtKB identifiers suitable for analysis (Online Resource 1). 47 of these proteins were previously identified as potential AF-risk genes [38] [34] (Online Resource 1). Protein class and cellular localization of 2308 proteins was determined using the Human Protein Atlas[44]. The largest group of proteins identified in our analysis were classified as enzymes (790). Our analysis also included 271 transporters and 29 transcription factors. The identified proteins were localized to the nucleoplasm, followed by the cytosol, mitochondria, plasma membrane, vesicles, endoplasmic reticulum, Golgi apparatus, nucleoli, and other subcellular structures (Figure 1). A comparison of detected proteins with a database of mitochondrial associated proteins (Ref [24], Table S30) suggests that nuclear-encoded mitochondrial-associated proteins accounted for 32% of all detected proteins.

Of the 3090 proteins identified in the LAA, 408 unique genes were differentially expressed in AF compared to sinus rhythm (unadjusted $p<0.05$) (Online Resource 2); 216 were down-regulated and 192 were up-regulated (Figure 2). Of these proteins, 115 were significant at a false discovery rate threshold of 0.10. The most significant differentially expressed protein in AF was enolase 3 (ENO3), a glycolytic enzyme, highlighting a key role for metabolic alterations in AF. In addition to ENO3, the most significant expressed proteins that were downregulated in AF included spermine synthase (SMS), galectin 3 (LGALS3), glycerol-3-phosphate dehydrogenase 2 (GPD2), myosin heavy chain 4 (MYH4), troponin C1 (TNNC1), NAD kinase (NADK2), acetyl-CoA carboxylase β (ACACB), coiled-coil domain containing 51 (CCDC51), and acylaminoacyl-peptide hydrolase (APEH). The most significant differentially expressed proteins that were upregulated in AF included collagen type VI alpha 1 chain (COL6A1), myosin light chain 5 (MYL5), myotilin (MYOT), catalase (CAT), NPC intracellular cholesterol transporter 2 (NPC2), lactotransferrin (LTF), Rho GDP dissociation inhibitor β (ARHGDIB), ADP-ribosylhydrolase like 1 (ADPRHL1), milk fat globule-EGF factor 8 protein (MFGE8), and S100 calcium binding protein A8 (S100A8). NADH:ubiquinone oxidoreductase core subunit 1 (MT-ND1), a mitochondrial DNA-encoded subunit of complex I, was the most significant differentially expressed electron transport chain protein. Together, these changes point to metabolism, contractile function, and calcium handling as key pathways that play a role in the response of the LA to AF.

Pathway Analysis

Ingenuity Pathway Analysis (IPA) was used to identify the cellular pathways impacted by the proteins that were differentially expressed in AF (unadjusted $p< 0.05$). This analysis identified 362 canonical pathways (Online Resource 3), of which 89 were significantly altered (false discovery rate $p<0.05$) (Figure 3), and highlighted key roles for metabolism, contractile function, and calcium signaling in AF. Mitochondrial Dysfunction, Epithelial Adherens Junction Signaling, Tight Junction Signaling, Oxidative Phosphorylation, and Glutathione Redox Reactions I were identified as the most significant pathways. Although the Oxidative Phosphorylation Pathway was predicted by IPA to be activated, additional metabolic pathways that were altered in AF (false discovery rate $p<0.05$) including NRF-2 Mediated Oxidative Stress Response, Sirtuin Signaling, Glycolysis I, Gluconeogenesis I, and Glycogen Degradation were predicted to be inhibited (Figure 3).

Proteins that were altered in the Epithelial Adherens Junction Signaling pathway included contractile proteins (ACTA2, ACTB, MYH2, MYH4, MYH6, MYL2, and MYL5) cytoskeletal proteins (CTNNA1, CTNNB1, RAC1, and RHOA, TUBB, TUBB2A, TUBB4B, TUBB8), and a protein involved in receptor-mediated endocytosis (EPN1) (Online Resource 3). Proteins that were altered in the Tight Junction Signaling pathway included many of the same proteins that were altered in the Epithelial Adherens Junction Signaling pathway including contractile proteins (ACTA2, ACTB, MYH2, MYH4, MYH6, MYL2, MYL5, MYLK), cytoskeletal proteins (CTNNA1, CTNNB1, RAC1, RHOA, SPTAN1, and VAPA), and CPSF6, a protein subunit of a cleavage factor complex that plays a role in 3’ RNA cleavage and polyadenylation processing. Changes in expression of many of these proteins in AF (false discovery rate $p<0.05$) are also reflected in identification of IPA pathways that include Remodeling of Epithelial Adherens Junctions, Cytoskeleton

Signaling, and Calcium Signaling. Although IPA predicted the Cytoskeleton Signaling pathway to be activated, the directionality of the other pathways was inconsistent and unable to be predicted (Figure 3).

IPA identified 133 proteins as potential upstream regulators of the proteins significantly altered by AF (false discovery rate $p<0.05$) (Online Resource 4). Tumor protein P53 (TP53), transforming growth factor β−1 (TGFB1), transducin-like enhancer of split 3 (TLE3), lysine demethylase 5A (KDM5A), and KRAS proto-oncogene, GTPase (KRAS) were the most significant upstream regulators.

Proteins in Metabolic Pathways

While IPA identified Mitochondrial Dysfunction and Oxidative Phosphorylation as top canonical pathways, significant changes were also evident in many proteins in other metabolic pathways. Of the 408 proteins that were differentially expressed in AF, 134 (33%) are nuclear-encoded mitochondrial associated[24] and 87 (21%) are localized to the mitochondria [5]. Among the proteins localized to the mitochondria, 55 were downregulated and 32 were upregulated. In this analysis, 35 upstream regulators were identified with electron transport chain proteins as potential downstream targets, including five that were likely regulators of MT-ND1 (Table 2). AMP-activated protein kinase catalytic subunit α (PRKAA1), peroxisome proliferator activated receptor (PPAR) δ, and PPARγ coactivator 1 α (PGC1α), known regulators of metabolic pathways, were also identified as upstream regulators of proteins differentially expressed in AF (Online Resource 4).

