Title
Unique Cardiovascular Risk Profiles in US South Asians: From Epidemiology to Epigenetic Biomarker Discovery

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Unique Cardiovascular Risk Profiles in US South Asians:

From Epidemiology to Epigenetic Biomarker Discovery

By

Elena Flowers

DISSERTATION

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DOCTOR OF PHILOSOPHY

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DEDICATIONS

This dissertation is dedicated to people afflicted with risk factors for cardiovascular disease, particularly those who face social and economic challenges affecting their health status. I hope that my research will make a difference in the lives of these individuals.

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ABSTRACT

Gene-environment interactions are increasingly understood to be a primary contributing factor in the etiology of cardiovascular disease. Environmental factors include person and societal-level variables. Genome factors include sequence variation and mechanisms controlling genome expression. These regulatory mechanisms are the nexus of gene-environment interactions, and can be transient or adaptive; the latter are termed epigenetics. Some genetic characteristics are highly homogeneous within ethnic groups, due to a combination of geographically derived natural selection and environmental factors. South Asians are a population with disproportionately high cardiovascular disease burden. A prevalence study of cardiovascular risk factors in this population provides evidence that common risk factors are alterations in cholesterol metabolism; specifically low high-density lipoprotein cholesterol with elevated triglycerides. These initial observations led to a second study yielding evidence that abdominal adiposity is not a necessary precursor to the development of this dyslipidemia, challenging previous assumptions. A third study found pro-atherogenic risk profiles in South Asians are present decades earlier than in other populations. While these risk factors have genetic underpinnings, the added effects of a changing environment (i.e., Westernization of South Asians) are not known. MicroRNA are an epigenetic post-transcriptional regulatory mechanism of messenger RNA translation, and are potential biomarkers of pathophysiology and response to interventions. MicroRNA in human blood are differentially expressed in numerous disease conditions, and *in vitro* studies indicate that microRNA play a role in regulation of cholesterol metabolism in response to the extracellular environment. Methods to quantify microRNA include microarray, in which a large number of microRNA targets are screened. Microarray results are then validated with the more sensitive quantitative polymerase chain reaction method. Using microRNA arrays and a case-control design of pooled bio-specimens, a fourth study identified 16 candidate microRNA biomarkers of this atherogenic profile, which were then verified by quantitative polymerase chain reaction. Several of these are known to target messenger RNA involved in cholesterol metabolism. These results are promising evidence for translational application of microRNA as clinical biomarkers elucidating underlying genetic determinants of atherogenic risk in South Asians. Further research is needed to determine whether microRNA expression in blood is sensitive to cardiovascular risk reduction interventions.

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My research interests include epigenetic regulation of gene expression and the development of risk factors for chronic disease, such as cardiovascular disease and type-2 diabetes, in response to social determinants of health. Prior to becoming a nurse, I worked as a research assistant on a pharmacogenetic study of medications for high cholesterol and hypertension, comparing response in Caucasians and African-Americans. As a result of this experience, I became attentive to the differences between these ethnic groups in health literacy and health status, and became very interested in knowing more about the impact of social determinants on health. During nursing school, I worked alongside a public health nurse performing home visits for people managing chronic disease, such as heart failure and asthma. Seeing patients in their home setting made it acutely clear to me that the environment has a profound effect on individual health status. I also witnessed first-hand the limitations to performing health-promoting behaviors that result from society-level factors such as neighborhood, access to care, education, and transportation. I subsequently developed a dissertation proposal aimed at studying mechanisms underlying cardiovascular risk in South Asians, an ethnic group disproportionately afflicted with elevated cardiovascular risk. This research has resulted in an enhanced description of the specific risk profile of South Asians living in the United States, contradicted a commonly held paradigm for the pathophysiologic mechanisms underlying cardiovascular risk in this population, and provided the first description of microRNA expression in peripheral blood in individuals with the common atherogenic dyslipidemia risk phenotype.

CHAPTER 1

INTRODUCTION

Over the past three decades, the debate regarding the role of nature versus nurture in disease etiology has given way to more complex models that accommodate a larger number of causal pathways and allow for interactions between risk factors on many levels. These pathways include both individual and environmental level factors, and incorporate the influences of both nature and nurture into a single comprehensive model. The examination of known environmental causes of disease, coupled with the recent scientific advances allowing for rapid and affordable detection of genetic variation, are improving our understanding of the individual and combined roles of genetic predisposition and environmental influences on cardiovascular disease. Interactions, including those occurring between an individual's psychosocial and physical environment and their unique genetic "makeup," are now accepted to have an important role in nearly all cardiovascular disease conditions and underlying biological processes.

Chapter 2 introduces the theoretical framework for this body of work. Unaccounted for gene-environment interactions are hypothesized to be a common alternative explanation for paradoxical findings about the etiology and prevention of cardiovascular disease. Environmental exposures can increase or decrease the effect of genetic predisposition, and vice versa. Lifestyle interventions to prevent and treat cardiovascular disease and its risk factors are in part unsuccessful because of incomplete understanding of the underlying biology of the disease and how manifestation of a disease is moderated by the environment. Thus, increasing the effectiveness of interventions may be possible through a more comprehensive understanding of the biology of cardiovascular disease, including interactions between genetic predisposition and environmental components such as lifestyle and behavioral variables.

One pathway to improving cardiovascular risk reduction is through community-based interventions like health coaching. Health coaching is a tool to engage patients in their health behaviors. As opposed to the traditional method of health care providers prescribing behavioral

recommendations, which is widely shown to result in poor adherence, coaching engages the patient in the process of recognizing their readiness to adopt new behaviors, reasons for ambivalence, and engaging in behavior change. Although previous studies of coaching interventions have been primarily conducted in Caucasian populations, there is growing interest in assessing the efficacy of this type of intervention for decreasing cardiovascular risk in other ethnic groups. The effects of ethnicity on health are complex, and likely arise from both genetic characteristics of individual ethnic groups and common cultural practices. Gene-environment interactions are almost certainly at play. One approach to cultural modification of a coaching intervention is to incorporate behaviors and beliefs common to a specific ethnic group. Chapter 3 describes an example of a culturally tailored community-based program aimed at reducing cardiovascular risk in South Asians. This program provides individualized, culturally specific dietary, physical activity, and stress reduction recommendations, and encouragement of behavior change and improvement in modifiable cardiovascular risk factors through regular discourse between participants and individually assigned coaches.

Compared to other ethnic groups, South Asians have at least two-fold increased risk for cardiovascular disease, making primary prevention of paramount importance in this population. Increased risk has been observed in both native and immigrant populations, and onset of risk factors and cardiovascular events often occurs as much as a decade earlier than in other ethnic groups, indicating that genetic predisposition plays a significant role. South Asians represent an increasingly large proportion of the United States population, with the highest concentrations living in urban areas. As described in greater detail in Chapter 4, many South Asians residing in the United States are first generation immigrants with a number of demographic characteristics that are typically protective from disease risk, such as marriage and a high level of education. Yet this group appears to have equal frequency of cardiovascular risk factors as the multi-ethnic United States population.

The causes underlying this risk are not fully understood, however atherogenic dyslipidemia, characterized by low level of high-density lipoprotein cholesterol (HDL-c) accompanied by elevated triglycerides, occurs far more commonly than other traditional cardiovascular risk factors (i.e., elevated low-density lipoprotein cholesterol, elevated blood pressure). Atherogenic dyslipidemia is associated with impaired insulin metabolism, leading to hyperglycemia and the development of type-2 diabetes. The clustering of these metabolic abnormalities, with or without the addition of hypertension, is termed Metabolic Syndrome. The underlying strata for development of Metabolic Syndrome is a topic of debate. Previous definitions of the Metabolic Syndrome necessitated abdominal obesity (measured by waist circumference) accompanied by at least two of four additional risk factors (low HDL-c, elevated triglycerides, elevated blood glucose, elevated blood pressure). More recently, a consensus statement from a number of national and international organizations defines the condition as three of the five risk factors listed above, without the prerequisite of abdominal adiposity.

Further complicating the picture, measures of adiposity may not perform equally well across ethnic groups. Asian populations appear to have a higher proportion of harmful visceral abdominal fat that is not readily measured by common measures of adiposity such as waist circumference. The relative excess of visceral adipose tissue compared to subcutaneous adipose tissue is hypothesized to be a mechanism by which South Asians are particularly susceptible to the development of metabolic abnormalities. As described in Chapter 5, we found evidence to the contrary, showing a significant proportion of overweight and obese individuals of South Asian ethnicity are metabolically normal, while multiple metabolic risk factors frequently occur in normal weight individuals. Thus the mechanisms underlying cardiovascular risk in this ethnic group may be unique and have yet to be fully elucidated.

Although South Asians are particularly susceptible to cardiovascular disease, and a common risk phenotype is present, the condition is still complex and undoubtedly arises from a combination of both genetic and environmental risk factors. As described in detail in Chapter 6,

microRNA (miR) are small molecules that mediate the relationship between the demands placed on an organism by the environment and expression of genes. Commonly categorized as an epigenetic mechanism, miR cause changes in gene expression and ultimately the phenotype without a requisite alteration of the underlying genetic code, and are hypothesized to regulate the development of complex chronic conditions like the metabolic syndrome. Preliminary data suggest a role for miR in the development and progression of obesity, dyslipidemia, elevated blood pressure, and impaired glucose metabolism. These observations beg the question of whether studies of miR may begin to disentangle the interplay between genetic predisposition and environmental exposures in the onset and progression of cardiovascular risk factors.

MiR are the subject of great interest, as they have potential to provide novel insights into mechanisms of disease, and have tremendous clinical translational potential. There are three implications for miR in clinical practice: (1) measurement of risk for significant clinical outcomes; (2) assessment of expression before and after risk reduction interventions (e.g., medication, diet, physical activity) to determine whether expression is differential, and whether miR are biomarkers for changes in clinical risk profile; and (3) as an intervention via either inhibition of endogenous miR or augmentation of miR levels with synthetic mimics. All three are relevant to cardiovascular risk factors, including atherogenic dyslipidemia. Prior studies provide strong support for the latter two applications, with exciting translational findings showing significant changes in cholesterol levels in large primates treated with a miR inhibitor. However to date, there is a dearth of research on miR as a biomarker for cardiovascular risk humans. Studies of this nature are the logical precursor to conduct of intervention studies in which miR are used as measures of response or as the intervention itself.

Standards for accurate and precise measurement are an important consideration in studies of miR expression, and are described in Chapter 7. The first level is collection from an appropriate tissue source and preservation of unstable miR molecules. They highly dynamic and specific nature of miR means that expression is variable between tissue types within a single

organism. Although measurement of miR from liver, for example, might be useful for understanding mechanisms of dyslipidemia, accessing human liver tissue is invasive and incurs health risk. However, blood, being a signaling medium that is routinely accessed in clinical care with minimal risk, is a realistic candidate for translational studies of miR expression. The second step is isolation of miR from other cellular components and larger RNA molecules. Once miR of acceptable quality and quantity is obtained, there are three common methods by which it can be quantified: Northern blot, quantitative polymerase chain reaction (qPCR), and microarray. Each method has strengths and weaknesses that are described in chapter seven. The selected measurement method also has implications for analysis, including standardization of starting quantity of miR isolated from tissue, and adjustment for comparison of up to 1,000 discrete miR species.

Chapter 8 describes a study comparing miR expression in blood between individuals with and without cardiovascular risk factors. Based on the finding that atherogenic dyslipidemia, with or without abdominal adiposity, is the most common cardiovascular risk phenotype in South Asians, this was selected as the phenotype of interest to begin investigating the role of miR in this unique population. The study utilized array-based methods to screen blood for prevalent and differentially expressed miR, followed by qPCR validation of targets with biologically plausible function. Eighty-five miR targets were screened, and 16 (19%) displayed at least two-fold differential expression that was statistically significant. Of those, expression differences were validated for three miR that likely target genes regulating lipid metabolism: miR-106b, miR-125b, and miR-21. The findings of this study support the hypothesis that miR expression in blood is a biomarker for cardiovascular risk factors. Future directions include delving into the specific molecular pathways targeted by these miR, longitudinal studies with repeated measures of miR expression and cardiovascular risk factors, and measuring miR before and after risk reduction interventions.

CHAPTER 2

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Gene-environment interactions in cardiovascular disease☆

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Abstract

Background: Historically, models to describe disease were exclusively nature-based or nurture-based. Current theoretical models for complex conditions such as cardiovascular disease acknowledge the importance of both biologic and non-biologic contributors to disease. A critical feature is the occurrence of interactions between numerous risk factors for disease. The interaction between genetic (i.e. biologic, nature) and environmental (i.e. non-biologic, nurture) causes of disease is an important mechanism for understanding both the etiology and public health impact of cardiovascular disease.

Objectives: The purpose of this paper is to describe theoretical underpinnings of gene–environment interactions, models of interaction, methods for studying gene–environment interactions, and the related concept of interactions between epigenetic mechanisms and the environment.

Discussion: Advances in methods for measurement of genetic predictors of disease have enabled an increasingly comprehensive understanding of the causes of disease. In order to fully describe the effects of genetic predictors of disease, it is necessary to place genetic predictors within the context of known environmental risk factors. The additive or multiplicative effect of the interaction between genetic and environmental risk factors is often greater than the contribution of either risk factor alone.

Keywords: Confounding; Environment and public health; Gene expression; Genetic variation; Methods; Phenotype

1. Introduction

Health promotion and disease prevention is a primary component of nursing practice. The cardiovascular disease pandemic necessitates that nursing practice focuses on prevention and treatment of cardiovascular disease through risk assessment and stratification, and risk reduction through lifestyle and behavioral change. Although progress has been made in treating cardiovascular disease and its risk factors, the prevalence of this largely preventable condition worldwide remains unacceptably high.

Over the past three decades, the nature versus nurture debate over disease etiology has given way to more complex models that accommodate a larger number of causal pathways and allow for interactions between risk factors on many levels. These pathways include both individual and environmental level factors, and incorporate the influences of both nature and nurture into a single comprehensive model. The examination of known environmental causes of disease, coupled with the recent scientific advances allowing for rapid and affordable detection of genetic variation, is advancing our understanding of the individual and combined roles of genetic predisposition

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and environmental influences on cardiovascular disease. Interactions, including those occurring between an individual's psychosocial and physical environment and their unique genetic "makeup," are now accepted to have an important role in nearly all cardiovascular disease conditions and underlying biological processes.

Unaccounted gene–environment interactions are hypothesized to be a common alternative explanation for paradoxical findings about the etiology and prevention of cardiovascular disease [1]. Environmental exposures can increase or decrease the effect of genetic predisposition, and genetic predisposition can modify the effects of the environment [2,4]. Lifestyle interventions to prevent and treat cardiovascular disease and its risk factors are in part unsuccessful because of incomplete understanding of the underlying biology of the disease and how manifestation of disease is moderated by the environment. Increasing the effectiveness of interventions may be possible through a more comprehensive understanding of the biology of cardiovascular disease, including interactions between genetic predisposition and environmental components such as lifestyle and behavioral variables. The aim of this paper is to describe theoretical underpinnings supporting the interaction between genetics and the environment and the onset and progression of cardiovascular disease.

2. Gene–environment interactions

Evidence supports the existence of gene–environment interactions for nearly every disease condition, including mental health disorders [5,6], cardiovascular and metabolic disease [7–12], infectious disease [13,14], and trauma and injury [15]. While the field of quantitative genetics aims to identify specific gene loci responsible for disease, genetic epidemiology places what is known about genetic predictors of disease in the context of a population, searching for mechanisms of disease that include both genetic predisposition and environmental factors. The purposes of studying gene–environment interactions are to understand the complete etiology of a disease inclusive of multiple discrete and interacting pathways, and to determine the public health impact of individual factors within a specific population so that interventions can be designed to maximize health and minimize disease.

When an interaction between the genotype and environmental factors is present, this interaction is said to exert a main effect on the likelihood of developing disease. Additional marginal effects result from the independent contributions of the genotype and the environmental factors. Studies of genetic determinants of disease or environmental risk factors for disease are often designed to assess marginal effects only; however in many cases, the main effect of the interaction is hypothesized to be a far greater contributor to disease than either marginal effect alone [1]. Inconsistent and inconclusive findings from studies of the marginal effects of genetic determinants of disease are common. Failure to identify the presence of a gene–environment interaction with significant main effects is a likely alternative explanation for incongruous findings [1].

2.1. Models of interaction

There are two commonly discussed types of interaction: statistical and biologic [3]. Statistical interaction is strictly a mathematical phenomenon, in which the measured effects of one variable depend on the level of a second variable. By contrast, biologic interaction refers to the intersection of what are considered to be discrete pathways relevant to the maintenance of homeostasis or even the onset and progression of a physiologic condition. Both of these concepts are central to consideration of gene–environment interactions. Because the aim of studying gene–environment interactions is to discover new mechanisms of disease or describe the causes of deviation from expected expression of disease, biologic interaction is the very definition of gene–environment interaction. In order to quantify the presence of biologic interaction and make meaningful inferences about observations of interactions, incorporation of statistical interaction should be included during data analysis in order to accurately model the true underlying condition.

Gene–environment interactions can be either complementary or antagonistic [16]. In the case of complementary interactions, both factors (i.e. environmental exposure and genetic predisposition) work in the same direction on disease risk. For example, an allele for the familial hypercholesterolemia (FH) gene might increase susceptibility to atherosclerosis by upregulating the production of low-density lipoprotein cholesterol, and high intake of saturated fat may increase the likelihood of atherosclerosis by increasing low-density lipoprotein cholesterol levels. These two factors act in complement to increase an individual's risk of developing atherosclerosis. In contrast, antagonist interaction occurs when the direction of effect of two variables opposes each other. In the case of hypertension, carrying one of the known risk alleles for essential hypertension will increase an individual's likelihood of developing high blood pressure, whereas engaging in moderate physical activity most days of the week exerts the opposing effect of decreasing lifetime risk of hypertension.

2.1.1. Gene–gene interactions

Implicit in the definition of multi-factorial traits is that risk factors, be they environmental or genetic, can interact. The interaction of two or more genes is termed epistasis. Procedurally, studying gene–gene interactions is similar to studying gene–environment interactions. Although epistasis is related to gene–environment interactions and shares underlying principles, it is beyond the scope of this paper, and will not be discussed in further detail. An excellent review of epistasis can be found elsewhere [17].

2.1.2. Gene–environment correlations

A similar but distinct phenomenon from gene–environment interactions is gene–environment correlations. Correlations

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occur when a genetic marker is highly associated with, and possibly causal of, a behavioral characteristic or exposure that predisposes the outcome of interest [18]. A thorough description of work describing gene–environment correlations, including clinical examples, can be found elsewhere [19].

2.2. Models of disease risk

The risk of disease in the presence of environmental and genetic risk factors can be depicted using a classic 2 × 2 table (Table 1). For simplicity, we consider both environmental and genetic predictors and the disease outcome to be dichotomous. When genetic and environmental risk factors interact, four scenarios are possible (Table 2).

There are two primary mathematical models of risk that describe the relationship between multiple predictors of human disease: additive and multiplicative. Additive models assert that the effects of each predictor are summed in order to determine an individual's likelihood of disease. Predictors can still be complementary or antagonistic, however the net effect of all predictors is the sum of each predictor's effect on the disease outcome. In the case of gene–environment interactions, the effect of the environment depends on the genotype of an individual [2], and a statistical interaction is considered to be present when there is departure from the simple additive model [4]. We can return to the atherosclerosis example from above and assume a simplified scenario in which there are only two predictors of disease: saturated fat intake and the FH risk allele (this example is chosen for simplicity only, and the following discussion does not encompass the entirety of biological mechanisms describing atherosclerosis risk). If no interaction is present, then an individual's relative risk of atherosclerosis (RR_a) is equal to the risk associated with their FH genotype (RR_g) plus the risk associated with their saturated fat intake (RR_e), which can be expressed mathematically as $RR_a = RR_g + RR_e$. Note that the mathematical term in this equation can be addition or subtraction, depending on whether the two effects are complementary or antagonistic. If an interaction is present, then a person who has a high saturated fat intake with no copies of the risk allele will have a relative risk for atherosclerosis equal to the harmful effect of saturated fat ($RR_a = RR_e$). However, for an individual who does carry the FH risk allele, the risk of saturated fat may no longer be significant, and atherosclerosis relative risk will be equal to the risk of the FH risk allele ($RR_a = RR_g$). In the presence of an interaction, the risk for atherosclerosis associated with saturated fat depends on FH genotype.

