Specific Aims

Atrial fibrillation (AF) is the most common arrhythmia encountered in the U.S.[1] and the growing burden of AF[2] has profound health implications due to the association of AF with an increased risk of stroke, heart failure, and mortality.[3-5] Despite the great need for better AF risk prediction, few biomarkers exist to identify individuals at risk for AF and its complications.

In order to **identify novel AF biomarkers** and **determine the key pathways regulating pathological atrial remodeling**, we will take advantage of recent developments in the field of gene regulation. MiRNAs, a class of endogenous non-coding RNA species, are key regulators of gene expression in cardiovascular development and disease (CVD).[6] Moreover, data indicate that miRNAs are exported from cells and can provide insights into gene regulatory events *in vivo*.[6-8] MiRNAs have been associated with different forms of heart disease, including atrial and ventricular arrhythmias (miR-1, miR-133), myocardial fibrosis (miR-21, miR-29), and cardiac hypertrophy (miR-208).[6-8] MiRNAs regulate genes important to cardiac conduction (*HCN2&4, GJA1*), some of which have been implicated in the pathogenesis of AF.[6-8] MiRNAs have ideal characteristics as biomarkers, since they are stable, easily detectable, and present in the peripheral circulation.[6-9] However, we do not know the extent to which atrial pathological events are reflected in the circulating miRNA pool or the time course(s) involved in miRNA release. Despite their potential prognostic utility, **no prior study has examined the relations between circulating miRNAs and development of incident or recurrent AF**.

Focusing on the 19 miRNAs known to be present in the heart, I propose to test the **central hypothesis that circulating cardiac miRNAs are associated with atrial injury and that key miRNAs are associated with increased risk for AF**.[6-8] In the 3 proposed investigations, I will also examine the relations of an additional 75 non-cardiac-specific circulating miRNAs to discover new associations between miRNAs and AF not constrained by existing knowledge. My approach will provide insights into potentially novel mechanisms underlying AF and will establish a mechanism-based framework for identifying new biomarkers for AF risk prediction and targeting of therapies. I will accomplish these goals through the following aims:

Aim 1. To determine the time-dependent changes in key circulating miRNAs after left atrial (LA) ablation and relate these changes to recurrence of AF in a prospectively recruited patient cohort.
Hypothesis 1: In 180 UMMS participants with intensive AF phenotyping and undergoing LA ablation, circulating levels of 19 cardiac miRNAs will be up- or down-regulated from baseline at 1-hour post-ablation and will relate to recurrence of AF over a 12-month follow-up period.

Aim 2. To evaluate the cross-sectional relations of key circulating miRNA levels with AF in a case-control study.
Hypothesis 2: In 360 UMMS participants (180 AF cases, 180 controls) with intensive AF phenotyping recruited over a 30-month period, circulating levels (plasma and whole-blood) of 19 cardiac miRNAs will differ between subjects with AF and age- and sex-matched controls without AF.

Aim 3. To evaluate the relations of key circulating miRNA levels with prevalent and incident AF, and chronic atrial remodeling, as assessed by echocardiographic LA dimension, in the Framingham Heart Study (FHS) Offspring Study, a community-based cohort.
Hypothesis 3: In 2,477 FHS participants, circulating levels of 19 cardiac miRNAs will be associated with prevalent AF, new-onset AF, and higher echocardiographic (echo) LA dimension.

Background and Rationale

Epidemiology and Public Health Impact of AF: AF is an important clinical and public health problem and the prevalence of AF is expected to rise from 3-6 million Americans today to 12 million in 2050.[1,2] AF is associated with an increased risk of stroke, heart failure, and all-cause mortality.[3,4] The estimated excess annual national cost from AF treatment is $26 billion.[10]

Importance of atrial injury and structural remodeling to development of AF: AF is a heterogeneous disorder with multiple initiating and perpetuating mechanisms, including reentry.[11] CVD risk factors such as hypertension cause increased LA wall stress, ischemia, inflammation, myocyte apoptosis and atrial fibrosis.[12] We have proposed a pathophysiologic model of AF that emphasizes the role of LA structural change over time in generating a substrate that is vulnerable to electrical anisotropy, reentry and AF.[13] Our model (**Figure 1**) is based on observations that pathologic LA structural remodeling reflects duration and intensity of exposure to CVD risk factors known to promote AF, precedes the development of AF, and is associated with increased risk of AF. An increased appreciation for the importance of LA structural remodeling to the pathogenesis of AF is reflected by the conceptual model proposed by a recently convened NHLBI working group on AF prevention.[14]

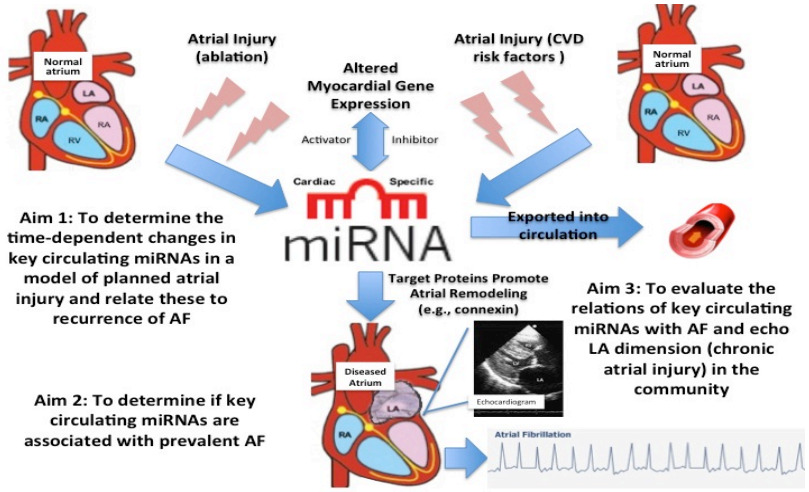

Figure 1. Conceptual Model

Pathophysiologic model of the progression from a normal atrium to a remodeled atrium vulnerable to AF as a result of atrial injury (from ablation and CVD risk factors). The importance of miRNA to the pathogenesis of atrial remodeling is emphasized.[6,7] The study aims are juxtaposed, illustrating how the proposed research plan relates to the model proposed by the NHLBI working group. Only pathways relevant to the research application are emphasized. RA=right atrium; LA=left atrium; RV=right ventricle; LV=left ventricle