Fourteen proteins differentially expressed in AF are components of electron transport chain complexes (Figure 4a). Increased expression of subunits of NADH dehydrogenase (complex I) (MT-ND1, NDUFA6, NDUFA7, NDUFB6, NDUFB9, NDUFB10, NDUFC2, NDUFS4, NDUFS5, NDUFV3), ubiquinol-cytochrome c reductase (complex III) (UQCR10), and cytochrome c oxidase (complex IV) (COX6A2, NDUFA4) was accompanied by decreased expression of a subunit of succinate dehydrogenase (complex II) (SDHB). These changes in expression of subunits of the electron transport chain complexes were accompanied by altered expression of proteins that play a critical role in assembly of the electron transport chain. Expression of complex I assembly factor NDUFAF4 was increased, though expression of NDUFAF5 and NDUFAF6 were decreased (Figure 4b). Translocase of inner mitochondrial membrane domain containing 1 (TIMMDC1), a chaperone protein that plays a role in the assembly of complex I, was increased. Apoptosis inducing factor (AIFM1) has roles in cell death and in complex I biogenesis [45]; its expression was decreased. Additionally, decreased expression of complex III assembly protein LYR motif containing 7 (LYRM7), complex IV assembly proteins COA4 and COA6, and HIG hypoxia inducible domain family member 1A (HIGD1A), a protein proposed to be a subunit of complex IV that plays a role in supercomplex assembly [43], was evident. Catalase (CAT) was one of the most significant differentially expressed proteins (Figure 2). Additionally, IPA identified Glutathione Redox Reactions I as a top canonical pathway (Figure 3) with altered expression of glutathione peroxidases (GPX1, GPX4), glutathione disulfide reductase (GSR), glutathione s-transferases (GSTK1, GSTM2, GSTT1) (Figure 4c).

Peroxiredoxin (PRDX1) and glutathione synthetase (GSS), proteins that play a role in the cell's response to oxidative stress, but not identified as proteins in the Glutathione Redox Reactions I pathway, were also altered in AF. Together, these changes highlight the key role these redox proteins play in modulating the atrial response to the increased metabolic demand of AF.

AF-related changes affecting mitochondrial protein expression were also evident in other metabolic pathways (Figure 5). GPD2, a component of the glycerol phosphate shuttle, was one of the most significantly decreased proteins in AF (Figure 2). Decreased expression of SDHB, a protein that serves a dual role as a subunit of complex II and a key enzyme in the citric acid cycle, was accompanied by reduced expression of fellow citric acid cycle-associated proteins pyruvate carboxylase (PC), succinate-CoA ligase (SUCLG2), and isocitrate dehydrogenase (IDH3G), but increased expression of the mitochondrial pyruvate carrier (MPC1) which transports pyruvate into the mitochondrial matrix. Propionyl-CoA carboxylase subunits α (PCCA) and β (PCCB), enzymes that play a role in catabolism of odd chain fatty acids and branched chain amino acids, were also decreased in AF. Changes in protein expression were also evident in mitochondrial pathways that include regulation of post-transcriptional mitochondrial gene expression (GRSF1), import of RNA into the mitochondria (PNPT1), mitochondrial protein synthesis (MRPL10, MPRL11, MRPL28, and MRPL53), mitochondrial protein import (TIMM8A, TIMM44, TIMMDC1), mitochondrial translation initiation (MTIF, CHCHD1), and DNA repair (REXO2).

Cytosolic proteins that play key roles in metabolism also were altered in AF. Notably, expression of AMP-activated protein kinase (AMPK) subunit β−2 (PRKAB2), an important regulator of the cell's response to energetic demand, was decreased. Decreased expression of ENO3, was accompanied by decreased expression of pyruvate kinase (PKM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), key enzymes in the glycolytic pathway. Expression of the monocarboxylate transporter (MCT) increased in AF. Additionally, increased expression of the plasma membrane fatty acid transporter CD36 and decreased expression of ACACB, acyl-CoA thioesterase 1 (ACOT1), and acyl-CoA synthetase short-chain family member 2 (ACSS2), key regulators of intracellular fatty acid utilization, were evident in AF. Expression of cytosolic adenylate kinase (AK1), which has a key role in cellular energy homeostasis, was also decreased in AF.

Ion Homeostasis

Expression levels of several proteins involved in cellular ion homeostasis were also altered. The pore subunit of the mitochondrial ATP-sensitive potassium channel (CCDC51) was decreased while the chloride channel (CLIC5) was increased. IPA identified Calcium Signaling as a significant canonical pathway ($p<1.23E-03$) in AF. Expression of CACNA2D2, a regulatory subunit of plasma membrane voltage-gated calcium channels (CACNA1C, CACNA1D) was increased, while expression of the mitochondrial calcium uniporter (MCU) was decreased. The integral role of calcium cycling in atrial function is also supported by altered expression of calcium binding proteins (S100A8, S100A13, THBS4) and proteins such as metabolic GPD2 whose activity is regulated by calcium.

Muscle Contraction

The changes detected in protein expression in the Calcium Signaling pathway highlighted the interactions between calcium regulation and muscle contraction, identifying changes in expression of proteins that play an integral role in cardiac muscle contraction. Of the 408 proteins differentially expressed in AF, 13 proteins are components of the sarcomere (Online Resource 2). Expression levels of myosin light chain kinase (MYLK, MYLK3), myosin heavy chain (MYH2, MYH4, MYH6), myosin XVIIIB (MYO18B), TNNC1, and tropomyosin (TPM2) were decreased in AF, while expression levels of actin (ACTA2), myosin light chain (MYL2, MYL5), myosin heavy chain (MYH7), and MYOT were increased (Figure 6a). The observed changes in MYH6 and MYH7 abundance are prototypical changes in AF that have been previously documented in right atrial tissues at both the protein [33] and mRNA levels [2].

AF Risk Genes

Several of the proteins that were differentially expressed in AF have been identified as potential AF risk genes [38] [34] (Figure 6b). Additionally, potential AF risk genes[38] [34] CRK proto-oncogene adaptor protein (CRK), estrogen receptor 2 (ESR2), insulin like growth factor 1 receptor (IGF1R), myocardin (MYOCD), NK2 homeobox 5 (NKX2–5), phospholamban (PLN), and T-box transcription factor (TBX5) were identified as upstream regulators of proteins altered by AF.

Persistent vs. Paroxysmal Atrial Fibrillation

We further analyzed differentially expressed proteins in AF patients who were in sinus rhythm at the time of tissue acquisition, but with a clinical history of either paroxysmal (n=4) or persistent AF (n=4). Of the 3090 proteins identified in the LAA, 499 unique genes were differentially expressed in persistent compared to paroxysmal AF (unadjusted $p<0.05$) (Online Resource 5); 247 were down-regulated and 252 were up-regulated with the most significant proteins identified in Supplemental Figure 1. Of these proteins, 97 were significant at a false discovery rate threshold of 0.10. Of the 499 proteins differentially expressed in atria from patients in sinus rhythm with a history of persistent vs. paroxysmal AF, 102 (20%) are localized to the mitochondria [5] (Online Resource 5). Among the differentially expressed proteins, 14 were previously identified as AF risk genes [34,38] (Online Resource 5).

IPA identified 104 canonical pathways that were significantly altered (false discovery rate $p<0.05$) (Online Resource 6). The proteins that were altered and their associated pathways also highlight key roles for metabolism, contractile function, and calcium signaling in AF, though the directionality of these changes were distinct from the above comparison of sinus rhythm to AF. Mitochondrial Dysfunction, Oxidative Phosphorylation, LXR/RXR Activation, FXR/RXR Activation, and EIF2 Signaling were identified as the most significant pathways. Although the Oxidative Phosphorylation pathway was predicted by IPA to be inhibited in persistent AF (false discovery rate $p<0.05$), the Sirtuin Signaling and Glycolysis I pathways were predicted to be activated (Supplemental Figure 2). Actin Cytoskeleton Signaling and Epithelial Adherens Junction Signaling pathways were also significantly affected (Supplemental Figure 2).