In contrast, multiplicative models assert that the effects of the genotype depend on the environment [2]. In the presence of a biologic interaction, departure from the multiplicative model will be observed [4]. If we apply a multiplicative model to the dichotomous atherosclerosis example for gene–environment interaction and again simplify to assume only two predictors, we can say that the effect of the FH risk allele depends on saturated fat intake. If no interaction is present, then an individual's relative risk of developing atherosclerosis is equal to the sum of the risk associated with their FH genotype and the risk associated with their saturated fat intake ($RR_a = RR_g + RR_e$). In the case of an interaction, the effect of the FH risk allele will depend on saturated fat intake. For an individual who consumes a large amount of saturated fat, carrying the FH risk allele would have no effect on the risk of atherosclerosis ($RR_a = RR_g \times RR_e$).

An alternative example for a continuous trait is blood pressure in individuals who experience a high level of autonomic stimulation during the workday (e.g. firefighters, air traffic controllers). An individual with no genetic predisposition to hypertension may have a normal blood pressure (e.g. 110/70 mmHg) even in the presence of a high level of autonomic stress. In contrast, the blood pressure of an individual who is genetically predisposed to hypertension will depend on the level of autonomic work-related stress. In the absence of autonomic stress, the relative risk of hypertension (RR_h) is equal to the genetic risk for hypertension ($RR_h = RR_g$), which may result in a moderately elevated blood pressure (e.g. 145/90 mmHg). For the individual who is genetically predisposed to hypertension and exposed to a high level of work-related autonomic stress, the relative risk of hypertension could be multiplicative ($RR_h = RR_e \times RR_g$), and blood pressure may be significantly

Table 1
Effects of genetic and environmental risk factors on relative risk of disease (RR) [a,b].

	High risk genotype present	High risk genotype absent
Environmental risk factor present	$RR_{ge} \gg 1$ [c]	$RR_e > 1$ [c]
Environmental risk factor absent	$RR_g > 1$ [c]	$RR = 1$ [c]

[a] Assumes a simple scenario in which the exposure and the genotype are dichotomous and result in a synergistic effect of both factors to increase the risk of disease.
[b] Adapted from Ottman, R. (1996). Gene–environment interaction: definitions and study designs. Prev Med, 25(6), 764–770.
[c] A relative risk of one is the level of risk for the general population.

Table 2
possible interaction effects between multiple risk factors on relative risk of disease (RR) [a].

Model of disease risk		
	Additive	Multiplicative
No interaction	$RR_{ge} = RR_g + RR_e - 1$	$RR_{ge} = RR_g \times RR_e - 1$
Complementary interaction	$RR_{ge} > RR_g + RR_e - 1$	$RR_{ge} > RR_g \times RR_e - 1$
Antagonistic interaction	$RR_{ge} < RR_g + RR_e - 1$	$RR_{ge} < RR_g \times RR_e - 1$

RR_g = relative risk of disease when genetic risk factor is present.
RR_e = relative risk of disease when environmental risk factor is present.
RR_{ge} = relative risk of disease when both genetic and environmental risk factors are present.
[a] Adapted from Ottman, R. (1996). Gene–environment interaction: definitions and study designs. Prev Med, 25(6), 764–770.

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elevated beyond the expected effects of either risk factor alone (e.g. 180/100 mmHg).

The choice of which model to consider depends on two primary considerations. Although a comprehensive discussion of model selection is available elsewhere [20], each will be summarized briefly here. The first is the biologic relationship between the predictors. Commonly, when two predictors act on the same pathway, a simple additive model is assumed [20]. Conversely, when two factors are thought to act on discrete physiologic pathways, the effect is often more than additive, and a multiplicative model is assumed [3,20]. In addition, the research question and aim of a study determine how a model is selected [2,20]. In the development of predictive models of disease for public health purposes and clinical decision-making, the underlying mechanisms of disease are not as important as the predictive capability of observable risk factors. In this scenario, an additive model that includes surrogate non-causal markers[1] will often suffice [3]. For studies of disease etiology, the aim is to understand the mechanisms by which disease is occurring, and multiplicative models may be necessary in order to correctly specify the relationship between predictors [3,20]. Often statistical modeling of both additive and multiplicative relationships is performed in order to determine the possible implications of model misspecification. In the case where additive and multiplicative models do not differ, the additive model is usually selected for simplicity and ease of interpretability.

2.2.1. Timing and spectrum of exposure

The occurrence of a gene–environment interaction is dynamic. As a result of changes in both gene expression and environmental exposure, interactions can occur at one time-point during the lifespan, periodically throughout life, or for longer durations. With regard to genetic exposure, a copy of the full genome is present in every cell of an organism; however not all genes are expressed at all times. Numerous genes are involved only during development and maturation, and once the adult stage is reached, expression of these genes is silenced. In contrast, some genes are only expressed in response to an environmental exposure or trigger. For example, a traumatic event such as a fracture will prompt localized expression of genes involved in inflammation and bone growth and remodeling that are not normally expressed in healthy osteocytes. Thus, the timing of an exposure can determine whether a gene–environment interaction occurs.

An organism's environment is typically in a state of constant flux. For humans, environmental factors can change throughout the day as well as over the course of a lifetime. Returning to the example of blood pressure and autonomic stimulation, work-related stress exerts immediate effects on hemodynamics and inflammatory processes, and individuals who are genetically predisposed to hypertension may be more likely to experience elevations in blood pressure during stressful work hours. In the case that this stress resolves at the completion of the workday, some physiologic phenomena, (e.g. hemodynamics) may return to a non-stressed baseline state, whereas others, (e.g. inflammation-induced damage to the arterial wall) are permanent. Repeated exposure to the environmental stimuli can result in cumulative permanent physiologic changes.

Alternatively, some individuals are exposed to second-hand cigarette smoke during childhood, and subsequently this exposure is removed from the environment during adulthood. Others will grow up in a smoke free environment but then partner with a smoker of cigarettes during adulthood. The effects of secondhand smoke exposure may differ for the developing pediatric vascular endothelium compared to adult vascular endothelium, and irreversible damage could occur during a critical developmental period that will not affect the adult exposed to second-hand smoke in the same way.

The spectrum of both genetic and environmental exposures can also determine whether an interaction occurs. Genetic dose is variable between individuals, and the individual's genotype will affect the level of exposure: some genes have dominant and recessive patterns of inheritance while others are co-dominant [21]. For the dominant inheritance pattern, an individual will be affected if they carry just one copy of the risk allele, whereas the recessive pattern of inheritance requires two copies of a risk allele for an individual to be affected. In the case of co-dominance, differing alleles for a given gene are equally expressed. Similarly, differing doses of environmental exposures exert differing effects. In some cases, there is a threshold effect in which no adverse consequences are observed until a threshold level of exposure is reached. In other cases, environmental exposure is continuous, and increasing doses will exert a linear increase in harmful or protective effects. Thus, quantifying the dose of both genetic and environmental exposure is important in order to detect the presence of gene–environment interactions.

2.3. Methodological issues

2.3.1. Study design

There are three primary epidemiologic study designs that are appropriate for studies of this nature: cohort, cross-sectional, and case-only (Table 3). The selection of study design depends on the aim of the study (i.e. investigations of disease etiology compared to assessing the impact of environmental exposure in the context of genetic predisposition), what is known about genetic determinants of the outcome of interest, the prevalence and/or incidence of the disease, and the resources required to perform the study. Prospective population-based cohort studies are the gold standard for gene–environment interaction studies, offering substantially decreased risk of measurement error and

[1] A known genetic locus or environmental measure that is not directly causal of disease but highly correlated with the causal region or exposure. Surrogate markers may be selected for reasons of cost or ease of measurement.

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Table 3
Gene–environment interaction study designs.

Study design	Features	Pros	Cons
Cohort	• Selection of sample occurs before onset of disease (case status unknown at outset of study) • Longitudinal follow up of entire sample	• A priori knowledge of causal regions of the genome not required • Can infer causal relationships between exposure and outcome • Accurate measurement timing of environmental exposure • Decreases likelihood of survivor and recall biases	• Time−consuming (often requires years of follow-up time) • Expensive • Require extremely large sample sizes to study rare or heterogeneous conditions
Case–control	• Purposeful sampling of individuals with outcome of interest and controls typically matched on pre-specified criteria • Can be cross-sectional or longitudinal	• Increased power to study rare conditions • A priori knowledge of causal regions of the genome not required • Less expensive and time-consuming than cohort design	• Cannot make causal inferences with cross-sectional time-frame • Difficult to determine selection criteria for appropriate controls • Possible confounding due to population stratification
Case-only	• Sample consists of only individuals known to have outcome of interest • Case status determined by genotype (presence of known genetic determinants of disease)	• Highly useful for studies of gene–environment interaction • Smaller sample size often possible • Eliminates problem of appropriate control selection in case–control design • Less expensive and time consuming than cohort design	• Requires knowledge of causal regions of the genome • Does not allow for estimation of the main effects of environmental and genetic exposure • Cannot make causal inferences with cross-sectional timeframe

subsequent bias, however they are generally extremely resource-intensive [22]. The classic case–control design can also be used for studies of gene–environment interactions, but are more susceptible to confounding compared to other study designs. For genetic association studies and gene–environment interaction studies, controls can be selected from among family members, which typically decreases the potential for confounding due to population stratification and can increase the power to detect an effect, but can result in the detection relationships that are less relevant at the population level [22,23]. A modification of the classic case–control design that is well suited to studies of gene–environment interactions is the case-only design. In case-only studies, inclusion criteria limit sample selection to individuals with the outcome of interest [24,25]. A limitation of the case-only design is that a priori knowledge of causal regions of the genome is required. A complete discussion of study designs appropriate for investigation of gene–environment interactions is available elsewhere [22–25].

2.3.2. Measurement

For studies of gene–environment interactions, the most common sources of measurement bias arise from misclassification of both environmental and genetic exposure. As discussed above, environmental exposures can vary over the course of a lifetime, which poses a challenge to accurate measurement (i.e., recall bias, biomarker stability). In some instances, individuals with a disease may be more likely to recall and/or report exposure because of their disease, resulting in differential misclassification and thus biased estimates of association and interaction [26,27]. Genotype is also subject to misclassification. When stringent quality control standards are implemented during laboratory analysis, the likelihood of misclassification is diminished for studies of relatively rare disorders (e.g., phenylketonuria) for which functional polymorphisms in single genes can be quantified [2,4]. However for disease conditions that are multifactorial, the principle of linkage disequilibrium is often exploited in order to identify regions of the genome that are associated with the outcome of interest. Genotype measurement by linkage disequilibrium is efficient and decreases genotyping costs. The principle of linkage disequilibrium is dependent on population substructure, or sub-populations determined by geographic ancestry that have shared common allele frequencies. The correlation between a region of the genome and an outcome is a group-averaged statistic for a given sub-population, which can result in misclassification of the genotype for an individual if they differ from the group norm [2,4]. When cases and controls are sampled from the same study base (sub-population), this type of misclassification is likely to occur with equal frequency, which will not result in biased estimates of association, but may decrease the likelihood of detection of a true interaction [26,27]. The least accurate method of quantifying genetic exposure is family history, which is also subject to misclassification as the actual genotype of the individual in unknown, and recall of family history can be incorrect [2,4]. Similar to recall of environmental exposure, accuracy of recall of family history may be differential between cases and controls, resulting in biased measures of association and interaction [26,27].

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2.4. Epigenetics and gene–environment interactions

Epigenetics is the study of mechanisms that result in changes to the phenotype or appearance of an organism that do not result from underlying changes to the genetic sequence. Commonly studied epigenetic mechanisms can affect the DNA, as in the case of DNA methylation[2] or histone acetylation,[3] or post-transcriptional modifications such as microRNA[4] regulation of gene expression. Traditional gene–environment interactions occur when the protein encoded by a particular gene interacts with an environmental exposure. Similarly, expression of a gene can be affected by exposure to an environmental factor, resulting in silencing or increased or decreased expression of a gene that may persist due to lasting but potentially reversible changes. For example, exposure to tobacco smoke may result in up-regulation of genes associated with platelet activation, and increased expression of these genes will persist while tobacco smoke is present at regular intervals. However, when this stimulus is removed for an extended period of time, epigenetic up-regulation of inflammatory genes will cease, and platelet activity will return to a normal, healthy physiologic state. In contrast, concomitant changes in genes mediating inflammation may not return to baseline due to changes in DNA methylation or histone acetylation. With regard to platelet activation, the deleterious effects of exposure to tobacco smoke are reversible; however for inflammation, there can be long-lasting or permanent alterations in genes expression, resulting in cumulative physiologic damage over the lifespan. The same principles that apply to gene–environment interactions, including timing and spectrum of environmental exposure, apply to interactions between epigenetic mechanisms and environmental exposures.

3. Conclusion

Humans exist within a dynamic environment and are subject to factors exerting effects on health outcomes on a number of levels. The current paradigm for understanding causes of cardiovascular disease within a complex system suggests that these conditions are rarely, if ever, the result of a single causal factor. The conceptual frameworks underlying cardiovascular disease postulate that these conditions occur in the presence of numerous genetic and environmental risk factors, that interactions between these factors occur on several levels, and that these interactions account for significant primary effects on the likelihood of disease occurrence [1,28,29]. In some cases, an interaction between individual gene loci and environmental exposure is believed to have a greater effect than the individual marginal effects of either factor alone [1]. Failing to account for the presence of a gene–environment interaction can result in incorrect conclusions about the etiology of cardiovascular disease, and is often attributed as a cause of incongruous study findings.

Gene–environment interactions have important implications for both nurse-clinicians and nurse-researchers. Worldwide, a current emphasis of nursing practice is to identify and treat individuals suffering from cardiovascular risk factors in order to prevent the onset and sequelae of cardiovascular disease. Throughout the twentieth century, nursing practice has focused largely on behavioral interventions and modification of the environment. The genomic era of healthcare both facilitates and necessitates that nurses also understand genetic predisposition for cardiovascular disease, and most importantly, place genetic predisposition within the context of an individual's environment.

[2] Addition of a methyl group to DNA that silences expression of some genes.

[3] Changes to the protein-DNA folding structure that prevent transcription of select regions of the genome.

[4] Small interfering RNA molecule that represses transcription of a messenger RNA sequence preventing polypeptide formation.

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CHAPTER 3

A CULTURALLY SPECIFIC HEALTH COACHING PROGRAM TO REDUCE CARDIOVASCULAR RISK IN SOUTH ASIANS

Background

Health coaching is a tool to engage patients in their health behaviors. As opposed to the traditional method of health care providers prescribing behavioral recommendations, which is widely shown to have poor adherence, coaching engages the patient in the process of recognizing their readiness to adopt new behaviors, reasons for ambivalence, and engaging in behavior change (1). The use of health coaching has been shown to be an effective strategy for reducing cardiovascular risk factors. A large randomized control trial in Australia found that telephone-based coaching by health personnel (nurses and dieticians) was effective at decreasing total cholesterol and other cardiovascular risk factors after one year compared to the usual care group (2). The coaching program was an iterative process of developing and executing a plan for behavior change (i.e. diet and physical activity), and did not directly involve prescription of medications (2). A randomized control trial in the United States provided a similar intervention delivered by nurse practitioners and community health workers, with incorporation of aggressive medication management, and also found significant improvement in cardiovascular risk factors in the intervention group (3). Preliminary evidence suggests that coaching strategies are also effective in management of related disease conditions, including type 2 diabetes (4) and obesity (5), and studies to determine the efficacy and required intensity of coaching interventions are ongoing (6, 7).

Compared to other ethnic groups, South Asians have at least two-fold increased risk for cardiovascular disease (8), making primary prevention of paramount importance in this population. Increased risk has been observed in both native and immigrant populations (9), and onset of risk factors and cardiovascular events often occurs as much as a decade earlier than in other ethnic groups (10, 11). The Indian sub-continent was projected to account for 60% of global cardiovascular burden by 2010 (9), and South Asians in the United States and Europe have a disproportionately high prevalence of cardiovascular risk factors (12, 13), despite the presence of protective demographic factors (14). In India, the cost of cardiovascular medications alone is estimated to be $450 million per year, with a projected cost of $3.8 billion annually if optimal therapy was achieved (15). Coaching performed by non-medically trained personnel is a promising and cost-effective complement to medical treatment for primary prevention of risk factors for cardiovascular disease.

Previous studies of coaching interventions have been primarily conducted in Caucasian populations (2, 16). There is growing interest in assessing the efficacy of this type of intervention for decreasing cardiovascular risk in ethnic sub-groups (4, 17), however to our knowledge, no prior studies have conducted a coaching intervention in the high risk South Asian population. Further, there is a paucity of evidence on the effect of providing culturally specific coaching that is tailored towards an individual's typical dietary and lifestyle patterns. The South Asian Heart Center has developed a coach-based cardiovascular risk reduction intervention called Heart Health Coaching. The aims of this intervention are two-fold: (1) to provide individualized, culturally specific dietary, physical activity, and stress reduction recommendations, and (2) to encourage behavior change and improvement in modifiable cardiovascular risk factors through regular discourse between participants and individually assigned coaches. The purpose of this paper is to describe the Heart Health Coaching intervention and report the number and characteristics of participants enrolled over five years.

Methods

Setting and Sample

The South Asian Heart Center, located at a community hospital in the Bay Area region of California, is a primary prevention program aimed at reducing cardiovascular risk in South Asians. Participants are recruited to the program through outreach events at community centers and corporations, physician referral, and word-of-mouth. Adults who self-identify as South Asian are eligible to undergo the cardiovascular disease risk screening process and participate in the coaching program.

Baseline Data Collection

Participants indicate their interest in the program by self-registering on the South Asian Heart Center's internet-based database. The initial screening consists of three components: Heart-Health Risk Assessment questionnaire, anthropometric measurements, and laboratory testing (Figure 1). The questionnaire consists of 70 questions regarding demographics, personal and family medical history, and behavioral and lifestyle information, and is obtained by via scripted telephone interview between the participant and a volunteer coach. This is a pre-scheduled conversation, lasting approximately 30 minutes. Trained staff members complete anthropometric measurements (height, weight, and waist circumference) and blood pressure and heart rate. Laboratory measurements are performed by Berkeley HeartLab (Alameda, CA) and include advanced lipoprotein testing, including low density lipoprotein cholesterol (LDL-c) sub-fractions, high density lipoprotein cholesterol (HDL-c) sub-fractions (segmented gradient gel electrophoresis), Apolipoprotein-B and Lipoprotein (a) (immunoturbidimetric assay), and other biomarkers associated with cardiovascular risk, including glucose, insulin, fibrinogen, homocysteine, and C-reactive protein. Participants pay insurance co-pay or subsidized full cost of the laboratory testing, depending on individual insurance coverage. At this initial visit, participants are asked if they would like to provide informed consent to participate in research.

All data reported here were obtained under the approval of the El Camino Hospital Institutional Review Board.

Risk Assessment and Recommendations

The South Asian Heart Center employs a Health Educator/Dietician, who uses the data collected in the initial questionnaire, labs, and anthropometric measurements to create an individual risk report. For the highest-risk patients, the Medical Director is also consulted. Participants return for a 30-minute results and recommendations consultation (Figure 1). During the results and recommendations consultation, the Health Educator reviews cardiovascular risk factors and makes specific lifestyle modification recommendations based on the participant's current reported diet and physical activity. Dietary recommendations are tailored to the participant's typical diet (i.e. South Asian versus Western). Every participant receives a Wellness Packet, providing information about nutrition, exercise, Type-A behavior modification, smoking cessation, Transcendental Meditation, and yoga. It also includes information on wellness programs offered in the local community. Although recommendations are individually tailored, the overall goals for diet are consumption of at least three fruits per day and at least four vegetables per day, and performing at least 150 minutes of physical activity per week. The physical activity goal is based on the United States Department of Health and Human Services' recommendation of performing at least 150 minutes of moderate intensity physical activity per week (18) and the American Heart Association/American College of Sports Medicine recommendation to perform at least 30 minutes of moderate-intensity physical activity five days per week (19). Dietary goals are taken from the United States Department of Agriculture and American Heart Association recommendations to consume 4-5 servings each of fruits and vegetables each day (20, 21).

At the completion of the results and recommendations consultation, participants are scheduled for an additional phone consultation with a registered dietitian for further review and planning, and have the opportunity for a phone follow-up with an exercise physiologist and a

group orientation with a Transcendental Meditation teacher. A follow-up testing schedule for laboratory measurements is determined from initial risk profile, ranging from three months to five years. Results of the baseline screening and the risk assessment are recorded in an internet-based database that is accessible to the South Asian Heart Center's staff and coaches. At the consent of the participant, results are also sent to the primary health care provider. The South Asian Heart Center does not prescribe medications, however all participants are strongly encouraged to follow up with their primary health care provider, particularly when medication may be indicated. For participants with at least one clinical risk factor, the health educator recommends participation in the one-year coaching program (Figure 1). Participants are provided with information about the role of the coaches, which includes tracking progress, providing help with overcoming barriers to making lifestyle changes, assessing adherence, and providing reminders about follow-up blood tests. The coaching program is provided at no cost to the particpant.