Genomics and transcriptomics of AF and atrial remodeling: AF is a heritable disorder.[15] Genome-wide association studies have identified several loci associated with an increased risk for AF.[16] Animal models of AF have shown changes in atrial transcripts involved in natriuretic peptides and fibrosis.[17] Human RNA studies have demonstrated atrial remodeling,[18] as evidenced by **up-regulation** of genes involved in calcium signaling, potassium channels, antioxidant activity, inflammation, metabolic processes, fibrosis, apoptosis,[19] and **down-regulation** of genes involved in signal transduction and cell communication.[20,21]

MiRNA as Biomarkers: MiRNAs are single-stranded, non-coding, highly conserved post-translational regulators found in a variety of tissues.[6] MiRNAs regulate gene expression transcriptionally and post-transcriptionally through inhibiting translation and inducing degradation of specific RNAs.[9] Certain miRNAs are expressed cell-type specifically, or in association with particular physiological processes. Remarkably, miRNAs can be detected circulating in blood plasma or serum, as a result of cellular damage or secretion.[22] Therefore, **circulating miRNAs have ideal characteristics as biomarkers**, since they are stable and easily detectable (miRNA are comprised of nucleic acids and their sequences can be amplified). They also provide insight into mechanisms underlying disease.[23] Intronic miRNA are coded within a host gene, often regulating similar pathways to those of the protein encoded by the host gene and frequently acting to regulate a single biological process. Since miRNAs may be functionally redundant and can target a number of related RNAs, miRNA can mitigate against biological perturbations, such as in the setting of end-organ injury and AF.[24]

MiRNA in CVD: MiRNAs contribute to cardiac development and remodeling, including embryonic stem-cell differentiation, cardiomyocyte proliferation, electrical remodeling, and cardiac conduction.[25] MiRNAs found in cardiac tissue show dynamic changes in heart disease, suggesting their involvement in the regulation of CVD.[7] MiRNAs play important roles in different forms of heart disease, including atrial and ventricular arrhythmias (miR-1, miR-133, miR-328), fibrosis (miR-21, miR-29), and ventricular hypertrophy (miR-208, miR-133).[9] In animal models, miRNAs regulate genes known to control cardiac conduction (*HCN2&4, GJA1, KCNJ2*, and *CACNA1C*) and, in some cases, implicated in the pathogenesis of AF.[6,8,26] MiRNA-208 transgenic mice have a prolonged PR interval (an AF phenotype related to atrial remodeling)[27,28] and *miR208a-/-* mice develop AF.[29] In human models of AF, miR-328 was up-regulated and, in mouse and canine models, forced expression of miR-328 enhanced vulnerability to AF.[30] Circulating miRNAs have been associated with CVD, including coronary artery disease, myocardial infarction and heart failure.[8] MiRNA are released or secreted by cardiomyocytes, and the cellular secretion of unique miRNA suggests process specificity.[6] Unique patterns of miRNA are associated with different forms of ventricular remodeling phenotypes.[7] This serves as an important **proof-of-concept** for the science proposed herein, namely that miRNAs can be used as *in vivo* diagnostic and/or prognostic tools.[7] Moreover, and in contrast to other cellular disease mediators, drugs can target miRNAs. Since miRNA can modulate disease pathways, miRNA-based therapeutics would theoretically enable modulation of the cardiac stress response in a unique and powerful manner.[31]

Background and Rationale Summary

- AF is a major health problem because of its increasing prevalence and associated risk of stroke, heart failure, and death.
- Exposure to CVD risk factors injures the LA and promotes pathological atrial remodeling.
- Variation in RNA and miRNA has been associated with CVD and its risk factors, including cardiac remodeling and AF.

- Collaborations across UMMS and the FHS will accelerate discovery of pathophysiologically significant miRNA contributing to atrial remodeling and AF risk.

Preliminary Studies

I have conducted research that demonstrates my experience in studying cardiac remodeling and circulating biomarkers (e.g., miRNAs) in relation to clinical outcomes.[32-36] I have published in the area of AF[13,34,37-39] and established the UMMS AF Treatment Registry. The characteristics of the 120 AF patients recruited into the Registry over the last 12 months are shown in **Table 1**.

Table 1. Characteristics of 120 Patients Enrolled in UMMS AF Registry	
Age, years, SD	59 ± 10
Male sex (%)	68 (64.8%)
Weight (kg), SD	97.7 ± 21.3
Paroxysmal AF (%)	68 (65%)
Coronary Artery Disease	20 (19%)
Diabetes Mellitus	18 (17%)
Hypertension	75 (71%)
Failed Anti-Arrhythmic Drugs, n (%)	
None	5 (5 %)
1	53 (50%)
2	36 (34%)
≥3	11 (10%)
AF Quality of Life Score	84 (23.3)
Echocardiography	
Ejection Fraction, %	58 ± 5%
LAD, mm, SD	42 ± 7
Post-ablation arrhythmias at 6-months	
Recurrent AF	25 (21%)
Atrial Flutter	24 (20%)

Cardiac remodeling and biomarkers in relation to clinical outcomes: I have examined the associations of cardiac remodeling phenotypes with the risk of heart failure and death. I found that an increase in ventricular volume after exercise was associated with total mortality.[40] In a 2nd investigation, I showed that increased ventricular volume was an independent predictor of hospitalization for heart failure.[39] In a 3rd study, I observed that, when compared to participants with preserved kidney function, those with reduced kidney function had a 3-fold greater odds of developing AF.[34] In a 4th study involving FHS Offspring participants, I demonstrated that higher circulating concentrations of adiponectin and resistin were associated with higher left ventricular mass and lower fractional shortening, respectively.[35] During my FHS fellowship (07/08-6/09), I examined the clinical correlates of echo LA enlargement over the adult lifecourse in the FHS.[36] The findings of these studies informed a model of LA remodeling that serves as the foundation for the current application.[13]

Plasma miRNA and atrial injury: To determine the time-dependent changes to miRNAs after LA injury, I conducted expression profiling of 382 circulating miRNAs before and after LA ablation in 8 subjects (Ambros laboratory). I found 17 miRNAs (**Figure 2**) that were up- or down-regulated at least 2-fold by 1-hour after LA injury (including 3 candidates selected for hypothesis testing). The link between these 17 miRNAs and LA injury is novel, but some are known to regulate relevant processes such as cardiovascular development, cardiometabolic disease, or end-organ injury response.[6] Although power was limited by the small sample size, these data emphasize the feasibility of my approach and suggest my findings will be novel and identify new molecular targets. I will expand upon these preliminary results as described in Aim 1.