IPA identified 108 molecules as potential upstream regulators of the proteins significantly altered by persistent AF (false discovery rate $p<0.05$) (Online Resource 7). The most significant upstream regulators were hepatocyte nuclear factor 4 α (HNF4A), amyloid β precursor protein (APP), RPTOR independent companion of MTOR complex 2 (RICTOR), MYC proto-oncogene, BHLH transcription factor (MYC), and huntingtin (HTT). It is noteworthy that PGC1α, a key regulator of mitochondrial biogenesis, was also a top upstream regulator with an impact on AF persistence (false discovery rate $p=0.00005$).

Discussion

The primary finding of this study is that the abundance of both metabolic and myofibrillar proteins is altered in human AF. While all patients in this analysis had a history of AF, the most significant differences were detected between patients in SR compared to AF at the time of cardiac surgery. Expression of electron transport chain subunit proteins was increased, yet the expression of several electron transport chain assembly factors was decreased (Figure 5). Integral components of the TCA cycle (SDHB, SUCLG2, PC, and L2HGH) were also decreased. Increased expression of the plasma membrane lipid transporter CD36 was not accompanied by an upregulation of proteins that facilitate fatty acid uptake into the mitochondria or utilization through β-oxidation, but was accompanied by decreased expression of key proteins in the utilization of fatty acids (ACSS2, ACOT, ACACB). Key enzymes in the glycolytic pathway also were decreased (ENO3, GPD2, GAPDH). Taken together, the increased expression of electron transport chain proteins may reflect an attempt to meet the increased energetic demand in AF, but because this increase was not accompanied by an upregulation of pathways that would provide reducing equivalents to the electron transport chain, it is likely challenging for the energetic demand to be met.

Discordant changes were also evident in myocardial contractile protein expression, with both up- and down-regulation of key components of the contractile unit. We further identified alterations in proteins that play a role in calcium signaling, supporting the interrelationship between contractile function, ATP production, and calcium signaling. These findings are congruent with a recent study by Wiersma and colleagues that documented increased ATP levels in the LAA of patients with persistent AF, accompanied by alterations in myofibrillar and mitochondrial structural organization [50]. Increased expression of mitochondrial, myofibrillar, and calcium signaling proteins may reflect an attempt to meet the energetic and contractile demands of AF; concomitant decreased expression of proteins in these pathways may reflect an inability of the tissue to direct a coordinated response to increased heart rate, thereby compromising efficiency and contributing to heterogeneous and reduced ATP availability and development of a substrate for AF.

Calcium cycling abnormalities have been identified as key mediators of arrhythmogenesis in AF [17], and calcium signaling is regulated by the energy sensor AMPK [16]. Calcium transients are determined in part by calcium channel activity; while few ion channels subunits were detected in this study (due to their low abundance), a loss of L-type calcium current has been consistently observed in atrial myocytes from patients with dilated or

fibrillating atria [27,48,41,13]. It is intriguing that one of the regulatory subunits of this channel, CACNA2D, was upregulated in AF. Calcium currents [7,18] and calcium release (via the ryanodine receptor) are sensitive to redox state [47,51], and several pathways associated with response to oxidative stress were downregulated in the LAA of AF patients in this study (Figures 3, 4c). It is important to note that no change in the abundance of calcium/calmodulin dependent protein kinase II (CaMKII)-delta was detected, and other important stress response genes such as mitogen-activated protein kinase 9 (MAPK9, also known as JNK2) were not detected.

Intracellular calcium levels are a reflection of the work that is being done by cardiac myocytes, and the MCU acts as a sensor to stimulate oxidative phosphorylation and NADH production[29]. Our observation that MCU abundance is reduced in the AF tissues is intriguing. The MCU is required for uptake of calcium into the mitochondria, and activation of the MCU complex has been shown to prevent atrial calcium cycling alternans in a mouse-derived atrial cell line model [35]. In tachypaced HL-1 cardiomyocytes, a modest decrease in MCU prevented a decrease in the mitochondrial calcium transient amplitude, an effect that was not evident when MCU was decreased further [50]. Additional studies with isolated human atrial myocytes may be warranted to address this question.

While several earlier studies have investigated atrial protein expression in individuals with a clinical history of AF [20,8,42,21,22,28,14], the LA of patients who developed post-operative AF[23,31], and animal models of AF[11], to the best of our knowledge, this is one of the first studies to assess protein expression in LA tissue of patients with atrial rhythm documented at the time of tissue acquisition. Our study suggests that patterns of gene and/or protein expression in patients with a history of AF reflects their AF status. The SR group is comprised of patients with a history of paroxysmal or persistent AF, but who were documented to be in sinus rhythm at the time of surgery (in some cases following successful electrical or pharmacologic cardioversion). The AF group is comprised of patients with a history of persistent AF, but who were documented to be in AF at the time of surgery. Therefore, the primary comparison in our data is not AF history, but the rhythm status at the time of surgery and the duration of AF. Thus, the changes in protein expression reported here likely reflect both the dynamic changes that occur in response to AF and the longer-term effects evident in patients with long-standing persistent AF. This likely explains the observation that, though more than 200 potential AF risk genes have been identified, only 11 were differentially expressed in our comparison of AF vs. sinus rhythm. The identification of AF risk genes such as MYOT, that are differentially expressed, nonetheless highlights the key role that these proteins could play in AF. Progression of AF from a paroxysmal to persistent state may be facilitated by changes in the activity of a variety of kinases, including Akt, and to activation of the heat shock response [32]. Changes in the cytoskeleton are likely initiated by changes in phosphorylation of cytoskeletal proteins. Actin cytoskeleton signaling and RhoA signaling were both activated in the AF vs. sinus rhythm comparison (Figure 3) and in the paroxysmal vs. persistent AF comparison (Supplemental Figure 1). Both Akt and CDK4 activation have been linked to RhoA activation, and RhoA activation is associated with formation of actin stress fibers as well as inhibition of the heat shock response.