Coaching

The coaching program utilizes volunteer coaches, who either initiate a relationship with the South Asian Heart Center, or are recruited by current volunteers. Coaches are expected to commit to a minimum of eight hours per week for one year. Some coaches have previous experience in healthcare settings, but none are trained healthcare professionals. Coaches attend training sessions delivered by Berkeley HeartLab clinical personnel, during which multiple case studies are analyzed and discussed. New coaches are assigned to a mentor who provides one-on-one training for 1-2 months. Additional training is provided by lectures from experts on health behavior change and motivation, including curriculum on therapeutic lifestyle changes offered by the South Asian Heart Center's Medical Director. Each coach has a caseload of approximately 40 participants. Individuals who opt in to the coaching program are assigned to a coach based on the level of risk, level of coaching required, and workload distribution.

Participants choose whether they would like to be contacted by their coach via phone or email. The coaches' initial contact with a participant occurs 2-4 weeks after the results and

recommendations consultation (Figure 2). During this initial email or phone call, participants are asked specific questions regarding their success in incorporating the Health Educator's suggested lifestyle changes, and coaches troubleshoot any reported difficulties. The coaches use several templates and scripts that are then personalized with relevant details for an individual participant. The South Asian Heart Center Medical Director and Health Educator compose and review all the information provided to participants. Coaches record participant responses in progress note format in the internet-based database.

Depending on the number of risk factors, subsequent communication between coaches and participants occurs in intervals of every 4-6 weeks or every 3 months for one year (Figure 2). At each point of contact, coaches continue to assess progress with behavioral recommendations, help troubleshoot obstacles, and provide encouragement. Troubleshooting includes identifying reasons why participants are not meeting goals, and providing alternative suggestions or short-term intermediate goals. For example, a commonly reported obstacle is lack of time to meet physical activity recommendations. Coaches will suggest three shorter brisk walks per day as an alternative to a dedicated 30-minute session; if this sounds difficult, the coach might suggest starting with one short walk per day, increasing to two and then three after several week intervals. For dietary recommendations, coaches will remind participants that they will benefit from an increase of one fruit or vegetable serving per day, and similar to physical activity, will suggest increasing the number of servings again after several weeks of successfully adding one serving per day. Information collected at each point of contact is recorded in the internet-based database in the form of coach-assessed level of adherence for five categories. Four are objective measures (adhering to dietary recommendations, performing physical activity, performing stress reduction activities, medication adherence (when relevant)), based on the goal of consuming at least three fruits per day, at least four vegetables per day, and

performing at least 150 minutes of physical activity per week (Table 1). Coaches subjectively score the fifth measure, which is overall adherence (Table 1). In the case of non-response by a participant, the coach makes three contact attempts before the participant is removed from the coaching program (Figure 2). Documentation of contact attempts is recorded in the internet-based database.

Follow Up Data Collection

For participants who opt in to the coaching program, the questionnaire is repeated after one year, again by a scheduled, scripted telephone interview (Figure 1). Participants who were not eligible or did not elect to engage in the coaching program are also re-contacted after one year, and a follow-up questionnaire is collected if the participant is willing. Repeated laboratory and anthropometric measures are offered to all participants on an annual basis.

Statistical Analysis

Descriptive statistics, Student's t-test, and Chi-square test of independence were performed using Stata version 11 (College Station, TX).

RESULTS

Participant Flow and Follow-Up

Over five years, 3,287 individuals completed baseline screening, including questionnaire, anthropometric measurements and blood pressure, laboratory tests, and the results and recommendations consultation. The great majority, 3,132 (98%) were candidates for the coaching program. Of those, 2,726 (87%) elected to participate in the coaching program, and 1,359 (50%) followed through with their participation. Among the non-participants, 112 (8%) dropped out during the first year, while 1,255 (92%) did not respond to the coaches' three attempts at contact, and were therefore removed from the coaching program. Over five years, 1,051 (39%) individuals

who opted in to the program have completed one year of coaching, and an additional 308 (11%) are active participants in the first year.

Participant Characteristics

The majority of participants in the coaching program are married (93%) immigrants from South Asia (92%) with college-level education (96%) in their forties (43 years ± 10) (Table 2). Few participants report a history of cardiovascular disease (3%) or type-2 diabetes (9%), but there is a very high prevalence of family history of these conditions (cardiovascular disease 37%, type-2 diabetes 57%). The prevalence of smoking is very low (4%). Few participants meet the recommended daily intake of fruits (17%) and vegetables (9%) or time spent each week performing physical activity (35%). With the exception of body mass index (26kg/m^2 ± 5), mean values for clinical variables are within normal ranges. Consistent with the eligibility criteria for the coaching program of having at least one cardiovascular risk factor, the coached group had more abnormal clinical values than the non-coached group. Compared to the coached group, the non-coached group is less likely to have college level education (90% versus 96%, $p<0.05$), and more likely to have been born in the United States (19% versus 4%, $p<0.05$). The non-coached group also has a lower prevalence of family history of cardiovascular disease (19% versus 37%, $p<0.05$) and type 2-diabetes (29% versus 57%, $p<0.05$).

Discussion

We described a culturally specific coaching intervention aimed at decreasing cardiovascular risk in South Asians. A convenience sample of individuals underwent a comprehensive risk assessment, and those with at least one cardiovascular risk factor were invited to participate in the coaching program. Accordingly, the group of people who were referred to, and elected to participate in the program had a higher level of cardiovascular risk than those who were not eligible or opted out. This program provided coaching by non-medically trained personnel at regular intervals over the course of one year to facilitate improvement in behavioral

risk factors, namely diet, physical activity, stress reduction, and medication adherence when appropriate. We found that a large number of people were interested in and completed the program, indicating this is a feasible and appealing intervention in this population. Attrition primarily occurred at initiation of the intervention, and only 4% of participants who enrolled failed to complete the program. Additional studies with a longitudinal time-frame are needed to determine whether this intervention is also effective at improving clinical risk factors and decreasing incidence of cardiovascular disease in the high-risk South Asian population.

To our knowledge, this is the first adaptation of a health coaching model to incorporate culturally-specific components with non-medically trained coaches, and the first program targeted the high risk South Asian population. While a general approach to effecting change in behavior through coaching is effective (22), we do not know if additional improvements might result from the tailoring of coaching interventions to address specific cultural dietary and behavioral patterns, or whether interest and adherence of participants would be improved. Cultural specification, or tailoring, of other health-related interventions, including education, prevention, screening, and provider communication, has been widely implemented. Strategies to successfully adapt practices and interventions to be culturally specific continue to be to subject of research. Coaching interventions can learn from the knowledge base on cultural specificity developed in other health-related programs, and outcomes data from culturally specific coaching interventions such as the program described here can add a new and novel facet to this knowledge base.

We found that the non-coached group, who by eligibility definition were at lower risk for cardiovascular disease, also had a markedly lower prevalence of family history of cardiovascular disease and type-2 diabetes. This suggests that either genetics or learned familial behaviors, or likely a combination of both, predispose cardiovascular risk in the South Asian population. The interaction between genetic predisposition and the environment, including learned behavior, is increasingly thought to be equally as important as either of these factors alone in the development of cardiovascular disease and it’s risk factors (23-28). However, this interaction has not been

specifically studied in South Asians, taking into account culturally specific aspects of diet and behavior. Interestingly, we also observed that the non-coached group were slightly less likely to have a college-level education, and more likely to have been born in the United States. While the impact of these factors on other populations has been studied (29, 30), further research is needed to disentangle the impact of social determinants of health, such as education level, socioeconomic status, and immigration, in the South Asian population.

Coaching interventions like the Heart Health Coaching program have the potential for tremendous cost savings in prevention and treatment of cardiovascular disease. Given the substantial financial burden of cardiovascular disease, as well as the increasing global incidence, determining financially sound methods of prevention and treatment are of paramount importance. As evidenced by the high global incidence and prevalence of cardiovascular disease, current modalities for prevention, in addition to being costly, have less than desirable efficacy (31, 32). In contrast, preliminary data from trials of coaching interventions indicate significant improvements in health, meaning coaching interventions have the dual promise of being effective in both clinical outcomes and cost measures (2, 4, 5). Longitudinal data are needed to determine whether coaching strategies can effect long-lasting improvement in health status, and whether these improvements will be associated with decreased incidence of cardiovascular disease and related costs.

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Table 1. Adherence Assessment by Coaches

Behavior	Description
DIET AND NUTRITION	
No New Input	Participant did not address diet and nutrition questions in responses to coach
0-4 Meals/Week	Participant is eating 0-4 meals/week in accordance with recommendations
5-17 Meals/Week	Participant is eating 5-17 meals/week week in accordance with recommendations
> 18 Meals/Week	Participant is eating more than 18 meals/week week in accordance with recommendations
PHYSICAL ACTIVITY	
No New Input	Participant did not address exercise questions in responses to coach
No Routine	Participant is not exercising
1-2 Times/Week	Participant is exercising 1-2 times/week
3 Times/Week	Participant is exercising 3 times/week
4-5 Times/Week	Participant is exercising 4-5 times/week
6-7 Times/Week	Participant is exercising 6-7 times/week
STRESS REDUCTION	
No New Input	Participant did not address stress questions in responses to coach
None	Participant is not interested in practicing recommended stress reduction techniques
Planned	Participant is planning to practice recommended stress reduction techniques in near future
Sometimes	Participant sometimes practices recommended stress reduction techniques
Mostly	Participant often practices recommended stress reduction techniques
Regularly	Participant regularly practices recommended stress reduction techniques
MEDICATION	
No New Input	Participant did not address medication questions in responses to coach
No Change	Medications have not changed since last point of contact
No Medication	Participant is not taking any medication
Dosage Change	Participant has changed medication dosage
Added Medication	Participant has added started taking new medication(s)
OVERALL	
Not Assessed	Coach did not assess overall adherence
Low (<20%)	Low adherence with recommendations
Some (20-80%)	Some adherence with recommendations
Mostly (>80%)	High adherence with recommendations
No Plan	No plan was recommended

Table 2. Baseline demographic, behavioral, and clinical characteristics

Characteristic Mean ± SD or n (%)	Coached Participants (n = 2726)	Non-Coached Opted Out (n = 406)	Non-Coached Ineligible* (n = 48)	p-value
Age (years)	43 ± 10	43 ± 10	36 ± 11	0.4
Gender (men)	1868 (69)	232 (57)	18 (38)	<0.05
Birth country				
South Asia	2502 (92)	367 (90)	35 (73)	<0.05
United States	96 (4)	23 (6)	9 (19)	<0.05
Education				
Bachelor's or higher	2616 (96)	384 (95)	43 (90)	<0.05
Married	2525 (93)	368 (91)	40 (83)	<0.05
Medical History				
Cardiovascular disease	87 (3)	10 (2)	0 (0)	0.3
Type 2 diabetes	233 (9)	35 (9)	1 (2)	0.2
Family History				
Cardiovascular disease	1082 (37)	159 (39)	9 (19)	<0.05
Type 2 diabetes	1392 (57)	205 (54)	13 (29)	<0.05
Behaviors				
Current smoking	107 (4)	14 (3)	1 (2)	0.7
>4 Vegetable servings/day	252 (9)	50 (12)	5 (10)	0.1
>3 Fruit servings/day	473 (17)	82 (20)	12 (25)	0.2
>150 Minutes physical activity	951 (35)	172 (42)	21 (44)	<0.05
Stress reduction practice	892 (33)	155 (38)	10 (21)	<0.05
Clinical Variables				
TC (mmol/L)	4.9 ± 1.0	4.7 ± 0.9	4.4 ± 0.6	<0.05
LDL (mmol/L)	3.0 ± 0.8	2.8 ± 0.8	2.5 ±0.5	<0.05
HDL (mmol/L)	1.2 ± 0.3	1.3 ± 0.4	1.5 ± 0.3	<0.05
TG (mmol/L)	1.6 ± 1.0	1.4 ± 0.8	0.9 ± 0.5	<0.05
Glucose (mmol/L)	5.1 ± 1.0	5.0 ± 0.9	4.6 ± 0.5	<0.05
Systolic blood pressure (mmHg)	121 ± 16	119 ± 17	113 ± 14	0.2
Diastolic blood pressure (mmHg)	76 ± 10	73 ± 10	70 ± 8	0.2
BMI (kg/m^2)	26 ± 5	25 ± 4	23 ± 3	<0.05
Waist circumference (cm)	89 ± 10	87 ± 10	79 ± 7	<0.05

*****no cardiovascular risk factors

TC, total cholesterol
LDL, low-density lipoprotein cholesterol
HDL, high-density lipoprotein cholesterol
TG, triglycerides
BMI, body mass index

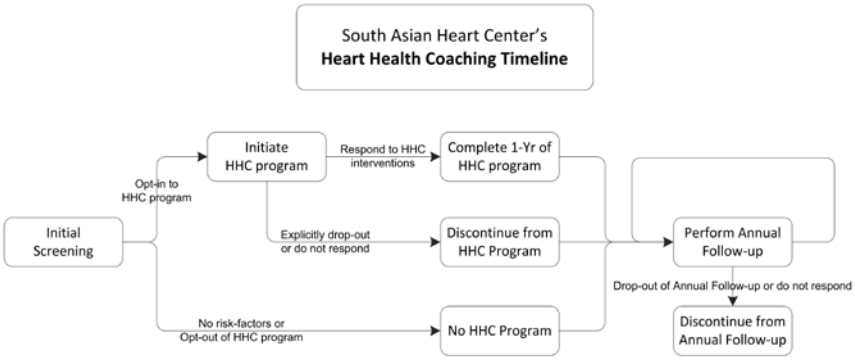

Figure 1. South Asian Heart Center Flow of Events

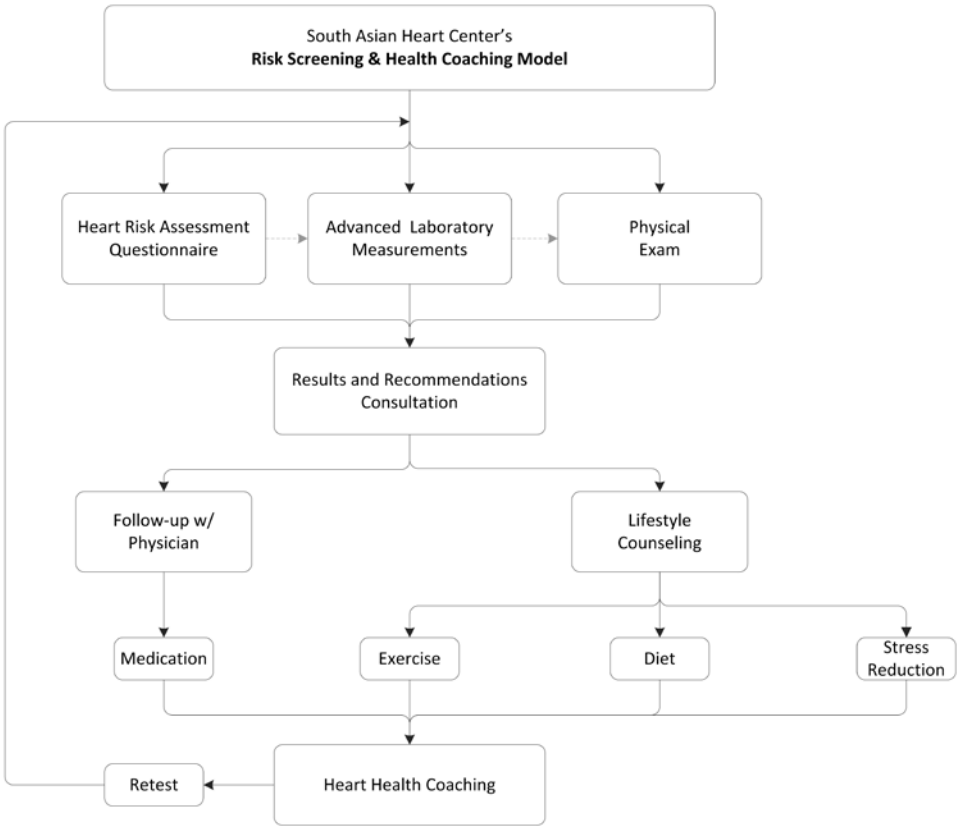

Figure 2. Coaching Flow of Events

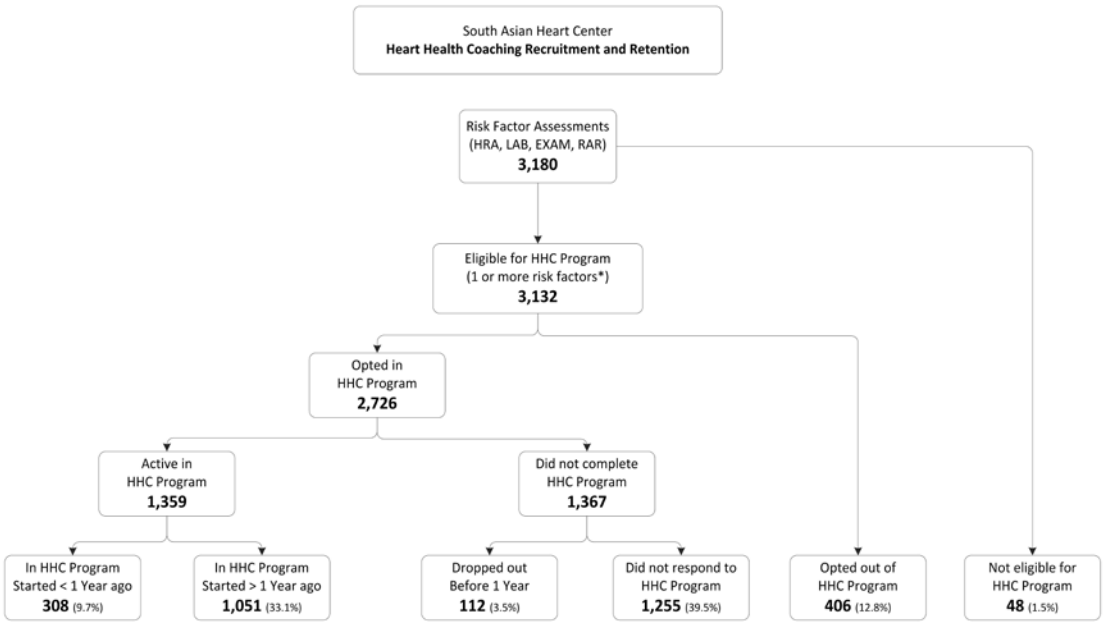

Figure 3. Number of participants in each category

*Risk factors include elevated total cholesterol, elevated LDL, low HDL, elevated triglycerides, elevated glucose, overweight, type 2 diabetes, family history of cardiovascular disease, smoking, elevated blood pressu

CHAPTER 4

METABOLIC SYNDROME AND RELATED DISORDERS
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Prevalence of Metabolic Syndrome in South Asians Residing in the United States

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Abstract

Aims/hypothesis: The aim of this study was to define the prevalence of the metabolic syndrome and its component risk factors among individuals of South Asian origin living in the United States.
Methods: We analyzed baseline data from 1,445 participants enrolled in a cohort study investigating risk factors for cardiovascular disease in South Asians. We defined the metabolic syndrome using the International Diabetes Federation criteria for waist circumference (>90 cm for men; >80 cm, women), triglycerides (>150 mg/dL), high-density lipoprotein cholesterol (HDL-C) (<40 mg/dL (men), <45 mg/dL (women)), blood pressure (>135/80 mmHg), and fasting glucose (>100 mg/dL).
Results: The mean age was 43 ± 10 years, and 30% of participants were women. The prevalence of metabolic syndrome was 27% (31% men vs. 17% women, $P < 0.05$). Fifty-nine percent of the cohort had high waist circumference (58% men vs. 62% women, $P =$ not significant [N.S.]), 47% had low HDL-C [46% men vs. 48% women (NS)], 19% had elevated triglycerides (23% men vs. 8% women, $P < 0.05$), 14% had hypertension (16% men vs. 9% women, $P < 0.05$), and 13% had elevated fasting glucose (18% men vs. 11% women, $P < 0.05$). The most common metabolic syndrome phenotype is low HDL-C with elevated triglycerides.
Conclusions: Although the prevalence of the metabolic syndrome is lower than previous reports of South Asians, the prevalence is still unacceptably high despite the presence of protective demographic factors.