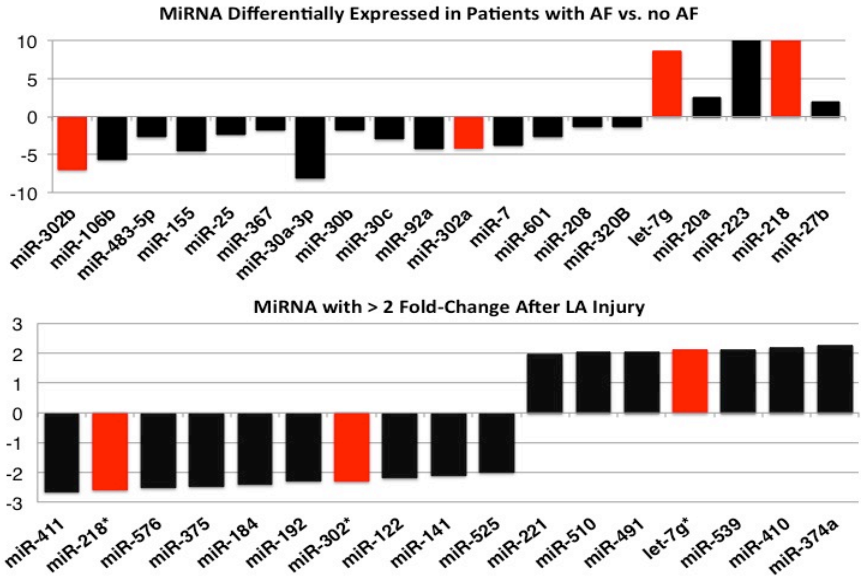

Figure 2. MiRNAs Differentially Expressed (Fold-Difference) in AF (top) and After LA ablation (bottom). MiRNAs up- or down-regulated in both experiments are highlighted in red. N.B., miR-223 and 218 expression was >10-fold higher in AF patients than in controls.

Plasma miRNA and AF: To establish the circulating miRNAs associated with AF, I also conducted expression profiling of 94 circulating miRNAs in 17 individuals with AF and 24 control subjects. I found that plasma levels of 20 miRNAs (**Figure 1**) differed significantly between those with and without AF (including 6 candidates selected for hypothesis testing). I observed overlap (3 miRNAs: miRs-218 and 302, let-7g) between miRNAs associated with AF and miRNAs up- or down-regulated after LA injury (labeled in red in **Figure 2**). These data support my hypothesis that **altered gene regulation after atrial injury plays an important role in the pathophysiology of AF**. Although power was limited by the modest sample size, these data support the rationale for using circulating miRNA as AF biomarkers. I will expand upon these results in Aims 2-3.

Circulating miRNA: To establish which miRNA are expressed in the circulation of patients with and without CVD, Dr. Freedman and I comprehensively profiled miRNAs (762) in the peripheral circulation of individuals with and without CVD. We found 75 miRNA expressed in plasma or whole blood. Only those miRNAs present in the peripheral circulation or associated with AF or atrial injury were included in our proposed investigations.

FHS miRNA profiling and QC: The Freedman laboratory (UMMS Gene Expression Core) assayed expression for 347 unique miRNAs, listed as a cycle threshold (CT) value with standard deviation, in 2,477 FHS Exam 8 participants. MiRNA expression profiling was conducted using whole blood from PAXgene tubes. The lab employed the TaqMan chemistry miRNA profiling system (used in Studies 1-3). The FHS steering committee has reviewed the QC measures and praised its excellent reproducibility (variation coefficient <10%).

Experimental Design

Aim 1. To determine the time-dependent changes in key circulating miRNAs after LA ablation and relate these changes to recurrence of AF in a prospectively recruited patient cohort.

Rationale and Hypothesis: The degree to which atrial pathologic events such as atrial myocyte injury, apoptosis, and fibrosis are manifest in the circulating miRNA pool, as well as the time course(s) involved in their release, are unknown. Although ≈40% of patients with AF experience a recurrence at 12-months after LA ablation,[41] the physiology underlying treatment failure also remains poorly understood.[41,42] It is also unknown whether or not baseline miRNA levels, or changes in miRNA levels after ablation, relate to AF recurrence in patients with AF undergoing catheter-based ablation. **I hypothesize that key circulating miRNAs are up- and down-regulated within 1-hour after atrial injury and relate to AF after LA ablation.**

Data Source: Individuals who present for index radiofrequency LA ablation at UMMC and are in sinus rhythm at the time of their procedure will be recruited by trained study staff prior to their procedure. All participants must be ≥ 21 years and must meet pre-defined inclusion-exclusion criteria (UMMS IRB #PRO00000027). Over a 4-month period, we have recruited 30 participants (80% of those eligible).

Study Design: The baseline clinical, electrophysiologic, echo, ablation, and laboratory characteristics of 180 study participants undergoing index LA ablation for AF will be abstracted from the UMMS AF Treatment Registry (**Table 1**). To enable comparison of results to those of Study 1 (whole blood), yet improve our ability to detect subtle changes in extracellular miRNA levels (plasma), study staff will collect both fasting whole blood and plasma. Whole blood (PAXgene) and plasma will be obtained just prior to LA ablation (baseline) as well as at 1-hour and 1-month after the initial atrial ablation. Plasma will be isolated from K_2EDTA venous blood samples via centrifugation by the PI or a Research Technician using a standardized laboratory protocol and stored at -80°C.[43] Participants undergo a standard clinical exam at 1- and 12-months after enrollment in the AF Treatment Clinic. History and 12-lead electrocardiogram (ECG) are obtained at this visit, with particular attention paid to medications used and any intervening symptoms consistent with arrhythmic episodes. Upon study completion, a trained study nurse or physician will review all clinical data (including any hospitalizations or physician visits related to possible AF recurrence) from the medical records of study participants. Since 90% of UMMC patients undergoing AF ablation follow-up in the AF Treatment Center and are re-hospitalized at the UMMC, we do not anticipate significant missing data or losses to follow-up. Participants will be consented for a telephone health history update and solicitation of outside records if they do not return to UMMC.

Selection and profiling of miRNA: The 19 most abundant miRNAs in the heart account for more than 90% of all cardiac miRNAs.[6] So far, a functional role has been ascribed to only the most enriched miRNAs, reflecting the fact that a threshold level of miRNA expression seems to be required for effective repression of target gene expression.[31] However, the degree to which cardiac-specific miRNAs are present in the circulation under stress-related conditions remains poorly defined. For hypothesis testing and to minimize multiple testing concerns, I will focus on 19 miRNAs expressed in the heart or associated with processes implicated in the pathogenesis of atrial remodeling (**Table 2**). For hypothesis generation, I will examine 75 non-cardiac miRNAs expressed in plasma to discover new associations not constrained by existing knowledge. I will use these data to identify a set of invariant miRNAs to use as normalization controls. The UMMS Gene Expression Core will help to isolate miRNAs and profile miRNA expression using the TaqMan chemistry miRNA profiling system.