Previous studies have demonstrated significant changes in metabolic protein expression in AF and have noted some changes in protein expression similar to those reported here. Studies have reported changes in metabolic proteins that include GAPDH [31,15], SUCLG2[15], isoforms of PK[15] and PRDX[15,31,23], the NADP(+)2 isoform of IDH[15], enolase [11,23] and subunits of electron transport chain complexes [11,14,31] in human and animal models of AF. Congruent with our findings, changes in the expression of contractile proteins [39], specifically MYL2 [11], MYH6 [14], and MYH7 [23], extracellular matrix protein COL1A2 [14], and mitochondrial transport protein TIMM8A [14] have been reported in humans with AF, as well as in animal AF models. AKT serine/threonine kinase (AKT1, AKT2), insulin receptor (INSR), and serine/threonine kinase 11 (LKB1), kinases that play a role in metabolic pathways and exhibit altered activity in a tachypacing-induced AF model [32], were also identified in our study as upstream regulators in AF (Online Resource 4). The directionality and magnitude of metabolic protein expression changes differ between some studies, however, this is likely a result of differences in the tissues analyzed and specific AF status. Our own secondary analysis of persistent vs. paroxysmal AF tissues from patients in sinus rhythm at surgery showed changes in metabolic gene expression that were distinct and in some cases directionally opposite to those in the AF vs. SR comparison (Supplemental Figures 1 and 2; Online Resource 5). Nonetheless, when taken together, these studies highlight the significant impact of AF on proteins that play a role in metabolism and contractile function. An intriguing observational study based on both human and mouse heart failure studies demonstrated a strong positive relationship between abundance of genes involved in oxidative phosphorylation and ion channel genes important in cardiac electrical activity [1]. We suggest that a similar relationship is relevant for atrial pathophysiology as well, and that dysregulation of atrial energetics likely contributes to the substrate for AF. While few ion channel proteins were detectable, this may reflect their lower abundance rather than the lack of differential expression.

Limitations

While this study matched important variables between groups, the small overall sample size (n=12), and the smaller subset sample size (n=4 per group) are important limitations of this study. Additionally, the relatively small number of proteins identified in the present study, relative to a previously published proteomic analysis [14] is a limitation. Proteomic studies are best able to detect proteins that are more abundant in the cells of the assayed tissue, such as mitochondrial and cytoskeletal proteins, whereas detection of ion channels, which are much less abundant in the cell, is limited.

Additional studies with a larger sample size and greater sensitivity to detect less abundant proteins would be valuable. It is important to note however, that the analysis nonetheless identified many proteins differentially expressed in patients documented to be in AF, including ion channels and proteins that play key roles in cellular energy production and muscle contraction. These findings can be used to guide further studies that address the role that these proteins play in AF. The lack of a control group with no history of AF is also a limitation of the current study, but the use of this group in future studies would facilitate evaluation of the proteins and pathways associated with the risk of AF.

Conclusion

In conclusion, in a comparison of LAA protein expression changes in a cohort of patients with a history of AF, we identified significant expression changes of proteins related to mitochondrial energy production, cardiac muscle contraction, and calcium signaling. These studies identify Mitochondrial Dysfunction, Epithelial Adherens Junction Signaling, Tight Junction Signaling, Oxidative Phosphorylation, and Glutathione Redox Reactions as key pathways in AF and highlight the complex interrelationships between metabolic, calcium signaling, and contractile function pathways in the LA. Many of the pathways differentially affected by AF vs. sinus rhythm were also affected in a comparison of history of persistent vs. paroxsymal AF, presenting for surgery in sinus rhythm. We also identified changes in expression of several potential AF risk genes, providing additional insight into current studies investigating the role of potential AF risk genes in the development and progression of AF. Together, these studies suggest that metabolic genes are likely an important target for therapy in AF patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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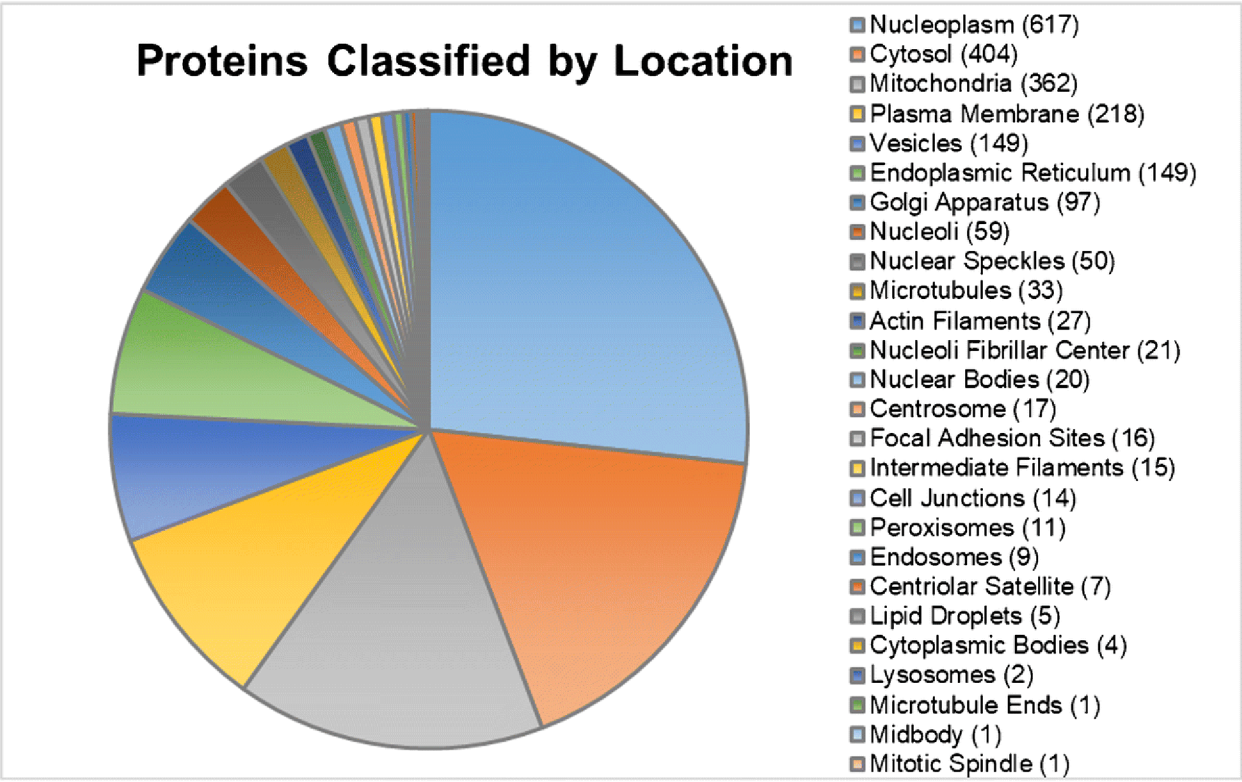

Fig. 1.

Classification of 2308 proteins identified by location using Human Protein Atlas. Actual values for each location are expressed as number of proteins.