Introduction

South Asians are individuals whose racial origins come from the Indian subcontinent, which includes India, Pakistan, Sri Lanka, Nepal, and Bangladesh. Compared to other ethnicities, South Asians have at least a two-fold increased risk of cardiovascular disease, myocardial infarction, type 2 diabetes, and cardiovascular death.[1–4] The Indian subcontinent accounts for 60% of the global cardiovascular burden.[5] High cardiovascular risk has been observed in both native and immigrant South Asian populations, indicating a genetic predisposition in addition to environmental effects.[3,6–10] Risk has been attributed to an excess of common risk factors that are more prevalent at a younger age.[11] A common metabolic and cardiovascular risk phenotype in South Asians includes components of the metabolic syndrome, a complex multifactorial precursor to cardiovascular disease and type 2 diabetes also arising from a combination of genetic and environmental risk factors.[12] The International Diabetes Federation (IDF) criteria define the essential component of the metabolic syndrome as high waist circumference, accompanied by at least two of four additional risk factors: Elevated fasting glucose, decreased high-density lipoprotein cholesterol (HDL-C), elevated triglycerides, and elevated blood pressure.[12]

Although the prevalence of the metabolic syndrome and obesity in the United States have increased over the past decade,[13,14] all U.S. studies have enrolled low numbers of South Asians.[15,16] Similarly, South Asian cohort studies have enrolled relatively few participants of South Asian origin from North America,[11] although there are over 2 million

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individuals of South Asian origin in the United States. The aim of this study was to define the prevalence of the metabolic syndrome and its components among individuals of South Asian origin living in the United States.

Methods

Design

The Bay Area South Asian Study is an ongoing prospective cohort study investigating risk factors for cardiovascular disease in South Asians. Between June, 2006, and December, 2008, 1,859 consecutive participants were enrolled in the South Asian Heart Center (SAHC) program at El Camino Hospital (Mountain View, CA). The SAHC is a not-for-profit organization that provides a screening cardiovascular risk evaluation, including a health risk assessment, physical examination, comprehensive laboratory testing, counseling, and case management for risk factor reduction.[17] Individuals are self-referred or referred by their health care providers. A total of 1,445 individuals (1,012 men, 433 women) completed the initial screening program, including laboratory testing, and are the subjects of this study. The study was approved by the local Institutional Review Board.

Measurements

As part of the SAHC clinical evaluation, demographic characteristics, medication use, habits including smoking, physical activity, and personal and family history including cardiovascular disease and risk factors, were measured by self-report using a standardized, scripted 30-min questionnaire administered via telephone by trained study personnel. Trained nurse practitioners and study personnel measured anthropometric and clinical variables including waist circumference, height, weight, and blood pressure. Hypercholesterolemia, hypertension, and type 2 diabetes were defined as abnormal clinical value (total cholesterol >200 mg/dL, systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg, or fasting blood glucose >125 mg/dL), or self-reported lipid-lowering therapy, antihypertensive medication use, or diabetes medication use. Baseline lipid measurements were obtained by peripheral venipuncture after 12 h of fasting. All laboratory assays were performed by a single Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (Berkeley Heart Lab, Berkeley, CA). Lipid fractionation was performed using a calorimetric method: Total cholesterol (TC), HDL-C, triglycerides, and glucose were measured using reagents from Roche Diagnostics (Indianapolis, IN) and performed on the Roche Modular PPP Analyzer.[18] Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula.[19] The metabolic syndrome components were defined using the IDF consensus definition of waist circumference >90 cm (men) or 80 cm (women), triglycerides >150 mg/dL, HDL-C <40 mg/dL (men) or 45 mg/dL (women), blood pressure >130/85 mmHg, and fasting glucose >100 mg/dL.[12]

Table 1. Demographic and Clinical Characteristics ($N = 1445$)

Characteristics	*Mean ± SD or* n *(%)*	*Men* (n = *1,012)*	*Women* (n = *433)*	P *value*
Age (years)	43 ± 10	43 ± 10	43 ± 11	0.6
Birth Country ($n = 849$)				
India	749 (88)	514 (69)	235 (31)	<0.05
Pakistan	9 (1)	7 (78)	2 (22)	0.1
Sri Lanka	5 (1)	5 (100)	0 (0)	
United States	40 (5)	26 (65)	14 (35)	0.07
Married	1343 (93)	947 (94)	396 (91)	0.2
Education				
Less than Bachelor's degree	52 (4)	21 (<1)	31 (7)	<0.05
Bachelor's degree	326 (23)	175 (17)	151 (35)	<0.05
Graduate/Master's degree	932 (65)	707 (70)	225 (52)	<0.05
Ph.D./post-graduate degree	132 (9)	106 (11)	25 (6)	<0.05
Behaviors				
Current smoking	54 (4)	49 (5)	5 (1)	<0.05
Former smoking	187 (13)	177 (17)	10 (2)	<0.05
Family history of cardiovascular disease				
Parent	811 (56)	560 (55)	251 (58)	0.3
Sibling ($n = 678$)	274 (40)	181 (39)	93 (43)	0.5
Clinical variables				
TC (mg/dL)	190 ± 37	192 ± 37	185 ± 35	<0.05
LDL-C (mg/dL)	116 ± 31	118 ± 32	111 ± 29	<0.05
HDL-C (mg/dL)	45 ± 12	42 ± 10	53 ± 13	<0.05
TG (mg/dL)	144 ± 93	159 ± 100	110 ± 63	<0.05
Glucose (mg/dL)	90 ± 16	92 ± 18	87 ± 12	<0.05
Systolic blood pressure (mmHg)	118 ± 17	120 ± 17	113 ± 17	<0.05
Diastolic blood pressure (mmHg)	76 ± 11	78 ± 11	72 ± 11	<0.05
BMI (kg/m^2)	25.7 ± 3.7	25.8 ± 3.5	25.6 ± 4.1	0.3
Waist circumference (cm)	88 ± 13	91 ± 12	82 ± 12	<0.05
Metabolic syndrome	387 (27)	315 (31)	72 (17)	<0.05

Abbreviations: TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; BMI, body mass index.

Table 2. Proportion of Participants with Abnormal Clinical Characteristics ($N = 1,445$)

Characteristic	n *(%)*	*Men* (n = *1,012)*	*Women* (n = *433)*	P *value*
TC > 200 mg/dL	544 (38)	414 (41)	130 (30)	<0.05
LDL-C > 160 mg/dL	129 (9)	110 (11)	19 (4)	<0.05
HDL-C <40 mg/dL (men) <50 mg/dL (women)	674 (47)	468 (46)	206 (48)	0.6
TG > 200 mg/dL	272 (19)	238 (23)	34 (8)	<0.05
Glucose > 126 mg/dL	44 (3)	39 (4)	5 (1)	<0.05
Blood pressure > 140/90 mmHg	203 (14)	165 (16)	38 (9)	<0.05
BMI > 25 kg/m^2	778 (54)	570 (56)	208 (48)	<0.05
Waist circumference >90 cm, men >80 cm, women	858 (59)	591 (58)	267 (62)	0.2
Metabolic syndrome	387 (27)	315 (31)	72 (17)	<0.05

Abbreviations: TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; BMI, body mass index.

Statistical analysis

The Student *t*-test was used for comparison of continuous variables and the chi-squared test for proportions. Logistic regression was used to compare the odds of metabolic syndrome and its components in men and women. Two-sided P values were calculated for all test statistics and $P < 0.05$ was considered significant unless otherwise indicated. Statistical analyses were performed using STATA Version 10 (College Station, TX).

Results

Demographic and clinical characteristics are shown in Table 1. The mean age was 43 ± 10 years and 30% of participants were women. The cohort primarily consisted of first-generation immigrants to the United States, with 89% born in South Asia. Sixty-nine percent reported a parent or sibling history of cardiovascular disease or cardiovascular risk. Four percent of men and 1% of women reported current smoking ($P < 0.05$) and 17% of men and 2% of women ($P < 0.05$) reported previous smoking. Ninety-three percent of participants are married. Overall, the cohort was highly educated, with 96% of the cohort reporting a Bachelor's-level education or higher, and 74% reporting a graduate-level education, defined as a Master's degree or doctorate. Men had a higher level of education than women. Other demographic characteristics were similar.

Self-reported prevalence of high total cholesterol was 56%, hypertension 22%, and type 2 diabetes 9%. The measured prevalence of clinical risk factors is shown in Table 2.

Table 3. Distribution of the Metabolic Syndrome Components

		Total n = *854* n *(%)*	*Men* n = *589* n *(%)*	*Women* n = *265* n *(%)*	P *value*
Pre – metabolic syndrome	Waist circumference (WC) only	213 (25)	106 (18)	107 (40)	<0.05
	Two risk factors				
	WC + HTN	62 (7)	46 (8)	16 (6)	0.5
	WC + HDL-C	102 (12)	59 (10)	43 (16)	<0.05
	WC + TG	64 (7)	46 (8)	18 (7)	0.7
	WC + glu	30 (4)	19 (3)	11 (4)	0.4
Metabolic syndrome	Three risk factors				
	WC + HTN + HDL-C	37 (4)	27 (5)	10 (4)	0.7
	WC + HTN + TG	28 (3)	23 (4)	5 (2)	0.2
	WC + HTN + glu	19 (2)	15 (3)	4 (2)	0.4
	WC + HDL-C + TG	138 (16)	112 (19)	26 (10)	<0.05
	WC + HDL-C + glu	15 (2)	10 (2)	5 (2)	0.8
	WC + TG + glu	23 (3)	17 (3)	6 (2)	0.7
	Four risk factors				
	WC + HTN + HDL-C + TG	57 (7)	48 (8)	9 (3)	<0.05
	WC + HTN + HDL-C + glu	10 (1)	9 (2)	1 (<1)	0.2
	WC + HTN + TG + glu	14 (2)	12 (2)	2 (<1)	0.2
	WC + HDL-C + TG + glu	22 (3)	20 (3)	2 (1)	<0.05
	Five risk factors				
	WC + HTN + HDL-C + TG + glu	24 (3)	22 (4)	2 (1)	<0.05

Abbreviations: WC, waist circumference (cm); HTN, hypertension (mmHg); HDL-C, high-density lipoprotein cholesterol (mg/dL); glu, fasting plasma glucose (mg/dL); TG, triglycerides (mg/dL).

Twenty-seven percent of the sample (31% men vs. 17% women, $P < 0.05$) meet the IDF criteria for the metabolic syndrome.[12] The most prevalent clinical risk factors were high waist circumference (59%, 58% men vs. 62% women, $P =$ not significant [N.S.]), high body mass index (BMI) (54%, 56% men vs. 48% women, $P < 0.05$), low HDL-C (47%, 46% men vs. 48% women, $P =$ N.S.), and high TC (38%, 41% men vs. 30% women, $P < 0.05$). Although the proportion of high waist circumference in men and women was similar, men had a significantly higher prevalence of the metabolic syndrome and its components ($P < 0.05$) (Table 2). Prevalence of the metabolic syndrome and cardiovascular and metabolic risk factors did not differ by country of origin or marital status. HDL-C levels were lower among college-educated participants (45 ± 11) than in those with less than a college level education (51 ± 15, $P < 0.05$); no other differences were found by education status.

We also analyzed the frequencies of the components of the metabolic syndrome among participants with the sentinel characteristic of high waist circumference ($n = 854$; Table 3). Among those with metabolic syndrome, high waist circumference with low HDL-C and elevated triglycerides was the most common phenotype for men and women. Low HDL-C is the most common risk factor, with 48% prevalence among the subgroup of participants with high waist circumference, followed by elevated triglycerides (44%). Hypertension (29%) and elevated blood glucose (20%) were less common. Overall, women most commonly presented with high waist circumference alone (40%), whereas men most commonly presented with metabolic syndrome (19%) determined by high waist circumference, low HDL-C, and elevated triglycerides.

Previous reports of cardiovascular risk in South Asians living in California from the Cardiovascular Health Among Asian Indians (CHAI) and California Health Interview Survey (CHIS) have calculated a cardiovascular risk score including hypertension, hypercholesterolemia, diabetes, history of myocardial infarction or coronary artery disease, and history of smoking.[8] To compare cardiovascular risk qualitatively in the SAHC cohort to previous population-based estimates, we calculated the same risk score (Table 4). We found the SAHC cohort to be at greater risk than previous observations from the CHAI and CHIS studies, with a higher proportion of participants having at least one cardiovascular risk factor (67%).

Discussion

To our knowledge, this is the largest cohort study of South Asians in North America. The principal finding is a 27% prevalence of the metabolic syndrome among South Asians living in the United States. Although women have a higher prevalence of abdominal adiposity (as measured by waist circumference) without the presence of components of the metabolic syndrome, men have a higher prevalence of most of the additional metabolic syndrome components and cardiovascular risk factors (Table 2). Dyslipidemia is common, with abnormal levels of HDL-C and triglycerides in addition to elevated total cholesterol and LDL-C, closely mirroring the metabolic syndrome phenotype. In contrast, the prevalence of hypertension among South Asians is low.[11,20,21]

In the presence of abdominal adiposity, dyslipidemia characterized by low HDL-C with accompanying elevated

TABLE 4. SUMMARY OF PREVALENCE STUDIES OF CARDIOVASCULAR RISK FACTORS AMONG SOUTH ASIANS IN CALIFORNIA

Study reference	*Study base and sampling method*	*Enrollment period*	*Sample size*	*Measurement*	*Age mean (range)*	*BMI mean ± SD*	*High cholesterol* n (%)	*High blood pressure* n (%)	*Diabetes* n (%)	*MI/angina* n (%)	*>1 Cardiovascular risk factor*[a] %
Flowers (2010)[b]	Convenience sample of South Asians living in the San Francisco Bay Area	2006–2008	1,445 (1,012 men, 433 women)	Clinical assessment and self-report	43 (21–82)	25.7 ± 3.7	984 (69)	395 (27)	124 (9)	43 (3)	67
Ivey et al. (2006)[8]	California population - based random sample	2001	769 (428 men, 341 women)	Self-report	41 (25–83)	24.5 ± 5.5	41/121[c] (31)	105 (13)	37 (4)	31 (6)	36
Ivey et al. (2006)[8]	Surname based sample from area codes regions with a high proportion of South Asians	1999–2001	304 (126 men, 178 women)	Self-report	25–80	25.7	106 (35)	62 (20)	32 (11)	10 (3)	34

[a]Cardiovascular risk factors include high blood pressure, diabetes, high cholesterol, current smoking or history of smoking more than 100 cigarettes in a lifetime, and history of myocardial infarction or angina.
[b]Flowers et al., unpublished.
[c]Question was asked only in participants who reported coronary heart disease and/or hypertension.
Abbreviations: BMI, body mass index; SD, standard deviation; MI, myocardial infarction.

Table 5. Summary of Prevalence Studies of the Metabolic Syndrome

Study reference	*Study base and sampling method*	*Enrollment period*	*Sample size*	*Age mean ± SD*	*Metabolic syndrome*[a] *%*	*Elevated waist circumference %*	*Low HDL-C %*	*Elevated triglycerides %*	*Elevated blood pressure %*	*Elevated fasting glucose %*
United States										
Flowers (2010)[b]	Convenience sample of South Asians living in the San Francisco Area	2006–2008	1,445 (1,012 men, 433 women)	43 ± 10	27 (30% men, 15% women)	59	42	37	23	16
Rianon and Rasu (2009)[10]	Convenience sample of Bangladeshi immigrant men in the US	2007	91 (91 men, 0 women)	46 ± 8	38	Not known	35	57	60	56
Misra et al. (2009)[9]	Population based sample of South Asians in the U.S.	Not known	1038 (609 men, 429 women)	46 ± 13	38 (38% men, 37% women)	61	38	42	36	63
India										
Deepa et al. (2007)[6]	Population based sample from India	2001–2002	2,350 (1,096 men, 1254 women)	40 ± 13	26 (23% men, 28% women)	49	64	25	31	21
United Kingdom										
Tillin et al. (2005)[3]	Population-based sample of South Asians in the U.K.	1988, 1991	1,603 (1,322 men, 281 women)	40 – 69[c]	29[d] (46% men, 31% women)	22[c]	59 [d, e]	59 [d, e]	52[d]	28[d]
United States										
Ervin (2009)[13]	Population based sample from U.S. (all ethnicities)	2003–2006	3,423 (1,794 men, 1,629 women)	>20	34 (40% men, 38% women)	53	25	31	40	39

[a]International Diabetes Federation criteria,[12] except where otherwise indicated.
[b]Flowers et al., unpublished.
[c]Range.
[d]National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome.[37]
[e]Reported as dyslipidemia.
Abbreviations: SD, standard deviation; HDL-C, high-density lipoprotein cholesterol.

triglycerides appears to be the driving component of onset of the metabolic syndrome for both men and women (Table 3). The high prevalence of this dyslipidemia pattern has previously been observed in individuals of South Asian origin, suggesting an important possible causal mechanism for cardiovascular risk in this ethnic group.[22] South Asians commonly are afflicted with abdominal adiposity accompanied by insulin resistance in addition to a dyslipidemia pattern characterized by the presence of high triglycerides and small, dense, dysfunctional HDL-C.[23–27] Women appear to be somewhat protected from developing the combined low HDL-C and elevated triglyceride dyslipidemia phenotype compared to men, which is also consistent with previous reports of cardiovascular and metabolic risk in South Asians,[28] and may indicate the need for important differences in risk assessment between men and women.

The prevalence of the metabolic syndrome in the SAHC cohort was modestly lower compared to other South Asian cohorts from the United States and Europe and to the overall U.S. population (Table 5). The differences were greater in women. There are several potential explanations. First, the SAHC cohort differs demographically from previous studies, with an increased prevalence of marriage and education, which are generally protective.[29–32] Second, there may be secular trends; recent U.S. data suggest a plateau in the rate or rise of obesity in America.[33] In men, the prevalence of the metabolic syndrome is higher in the SAHC cohort than in population studies of the country of India (Table 5). For many ethnic groups, acculturation to a Westernized lifestyle is associated with increased risk for coronary artery disease, obesity, and type 2 diabetes, with markedly higher prevalence of disease among first-generation offspring of immigrants than their parents.[34–36] In contrast, the prevalence in women is lower in the SAHC cohort than in native Indians (Table 5). The majority of women in the cohort are premenopausal (71%), which may in part explain the observed differences in risk by gender; however, our findings also indicate that characteristics of South Asian immigrants including education level and smoking habits may differ between men and women (Table 1).

Nonetheless, the prevalence of the metabolic syndrome in South Asians is still unacceptably high, and the causes of this high prevalence are unclear. Further study of the differences between the SAHC cohort and other groups provides an opportunity to investigate protective factors for cardiovascular and type 2 diabetes risks in this highly susceptible population.

Limitations

This was a cross-sectional prevalence study and was not designed to assess the risk of the metabolic syndrome on cardiovascular events. All participants were enrolled from a screening program. Therefore, sampling bias may be a potential concern. However, our estimates are similar to previous population-based estimates for cardiovascular events and type 2 diabetes in immigrant South Asians residing the same geographic region,[8] suggesting that this source of bias is unlikely or modest. Measurement of demographics, personal and family history, medication use, and habits were done by self-report. However, we validated self-reported data of medical history with the reported medications and laboratory values and found 96% agreement for self-reported dyslipidemia, diabetes, and hypertension (data not shown).

In conclusion, we found that South Asians in the United States have a high prevalence of cardiovascular and metabolic risk factors at a young age despite seemingly protective demographic characteristics. Early screening of this population for obesity, hypertension, insulin resistance, lipid disorders, and the metabolic syndrome is advisable for early risk factor modification. Further research investigating the specific mechanisms of risk in South Asians is needed to develop ethnicity-specific screening parameters for cardiovascular and metabolic risk, to determine how lifestyle and behavioral factors are affected by immigration and acculturation, and to understand if lifestyle and behavioral interventions may be adequate to decrease risk among individuals with moderately elevated cardiovascular risk or substantially decrease risk as adjuvant therapy to medication among high-risk individuals.

Author Disclosure Statement

The authors have nothing to disclose.

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CHAPTER 5

ADIPOSITY AND HEART DISEASE RISK CLUSTERING IN SOUTH ASIANS

Introduction

Wildman and associates [1] used data from the National Health and Nutrition Examination Surveys 1999-2004, and six criteria, to analyze the relationship between different degrees of adiposity and cardio-metabolic risk factors associated with insulin resistance in three different racial groups. Four of the criteria of cardio-metabolic risk were those, excluding waist circumference (WC), used to diagnose the metabolic syndrome [2], with the other two being high sensitivity C-reactive protein (hs-CRP) and homeostasis model assessment of insulin resistance (HOMA-IR). If two or more of the criteria were met, the subject was classified as being abnormal. Their results demonstrated that a substantial number of subjects whose body mass index (BMI) was ≤ 25 kg/m² were abnormal, and a comparable number whose BMI was ≥ 30 kg/m² were normal; findings consistent with previous publications [3, 4]. What makes the findings of Wildman, et. al. [1] unique is that the heterogeneity they described in the relationship between adiposity and cardio-metabolic risk was reasonably comparable in all three of the racial groups studied.