Timing of miRNA assessment: Since circulating miRNA levels can change rapidly after organ injury, I will assess miRNA levels at baseline and 1-hour (3-hours after first ablative lesion administered) after LA ablation. Because we are also interested in establishing the miRNAs associated with atrial structural remodeling, a process which can take weeks to occur, we will also measure miRNA levels at 1-month after ablation.

Feasibility/Attrition: This study has been designed to minimize patient inconvenience (only 1 f/u exam) and

costs, as outlined in the budget justification. Biospecimens from study participants (DNA, RNA, miRNA) will be de-identified and stored at no cost to the investigator or patient (**Mentor's Letter**). I anticipate a 10% attrition rate based on the literature and AF Treatment Center experience.[44] This application includes strategies to reduce attrition, establishing multiple ways to contact the participant (phone, email) and phone calls to participants to remind them of follow-up exams. To date, 80% of eligible participants have been recruited with no attrition.

Aim 1 Analytical Approach: I will describe fold-change in miRNA expression from baseline to 1-hour and 1-month post-ablation. MiRNA expression will be listed as a CT value, consistent with RT-PCR-based data. To test the hypothesis that miRNAs up- or down-regulated by 1-hour after AF ablation relate to recurrence of AF, I will describe how fold-change in miRNA expression (**Table 2**) from baseline to 1-hour after ablation relates to AF recurrence over a 12-month follow-up (dependent variable) using Cox proportional hazards modeling, adjusting for duration of ablation, number of ablative lesions, and factors associated with LA dimension or AF.[28,36] Secondarily, similar analyses will be performed for changes from baseline to 1-month. Patterns of miRNA expression change from baseline to 1 hour and 1 month will be classified and associated with time to AF recurrence as an exploratory analysis.

Table 2. Putative Functions and Phenotypes Associated with 19 Cardiac-Enriched or Specific miRNAs

MIRNA	FUNCTION (TARGET GENES)	ASSOCIATED PHENOTYPE**
miR-1	Cell cycle regulation; (Ion Channels and gap junction genes, GJA1, KNJ2)	Cardiac arrhythmia, cardiac development
miR-21	Upregulation of the protein sprouty (ERK-MAPK)	Anti-apoptotic factor, cardiac stress response
miR-23a	Inhibition of ubiquitin proteolysis	Regulates cardiac hypertrophy
miR-29	Inhibition of collagen and extracellular matrix proteins (ELN, FBN1, COL1A1)	Cardiac fibrosis
miR-92a*	Inhibition of neorevascularization (integrin subunit α5 and eNOS)	Reduction in cellular apoptosis and improved cardiac function
miR-122*	fatty acid beta-oxidation	endothelial dysfunction
miR-133a	Cell cycle regulation; (Ion Channel genes HCN2, HCN4)	Cardiac arrhythmia, cardiac development
miR-195	Phosphatidylserine decarboxylase	Cardiac growth and heart failure in transgenic mice
miR208a*	Transcriptional regulator, cardiac stress response (α-MHC, Connexin-40 (GJA5).	Cardiac hypertrophy; cardiac arrhythmia
miR-208b*	Controls myosin isoform switching; Intronic to Myh7	Cardiac hypertrophy, fibrosis
miR-218*	Suppresses Robo1 and 2	Cardiac development
miR-221*	P27(Kip1) and p57(Kip2)	Vascular smooth muscle growth
miR-223*	Regulates GLUT4	AF in dog model
miR-302*	Regulates E-cadherin	Cell–cell adhesion
miR-320	Pro-apoptosis (HSP20 levels); Increases expression of insulin-like growth factor-1	Down-regulated after ischemia reperfusion injury; down-regulated in AF
miR-328	Shortened atrial action potential (CACNA1C)	AF in rheumatic mitral valve disease
miR-499	Intronic to Myh7b	Myosin function; down-regulated in AF models
miR-590	TGF-beta1 and betaRII	Increases AF vulnerability
miR-664	Heparan sulfate 6-O-sulfotransferase 3	AF in dog model

* Associated with atrial injury or AF in preliminary studies.
** References[6-8,18,19,22,23,26,29-31,46,49]

Aim 1 Power: For a given number of events, the standard error of β coefficient per standard deviation for a continuous variable in a Cox proportional hazard model was empirically estimated from FHS exam 7 biomarker data (standardized to mean=0, SD=1). The standard errors of the β coefficients per standard deviation of change (from baseline to 1-hour) in miRNA levels were rescaled according to the number of expected events. Bonferroni correction was used to account for inflated type I error by multiple testing. The minimum detectable hazard ratios with 80% and 90% power are 1.61 and 1.70 respectively, assuming an AF recurrence rate of 40% at 12-months[45] and an overall 0.05 level of significance, i.e. alpha = 0.05/19 = 0.0026 for each test.

Anticipated Results and Future Directions: I anticipate that biologically important, circulating miRNA will be up- or down-regulated by 1-hour post-ablation and will relate to recurrence of AF. This work will: 1) determine the time-dependent changes to circulating miRNA in response to acute atrial injury; 2) examine the associations between baseline levels of key plasma miRNAs and factors associated with recurrence of AF; and 3) evaluate the relations between miRNA up- or down-regulated after ablation and recurrence of AF. I anticipate that the validation of our study findings will be necessary. I have secured the MGH AF Cohort as a potential replication partner for the proposed investigation (Dr. Patrick T. Ellinor III, Principal Investigator MGH AF Cohort, signed letter of collaboration). The proposed studies will lead to the identification of circulating biomarkers related to atrial injury that may have diagnostic and or prognostic value in AF. Mechanistic inferences will be made about the kinds of injury responses seen on the basis of evaluating which miRNAs are up- or down-regulated early after atrial injury vs. later. MiRNA found to be in association with LA injury after ablation will be compared with miRNA related to AF and increased LA dimension in the community (Aim 3). Gene targets will be determined using TARGETSCAN 6.0 and miRanda.

Potential Problems and Solutions:

1. Hypothesis-testing vs. hypothesis-generation: I will focus on 19 miRNAs associated with processes implicated in the pathogenesis of atrial remodeling or AF. I will update the 19 miRNAs examined if interim publications suggest other miRNA putatively related to AF. Because existing data are limited, I will conduct a secondary analysis examining 75 non-cardiac miRNAs expressed in circulating blood to discover new mechanisms related to the development of AF.