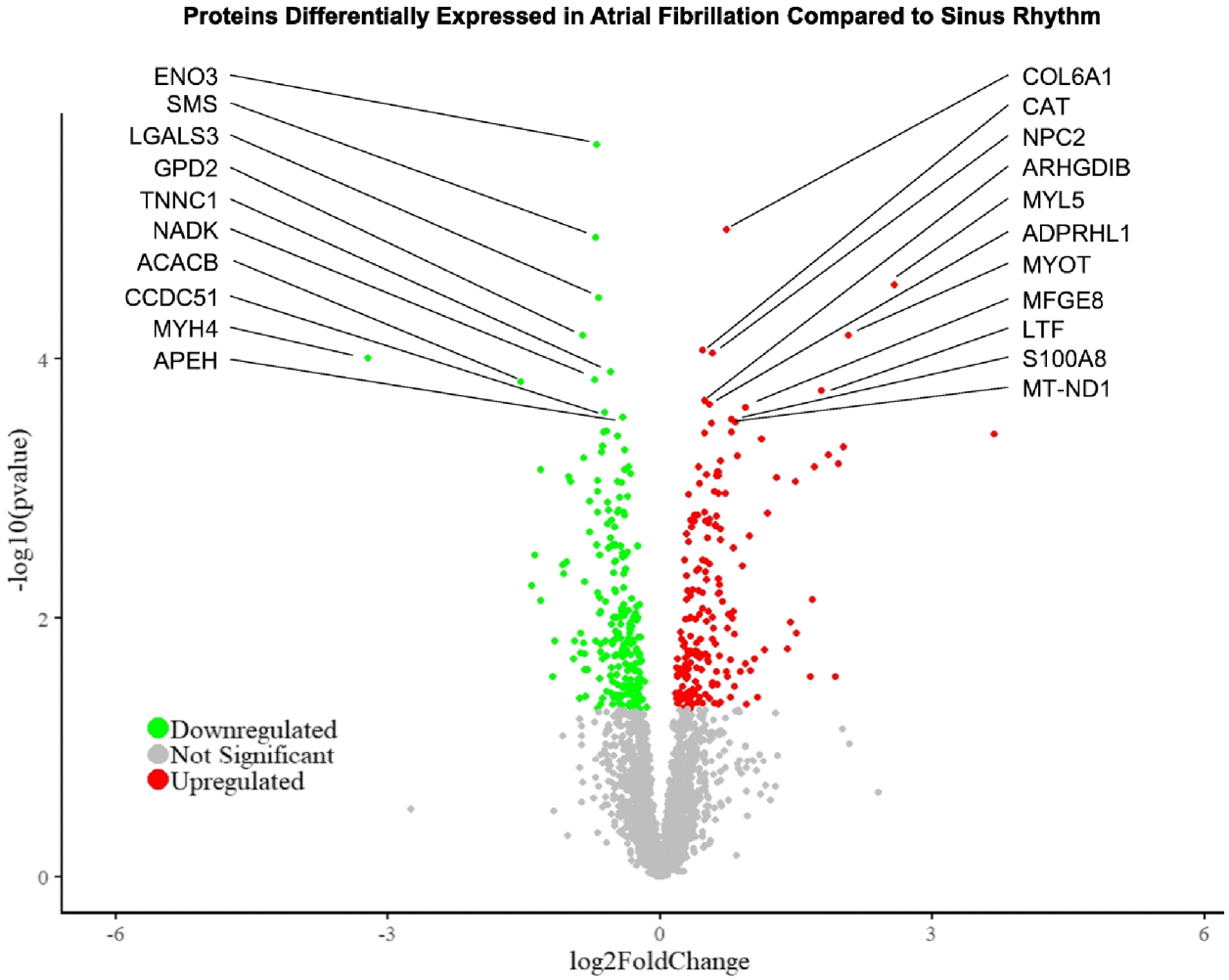

Fig. 2.

Proteins differentially expressed in human left atrial appendage tissue of patients in atrial fibrillation compared to sinus rhythm. Upregulated proteins are designated red, downregulated are designated green. Abbreviations used: acetyl-CoA carboxylase β (ACACB), ADP-ribosylhydrolase like 1 (ADPRHL1), acylaminoacyl-peptide hydrolase (APEH), rho GDP dissociation inhibitor β (ARHGDIB), catalase (CAT), coiled-coil domain containing 51 (CCDC51), collagen type VI alpha 1 chain (COL6A1), enolase 3 (ENO3), glycerol-3-phosphate dehydrogenase 2 (GPD2), galectin 3 (LGALS3), lactotransferrin (LTF), milk fat globule-EGF factor 8 protein (MFGE8), mitochondrial-encoded NADH:ubiquinone oxidoreductase core subunit 1 (MT-ND1), myosin heavy chain 4 (MYH4), myosin light chain 5 (MYL5), myotilin (MYOT), AD kinase (NADK2), NPC intracellular cholesterol transporter 2 (NPC2), S100 calcium binding protein A8 (S100A8), spermine synthase (SMS), and troponin C1 (TNNC1).