The current analysis is an effort to extend the findings of Wildman and associates [1], and had three major goals that differentiate it from their study. Firstly, we believed it important to evaluate the relationship between degree of adiposity and cardio-metabolic risk in apparently healthy individuals and for that reason excluded subjects with known cardiovascular disease (CVD), diabetes, hypertension, or dyslipidemia. Secondly, we wished to consider the possibility that the overall thrust of the findings of Wildman and colleagues [1] might have been somewhat confounded by their use of BMI as the primary index of adiposity, rather than waist circumference (WC). For example, It has been argued by Després, et. al. [5] that WC can differ significantly at a given BMI, and that the greater the WC, the more visceral adiposity –

presumably, the major factor leading to insulin resistance and associated metabolic abnormalities. Thus, we thought it worthwhile to address the relationship between adiposity and cardio-metabolic risk with both BMI and WC as our primary indices of adiposity, using ethnic specific criteria for abdominal obesity. Thirdly, it seemed important to evaluate a different ethnic/racial group than the three studied by Wildman, et. al. [1], and, in particular, a group in whom the findings might vary as a function of index of adiposity. We chose South Asians for this purpose as they have an increased prevalence of CVD, insulin resistance, hyperinsulinemia, and high triglycerides (TG), and low high-density lipoprotein cholesterol (HDL-C) concentrations [6-8], and it has been suggested that abdominal obesity accounts for these metabolic abnormalities and increased risk of CVD in these individuals [9-11].

Methods

Study Subjects

The study sample consisted of 463 women and 552 men, part of a larger group of volunteers (n = 4797) evaluated for cardio-metabolic risk at the South Asian Heart Center; a not-for-profit organization providing CVD risk assessment and counseling to South Asians in the San Francisco Bay Area. The Institutional Review Board of El Camino Hospital, Mountain View, California approved the study. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology: Shewan LG and Coats AJ. Ethics in the authorship and publishing of scientific articles. Int J Cardiol 2010;144:1-2. All participants were in generally good health and older than 18 years. Individuals taking drugs to lower blood pressure, glucose, or lipid levels were excluded, as were those not fasting for at least 10 hours. Glucose tolerance tests were not performed, but volunteers whose fasting plasma glucose concentration $\geq$ 7.0 mmol/L were considered to have diabetes and excluded from analysis, as were participants with a known history of hypertension, abnormal cholesterol, or CVD.

Anthropometric Measurements

Height and weight were determined with subjects in light clothing and without shoes, and BMI calculated by dividing weight (kilograms) by height (meter squared). WC was measured using the National Health and Nutrition Examination Survey III protocol during normal minimal respiration by placing a measuring tape around the waist just above the uppermost lateral border of the iliac crest [12]. Participants were classified as being normal weight, overweight, or obese on the basis of their BMI, and abdominally obese or abdominally normal on the basis of their WC [1, 2,12]. Blood pressure was measured with an automatic blood pressure recorder, using an appropriately sized cuff, with subjects sitting in a chair with feet on the floor and arm supported at heart level.

Laboratory Measurements

After an overnight fast, blood samples were drawn for measurement of plasma glucose, insulin, TG, HDL-C, and hs-CRP concentrations at the Berkeley Heart Lab [13]. Specifically, glucose concentrations were measured by enzymatic rate reaction; insulin by electrochemiluminescence immunoassay; triglycerides by blanked enzymatic method; HDL-C by a homogeneous direct assay, and hs-CRP by particle-enhanced immunoturbidimetric assay. The insulin assay had 100% cross-reactivity with human insulin and 5% with human pro-insulin. The total and within-run precisions (%) of the laboratory assays were as follows: glucose (2.03, 0.82); insulin (2.67, 1.35); TG (2.43, 0.90); HDL-C (3.25, 0.98); and hs-CRP (1.96, 0.52). HOMA-IR was calculated from fasting glucose and insulin concentrations using the formula: ([fasting insulin (μU/ml)]*[fasting glucose (mmol/L)])/22.5 [14]. The six criteria for identifying a cardio-metabolic abnormality were those used by Wildman, et al [1]. Criteria and cut-points are given in Table 1, and subjects were classified as metabolically healthy (<2 abnormal findings) or metabolically abnormal (≥2 abnormities) on the basis of these definitions.

Statistical Analysis

Descriptive statistics were used to provide means, ranges, standard deviations, and proportions for demographic and clinical variables. Student's t-test was used to assess for differences between continuous variables, and Pearson's Chi-square was used to test for differences in proportions. All statistical tests were performed using STATA version 11 (College Station, TX).

Results

Table 2 lists the demographic and metabolic characteristics of the metabolically healthy and metabolically abnormal groups based on their BMI category. Approximately one quarter of the population was metabolically abnormal, and 32% of these individuals were of normal weight. Of the 75% of individuals classified as metabolically healthy, 37% were overweight/obese. It can be seen that the vast majority of subjects were non-smokers, did not consume alcohol, and participated in some degree of physical activity on a weekly basis. Not surprisingly, the values of the 6 risk factors being evaluated were higher in the metabolically abnormal group. It should also be noted that essentially all of the overweight/obese individuals, metabolically healthy or abnormal, were also abdominally obese (elevated WC).

Table 3 lists the demographic and metabolic characteristics of the metabolically healthy and abnormal groups based on their WC category. In general, the comparisons are similar to those presented in Table 2 when BMI was used to classify individuals. Thus, 28% of the metabolically abnormal individuals had a normal WC, and 42% of the metabolically healthy group was abdominally obese. As in Table 1, values of the 6 risk factors were higher in the metabolically abnormal group

Figure 1 illustrates the prevalence of metabolically healthy and metabolically abnormal men (Panel A) and women (Panel B) when classified on the basis of BMI. The data in Panel A demonstrate that 23% of normal weight men are metabolically abnormal, and 21% of obese men are metabolically healthy. It can also be seen that the prevalence of metabolically abnormal

individuals essentially doubles as you go from normal weight to overweight, and again going from overweight to obese.

The data in Panel B show a quite different pattern of prevalence of the metabolically healthy and abnormal groups in women. At the simplest, the prevalence of metabolically abnormal women is much less in any BMI category when compared to men; thus only 7% of normal weight women are metabolically abnormal vs. 23% of men, and more than twice as many obese women are metabolically healthy as compared to obese men (50% vs. 21%).

Figure 2 compares the prevalence of metabolically healthy and metabolically abnormal men (Panel A) and women (Panel B) when classified on the basis of WC. In certain respects these findings reflect the results in Figure 1. Thus, 21% of men with a normal WC were metabolically abnormal, as compared to 23% of men with a normal BMI (Figure 1, Panel A). Furthermore, the prevalence of being metabolically abnormal was again much less in women than in men; 6% vs. 21% in those with a normal WC and 23% vs. 55% in individuals with an abnormal WC.

Figure 3 displays the relationship between measure of adiposity and number of abnormalities. Not surprisingly, the more abnormalities present, the more obese the individual. Thus, approximately one-third of those without any abnormality were obese/overweight by BMI classification or abdominally obese on the basis of their WC. At the other extreme, approximately 90% of those with 5 abnormalities were obese/overweight or abdominally obese. Intermediate were those with two abnormalities, and it can be seen that approximately two-thirds of the group with only two abnormalities had either an abnormal BMI or WC.

The results in Table 4 provide a more extensive analysis of the participants with 2 abnormalities by displaying the prevalence of the risk factor clustering that defined them as being metabolically abnormal. These data clearly identify dyslipidemia as the most common abnormality. Thus, the combination of a high TG and a low HDL-C concentration were present in approximately one-third of this population, and one or the other of these abnormalities was present in another approximately one-third of individuals with two abnormalities.

Discussion

At the simplest level the current results generally support the findings of the relationship between adiposity and presence of cardio-metabolic risk in non-Hispanic whites, non-Hispanic blacks, and Mexican-Americans described by Wildman, et. al. [1] in a fourth racial group – South Asians. Specifically, their results and our findings demonstrate that substantial numbers of individuals who are overweight/obese by BMI criteria can be metabolically healthy, and individuals with a normal BMI can be metabolically abnormal.

On the other hand, there are differences between the two studies. In the first place, we stratified participants into degrees of adiposity using conventional criteria based on both BMI and WC [1, 2]. This decision was based on the view that abdominal obesity is more powerful than overall obesity as the link between excess adiposity and cardio-metabolic risk in South Asians [9-11]. By so doing so, our results differed somewhat from what we had anticipated in that measurements of WC did not seem particularly advantageous as compared to determining BMI in identifying South Asians who were metabolically abnormal. Specifically, 502 participants were abdominally obese (an elevated WC), and 189 of them were metabolically abnormal (38%). Somewhat fewer (455) of the population were classified as being either overweight or obese by BMI criteria, and a similar proportion – 179 (39%) of them were metabolically abnormal. We cannot entirely place these data into the context of the findings of Wildman, et. al. [1] in the 3 racial/ethnic groups they studied, but they appear to be somewhat different. For example, Wildman, et. al. [1] state that "36.4 % of individuals with abdominal obesity expressed the metabolically healthy phenotype." By implication, it appears that ~64% of those with abdominal obesity were metabolically abnormal as compared to the ~40% abdominally obese South Asians who were metabolically abnormal in our study. In any event, it appears that measurements of BMI *or* WC in South Asians provide similar information as to the adverse impact of excess adiposity on cardio-metabolic risk.

A more dramatic difference between our results and those of Wildman, et. al. [1] is the apparent impact of sex on the relationship between adiposity and cardio-metabolic risk. Thus, Wildman, et. al. [1] found that "normal-weight men were 34% more likely than normal-weight women to have 2 or more metabolic abnormalities." In contrast, the comparisons in Figure 1A and 1B indicate that normal-weight South Asian men are approximately 3-times more likely to be metabolically abnormal (23% vs. 7%) than normal-weight, South Asian women. Furthermore, South Asian men with a normal WC were also approximately 3-times more likely to be metabolically abnormal than were South Asian women with a normal WC (21% vs. 6%). Thus, in this relative young group of South Asians (mean age of approximately 40 years), the adverse impact of excess adiposity on cardio-metabolic risk was greatly attenuated in women, whether estimated by BMI or WC.

Another, and not unexpected, difference between our findings in South Asians and those of Wildman, et. al. [1] in the 3 racial/ethnic groups they analyzed were the specific abnormalities that clustered together. Thus, they state that the 2 most common combinations were "a high triglyceride level/low HDL-C level and high blood pressure/high glucose level". It is obvious from Table 4 that our findings were similar in regards to the high TG and low HDL-C cluster, but without any predilection of the glucose and blood pressure combination. Given the increased prevalence of these lipid changes in South Asians [6-11] it is not surprising that they were commonly present in the metabolically abnormal individuals in the current study. It should also be noted that we excluded patients with known hypertension and diabetes from our study group, and this may well explain why did not observe an increased clustering of high glucose with high blood pressure.

Although our findings seem relatively straight-forward, they need to be in viewed within the limitations of our study protocol. Thus, the population was not selected at random, but had responded based on their awareness of a screening program being conducted to identify cardio-metabolic risk factors in South Asians. Although we were able to use ethnic specific criteria for

classifying individuals on the basis of WC [2], we are unaware of any alternative, generally accepted BMI criteria to classify South Asians as being normal-weight, overweight, or obese. Furthermore, the decision to use the six criteria employed to define cardio-metabolic risk was based on the prior publication of Wildman, et. al. [1], and there is no a priori evidence that this is the "best" approach to evaluate the relationship between excess adiposity and cardio-metabolic risk. Finally, limiting our analysis to apparently healthy individuals had the advantage of identifying disease risk, rather than disease, but it contributed to the relatively young age of our study group. Thus, they had a mean age of ~40 years, and at least 80% of the women were pre-menopausal.

On the other hand, to the best of our knowledge, our report represents the largest study in which standard values for cardio-metabolic risk have been reported in South Asians, and the relationship of these abnormities to both BMI and WC quantified. Furthermore, by excluding subjects with known disease, and of relatively young age, we have been able to provide an estimate of cardio-metabolic risk in an apparently healthy population of South Asians, a group recognized to be at high risk to develop type 2 diabetes and/or CVD [6-11]. Finally, from a public health perspective, our data support two clinically useful conclusions that seem to apply to a relatively young and apparently healthy population of South Asians: 1) measurements of BMI or WC are comparable in identifying those with a metabolically abnormal phenotype; and 2) at a given index of BMI or WC, men are at much greater cardio-metabolic risk than women.

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Table 1. Criteria for Defining a Cardio-Metabolic Abnormality

Measurement	Cut-point
Elevated blood pressure	Systolic/diastolic blood pressure ≥130/85 mm Hg
Elevated glucose level	Fasting plasma glucose concentration ≥100 mg/dL
Elevated triglyceride level	Fasting plasma triglyceride concentration ≥150 mg/dL
Decreased HDL-C	Fasting plasma HDL-C < 40 (men) or <50 mg/dL (women)
Insulin resistance	HOMA-IR>3.77 (upper 10^{th} percentile)
Systemic inflammation	hsCRP>5.5mg/L (upper 10^{th} percentile)

High-density lipoprotein cholesterol: HDL-C

Homeostasis model assessment insulin resistance: HOMA-IR

High sensitivity C-reactive protein: hsCRP

Table 2. Demographic and Cardio-Metabolic Characteristics by Body Mass Index (Mean ± SD or n (%))

		Metabolically Normal			Metabolically Abnormal		
Characteristics	Overall *n* = 1015	Normal Weight *n* = 477	Overweight *n* = 240	Obese *n* = 36	Normal Weight *n* = 83	Overweight *n* = 116	Obese *n* = 63
Age (years)	39 ± 9	39 ± 9	39 ± 9	41 ± 7	39 ± 8	39 ± 8	40 ± 9
Men (%)	526 (52)	213 (45)	115 (48)	10 (28)	63 (76)	88 (76)	37 (59)
Smoking Status							
None	911 (90)	444 (93)	220 (92)	32 (89)	72 (87)	93 (80)	50 (79)
Former	65 (6)	23 (5)	12 (5)	2 (6)	7 (8)	15 (13)	6 (10)
Current	39 (4)	10 (2)	8 (3)	3 (6)	4 (5)	8 (7)	7 (11)
Alcohol Intake							
<1 drink/day	977 (96)	456 (96)	234 (98)	36 (100)	81 (98)	110 (95)	60 (97)
1 drink per day	12 (1)	6 (1)	3 (1)	0	0	1 (1)	2 (3)
>1 drink per day	24 (2)	14 (3)	3 (1)	0	2 (2)	5 (4)	0
Physical Activity							
None	198 (20)	83 (18)	43 (19)	12 (35)	20 (26)	25 (23)	15 (25)
1-2 days/week	213 (22)	105 (23)	51 (22)	2 (6)	23 (30)	23 (21)	9 (15)
3 days/week	222 (23)	108 (23)	49 (21)	8 (24)	12 (15)	31 (28)	14 (23)
>4 days/week	344 (35)	166 (36)	88 (38)	12 (35)	23 (29)	32 (29)	23 (38)
Systolic blood pressure (mmHg)	116 ± 14	110 ± 10	115 ± 12*	120 ± 11**	121 ± 15	126 ± 15*	129 ± 14*
Diastolic blood pressure (mmHg)	73 ± 9	69 ± 7	73 ± 8**	73 ± 7**	76 ± 10	78 ± 10	79 ± 10*
Elevated blood pressure (≥130/85 mmHg)	194 (19)	42 (9)	26 (11)	6 (17)	26 (31)	57 (49)*	37 (59)*
HDL-C (mg/dL)	48 ± 13	52 ± 13	49 ± 10*	52 ± 11	39 ± 9	40 ± 8	40 ± 9
Low HDL-C (<40mg/dL men, <50mg/dL women)	260 (26)	60 (13)	28 (12)	1 (3)	58 (70)	72 (62)	41 (65)
Triglycerides (mg/dL)	114 ± 63	90 ± 36	100 ± 42*	110 ± 39*	185 ± 85	165 ± 79	170 ± 71
Elevated triglycerides (≥150mg/dL)	210 (21)	22 (5)	21 (9)*	6 (17)*	57 (69)	70 (60)	34 (54)
Glucose (mg/dL)	86 ± 10	84 ± 9	85 ± 8	84 ± 10	89 ± 11	90 ± 11	93 ± 10*
Elevated Glucose (≥100mg/dL)	85 (8)	15 (3)	4 (2)	1 (3)	19 (23)	32 (28)	14 (22)
Insulin (μU/mL)	9.7 ± 5.7	7.3 ± 3.4	9.4 ± 3.9**	11.5 ± 4.8**	11.4 ± 5.1	13.9 ± 6.8*	18.5 ± 8.8**
HOMA-IR	2.1 ± 1.3	1.5 ± 0.7	2.0 ± 0.9**	2.4 ± 1.0**	2.5 ± 1.2	3.1 ± 1.5*	4.3 ± 2.1**
Elevated HOMA-IR (>3.77)	96 (10)	4 (1)	7 (3)*	5 (14)**	17 (20)	33 (28)	30 (48)**
Body mass index (kg/m^2)	25 ± 4	22 ± 2	27 ± 1**	33 ± 3**	23 ± 2	27 ± 1**	33 ± 3**
Waist circumference (cm)	86 ± 11	78 ± 8	89 ± 8**	98 ± 10**	85 ± 7	93 ± 6**	104 ± 10**
Elevated waist circumference (≥90cm men, ≥80cm women)	502 (49)	106 (22)	172 (72)**	35 (97)**	28 (34)	98 (84)**	63 (100)
hsCRP (mg/L)	2.5 ± 3.7	1.7 ± 2.8	2.7 ± 3.9**	3.4 ± 3.1*	2.6 ± 4.2	3.4 ± 3.8	5.8 ± 5.2**
Elevated hsCRP (>5.5mg/L)	98 (10)	13 (3)	22 (9)**	7 (19)**	11 (13)	21 (18)	24 (38)*

Metabolically healthy: 0-1 cardio-metabolic abnormalities
Metabolically abnormal: 2-6 cardio-metabolic abnormalities
Cardio-metabolic abnormalities include blood pressure ≥ 130/85mmHg, triglycerides ≥ 150mg/dL, HDL-C < 40mg/dL (men) or < 50mg/dL (women), blood glucose ≥ 100mg/dL, HOMA-IR > 3.77 (90th percentile), and C-reactive protein > 5.5mg/dL (90th percentile)
Normal weight: BMI>25kg/m^2
Overweight: BMI 25-30kg/m^2
Obese: BMI>30kg/m^2
*p<0.05 compared to normal weight group
**p<0.001 compared to normal weight group

Table 3. Demographic and Metabolic Characteristics by Waist Circumference (Mean ± SD or n (%))

	Metabolically Healthy		Metabolically Abnormal	
Characteristics	Normal Waist Circumference *n* = 440	Elevated Waist Circumference *n* = 313	Normal Waist Circumference *n* = 73	Elevated Waist Circumference *n* = 189
Age (years)	38 ± 8	41 ± 9**	37 ± 8	40 ± 8*
Men (%)	232 (53)	106 (34)	60 (82)	128 (68)
Smoking Status				
None	407 (93)	289 (92)	65 (89)	150 (79)
Former	22 (5)	15 (5)	3 (4)	25 (13)
Current	11 (3)	9 (3)	5 (7)	14 (7)
Alcohol Intake				
<1 drink/day	422 (96)	304 (97)	70 (96)	181 (96)
1 drink per day	5 (1)	4 (1)	1 (1)	2 (1)
>1 drink per day	12 (3)	5 (2)	2 (3)	5 (3)
Physical Activity				
None	68 (16)	70 (23)*	15 (22)	45 (25)
1-2 days/week	104 (24)	54 (18)*	21 (30)	34 (19)
3 days/week	97 (23)	68 (23)	11 (16)	46 (25)
>4 days/week	160 (37)	106 (36)*	22 (32)	56 (31)
Systolic blood pressure (mmHg)	113 ± 12	114 ± 12	122 ± 14	126 ± 15
Diastolic blood pressure (mmHg)	70 ± 8	72 ± 8*	76 ± 9	78 ± 10
Elevated blood pressure (≥130/85 mmHg)	40 (9)	34 (11)	27 (37)	93 (49)
HDL-C (mg/dL)	52 ± 13	51 ± 11	40 ± 9	39 ± 8
Low HDL-C (<40mg/dL men, <50mg/dL women)	55 (13)	34 (11)	43 (59)	128 (68)
Triglycerides (mg/dL)	91 ± 37	98 ± 40*	181 ± 90	169 ± 75
Elevated triglycerides (≥150mg/dL)	21 (5)	28 (9)*	50 (68)	111 (59)
Glucose (mg/dL)	84 ± 9	85 ± 9	89 ± 11	91 ± 10
Elevated Glucose (≥100mg/dL)	12 (3)	8 (3)	17 (23)	48 (25)
Insulin (μU/mL)	7.2 ± 3.3	9.5 ± 4.2**	11.3 ± 5.1	15.3 ± 7.8**
HOMA-IR	1.5 ± 0.7	2.0 ± 0.9**	2.5 ± 1.2	3.4 ± 1.8**
Elevated HOMA-IR (>3.77)	4 (1)	12 (4)*	16 (22)	64 (34)*
Body mass index (kg/m^2)	23 ± 2	27 ± 3**	24 ± 2	29 ± 4**
Waist circumference (cm)	78 ± 7	90 ± 7**	83 ± 6	97 ± 9**
Elevated waist circumference (≥90cm men, ≥80cm women)	0	313 (100)	0	189 (100)
hsCRP (mg/L)	1.7 ± 2.9	2.7 ± 3.6**	2.3 ± 3.6	4.3 ± 4.6*
Elevated hsCRP (>5.5mg/L)	12 (3)	30 (10)**	9 (12)	47 (25)*