2. Radiofrequency ablation as a model of LA injury: In order to investigate time-dependent changes in circulating miRNAs after LA injury, I will use radiofrequency LA ablation as an *in vivo* model for atrial injury. Although LA injury in the community, like LA injury from ablation, is associated with myocyte apoptosis and enhanced fibrosis, the generalizability of this model to chronic atrial injury from CVD risk factors such as hypertension remains unclear. However, I will validate Study 1 findings in the community by examining the associations between the same cardiac miRNAs and echo markers of chronic LA injury in Study 3.

3. Causation vs. association: Gene targets of miRNAs associated with AF will be determined. However, since miRNAs can affect several genes, the mechanisms underlying these associations will require further exploration. Recognizing this, as part of a future R03 application, Dr. Chinmay Trivedi (UMMS) and I have begun to transfect candidate miRNAs (e.g., miR-302) into atrial myocytes (H2c9) in order to ascertain the effects of miRNA gain-of-function on target gene expression. We will also examine the *in vitro* effects of miRNA loss-of-function using this model.

Aim 2. To evaluate the cross-sectional relations of key circulating miRNA levels with AF in a case-control study.

Rationale and Hypothesis: MiRNAs regulate gene expression in a number of diseases, including AF.[24] MiRNAs have been detected in cell-free human plasma preparation, where they appear to be stable and protected from endogenous RNase activity.[46] Although experimental models suggest dynamic regulation of miRNAs by the heart in certain pathologic states, it is unknown if circulating levels of key cardiac-specific or enriched miRNAs differ between individuals with AF and those in normal rhythm. **I hypothesize that plasma levels of cardiac-specific or enriched miRNAs will differ between individuals with AF and controls.**

Data Source: Individuals with a history of paroxysmal or persistent AF who present for a LA ablation at UMMC and are in sinus rhythm at the time of their procedure will be recruited by trained study staff in the cardiac short-stay area. Individuals who are hospitalized at the UMMC, are in normal sinus rhythm, have no evidence of an acute coronary syndrome, and do not have a history of AF will be recruited by trained study staff in their hospital room and will comprise the control group. All participants must be ≥ 21 years and must meet pre-defined inclusion/exclusion criteria (Human Subjects Protection; UMMS IRB #PRO00000027). Over the past 3 months, we have recruited 30 participants with AF (80% of all eligible) and 27 controls.

Study Design: The baseline clinical, demographic, electrophysiologic and laboratory characteristics of 180 study participants with paroxysmal or persistent AF (cases) will be abstracted from the UMMS AF Treatment Registry (**Table 1**). Hospital electronic records will be used to abstract data on 180 controls. To enable comparison of results to those of Study 3 (whole blood), yet improve our ability to detect subtle changes in extracellular miRNA levels (plasma), staff will collect both fasting whole blood and plasma. Whole blood and plasma will be obtained on participants with AF prior to their LA ablation. Whole blood and plasma will be obtained on controls in the same manner. Plasma will be isolated from K_2EDTA venous blood samples using the laboratory methods described in Study 1.[43] MiRNA expression profiling methods are described in Study 1.

miRNA profiling: The Freedman laboratory will assay miRNA expression for a total of 94 miRNAs, listed as a cycle threshold (CT) value with accompanying standard deviation. The lab will employ the TaqMan chemistry miRNA profiling system described in **Preliminary Studies** and Study 1.

Feasibility: Given the minimal risks of the proposed study, the number of patients with AF treated in the electrophysiology lab (≈200/year), the committed assistance of a trained study technician (**Mentor's Letter**), UMMS co-investigators with expertise in miRNA isolation and expression profiling (Ambros, Freedman), the present study is feasible. Attrition is not a concern given the single study visit required for study completion. The study has been designed to minimize patient inconvenience and costs, as outlined in the budget justification. Biospecimens from study participants (DNA, RNA, miRNA) will be de-identified and stored at no cost to the investigator or patient (**Mentor's Letter**).

Aim 2 Analytical Approach: I will test the hypothesis that cardiac-specific or enriched circulating miRNAs are associated with paroxysmal or persistent AF by conducting a logistic regression analysis, adjusting the relative

effect of miRNA expression for several potential confounders. MiRNA expression will be listed as a CT value, consistent with RT-PCR-based data. AF risk factors will be adjusted for (e.g., age, sex, heart rate, blood pressure, body mass index, diabetes mellitus, prevalent myocardial infarction and heart failure) and AF characteristics (e.g., AF duration, or AF classification) when estimating the association of each of the 19 miRNA candidate levels (range 3-28 CT) with AF. For hypothesis testing, as described in Study 1, I will focus on 19 miRNAs expressed in cardiac tissue and/or associated with processes implicated in the pathogenesis of atrial remodeling (**Table 2**).[6,47] For hypothesis generation, I will examine 75 miRNAs expressed in plasma (as described in Study 1) to discover new associations not constrained by existing knowledge.

Aim 2 Sample Size/Power: For this study, the total sample size is 360, with 180 cases of AF and 180 individuals in the comparison group (no AF). For each of the 19 miRNA expression variants, I have 80% power to detect an odds ratio of ≥ 1.56 or 90% power to detect an odds ratio of ≥ 1.65 for AF for a 1 standard deviation increment in miRNA at alpha=0.0026.

Anticipated Results and Future Directions: I anticipate that biologically important, cardiac-specific or enriched circulating miRNA will be associated with AF. I also anticipate that the validation of our study findings will be necessary. The FHS Offspring Study will serve as an internal validation cohort (Study 3). The Multi-Ethnic Study of Atherosclerosis (MESA) will serve as an external replication partner (Dr. Liu, collaboration aggreement). MiRNA associated with AF will be compared with miRNA related to atrial injury in Study 1. We will study the RNA targets of miRNAs associated with atrial injury and/or AF by reviewing current publications and using algorithms such as TARGETSCAN 6.0 and miRanda.[48] The proposed studies are complementary and will lead to the identification of circulating biomarkers that may have diagnostic value in AF.

Potential Problems and Solutions:

1. Whole Blood vs. Plasma miRNA Profiling: Preliminary data suggest that plasma and whole blood miRNA profiles may differ in patients with CVD (**Preliminary Data**). In order to maintain uniformity of methods between the 3 proposed investigations, promote comparability of findings, and enhance the clinical applicability of results, I will analyze miRNA from whole blood in all Study 2 participants. To maximize my ability to detect subtle changes in extracellular miRNAs released into the circulation, I will also analyze miRNA from plasma.[49]

2. Novelty vs. replication: With all transcriptomic studies, replication is critical. I will internally replicate findings using data from the FHS Study as outlined in Study 3. I will also leverage my affiliation with the CHARGE-AF Consortium to recruit additional replication partners (e.g., MESA, MGH AF cohort).