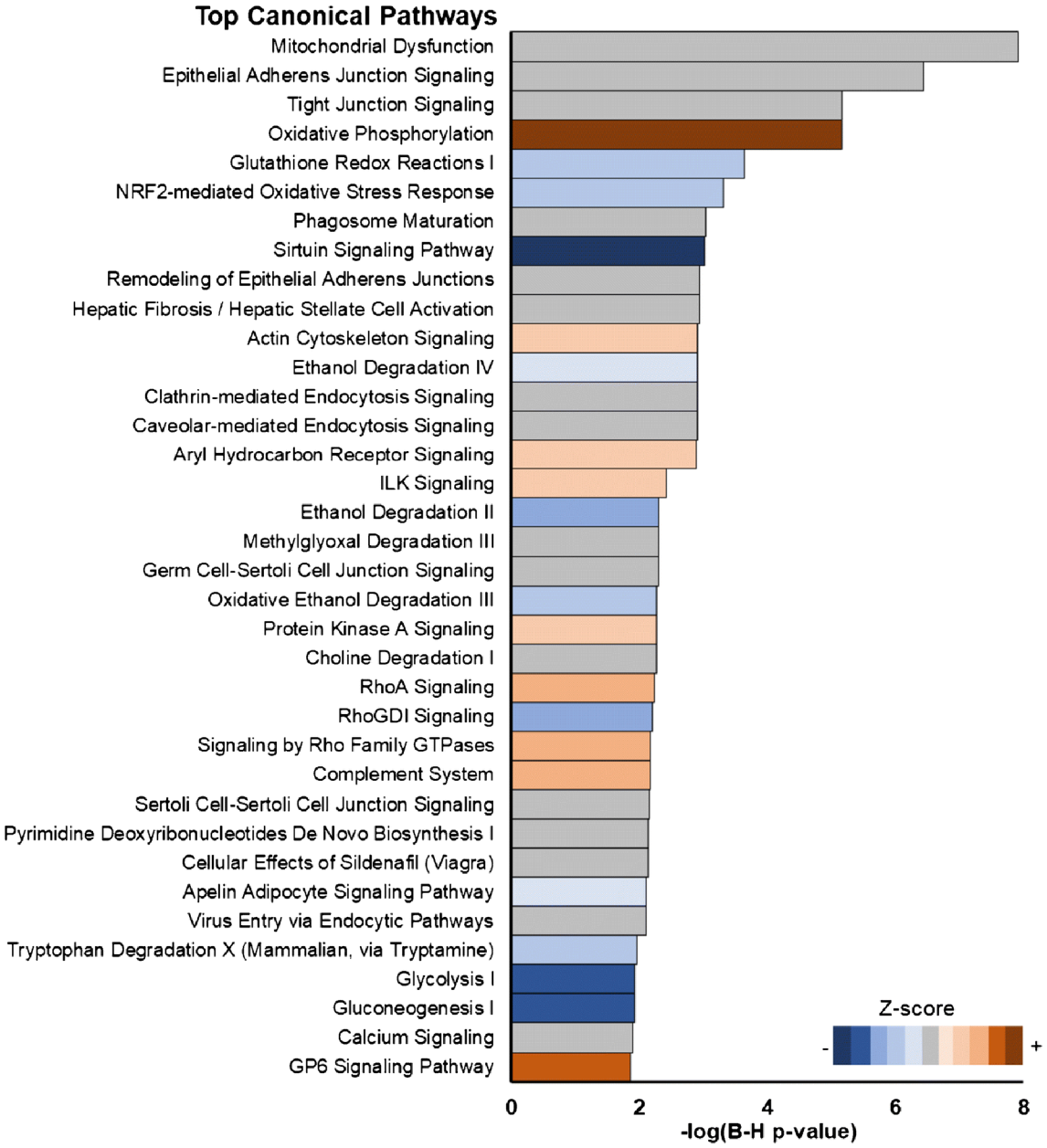

Fig. 3.

Top 35 canonical pathways identified by Ingenuity Pathway Analysis of proteins differentially expressed in atrial fibrillation compared to sinus rhythm. Pathways are presented in order of significance, with the most significant pathway listed first. Significance was determined using a false discovery rate $p<0.05$. Pathways that are predicted to be activated are designated in orange shades. Pathways that predicted to be inhibited are designated in blue shades. Grey designates pathways for which there was not sufficient information to predict directionality.

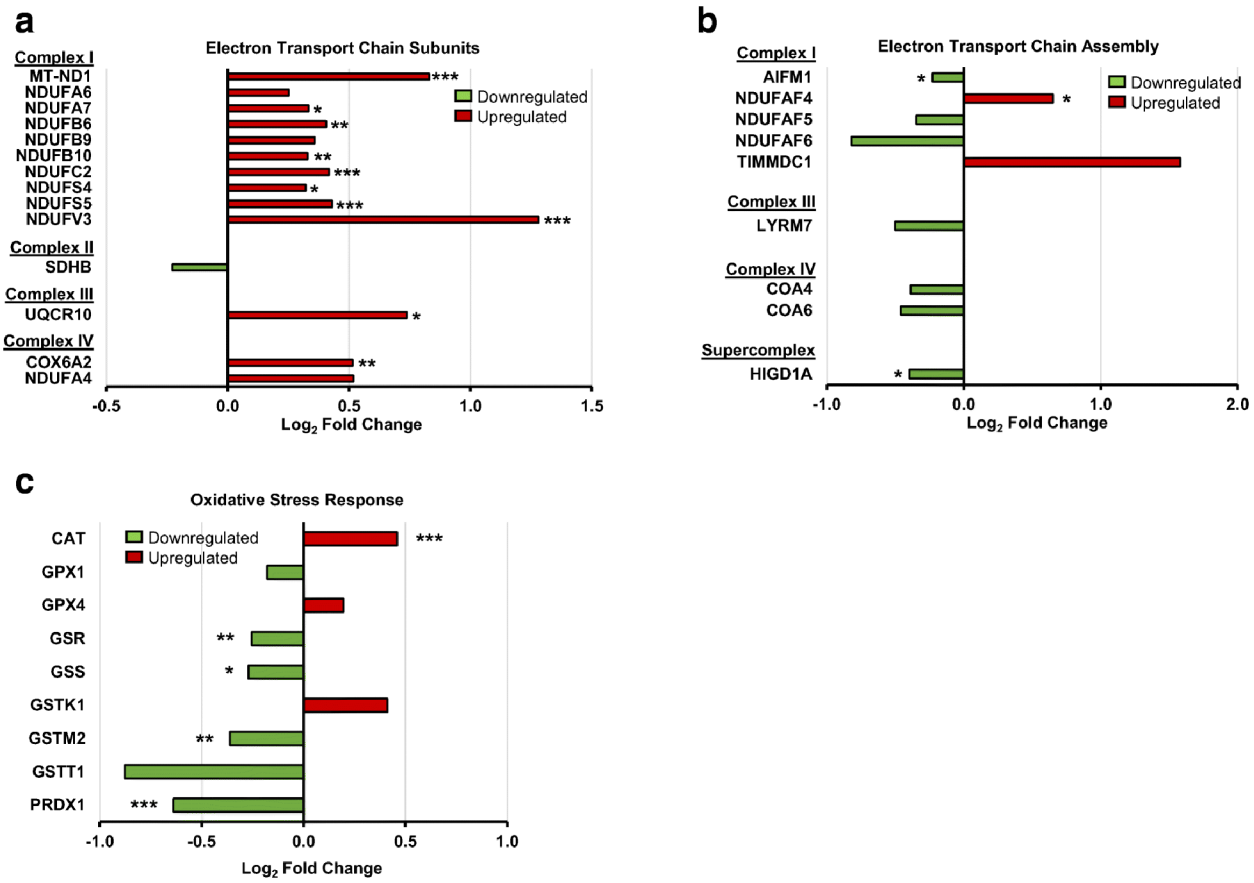

Fig. 4.

Expression of a) electron transport chain proteins, b) electron transport chain assembly proteins, and c) oxidative stress response proteins. Data is expressed as protein expression $\log_2$ fold change. Proteins that are upregulated are designated red. Proteins that are downregulated are designated green. *** indicates adjusted p value <0.05. ** indicates adjusted p value <0.10. * indicates adjusted p value <0.20. Abbreviations used: apoptosis inducing factor mitochondria associated 1 (AIFM1), catalase (CAT), cytochrome c oxidase assembly factor (COA4, COA6), cytochrome c oxidase subunit 6A2 (COX6A2), glutathione peroxidase (GPX1, GPX4), G-rich RNA sequence binding factor 1 (GRSF1), glutathione-disulfide reductase (GSR), glutathione synthetase (GSS), glutathione s-transferase (GSTK1, GSTM2, GSTT1), HIG1 hypoxia inducible domain family member 1A (HIGD1A), LYR motif containing 7 (LYRM7), mitochondrial-encoded NADH:ubiquinone oxidoreductase core subunit 1 (MT-ND1), NADH:ubiquinone oxidoreductase subunit (NDUFA4, NDUFA6, NDUFA7, NDUFB6, NDUFB9, NDUFB10, NDUFC2, NDUFS4, NDUFS5, NDUFV3) NADH:ubiquinone oxidoreductase complex assembly factor (NDUFAF4, NDUFAF5, NDUFAF6), peroxiredoxin (PRDX1), succinate dehydrogenase complex iron sulfur subunit B (SDHB), translocase of inner mitochondrial membrane domain containing 1 (TIMMDC1), ubiquinol-cytochrome c reductase, complex III subunit X (UQCR10).