Metabolically healthy: 0-1 metabolic abnormalities
Metabolically abnormal: 2-6 metabolic abnormalities
Metabolic abnormalities include blood pressure ≥ 130/85mmHg, triglycerides ≥ 150mg/dL, HDL-C < 40mg/dL (men) or < 50mg/dL (women), blood glucose ≥ 100mg/dL, HOMA-IR > 3.77 (90^{th} percentile), and C-reactive protein > 5.5mg/L (90^{th} percentile)
Normal waist circumference < 90 cm (men), <80 cm (women)
Elevated waist circumference ≥ 90 cm (men), ≥ 80 cm (women)
*p<0.05 compared to normal weight group
**p<0.001 compared to normal weight group

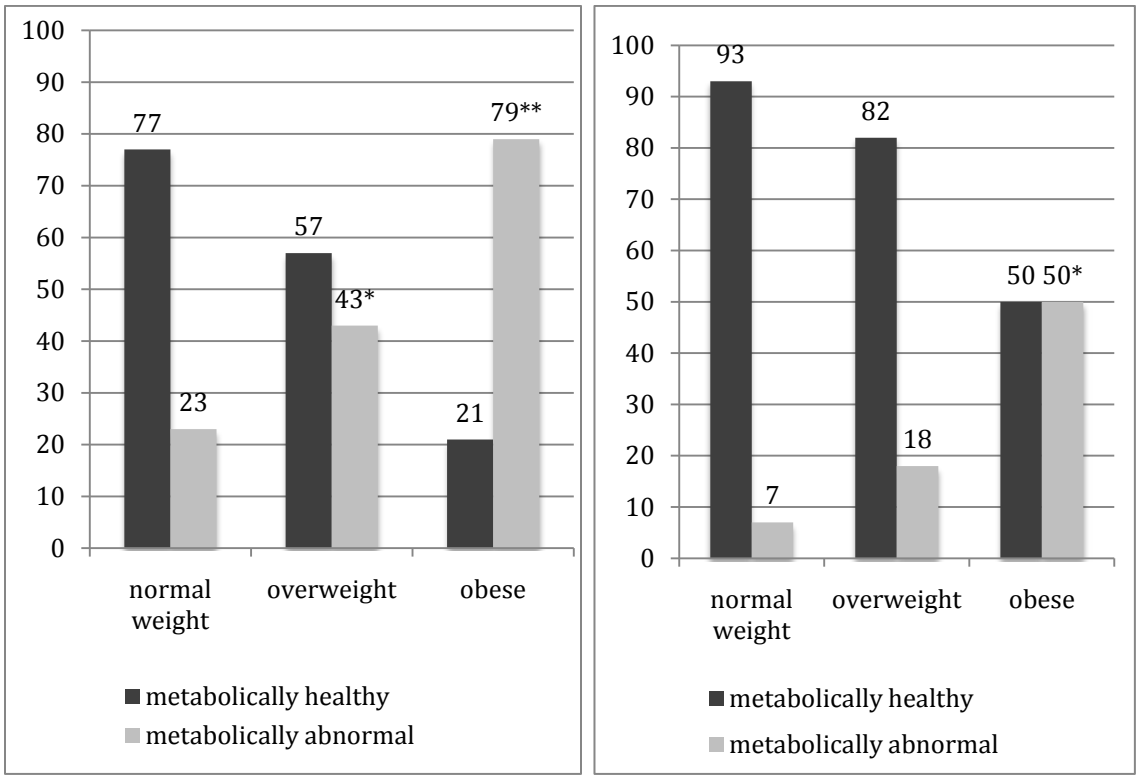
Table 4. Clustering of Two Cardio-Metabolic Risk Factors

Cardio-Metabolic Risk Factor Cluster	Frequency (*n* (%))
Low HLD-c and elevated triglycerides	59 (34)
Low HDL-c and elevated blood pressure	14 (8)
Low HLD-c and elevated blood glucose	14 (8)
Elevated triglycerides and elevated blood pressure	14 (8)
Low HDL-c and insulin resistance	11 (7)
All other combinations	57 (34)

Elevated blood pressure ≥130/85 mmHg
Low HDL-c <40mg/dL men, <50mg/dL women
Elevated triglycerides ≥150mg/dL
Elevated Glucose ≥100mg/dL
Elevated HOMA-IR >3.77 (upper 10^{th} percentile)

Figure 1. Prevalence of Cardio-Metabolically Healthy and Cardio-Metabolically Abnormal by BMI Group (panel A, men; panel B, women)

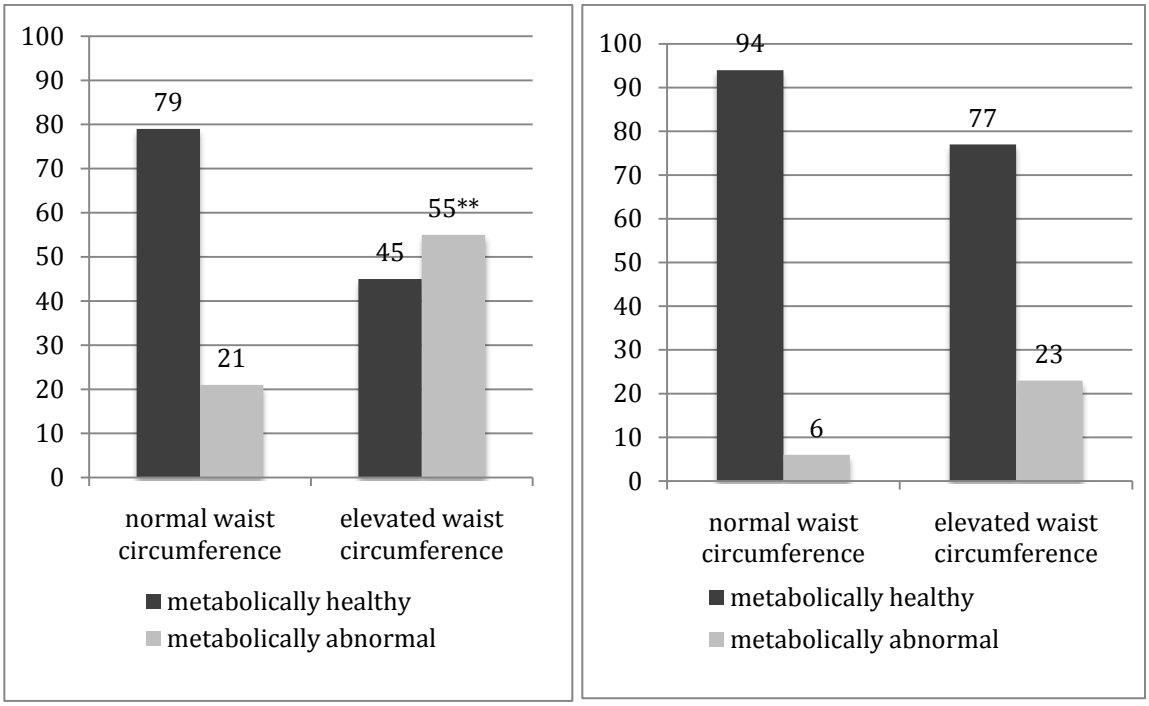
A **B**



*p<0.05 compared to normal weight group
**p<0.001 compared to normal weight group

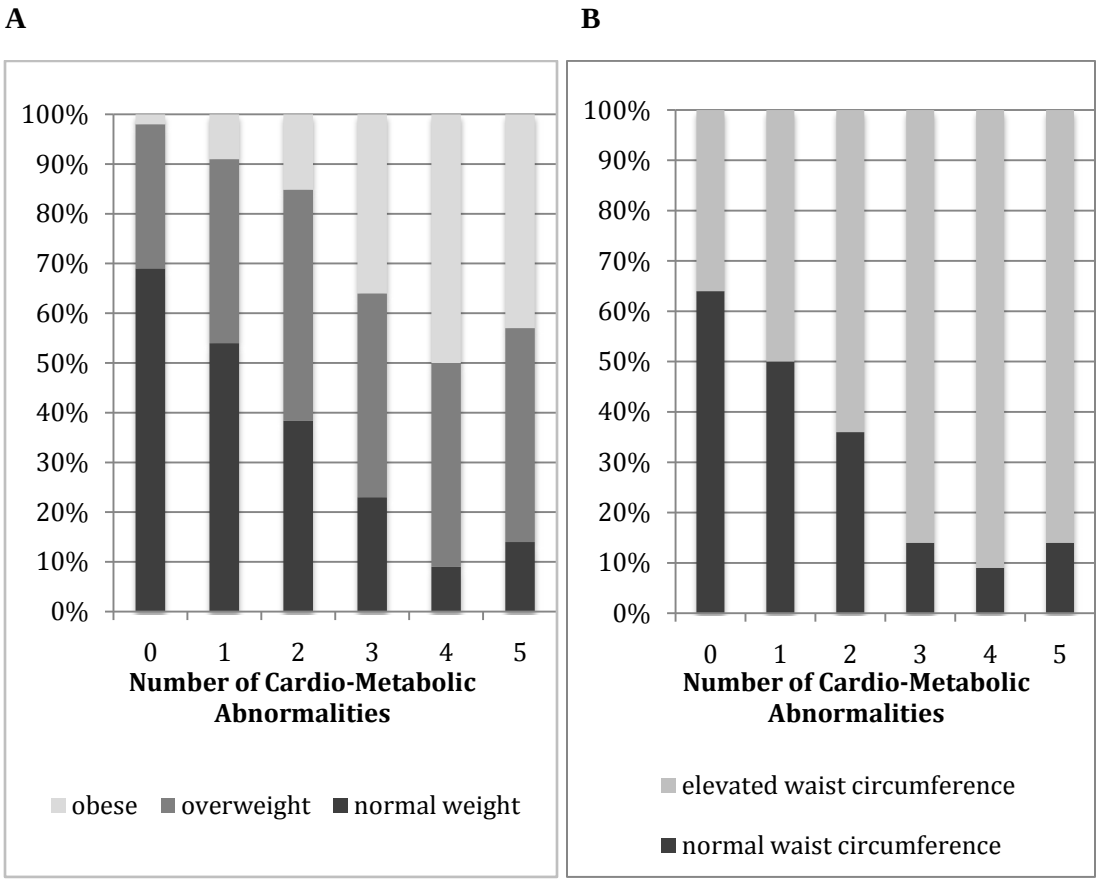
Figure 2. Prevalence of Cardio-Metabolic Healthy and Cardio-Metabolically Abnormal by Waist Circumference. (panel A, men; panel B, women).

A **B**



**p<0.001 compared to normal weight group

Figure 3. Number of Cardio-Metabolic Abnormalities by Adiposity (panel A, body mass index, panel B waist circumference)



Normal weight: BMI>25kg/m^2
Overweight: BMI 25-30kg/m^2
Obese: BMI>30kg/m^2

Normal waist circumference < 90 cm (men), <80 cm (women)
Elevated waist circumference ≥ 90 cm (men), ≥ 90 cm (women)

CHAPTER 6

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MicroRNA regulation of lipid metabolism

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ABSTRACT

MicroRNAs are structural components of an epigenetic mechanism of post-transcriptional regulation of messenger RNA translation. Recently, there is significant interest in the application of microRNA as a blood-based biomarker of underlying physiologic conditions, and the therapeutic administration of microRNA inhibitors and mimics. The purpose of this review is to describe the current body of knowledge on microRNA regulation of genes involved in lipid metabolism, and to introduce the role of microRNA in development and progression of atherosclerosis.

1. Introduction

Dyslipidemia is a major risk factor for atherosclerosis, and reductions in levels of atherogenic lipoproteins substantially decrease risk. The current selection of behavioral and pharmacological interventions has variable and often inadequate effects on improving lipoprotein profiles. MicroRNA (miR) is a regulatory mechanism effecting mRNA translation, with downstream alterations in lipoprotein synthesis, reverse cholesterol transport and insulin signaling, making miR a potential biomarker of underlying pathology and therapeutic target and/or agent for regulating lipoprotein metabolism. Thus far, two miRs are proven to have a direct role in lipoprotein metabolism [1,2], and a number of others have been implicated and warrant investigation. This paper will summarize the current knowledge of miR expression and function in lipid metabolism. Because the body of knowledge is limited, we will include miR findings from both *in vitro* and *in vivo* studies of animal models and humans.

2. MicroRNA Biogenesis

MicroRNAs are nucleotide sequences 18–25 nucleotides in length that are composed of ribonucleic acid and possess a regulatory role in mRNA translation. The first observation of miR described binding of short antisense RNA to mRNA in the cellular cytoplasm of *Caenorhabditis elegans* [3]. Subsequently, numerous miRs have been found in diverse species, including humans, the sequences of which are highly conserved across species, supporting a critical role of these molecules in post-transcriptional gene regulation [4–6]. The first description of miR in metabolic processes in animals was in *Drosophila melanogaster* [7].

Abbreviations: miR, microRNA; mRNA, messenger RNA; RNA, ribosomal nucleic acid; pri-miR, primary microRNA; DGCR8, DiGeorge syndrome critical region gene 8; RISC, ribonucleoprotein miR-induced silencing complex; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; SREBF, Sterol Regulatory Binding Element Factor; ABCA1, Adenosine Triphosphate Binding Cassette Transporter.

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MiR sequences originate in the cell nucleus, where miR encoding genes are transcribed, generating miR precursors (Fig. 1). Initially, a primary miR (pri-miR) transcript several thousand base pairs in length is formed, which adopts a double strand stem-loop structure for stability [8]. Pri-miR is processed into a 60–70 nucleotide molecule called pre-miR by a nuclear ribonuclease called Drosha [9,10]. Pre-miR is then exported to the cytoplasm where it is further modified by the Dicer ribonuclease into a double stranded nucleotide sequence 18–25 nucleotides in length [11]. This double stranded molecule is cleaved into a functional strand called the "guide strand" and the complementary strand termed the "passenger strand," which is subsequently degraded by the DiGeorge syndrome critical region gene 8 (DGCR8) protein, commonly called Pasha [12], yielding the mature single strand miR [13,14]. In order to preserve these molecules from degradation in the cytoplasm, the functional miR is bound to DGCR8 protein to form a ribonucleoprotein called the ribonucleoprotein miR-induced silencing complex (RISC). RISCs accumulate in cytoplasmic foci called P-bodies [15–17]. In addition to storing miR molecules, RISCs contain enzymes necessary for mRNA degradation following cleavage caused by miR binding [17]. Ultimately, RISCs bind to the 3' un-translated region of mRNA in order to inhibit initiation of translation, thus acting to modulate the quantity and rate of protein translation [18]. MiRs that are perfectly complimentary to the mRNA sequences cause mRNA cleavage, whereas imperfect complementarity causes reversible inhibition of mRNA translation [19].

Individual MiR species are assigned a numeric name in sequential order by date of discovery and classification [20]. The full name of a miR species is preceded by a three-letter prefix designating the species (e.g., hsa-miR-101 was the 101st

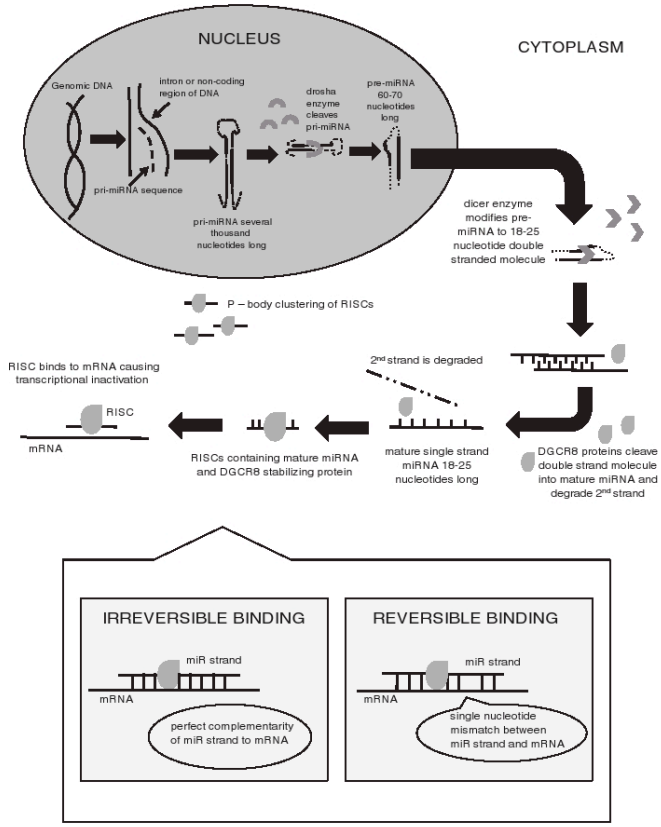

Fig. 1 – MicroRNA processing pathways in the nucleus and cytoplasm. Dotted lines: segments of RNA that are cleaved from precursor molecules during processing of mature microRNA. Solid lines: segments of RNA that are retained during processing and incorporated in mature microRNA. RISC: ribonucleoprotein miR induced silencing complex. DGCR8 protein: DiGeorge syndrome critical region gene 8, whish is an essential cofactor protein for primary miR processing.

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miR to be reported in *Homo sapiens*) [20]. Orthologous, or identical miR sequences found in multiple species are assigned the same numeric value with a species-specific prefix [20]. For example, the mouse orthologue to the human example given above is mmu-miR-101 (*Mus musculus* miR-101). MiR species whose sequence differs at only one or two locations are called paralogues and are given a single letter suffix (e.g. hsa-miR-30a, hsa-miR30b, hsa-miR30c) [20].

MiRs regulate numerous disease processes, including cell differentiation and proliferation in cardiac tissue during fetal cardiac development and cardiac hypertrophy, conduction physiology via potassium channels, angiogenesis in myocardium, glucose metabolism, lipid metabolism, and adipocyte differentiation [21,22]. The first studies of miR and metabolism were performed in fruit flies and determined that dme-miR-14 regulates fat metabolism by down-regulating the production of triacylglycerol and diacylglycerol [7]. Further work demonstrated that flies lacking dme-miR-278 become insulin resistant with an increased insulin production accompanied by elevated circulating glucose [23]. MiR expression patterns can differ by tissue type, suggesting that this mechanism of gene expression regulation is dynamic and can be highly specific [24]. Because miR regulation of mRNA translation is a highly dynamic process, it is thought that miR activity may be triggered by external stimuli, mediating an organism's response to changes in its environment. Thus there is potential for miRs to be biomarkers for disease progression and/or response to therapy, and ultimately targets of therapeutics for cardiovascular and metabolic disorders.