3. Cell-type specificity vs. generalizability: Although dynamic regulation and cellular secretion of miRNAs suggests process specificity,[9] the present study will not confirm whether miRNAs associated with AF are secreted/released by atrial myocytes. I will, however, compare miRNAs associated with AF from Aim 2 with those released after LA injury (Aim 1) and/or in association with echo markers of chronic LA injury (Aim 3).

Aim 3. To evaluate the relations of key circulating miRNA levels with prevalent and incident AF, and chronic atrial remodeling, as assessed by echocardiographic LA dimension, in the FHS Offspring Study, a community-based cohort.

Rationale and Hypothesis: Data are limited on how miRNAs relate to pathologic changes in LA structure and AF, especially in the broader community setting. To further explore the hypothesis that miRNAs contribute to LA structural remodeling and AF, I will examine the relations of the same set of 19 cardiac-specific or enriched miRNAs with echo LA dimension (a marker of chronic LA injury) and AF. **I hypothesize that key circulating miRNAs are associated with AF and increased echo LA dimension.** The analyses outlined in this application have undergone peer-review and have been approved by the FHS Executive Committee.

Study Sample: The study sample will be comprised of FHS Offspring Exam 8 (n=2,608, 2005-08) participants with available echo, electrocardiographic, and miRNA data (n=2,477). All FHS blood samples are obtained and stored using methods proven to maintain miRNA stability. The average age of FHS Exam 8 participants is 66 years, 55% are women, 7% have a history of myocardial infarction, 3% have a history of heart failure, and 7% have been diagnosed with AF. Participants undergo an exam at the FHS once every 4-8 years.

FHS AF: Participants are asked about AF at exams and at biennial health updates. If AF is reported, records are sought. Presence of AF is determined from multiple sources: 12-lead ECGs obtained at each FHS exam and from all CVD-related hospitalizations and clinician visits. Cases of suspected AF undergo rigorous adjudication. 188 Exam 8 participants have AF. 223 incident AF cases (9%) are projected for analysis in 2014.

FHS Echo: At Exam 8, participants underwent resting echo evaluation. Echos were performed by experienced technicians and evaluated using a standardized protocol with excellent reproducibility.[36] LA dimension was

determined from M-mode in accordance with Society guidelines.[50,51] FHS also allows for off-line analyses of digitized echos to augment the standardized protocol. The archived digital recordings (2- and 4-chamber views) of 300 FHS subjects with no prior AF will be used for a focused measurement of LA volume (LAV) at end-ventricular systole and diastole (then indexed to height and adjusted for BMI to calculate LAV index), LA emptying fraction (LA-EF; LAVmax-LAVmin/LAVmax), and tissue Doppler A', using standard techniques in accordance with guidelines.[52] LAV index, LA-EF, and tissue Doppler A' have been selected because they share strong and independent associations with AF[53-55] and reflect distinct aspects of LA structure or function.[56] The 3 echo variables will be available for a subset of the sample because the PI or a technician must measure archived images.

miRNA profiling: For hypothesis testing, I will focus on 19 miRNA abundantly expressed in the heart and/or associated with processes implicated in the pathogenesis of atrial remodeling or AF (**Table 2**).[6] For hypothesis generation, and because these analyses can be performed at no additional cost, I will examine 329 non-cardiac SABRe miRNAs to discover new associations not constrained by existing knowledge.

Aim 3 Analytical Approach: Regression models with mixed effects will be used, in which random effect will be used to account for sibling correlation among FHS Offspring cohort enrollees. Linear mixed effect regression models will be used for associating miRNA with quantitative variables (e.g., LA dimension), generalized linear mixed models will be used for binary outcomes (e.g., prevalent AF), and Cox proportional hazards models with robust sandwich estimators will be used for time-to-event analyses (e.g., incident AF). Standardized miRNA data will be used in all association tests. AF risk factors (including age, blood pressure, body mass index, prevalent myocardial infarction and heart failure) will be adjusted for in estimating the association of each of the 19 miRNA levels with prevalent or incident AF. Death before new-onset AF will be treated as a censoring factor in time to AF analysis. For miRNA associated with AF, I will test for effect modification by age, sex, and body mass index by including interaction terms with these variables and miRNA (using 1 miRNA at a time). Kaplan-Meier curves will be used to depict the probability of developing AF according to miRNA tertiles. Since I hypothesize that the relation between miRNA and AF may be mediated by the influence of miRNA on LA structure, I will construct additional models with adjustment for LA dimension. In order to develop a more complete picture of the mechanisms underlying LA remodeling, I will relate miRNA expression for 19 candidates (independent variables) with echo LA structure or function in 300 FHS Offspring participants with intensive echo phenotyping using linear regression for each of the specified dependent variables **(**LAV index, LA-EF, and tissue Doppler A') as exploratory analyses.

Aim 3 Power: An attrition rate of 10% was applied to all the estimated samples sizes, including the numbers of prevalent or projected events for power calculations, because a few samples could fail to generate valid miRNA data (per FHS investigators). Since 3 primary outcomes will be studied in this aim, the Bonferroni approach corrected alpha level is 0.05/19/3=0.0009. For examining the association between miRNA expression and LA dimension, power calculations are based on established methods.[57] We calculated the minimum detectable effect size in terms of partial R^2, i.e. the percentage of variability in LA dimension that each of the 19 miRNAs could explain in addition to covariates. Using our available sample size of 2,230 participants, we will have 80% and 90% to detect partial R^2 of 0.8% and 0.9%, respectively. In testing the hypothesis that key circulating miRNAs are associated with prevalent AF, I have over 80% and 90% power to detect odds ratios of 1.42 and 1.47, respectively, for a 1 standard deviation increment in miRNA with a total sample size of 2,347 and 169 prevalent cases of AF at alpha=0.0009. For the test of linking miRNA expression and time to new-onset AF, the method for power calculation is the same as that in Aim 1. Assuming the sample size of 2,347 and 201 cases of incident AF, I have 80% and 90% power to detect hazard ratios of 1.36 and 1.41, respectively, for a 1 standard deviation increment in miRNA expression at alpha=0.0009.