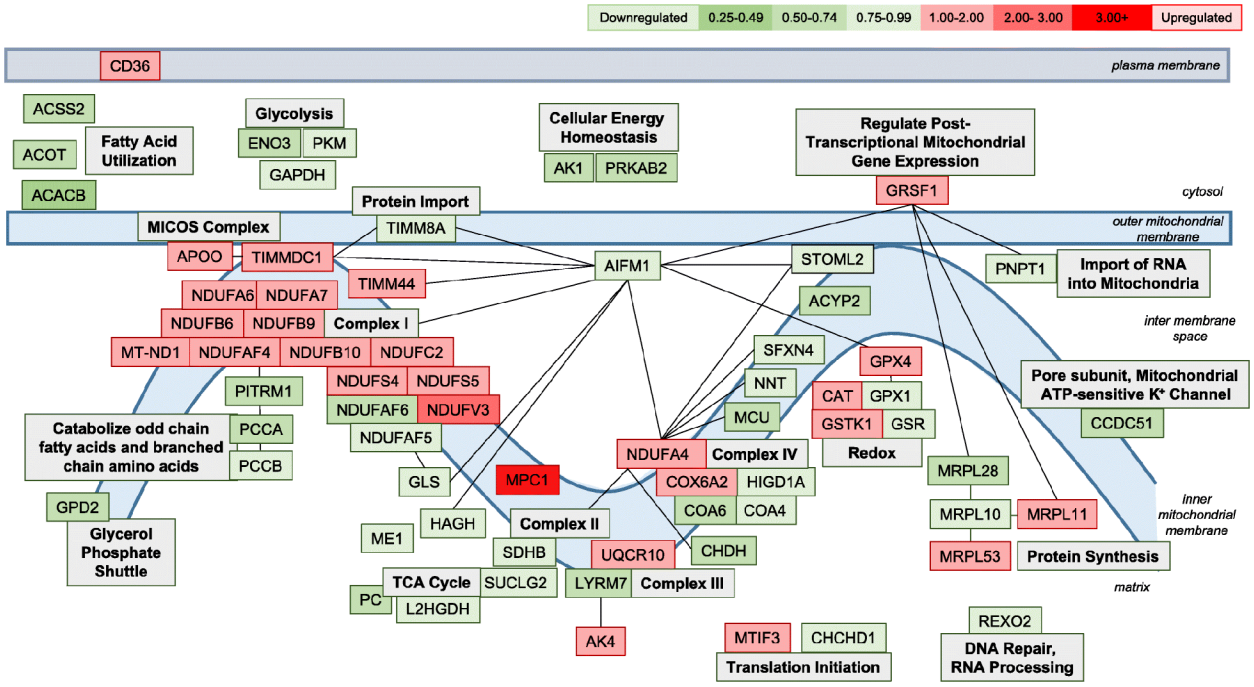

Fig. 5.

Expression of proteins in metabolic pathways. Data is expressed as protein expression fold change. Proteins that are upregulated are designated red. Proteins that are downregulated are designated green. Relationships between proteins, identified by Ingenuity Pathway Analysis, are designated by black lines connecting proteins. Significance was determined as $p<0.05$. Abbreviations used: acetyl-CoA carboxylase β (ACACB), acyl-CoA thioesterase (ACOT), acyl-CoA synthetase short chain family member 2 (ACSS2), acylphosphatase 2 (ACYP2), adenylate kinase 4 (AK4), apoptosis inducing factor mitochondria associated 1 (AIFM1), apolipoprotein O (APOO), catalase (CAT), coiled-coil domain containing 51 (CCDC51), coiled-coil-helix-coiled-coil-helix domain containing 1 (CHCHD1), choline dehydrogenase (CHDH), cytochrome c oxidase assembly factor 4 homolog (COA4, COA6), cytochrome c oxidase subunit 6A2 (COX6A2), enolase 3 (ENO3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutaminase (GLS), glycerol-3-phosphate dehydrogenase 2 (GPD2), glutathione peroxidase (GPX1, GPX4), G-rich RNA sequence binding factor 1 (GRSF1), glutathione-disulfide reductase (GSR), glutathione s-transferase kappa 1 (GSTK1), hydroxyacylglutathione hydrolase (HAGH), HIG1 hypoxia inducible domain family member 1A (HIGD1A), isocitrate dehydrogenase (IDH3G), L-2-hydroxyglutarate dehydrogenase (L2HGDH), LYR motif containing 7 (LYRM7), malic enzyme 1 (ME1), mitochondrial contact site and cristae organizing system (MICOS), mitochondrial calcium uniporter (MCU), mitochondrial ribosomal protein (MRPL10, MRPL11, MRPL28, MRPL53), mitochondrial translation initiation factor (MTIF), mitochondrial-encoded NADH:ubiquinone oxidoreductase core subunit 1 (MT-ND1), mitochondrial pyruvate carrier 1 (MPC1), mitochondrial ribosomal proteins (MRPL10, MPRL11, MRPL28, MRPL53), NADH:ubiquinone oxidoreductase subunit (NDUFA4, NDUFA6, NDUFA7, NDUFB6, NDUFB9, NDUFB10, NDUFC2, NDUFS4, NDUFS5, NDUFV3) NADH:ubiquinone oxidoreductase complex assembly factor (NDUFAF4, NDUFAF5, NDUFAF6), nicotinamide

nucleotide transhydrogenase (NNT), pitrilysin metallopeptidase 1 (PITRM1), propionyl-CoA carboxylase subunits α (PCCA) and β (PCCB), pyruvate carboxylase (PC), pyruvate kinase M1/2 (PKM), polyribonucleotide nucleotide nucleotidyltransferase 1 (PNPT1), RNA exonuclease 2 (REXO2), sideroflexin 4 (SFXN4), stomatin like 2 (STOML2), succinate dehydrogenase complex iron sulfur subunit B (SDHB), succinate-CoA ligase (SUCLG2), translocase of inner mitochondrial membrane (TIMM8A, TIMM44) translocase of inner mitochondrial membrane domain containing 1 (TIMMDC1), ubiquinol-cytochrome c reductase, complex III subunit X (UQCR10).