3. MiR-33

The miR showing the greatest therapeutic promise to date is miR-33 [25]. The transcripts for the two hsa-miR-33 isoforms (a and b) are located in a non-coding region of Sterol Regulatory Binding Element Factor (SREBF) genes, which regulate cholesterol synthesis by several mechanisms, including transcriptional regulation of LDL-c receptors (Table 1, Fig. 2) [2]. MiR-33 is highly conserved across species, and can be found in numerous cell types, including macrophages, hepatocytes, and endothelial cells [2]. Microarray experiments found hsa-miR-33 to be differentially expressed in response to cholesterol enrichment or depletion in human macrophage cells *in vitro* [2]. Hsa-miR-33-a expression is directly correlated with levels of SREBF-2, and inversely correlated with expression of Adenosine Triphosphate Binding Cassette Transporter (ABCA1), a cholesterol efflux pump [2]. Similar findings were observed in mouse models of hypercholesterolemia (i.e., homozygous LDL-c receptor knockout and homozygous apolipoprotein E knockout) [2]. Also, mmu-mir-33 was inversely associated with serum cholesterol, with decreased expression in homozygous LDL-c receptor knockout mice fed high fat diets compared to normal diet. Corresponding changes in SREBF-2 (positive) and ABCA1 (negative) were observed. Importantly, these findings prove that miR-33 is responsive to environmental changes (i.e. alterations in cholesterol levels secondary to diet or medication), making miR potential biomarkers of response to environmental stimuli and targets of therapeutic interventions.Additional putative binding sites for miR-33 occur on ATP-Binding Cassette, Sub-Family G (ABCG1) and Neimann Pick type C1 (NPC1) mRNA. *In vitro* transfection studies demonstrated that miR-33 decreased ABCA1 and NPC1 mRNA, but not ABCG1 mRNA, in human macrophages. Finally, lentivirus transfection of mice with mmu-miR-33 substantially decreased ABCA1, resulting in 22% decrease in plasma high-density lipoprotein cholesterol (HDL-c), while transfection of mmu-miR-33 inhibitor resulted in increased expression ABCA1 and accompanying 25% increase in HDL-c [2]. Increased HDL-c in response to miR-33 inhibitors corresponds with several anti-atherogenic events, including changes in reverse cholesterol transport, decreased plaque size and lipid accumulation in foam cells, decreased number of macrophages, and increased plaque stability in the aortic root of mouse hearts [26]. An intriguing observation is that the transfected miR-33 inhibitors were found to actually enter atherosclerotic lesions, and increase expression of ABCA1 in macrophages residing in the plaque [26]. The same macrophages displayed changes in expression of inflammatory gene [26].

MiR-33 also regulates mRNA associated with β-oxidation of fatty acids, including Hydroxylacyl-CoA Dehydrogenase/3 Ketoacyl-CoA Thiolase (HADHB), Carnitine O-Octanoyltransferase (CROT), and Carnitine Palmitoyltransferase 1A (CPT1A) (Table 1) [27]. Overexpression of miR-33b in radioactively labeled hepatocytes (Huh7 and HepG2) decreased the rate of oxidation, while inhibition induced β–oxidation [27]. In Huh7 cells, transfection with miR-33b also led to accumulation of a greater number of triglycerides and larger lipid droplets [27].

Until recently, studies of miR-33 function were stymied by the lack of the presence of both the miR-33b isoform and the SREBF1 gene in mice. A recent study of African green monkeys, who, like humans, have the SREBF1 gene and sae-miR-33b isoform, found substantial alterations in gene expression and lipoprotein levels after sae-miR-33-b inhibition [28]. Animals receiving sae-miR-33b inhibitor had decreased triglycerides (50%) and increased HDL-c (50%) after 12 weeks of treatment, with no apparent side effects [28]. Numerous corresponding changes in mRNA involved in regulation of lipoprotein levels with putative binding sites for sae-miR-33b, including ABCA1, CROT, HADHB, SREBF1, Protein Kinase AMP-Activated 1 (AMPK), and CPT1A were associated with changes in lipoprotein levels (Table 1, Fig. 2) [28].

In both *in vitro* studies of Huh7 cells and *in vivo* studies of mice, miR-33 appears to inhibit translation of Insulin Receptor Substrate 2 (IRS2) mRNA, with accompanying downstream alterations in IRS2 targets (Table 1) [27]. Similarly, the expression of IRS2 is increased in African Green Monkeys treated with sae-miR-33b inhibitor [28]. IRS2 is a mediator of insulin and insulin-like growth factor 1, and decreased levels are associated with development of insulin resistance [29]. Impaired insulin metabolism is widely known to be a risk factor for cardiovascular disease, and is associated with lipoprotein metabolism in that impaired insulin secretion resulting from insulin resistance also leads to decreased lipoprotein lipase response and accumulation of plasma fatty acids [30]. Correspondingly, *in vitro* inhibition of miR-33a and miR-33b up-regulates fatty acid oxidation and hepatocyte insulin response [27]. Similar to studies of miR-mediated lipoprotein metabolism, further studies of insulin

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Table 1 – Gene Targets of MicroRNA Regulating Lipid Metabolism.

miR species	Direction of Effect	Observed Targets	Target Gene Function	Citation
33	↓	Adenosine Triphosphate Binding Cassette Transporter (ABCA1)	Cholesterol efflux pump; initiation of HDL-c formation	Rayner et al, 2010, Rayner, Sheedy, et al, 2011, Rayner, Esau, et al, 2011
33	↓	Neimann Pick Type C1 (NPC1)	Transports LDL-c to endosomes/lysosomes for hydrolysis to free cholesterol	Rayner et al, 2010
33	↓	Hydroxylacyl-CoA Dehydrogenase/3 Ketoacyl-CoA Thiolase, beta subunit (HADHB)	Mitochondrial oxidation of fatty acids	Rayner, Esau, et al, 2011; Dávalos et al, 2011
33	↓	Carnitine O-Octanoyltransferase (CROT)	Mitochondrial oxidation of fatty acids	Rayner, Esau, et al, 2011; Dávalos et al, 2011
33	↓	Carnitine Palmitoyltransferase 1A (CPT1A)	Mitochondrial oxidation of fatty acids	Rayner, Esau, et al, 2011; Dávalos et al, 2011
33	↑	Sterol Regulatory Element Binding Transcription Factor (SREBF)	Regulates transcription of LDL receptor, fatty acids, and cholesterol synthesis pathways	Rayner, Esau, et al, 2011
33	↓	AMP Kinase (AMPK)	Regulates ATP-consuming biopathways to prevent cellular ATP depletion	Rayner, Esau, et al, 2011; Dávalos et al, 2011
33	↓	Insulin Receptor Substrate 2 (IRS2)	Mediates effects of insulin	Rayner, Esau, et al, 2011; Dávalos et al, 2011
122	↓	Glycogen Synthase 1(GYS1)	Catalyzes the addition of glucose to glycogen	Esau et al, 2006
122	↓	Solute Carrier Family 7 (SLC7A1, CAT1)	Transport of cationic amino acids in hepatic cells	Esau et al, 2006; Cirera et al, 2010
122	↓	Misshapen/NIK-Related Kinase 1 (MINK1)	Activates JNK and p38 pathways, which are involved in stress response	Esau et al, 2006
122	↓	Aldoase A, Fructose-Biphosphate (ALDOA)	Catalyzes conversion of fructose to glyceraldehydes	Esau et al, 2006
122	↓	Cyclin G1 (CCNG1)	DNA repair	Esau et al, 2006
122	↓	Procollagen-Proline, 2-Oxoglutarate-4-Dioxygenase (P4HA1)	Post-translational modification of collagens	Esau et al, 2006
122	↑	Sterol Regulatory Element Binding Transcription Factor (SREBF)	Regulates transcription of LDL receptor, fatty acids, and cholesterol synthesis pathways	Iliopoulos et al, 2010
122	↑	Diacylglycerol O-Acyltransferase 2 (DGAT2)	Catalyzes synthesis of triglycerides	Iliopoulos et al, 2010
122	↑	Fatty Acid Synthase (FASN)	Synthesis of long chain fatty acids	Iliopoulos et al, 2010
122	↑	Nuclear Receptor Subfamily 1, group H, member 3/Liver X Receptor α (NR1H3)	Cholesterol homeostasis	Iliopoulos et al, 2010
122	↑	Acetyl-CoA Carboxylase α (ACACA)	Fatty acid synthesis	Iliopoulos et al, 2010
122	↑	Cholesterol 7α-hydroxylase (CYP7A1)	Bile acid production, absorption of cholesterol	Song et al, 2010
370	↓	Carnitine Palmitoyltransferase 1A (CPT1A)	Mitochondrial oxidation of fatty acids	Iliopoulos et al, 2010
370	↑	Sterol Regulatory Element Binding Transcription Factor 1c (SREBF1c)	Regulates transcription of LDL receptor, fatty acids, and cholesterol synthesis pathways	Iliopoulos et al, 2010
370	↑	Diacylglycerol O-Acyltransferase 2 (DGAT2)	Catalyzes synthesis of triglycerides	Iliopoulos et al, 2010
370	↑	Fatty Acid Synthase (FASN)	Synthesis of long chain fatty acids	Iliopoulos et al, 2010
370	↑	Nuclear Receptor Subfamily 1, group H, member 3/Liver X Receptor α (NR1H3)	Cholesterol homeostasis	Iliopoulos et al, 2010
370	↑	Acetyl-CoA Carboxylase α (ACACA)	Fatty acid synthesis	Iliopoulos et al, 2010
758	↓	Adenosine Triphosphate Binding Cassette Transporter (ABCA1)	Cholesterol efflux pump; initiation of HDL-c formation	Ramirez et al, 2011

metabolism in animal models are limited by the lack of miR-33b in traditional animal models of diabetes (i.e. mice, rats).

4. MiR-122

MiR-122 accounts for 70% of all miRs in the adult mouse liver and is conserved in species ranging from fish to vertebrate mammals [24,31]. Several experimental studies of miR-122 indicate a regulatory role in lipid metabolism. Transfection of an anti-sense oligonucleotide inhibitor of mmu-miR-122 into a mouse hepatocyte-derived cell line (AML12) caused an increase in six mRNA (Glycogen Synthase 1 (GYS1), Solute Carrier Family 7 (SLC7A1), Misshapen/NIK-Related Kinase 1 (MINK1), Aldoase A, Fructose-Biphosphate (ALDOA), Cyclin G1 (CCNG1), and Procollagen-Proline, 2-

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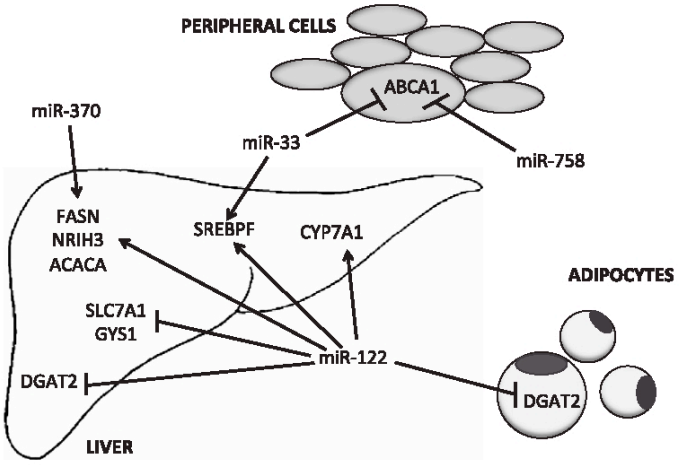

Fig. 2 – Messenger RNA targets of microRNA that are involved in regulation of lipoprotein metabolism. Pointed tip arrows: up-regulation of target mRNA expression. Blunted tip arrows: down-regulation of target mRNA expression. ABCA1: Adenosine Triphosphate Binding Cassette Transporter, SREBPF: Sterol Regulatory Element Binding Transcription Factor; DGAT2: Diacylglycerol O-Acyltransferase 2; CYP7A1: Cholesterol 7α-hydroxylase. FASN: Fatty Acid Synthase; NRIH3: Nuclear Receptor Subfamily 1, group H, member 3/Liver X Receptor α; ACACA: Acetyl-CoA Carboxylase α; SLC7A1: Solute Carrier Family 7; GYS1: Glycogen Synthase 1.

Oxoglutarate-4-Dioxygenase, Alpha Subunit, Isoform 1 (P4HA1)) [1], some of which may have indirect effects on lipid metabolism (Table 1, Fig. 2). The same results were observed with mmu-miR-122 inhibition in mice, and in both experiments, an inhibitor dose-dependent response of mRNA expression was observed [1]. Importantly, the mmu-miR-122 inhibited mice had a significant decrease in plasma total cholesterol and plasma triglycerides compared to controls [1].

Similarly, hsa-miR-122, along with hsa-miR-422, inhibits translation of the Cholesterol 7α-hydroxylase (CYP7A1) mRNA, and therefore bile acid synthesis [32]. In a human hepatocyte-derived cell line (HepG2), cells treated with hsa-miR-122 inhibitor displayed increased bile acid concentrations compared to untreated controls, implicating hsa-miR-122 regulation of CYP7A1 as a possible mechanism for decreasing serum cholesterol and triglycerides [32]. In addition, inhibition of hsa-miR-122 in Hep2G cells decreased the expression of SREBF-1c, a gene that regulates transcription of the low density lipoprotein cholesterol (LDL-c) receptor and cholesterol synthesis pathway, along with other enzymes known to regulate fatty acid and triglyceride accumulation in the liver [33]. The activity of hsa-miR-122 in this context appears to be regulated by hsa-miR-370, making the latter an additional potential therapeutic target [33]. In a porcine model of obesity, liver levels of ssc-miR-122 were down regulated in response to a high cholesterol diet compared to normal diet, and corresponding increases in body weight, total cholesterol, and HDL-c were observed [34]. However, no changes in SLC7A1, a target of miR-122 with a regulatory role in endothelial function, were observed.

5. MiR-370

As described above, miR-370 has an indirect effect on lipid metabolism via its up-regulation of miR-122 and its targets. Hsa-miR-370 has a direct inhibitory effect on CPT1A in Hep2G cells, which facilitates β-oxidation and is also a target of miR-122, as well as direct effects on SREBF-1c (Table 1, Fig. 2) [33]. Inhibition of three enzymes (diacylglycerol O-acyltransferase 2 (DGAT2), fatty acid synthase (FASN), and acetyl-CoA carboxylase 1 (ACACA)) is also seen in Hep2G cells *in vitro*, and it appears that changes in FASN and ACACA are mediated by the effects on SREBF and DGAT2 (Table 1, Fig. 2) [33]. Transfection of mmu-miR-370 in mice resulted in increased hepatic triglyceride and cholesterol levels, but corresponding changes in mRNA targets were not described [33].

6. Emergent Candidate MiR and Lipoprotein Regulation

Several additional miRs show preliminary evidence of a role in the regulation of lipoprotein metabolism. MiR-758 was a bioinformatically predicted regulatory agent for ABCA1, the protein enabling cellular cholesterol efflux by apolipoprotein AI containing particles (i.e., HDL-c). *In vitro* studies of mouse

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(J774 cells) and human (THP-1 cells) macrophages revealed miR-758 is inversely associated with ABCA1, with inhibition of miR-758 causing increased ABCA1 expression, and administration of a miR-758 mimic causing decreased expression [35]. Further study of an animal model found expression of mmu-miR-758 was decreased in peritoneal macrophages from mice fed a high fat diet, with accompanying increases in the expression of ABCA1 [35].

MiR-106b also appears to target ABCA1, impairing cellular cholesterol efflux in mouse neuroblastoma (Neuro2a) cells treated with radioactively labeled cholesterol [36]. This miR shows important neurologic implications as amyloid β peptide, a precursor to Alzheimer's Disease that appears to be regulated by ABCA1, is substantially increased by transfection of miR-106b *in vitro* [36].

7. Emergent Candidate MiR and Atherosclerosis

A primary consequence of dyslipidemia (i.e., elevated non-HDL-c) is the development of atherosclerosis. Thus miR-modulated regulation of lipid metabolism influences the development of atherosclerotic lesions. Several studies show that miRs influence the development and progression of atherosclerotic lesions via a number of mechanisms, including inflammation of macrophages and foam cell development, smooth muscle cell proliferation and migration, and apoptosis. Here we provide a brief summary of miRs involved in atherosclerosis. Additional review of this body of literature can be found elsewhere [37].

The hypothesis that miR may have overlapping regulatory functions in lipid metabolism and atherosclerosis is supported by the observation that miR-21 is a prevalent miR in cardiomyocytes, regulating apoptosis and vascular smooth muscle cell proliferation [38]. MiR-21 also appears to have a role in liver function and cholesterol regulation. Hepatic expression of miR-21 is up-regulated by free unsaturated fatty acids, with increased expression in the livers of rats fed a high fat diet and human liver tissue of obese individuals [39]. The mechanism of action of miR-21 in this setting is down-regulation of expression of the Phosphatase and Tensin Homolog protein, a tumor suppressor involved in early hepatic insulin hypersensitivity [39]. These findings provide additional evidence for dynamic activity of miR in response to environmental stimuli, resulting in alterations of metabolic function. In humans, has-miR-21 is detectable in blood [40,41], and therefore feasibly measured in a research context, making this an ideal target for testing whether variable expression is associated with risk reduction interventions.

Microarray measurement of miR in human monocytes *in vitro* exposed to oxidized LDL-c showed differential expression of several miRs, with as much as 11-fold change in expression (hsa-miR-125-5p) in exposed cells versus controls [42]. Oxidized LDL-c is a pro-atherogenic species that has a high affinity for macrophages, facilitating in the development of foam cells and atherosclerotic lesions. Administration of hsa-miR-125-5p inhibitors in human monocytic leukemia (THP-1) cells results in significant increases in total cholesterol after exposure to oxidized LDL-c, as well as decreased oxidized LDL-c uptake in macrophages [42]. Inhibition studies also show alterations in inflammatory markers associated with atherosclerosis, including Interleukin-6, Interleukin-2, Transforming Growth Factor-β, and Tumor Necrosis Factor-α [42]. Ischemia-induced arterial injury is associated with changes in miR expression in endothelial cells in rats, including up-regulation of rno-miR-21 and down-regulation of rno-miR-125 [43]. Numerous additional studies report that miR expression is associated with changes in vascular smooth muscle cell function in mammalian species, particularly mmu-miR-143, mmu-miR-145, rno-miR-145 [44–47], rno-miR-221, and rno-miR-222 [48].

Further research is needed to identify where atherosclerosis-associated differential expression of miR occurs in tissues associated with this disease (e.g. endothelial cells, smooth muscle cells, macrophages). Furthermore, understanding of all of the regulatory effects of miR on expression of genes associated with atherosclerosis mechanisms, including which mRNA are targeted, remains incomplete. And finally, the temporally and spatially (i.e., tissue type, cell type) specific expression represents a critical additional layer of complexity that warrants detailed study.

8. Conclusions

MiRs are a class of small RNA molecules with reversible regulatory actions on gene expression. MiRs regulate a wide variety of physiologic processes including dyslipidemia, a major risk factor for atherosclerosis. MiR-33 targets ABCA1, and has tremendous prospective therapeutic implications, given its direct role on HDL-c, non-HDL-c, and triglycerides. Importantly, exogenous miR-33 treatment has recently been studied in large primates, with dramatic improvements in lipoprotein levels and no apparent short-term side effects. In addition, inhibition of miR-33 appears to improve insulin sensitivity.

MiR-122 is the most prevalent miR in the livers of many species, and targets several genes that control cholesterol metabolism. Moreover, miR-122 appears to act in tandem with other miR (i.e. mir-370) to modify lipoprotein metabolism in the liver. The aggregate effects of miR-122 appear to be decreased total cholesterol and triglycerides and decreased production of the LDL-c receptor. There are several additional miRs with early evidence suggesting functional roles in cholesterol metabolism, and the activity of miRs appears to be responsive to changes in the environment. Thus, these miRs may represent plausible anti-atherogenic therapeutic targets.

9. Clinical implications

There are three potential implications for miR in clinical practice. The first is measurement of risk for significant clinical outcomes. Taking miR-33 as an example, differential expression is observed in genetically altered mice lacking LDL-c receptors compared to controls. Importantly, these differences were observed in hepatic tissue. However, in order for miR expression to be clinically practical in humans, differential expression must be observed in readily accessible tissue, such as blood. Measurement of miR in peripheral blood

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leukocytes for a wide array of conditions including cancer [49,50], myocardial infarction and injury [51,52], stroke [53], and sepsis [54], shows distinct patterns of expression in disease states compared to controls. These findings suggest that use of differential miR expression patterns in peripheral blood of humans with abnormal lipid metabolism is a plausible clinical biomarker. To our knowledge, no studies of human miR expression in dyslipidemia have been reported (Table 2). There are three possible implications for measurement of miR profiles in peripheral blood: (1) indirect markers of underlying pathology, (2) direct markers of pathology, and (3) reflective makers of pathology. In order to study the latter, studies measuring simultaneous expression of miR in blood and organs are needed. No studies comparing miR expression levels in blood to organ specific (e.g. macrophages and liver) expression were found, however comparative studies in animals have been reported for other disease processes [55].

Secondly, a translational application for blood-based miR measurement is to assess expression before and after risk reduction interventions (e.g., medication, diet, physical activity) to determine whether blood-based expression is differential, and whether miRs are biomarkers for changes in clinical risk profile. Data suggest that miRs are highly responsive to changes in the *in vitro* cellular environment and to an organisms environment. Keeping with the miR-33 example provided above, differential expression has been observed between mice fed high fat compared to normal diets. Studies report encouraging preliminary findings supporting environment-induced response in human-derived cell lines *in vitro* (Table 2). Future studies employing repeated measures designs for miR expression in humans are warranted in order to determine whether miR can serve as a meaningful measure of the effects of interventions and underlying physiologic changes.