Anticipated Results and Future Directions: I anticipate that biologically important miRNA will be related to prevalent and incident AF as well as greater LA dimension. We will study the RNA targets of miRNAs associated with atrial remodeling or AF by reviewing current publications and using algorithms such as TARGETSCAN 6.0 and miRanda.[48] These investigations will provide insight into which genes are involved in atrial remodeling and AF and identify biomarkers with potential prognostic or diagnostic importance in AF.

Potential Problems and Solutions

1. Whole Blood (PAXgene) miRNA Profiling: RNA signatures may vary by cell type and patterns of miRNA expression may differ between blood components. Utilization of whole blood preparations for the proposed miRNA analyses will not provide the specific source of miRNA. Recognizing this, this project is designed to be resource effective, utilizing existing miRNAs on over 2,000 FHS participants. Plasma from FHS participants is

stored and the FHS Executive Committee will be petitioned for its use for miRNA analyses if necessary.
2. Classification of AF Type: The methods used to define incident and prevalent cases of AF in the FHS are well validated.[29] They do not, however, enable classification of AF based on contemporary criteria (paroxysmal, persistent, permanent) due to limitations in the methods of data abstraction. The UMMS AF Treatment Cohort employed for Studies 1 and 2 complements the FHS dataset and will allow us to examine associations between AF type and/or duration of AF with miRNA profiles. Associations between miRNAs and AF will also be validated in external replication partners (MGH AF Cohort, MESA) with robust AF phenotyping.
3. Use of M-mode as a marker of LA enlargement: The LA dimension provided by M-mode and used for the determination of LA enlargement may underestimate atrial size.[56] M-mode based LA assessment is, however, a valid measure of LA size.[56] To address this potential limitation, I will measure LAV index, LA-EF, and tissue Doppler A' in a subset of FHS participants, thereby allowing me to relate miRNAs to contemporary atrial remodeling traits.

Study Strengths: This set of studies uses a novel transcriptional regulator, miRNA, to **directly assess** regulatory **mechanisms underlying atrial remodeling and AF**. The investigations are further bolstered by the **hypothesis-driven selection** of cardiac-specific or enriched miRNAs for study. The measurement of serial miRNAs at baseline and at several time points post-ablation further strengthens Study 1, since each patient will serve as his/her own control, thereby diminishing differences in miRNA expression extrinsic to the exposure of interest and enabling us to assess temporal changes in plasma miRNA expression in the short- and medium-term. The use of a relatively large sample of patients with **symptomatic and well-characterized AF** and **analysis of miRNAs in both plasma and whole blood** strengthens Study 2. The use of a well-characterized, large sample from a seminal longitudinal study with standardized miRNA assessment and contemporaneous ascertainment of echo traits strengthens Study 3.

Impact and Future Directions: AF is highly prevalent and the burden of AF is increasing.[2] New avenues are needed to identify cellular and developmental pathways for AF that incorporate epidemiologic, genetic and transcriptomic data.[58-60] At the end of the grant period, I will have established a clinical and translational UMMS AF research program focused on advancing our understanding of the mechanisms underlying atrial structural remodeling and AF. I will build a UMMS AF cohort with extensive phenotyping and will add to the FHS by better characterizing LA structure and function in selected participants. This research will be used to generate new insights into the pathogenesis, risk prediction, and therapeutic management of AF.

Bibliography

1. Go AS, Hylek EM, Phillips KA, et al. Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. *JAMA* 2001;285:2370-5.
2. Miyasaka Y, Barnes ME, Gersh BJ, et al. Secular trends in incidence of atrial fibrillation in Olmsted County, Minnesota, 1980 to 2000, and implications on the projections for future prevalence. *Circulation* 2006;114:119-25.
3. Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. *Stroke* 1991;22:983-8.
4. Benjamin EJ, Wolf PA, D'Agostino RB, et al. Impact of atrial fibrillation on the risk of death. *Circulation* 1998;98:946-52.
5. Wang TJ, Larson MG, Levy D, et al. Temporal relations of atrial fibrillation & congestive heart failure & their joint influence on mortality: the Framingham Heart Study. *Circulation* 2003;107:2920-5.
6. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. *Nature*;469:336-42.
7. Laterza OF, Lim L, et al. Plasma MicroRNAs as sensitive & specific biomarkers of tissue injury. *Clin Chem* 2009;55:1977-83.
8. Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. *Circulation*;121:1022-32.
9. McManus DD, Ambros V. Circulating MicroRNAs in cardiovascular disease. *Circulation*;124:1908-10.
10. Coyne KS, Paramore C, et al. Assessing the direct costs of treating nonvalvular atrial fibrillation in the US. *Value Health* 2006;9:348-56.
11. Nattel S, Burstein B, Dobrev D.Atrial remodeling & atrial fibrillation:mechanisms & implications.*Circ Arrhythm Electrophysiol* 2008;1:62-73.
12. Burstein B, Nattel S. Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation. *J Am Coll Cardiol* 2008;51:802-9.
13. McManus DD, Shaikh AY,et al.Atrial fibrillation & heart failure parallels:lessons for atrial fibrillation prevention.*Crit Pathw Cardiol*;10:46-51.
14. Benjamin EJ, Chen PS, Bild DE, et al. Prevention of atrial fibrillation. *Circulation* 2009;119:606-18.
15. Fox CS, Parise H, D'Agostino R, et al. Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring. *JAMA* 2004;291:2851-5.
16. Ellinor PT, Lunetta KL, Glazer NL, et al. Common variants in KCNN3 are associated with lone atrial fibrillation. *Nat Genet*;42:240-4.
17. Mace LC, Yermalitskaya LV, Yi Y, et al. Transcriptional remodeling of rapidly stimulated HL-1 atrial myocytes exhibits concordance with human atrial fibrillation. *J Mol Cell Cardiol* 2009;47:485-92.
18. Nattel S, Frelin Y, Gaborit N, et al. Ion-channel mRNA-expression profiling: Insights into cardiac remodeling and arrhythmic substrates. *J Mol Cell Cardiol*;48:96-105.
19. Kim NH, Ahn Y, Oh SK, et al. Altered patterns of gene expression in response to chronic atrial fibrillation. *Int Heart J* 2005;46:383-95.
20. Ohki R, Yamamoto K, Ueno S, et al. Gene expression profiling of human atrial myocardium with atrial fibrillation by DNA microarray analysis. *Int J Cardiol* 2005;102:233-8.