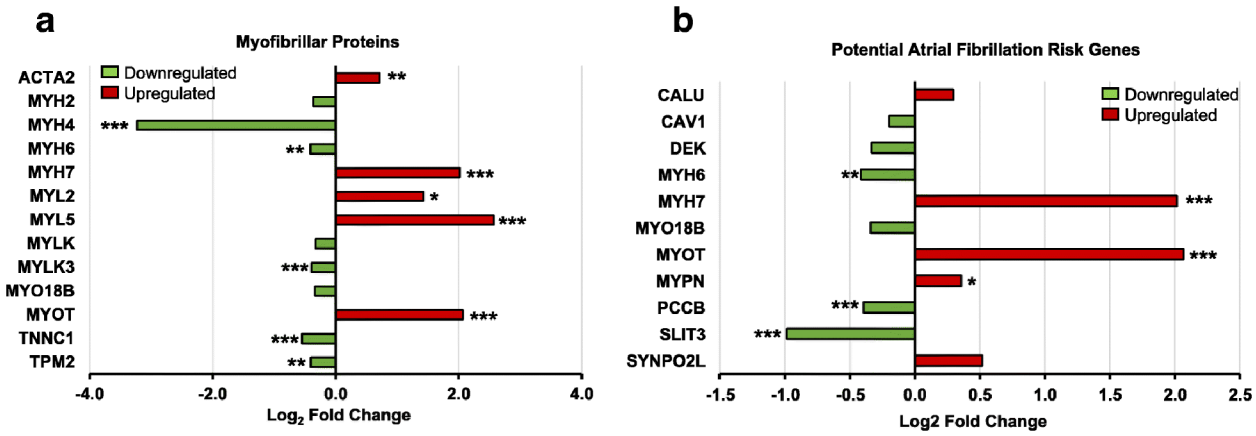

Fig. 6.

Protein expression of a) myofibrillar proteins and b) potential atrial fibrillation risk genes. Data is expressed as protein expression $\log_2$ fold change. Proteins that are upregulated are designated red. Proteins that are downregulated are designated green. *** indicates adjusted p value <0.05. ** indicates adjusted p value <0.10. * indicates adjusted p value <0.20. Abbreviations used: actin alpha 2 (ACTA2), calumenin (CALU), caveolin 1 (CAV1), DEK proto-oncogene (DEK), myopalladin (MYPN), myosin heavy chain (MYH2, MYH4, MYH6, MYH7), myosin light chain 2 (MYL2, MYL5), myosin light chain kinase (MYLK, MYLK3), myosin XVIIIB (MYO18B), myotilin (MYOT), propionyl-CoA carboxylase subunit β (PCCB), slit guidance ligand 3 (SLIT3), synaptopodin 2 like (SYNPO2L), troponin C1 (TNNC1), and tropomyosin (TPM2).

Table I.

Demographics of patients in sinus rhythm (SR) or atrial fibrillation (AF) at the time of surgery. All patients had a history of AF. Patients were matched by sex (M/F), age (years), body mass index (BMI), mitral valve disease, aortic valve disease, diabetes, and heart failure. AF diagnosis of Persistent, but in SR at the time of surgery indicated patients with prior history of persistent AF, but who had been cardioverted to SR by the time of surgery.

Rhythm at time of Surgery	AF duration (months)	AF Diagnosis	Sex	Age	BMI	Mitral Valve Disease	Aortic Valve Disease	Diabetes	Heart Failure
SR	3	Paroxysmal	M	60	28	Yes	No	No	No
SR	33	Paroxysmal	M	56	27	Yes	No	No	No
SR	4	Paroxysmal	F	64	18	Yes	No	No	No
SR	30	Paroxysmal	F	49	24	Yes	No	No	No
SR	1	Persistent	M	56	27	Yes	No	No	No
SR	8	Persistent	M	65	21	Yes	No	No	No
SR	4	Persistent	F	52	29	Yes	No	No	No
SR	4	Persistent	F	62	21	Yes	No	No	No
AF	65	Persistent	M	53	25	Yes	No	No	No
AF	108	Persistent	M	75	27	Yes	No	No	No
AF	84	Persistent	F	66	22	Yes	No	No	No
AF	240	Persistent	F	67	21	Yes	No	No	No

Table II.

Upstream regulators identified by Ingenuity Pathway Analysis with electron transport chain proteins as potential downstream targets. Upstream regulators that are likely regulators of MT-ND1, a subunit of complex I that is encoded by mitochondrial DNA are designated by *. Significance was determined using a false discovery rate $p<0.05$.

Protein Name	Gene ID	P value
* Amyloid Beta Precursor Protein	APP	1.07E-03
Calcium Binding Protein 39 Like	CAB39L	6.26E-05
Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit	CLPP	2.47E-02
Cytochrome P450 Family 1 Subfamily A Member 1	CYP1A1	4.24E-02
* Dystrophin	DMD	2.98E-05
Epidermal Growth Factor Receptor	EGFR	8.63E-04
Erb-B2 Receptor Tyrosine Kinase 2	ERBB2	9.17E-03
Estrogen Receptor 1	ESR1	4.26E-03
Forkhead Box O1	FOXO1	8.93E-03
Hemoglobin Subunit Alpha 1/ Hemoglobin Subunit Alpha 2	HBA1/HBA2	7.09E-03
Hepatocyte Nuclear Factor 4 Alpha	HNF4A	2.03E-03
Huntingtin	HTT	2.16E-06
Insulin Like Growth Factor 1 Receptor	IGF1R	3.94E-06
Insulin Receptor	INSR	7.87E-03
*Insulin	insulin	8.59E-04
Lysine Demethylase 5A	KDM5A	1.69E-06
Microtubule Associated Protein Tau	MAPT	3.35E-04
MYC Proto-Oncogene, BHLH Transcription Factor	MYC	1.21E-05
Nuclear Receptor Subfamily 4 Group A Member 1	NR4A1	9.98E-04
Nuclear Receptor Subfamily 4 Group A Member 3	NR4A3	2.38E-02
O-GlcNAcase	OGA	1.07E-03
Pyruvate Kinase M1/2	PKM	8.93E-03
Phospholipase A2 Receptor 1	PLA2R1	8.95E-04
PPARG Coactivator 1 Alpha	PPARGC1A	2.92E-04
Presenilin 1	PSEN1	5.07E-04
RB Transcriptional Corepressor 1	RB1	3.66E-03
RPTOR Independent Companion Of MTOR Complex 2	RICTOR	3.34E-05
Ryanodine Receptor 1	RYR1	1.35E-02
* SET And MYND Domain Containing 1	SMYD1	1.22E-02
Serine/Threonine Kinase 11	STK11	8.23E-04
Transforming Growth Factor Beta 1	TGFB1	3.12E-07
* TLE Family Member 3, Transcriptional Corepressor	TLE3	3.91E-07
Tumor Protein P53	TP53	2.43E-09
Uncoupling Protein 1	UCP1	2.13E-02
Zinc Finger Protein 106	ZNF106	2.47E-02