21. Barth AS, Merk S, Arnoldi E, et al. Reprogramming of the human atrial transcriptome in permanent atrial fibrillation: expression of a ventricular-like genomic signature. *Circ Res* 2005;96:1022-9.
22. De Rosa S, Fichtlscherer S, Lehmann R, et al. Transcoronary concentration gradients of circulating microRNAs. *Circulation*;124:1936-44.
23. Xiao J, Liang D, Zhang Y, et al. MicroRNA expression signature in atrial fibrillation with mitral stenosis. *Physiol Genomics*.
24. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-97.
25. Zhao Y, Ransom J et al. Dysregulation of cardiogenesis, cardiac conduction, & cell cycle in mice lacking miRNA1-2.*Cell* 2007;129:303-17.
26. Luo X, Zhang H, Xiao J, et al. Regulation of human cardiac ion channel genes by microRNAs. *Cell Physiol Biochem*;25:571-86.
27. Pfeufer A, van Noord C, Marciante KD, et al. Genome-wide association study of PR interval. *Nat Genet*;42:153-9.
28. Schnabel R, Sullivan L, et al. Development of a risk score for atrial fibrillation: a community-based cohort study. *Lancet* 2009;373:739-45.
29. Callis TE, Pandya K, Seok HY, et al. MicroRNA-208a is a regulator of cardiac hypertrophy & conduction in mice. *J Clin Invest* 2009;119:2772-86.
30. Lu Y, Zhang Y, Wang N, et al. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. *Circulation*;122:2378-87.
31. Brown BD, Naldini L. Exploiting & antagonizing microRNA regulation for therapeutic & experimental applications. *Nat Rev Genet* 2009;10:578-85.
32. McManus D, Shlipak M, Ix JH, et al. Association of cystatin C with poor exercise capacity and heart rate recovery: data from the heart and soul study. *Am J Kidney Dis* 2007;49:365-72.
33. McManus DD, Aslam F, Goyal P, et al. Incidence, prognosis, and factors associated with cardiac arrest in patients hospitalized with acute coronary syndromes (the Global Registry of Acute Coronary Events Registry). *Coron Artery Dis*.
34. McManus DD, Corteville D, Shlipak M et al. Relation of kidney function & albuminuria with atrial fibrillation. *Am J Cardiol* 2009;104:1551-5.
35. McManus DD, Lyass A, Ingelsson E, et al. Relations of Circulating Resistin and Adiponectin and Cardiac Structure and Function: The Framingham Offspring Study. *Obesity (Silver Spring)*.
36. McManus DD, Xanthakis V, et al. Longitudinal tracking of left atrial diameter over the adult life course. *Circulation*;121:667-74.
37. Lee J, McManus D, Merchant S, et al. Automatic Motion and Noise Artifact Detection in Holter ECG Data using Empirical Mode Decomposition and Statistical Approaches. *IEEE Trans Biomed Eng*. 2011; epub ahead of print.
38. Magnani JW, Rienstra M, Lin H, et al. Atrial fibrillation: current knowledge and future directions in epidemiology and genomics. *Circulation*;124:1982-93.
39. McManus DD, Shah SJ, Fabi MR, et al. Prognostic value of left ventricular end-systolic volume index as a predictor of heart failure hospitalization in stable coronary artery disease: data from the Heart and Soul Study. *J Am Soc Echocardiogr* 2009;22:190-7.
40. Turakhia MP, McManus DD, Whooley MA, et al. Increase in end-systolic volume after exercise independently predicts mortality in patients with coronary heart disease: data from the Heart and Soul Study. *Eur Heart J* 2009;30:2478-84.
41. Balk EM, Garlitski AC, Alsheikh-Ali AA, et al. Predictors of atrial fibrillation recurrence after radiofrequency catheter ablation: a systematic review. *J Cardiovasc Electrophysiol*;21:1208-16.
42. Okumura Y, Watanabe I, Nakai T, et al. Impact of Biomarkers of Inflammation and Extracellular Matrix Turnover on the Outcome of Atrial Fibrillation Ablation: Importance of Matrix Matalloproteinase-2 as a Predictor of Atrial Fibrillation Recurrence. *J Cardiovasc Electrophysiol*.
43. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. *Science* 2001;294:862-4.
44. Elesber AA, Rosales AG, Herges RM, et al. Relapse and mortality following cardioversion of new-onset vs. recurrent atrial fibrillation and atrial flutter in the elderly. *Eur Heart J* 2006;27:854-60.
45. Weerasooriya R, Khairy P, Litalien J, et al. Catheter ablation for atrial fibrillation: are results maintained at 5 years of follow-up? *J Am Coll Cardiol*;57:160-6.
46. Ji X, Takahashi R, Hiura Y, et al. Plasma miR-208 as a biomarker of myocardial injury. *Clin Chem* 2009;55:1944-9.
47. van Rooij E, Olson EN. MicroRNAs: powerful new regulators of heart disease & provocative therapeutic targets. *J Clin Invest* 2007;117:2369-76.
48. Griffiths-Jones S. miRBase: the microRNA sequence database. *Methods Mol Biol* 2006;342:129-38.
49. Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res*;107:810-7.
50. Feigenbaum H. Echocardiography. Philadelphia, Pa: Lea & Febiger.
51. Schiller NB, Shah PM, Crawford M, et al. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American Society of Echocardiography. *J Am Soc Echocardiogr* 1989;2:358-67.
52. Knutsen KM, Stugaard M, Michelsen S, et al. M-mode echocardiographic findings in apparently healthy, non-athletic Norwegians aged 20-70 years. Influence of age, sex and body surface area. *J Intern Med* 1989;225:111-5.
53. Abhayaratna WP, Fatema K, Barnes ME, et al. Left atrial reservoir function as a potent marker for first atrial fibrillation or flutter in persons > or = 65 years of age. *Am J Cardiol* 2008;101:1626-9.
54. Erdei T, Denes M, Kardos A, et al. Left atrial & left atrial appendage function in paroxysmal atrial fibrillation. *Acta Physiol Hung*;98:137-46.
55. Shin SY, Lim HE, et al. Impaired transport function of the left atrium in patients with lone paroxysmal atrial fibrillation. *Echo*; 28:44-51.
56. Abhayaratna WP, Seward JB, et al. Left atrial size: physiologic determinants and clinical applications. *J Am Coll Cardiol* 2006;47:2357-63.
57. Cohen J. A power primer. *Psychol Bull* 1992;112:155-9.
58. Pereira-Leal JB, Enright AJ, Ouzounis CA. Detection of functional modules from protein interaction networks. *Proteins* 2004;54:49-57.
59. Spirin V, Mirny LA. Protein complexes and functional modules in molecular networks. *Proc Natl Acad Sci* 2003;100:12123-8.
60. Snel B, Bork P, et al. The identification of functional modules from the genomic association of genes. *Proc Natl Acad Sci* 2002;99:5890-5.