Finally, there are currently two plausible mechanisms for miR as an intervention. The first is administration of antisense oligonucleotide inhibitors to specific miR (also termed antagomiRs), into the peripheral circulation. Inhibitors block the activity of the endogenously occurring miR, resulting in alteration of gene expression at a specific location. Animal studies have demonstrated successful inhibition of miR-33 activity following inhibitor administration, accompanied by substantial changes in lipoprotein profile. The second application is miR mimics, which are synthesized and chemically modified copies of miR species that can be administered into the peripheral circulation in order to enhance the overall effect of a specific miR. Both applications are effective in *in vitro* studies of human-derived cells (Table 2). A review of these methods is described in detail elsewhere [56].

10. Strengths and weaknesses

Significant strengths of miR research are the potential for an important complementary approach to assessing cardiovascular risk, measurement of response to behavioral and pharmacologic interventions, and as therapeutic targets for cardiovascular risk reduction. MiRs are highly dynamic, and alterations in expression are likely to precede changes in current standard clinical biomarkers. A limitation to clinical translation of miR research to the clinical setting is that the

Table 2 – Evidence for Clinical Applications of microRNA in *in vitro* Studies of Human-Derived Cell Lines and *in vivo* Studies of Humans and Related Primates.

miR species	Markers of response	Targeted interventions
33	miR-33 is down regulated by cholesterol enrichment in human THP-1 macrophages *in vitro* (Rayner et al, 2010)	miR-33 inhibition decreased serum triglycerides and increased HDL-c in African Green Monkeys and upregulated fatty acid oxidation and hepatic insulin response in Huh2 hepatocytes *in vitro* (Rayner et al, 2011); miR-33 inhibition decreased expression of Insulin Receptor Substrate 2 mRNA in HepG2 hepatocytes *in vitro* (Davalos, et al, 2011)
122		miR-122 inhibition decreased expression of Sterol Regulatory Element Binding Factor 1c mRNA (Iliopoulos, et al, 2010) and increased bile acid concentrations in HepG2 hepatocytes *in vitro* (Song et al, 2010)
370		miR-370 transfection upregulated Sterol Regulatory Binding Factor 1c, Diacylglycerol O-Acyltransferase 2, Fatty Acid Synthase, and acyl-CoA carboxylase 1 in HepG2 hepatocytes *in vitro* (Iliopoulos et al, 2010)
758		Inhibition of miR-758 decreased Adenosine Triphosphate Binding Cassette Transporter mRNA in THP-1 macrophages *in vitro* (Ramirez et al, 2011)
21	miR-21 is upregulated by elevated free fatty acids secondary to obesity in human liver (Vinciguerra et al, 2009)	
125-5p	miR125-5p is upregulated in THP-1 monocytes exposed to oxidized LDL-c *in vitro* (Chen et al, 2009)	Inhibition of mir-125-5p increased total cholesterol and oxidized LDL-c uptake, and altered expression of inflammatory markers in THP-1 monocytes *in vitro* (Chen et al, 2009)

microRNA (miR).
High-density lipoprotein cholesterol (HDL-c).
Messenger RNA (mRNA).
Low-density lipoprotein cholesterol (LDL-c).

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majority of experiments on miR expression and lipoprotein metabolism were performed on tissues that are not feasible to access in routine clinical care (e.g. liver, endothelium). In order for a biomarker to be clinically useful, the tissue source must be easily obtained, and further research on feasibility and utility of blood-based detection in humans is needed.

Author Contributions

Elena Flowers conducted literature search, synthesized data, composed manuscript, and created tables and figures. Erika S. Froelicher advised first author on manuscript composition, editorial process, and provided critical review of content. Bradley E. Aouizerat provided expert review and content checking, and advised first author on manuscript composition and scope.

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Conflicts of interest

None.

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CHAPTER 7

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Measurement of MicroRNA: A Regulator of Gene Expression

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Abstract

MicroRNAs (miRs) are epigenetic regulators of messenger RNAs' (mRNA) expression of polypeptides. As such, miRs represent an intriguing mechanism by which gene–environment interactions are hypothesized to occur on the level of epigenetic control over gene expression. In addition to promising findings from in vitro studies indicating that miRs have the potential to function as therapeutic agents in modifying the course of pathophysiologic conditions, recent human studies revealed changes in miR expression patterns in response to behavioral interventions. The authors provide an overview of how miRs are preserved and isolated from other genetic material and describe commonly used methods for measuring miR in the research setting, including Northern blot, polymerase chain reaction, and microarray. The authors also introduce bioinformatic approaches to analysis of high-throughput miR expression and techniques used to create predictive models of miR-mRNA binding to describe possible physiologic pathways affected by specific miRs.

Keywords

microRNA, epigenetics, measurement, microarray

Gene–environment interactions are increasingly recognized as an important mechanism in the development and progression of all disease conditions, ranging from common, complex disease (e.g., cardiovascular disease, type 2 diabetes) to mental health disorders (e.g., schizophrenia, antisocial personality disorder) to trauma (e.g., gunshot wounds). The theory of gene–environment interaction posits that the expression and effects of the individual genome can be modified by environmental stimuli. These interactions can occur at the gene level, when specific risk or protective alleles are present and interact with environmental factors, or at the epigenetic[1] level, when the expression of genes controlling normal physiologic processes is up- or downregulated in response to environmental factors.

A primary purpose of studying gene–environment interactions is to determine the public health impact of individual risk factors within a specific population, facilitating development of targeted interventions to maximize health and minimize disease. Importantly, however, population-based interventions to target risk factors for adverse health outcomes often show less than desirable effectiveness. Improved risk stratification and early detection of risk may help to prevent onset of disease and prevent secondary outcomes. While clinical outcomes associated with interventions to change lifestyle and behavioral characteristics have been well described, the mechanisms by which health outcomes are modified are not fully understood. A deeper understanding of the mechanisms by which risk factors act, including epigenetic modification of gene expression, may guide therapies and interventions in order to increase efficacy. This type of health promotion and disease prevention is a primary component of nursing practice.

MicroRNAs (miRs), a recently described epigenetic phenomenon, regulate gene expression. MiR activity regulates numerous disease conditions in humans, including cardiovascular and metabolic diseases, cancers, and sequelae from trauma. Insights from miR studies have confirmed previously hypothesized physiologic mechanisms and shed light on possible new pathways of disease. MiR activity often reflects change in a physiologic condition before overt effects are observed in the clinical setting and may serve as a sensitive biomarker for these conditions. Importantly, miR activity is highly dynamic

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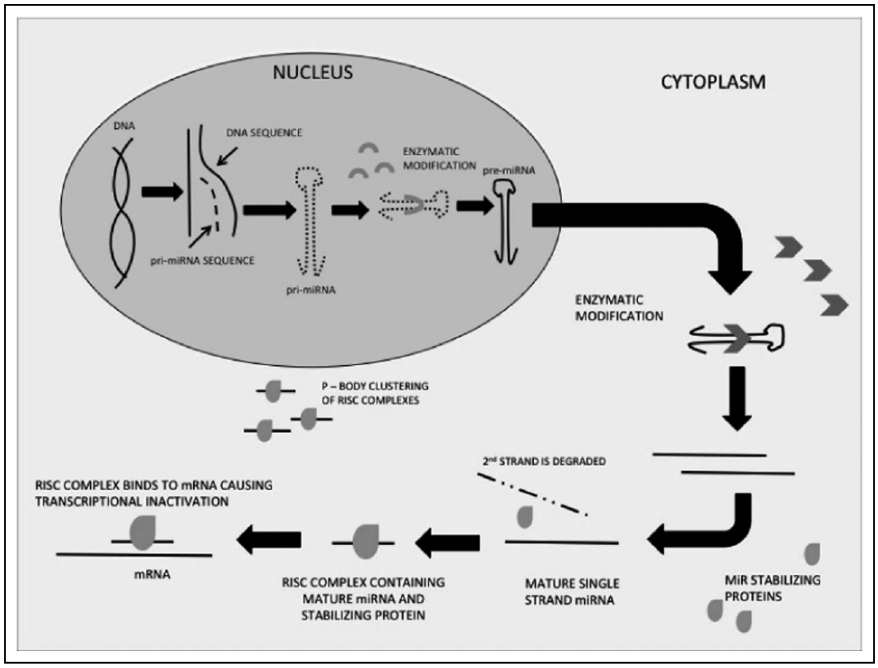

Figure 1. MicroRNA (miR) processing in the nucleus and cytoplasm, which portrays the origination of miR from deoxyribonucleic acid (DNA) in the nucleus, modification into mature miR, and activity in the cytoplasm.

and responds to environmental stimuli, resulting in alterations of gene expression. This characteristic of miR activity makes it particularly appealing for further study as a marker for response to therapies and interventions, an important and promising frontier in nursing research.

Our purpose in this article is to describe methods for detection and quantification of miR. Reliable and valid methods for measurement of miR are available in the research setting. MiR is a short, single-strand sequence of nucleotides found most commonly in the cytoplasm of cells. MiR measurement in humans requires access to a source of cells, from which miRs are isolated and preserved to ensure stability during storage prior to measurement. Methods of accessing the miR sources can be noninvasive (e.g., with saliva) or invasive (e.g., with neural tissue). We describe three methods for detection and quantification of specific miR species: Northern blot, targeted polymerase chain reaction (PCR), and microarray. Simultaneous detection and quantification of numerous miR species are possible with high-throughput PCR and microarray methods. These methods result in vast amount of data, presenting analytic and computational challenges. Thus, we also briefly present methods for decreasing the likelihood of false positives while maintaining high sensitivity and clustering algorithm methods to allow for rational categorization and interpretation of large amount of data.

miR

MiRs are short sequences (18–25 nucleotides) of RNA found in the cellular cytoplasm. MiRs originate from the transcription of deoxyribonucleic acid (DNA) in the cell nucleus (Figure 1). The initial single-stranded product that forms a stem–loop structure that includes a double-strand region is modified by a series of enzymatic reactions into the mature single-strand miR molecule. Mature miRs bind to stabilizing proteins to form structures called ribonucleoprotein miR–induced silencing complexes, which collect in the cytoplasm in structures termed p-bodies. MiRs exert their regulatory effects by binding to messenger RNA (mRNA), inhibiting translation of mRNA into polypeptides.

MiRs are highly dynamic, showing variable expressivity over time, which is associated with variable concentrations of protein products regulated by miR. The triggers of variation in miR expression are not fully understood, however, changes to both the intracellular and the external environment appear to have important effects. This property makes miR an important

candidate for studies of gene–environment interactions. Studies of miR activity in cells isolated from the pancreases of mice exposed to a high glucose stimulus showed threefold changes in individual miR's expression (Tang, Muniappan, Tang, & Ozcan, 2009), and similarly, rats fed a high-fat diet and overweight humans exhibit elevations in concentrations of specific miRs compared to normal-weight controls (Vinciguerra et al., 2009). Healthy humans exhibit changes in the expression of miRs in circulating neutrophils following an exercise intervention (Radom-Aizik, Zaldivar, Oliver, Galassetti, & Cooper, 2010). These findings further support the role of miR as a mediator of gene expression changes in response to the environment and substantiate the possibility of using miR to measure the effects of behavioral changes, permitting a deeper understanding of the mechanisms by which behavioral changes function.

Studies of the role of miR regulation in disease most commonly incorporate two aims. The first is to detect which of several hundred known miR species are present in a given tissue, in effect determining the miR fingerprint that characterizes a specific tissue. The type and number of miRs present depends on the physiologic needs of the tissue, and this fingerprint differs between individuals with normal physiologic function and individuals with a pathologic condition predisposing to or causing a disease. Therefore, the second aim is to quantify the level of prevalent miR species so that comparative analysis between individuals can be performed. In order to accomplish these aims, the researcher must first obtain tissue. The choice of tissue source depends on the disease condition of interest and feasibility of access. The extracted tissue must then be preserved, and the miRs must be isolated from other cellular and genetic material before detection and quantification.

Tissue Sources of miR

The central dogma of molecular biology (Figure 2) tells us that genes exist as sequences of nucleotides that are transcribed to an intermediate messenger molecule aptly called mRNA, which is then translated into amino acids and synthesized into polypeptides, the building blocks of proteins. However, not all genes are expressed at all times. Some genes, known as housekeeping genes, are essential to cellular function, regardless of cell type, whereas other genes encode for specific polypeptides forming proteins only needed in certain physiologic states or tissues. Further, the function of some genes is to express RNA (including miR) that is never translated into polypeptides. For example, some genes encode for the machinery necessary to perform transcription and translation. A form of RNA called transfer RNA (tRNA) binds individual amino acids and facilitates polypeptide synthesis during transcription of mRNA. Ribosomal RNA (rRNA) is another helper in the process of transcription, and both tRNA and rRNA are created by the process of transcription and translation of their specific genes. In contrast, melanin, a protein that causes skin pigmentation, is continually expressed in dermal cells but is not expressed in neural cells where its function would be inappropriate. We

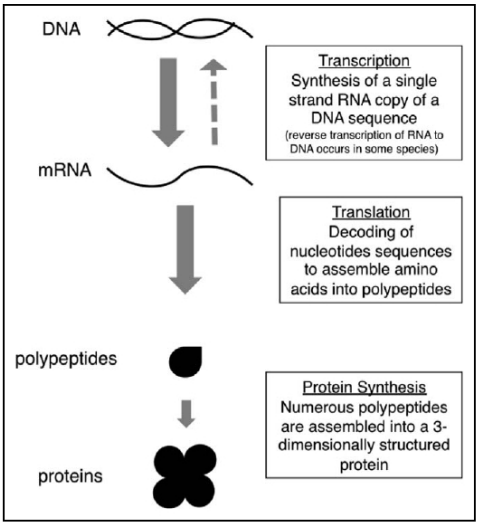

Figure 2. Central dogma of molecular biology, which depicts the process by which deoxyribonucleic acid (DNA) is converted into RNA and polypeptides, the building blocks of amino acids and proteins.

observe varying degrees of skin pigmentation between different ethnic and geographic populations, suggesting that melanin expression is variable among individuals and is dependent on some degree of predetermined genetic control as well as environmental exposure in the form of ultraviolet radiation that composes sunlight. Melanin, then, is an example of dynamic gene expression that is tissue specific, as well as an interaction between genetic predisposition and the environment.

Because of the specific nature of gene expression associated with discrete physiologic pathways, studies of gene expression require access to tissues in which these pathways are occurring. Early investigation of gene expression and epigenetic mechanisms regulating gene expression are generally performed in cultured cells in vitro followed by studies in animal models in tightly controlled experimental settings. For numerous disease conditions, miR expression varies within an organism by tissue type (Ikeda et al., 2007; Kloting et al., 2009; Lagos-Quintana et al., 2002; Liu & Kohane, 2009; Poy et al., 2004). Translation of these findings to human pathophysiology is often limited by feasibility of access to tissues involved in specific pathways of interest. For example, studies of genes expressed in heart failure can be performed on cardiomyocyte cell lines in vitro, but translation to human studies requires cardiac biopsy, involving extremely invasive cardiac catheterization. In some settings, cardiac biopsies may be performed on individuals with heart failure; however, it would be ethically inappropriate to expose healthy controls to the same procedure, making comparative analysis of cases

and controls in order to determine pathophysiologic miR profiles infeasible.

The practical and ethical challenges associated with accessing tissue sources limit the clinical utility of measuring RNA. However, most disease conditions involve signaling, increased systemic inflammation, and/or physiologic stress in the peripheral circulation. Preliminary data indicate that plasma measurements of miR expression can effectively detect differences between diseased and healthy individuals for a number of disease conditions.

Investigators in studies of numerous disease conditions have quantified miR expression in blood. MiRs in peripheral blood can be isolated from leukocytes, which may capture miR function in immune activity. However, miRs have also been detected in the plasma component of blood (Chen et al., 2008). Measurement studies of plasma demonstrated that miR is stable and reproducible using common RNA quantification methods (Chen et al., 2008). Much of the work describing miR expression in plasma to date has been performed on humans and other animals with cancer, and researchers have observed differential expression of miRs between humans with and without cancer (Lodes et al., 2009). In one example, investigators observed that miR expression in plasma differed between individuals with and without pancreatic cancer, and a panel of four miR species exhibited a sensitivity of 64% and sensitivity of 89% for detecting pancreatic cancer compared to controls (Wang et al., 2009). Preliminary research has shown that miR expression also differs in the plasma of humans with disease conditions other than cancer (Liu et al., 2009; Tan et al., 2009; Vasilescu et al., 2009), including diabetes (Chen et al., 2008). Chen et al. (2008) observed unique expression of 17 (serum) and 27 (blood cell) miRs in individuals with diabetes compared to healthy controls. They also found that the serum miR expression better differentiated diabetic individuals from healthy controls than miR expression from blood cells. Based on the studies cited above, blood-based measurement of miR expression appears to be feasible and shows promise for detection and discrimination of disease states.

Preservation

MiR is an unstable molecule, prone to degradation by ubiquitous ribonuclease enzymes that rapidly degrade RNA. As a result, studies of RNA, including miR, require immediate preservation of tissue specimens in order to accurately capture miR levels present at the time of sample collection. Common preservation techniques include freezing complete specimens in liquid nitrogen or on dry ice or placing the specimen in an aqueous salt buffer, which precipitates ribonuclease enzymes, preventing degradation of RNA, including miR (Allewell & Sama, 1974). Freezing methods have limitations including the need for specialized equipment and resources, trained personnel, and cold storage. Salt-buffer preservation methods are simple to perform and have shown comparable results from RNA isolation and quantification methods when compared to fresh-frozen methods (Brown & Smith, 2009; Florell et al., 2001; Mutter et al., 2004). Salt-buffer-preserved RNA specimens can be stored under standard refrigeration (4 °C) for up to 30 days or indefinitely at −20 °C or −80 °C (Ambion, 2008b).

Isolation

Commercial kits are widely used for isolation of total RNA from tissues and other materials. Briefly, the methods employed by these kits are as follows: (a) incubation of tissue with a lysis buffer to disrupt cell membranes, (b) extraction of RNA with acid guanidinium thiocyanate-phenol-chloroform reagent (commercially available as Trizol [Invitrogen, Carlsbad, CA] or TRI-Reagent [Sigma-Aldrich, St. Louis, MO]), (c) washing of the precipitate with an ethanol solution, (d) centrifugation in a spin column tube containing a filter to isolate RNA precipitate, and (e) elution of the precipitate in ribonuclease-free water resulting in an aqueous solution of isolated RNA (Ambion, 2008a; Chomczynski & Sacchi, 1987). Extraction of small RNA molecules including miR requires some modification of this procedure by altering the concentration of ethanol wash and using a different column filtration device containing a glass fiber filter (Ambion, 2008a; Mraz, Malinova, Mayer, & Pospisilova, 2009). A higher concentration of ethanol renders larger RNA molecules (>200 nucleotides) immobilized and unable to pass through the filter (Ambion, 2008a). The ethanol concentration of this small RNA-enriched solution is increased again and passed through a second filter, rendering the small RNA immobilized and isolated above the filter. Quantitative analyses of miR expression in fresh compared with properly preserved and stored tissue samples have shown comparable results (Mraz et al., 2009). Properly isolated and preserved miRs can then be further analyzed in order to detect the presence of and quantify the individual miR species.

Detection and Quantification

In order to investigate the role of miR regulation of gene expression in a given pathological process, it is typically necessary to first determine which of the several hundred individual miR species are present in a specific tissue source. Second, investigators must quantify prevalent miR species in order to compare expression levels between tissue types and between individuals with differing phenotypes. There are three primary methods for detecting and quantifying miR expression: Northern blot, PCR, and microarray. Selection of an appropriate method depends on the aim of the research study and type of data sought for downstream analysis (Table 1).

Northern Blot

Northern blotting is an electrophoretic method for separating individual RNA molecules on the basis of size, shape, and/or electrical charge and detecting individual RNA sequences using binding to radioactive or chemiluminescent-labeled[2] oligonucleotides[3] called probes (Alwine, Kemp, & Stark, 1977;

Table 1. Comparison of MicroRNA (miR) Measurement Methods

Method	Strengths	Weaknesses
Northern blot	Allows for detection of miR size	Requires a large amount of starting material Requires stringent oligonucleotide[a] hybridization Only semiquantitative; poorest sensitivity and specificity
Polymerase chain reaction (PCR)	Multiplex simultaneous amplification of several miR species Small amount of starting material required Comparative analysis of quantity of several miRs possible Can be automated, decreasing human error Improved sensitivity and specificity compared to Northern blot	Targeted study of hypothesized miRs only Specially shaped specific sequence primers required for amplification to occur
Microarray	Multiplex simultaneous amplification of several miR species Hypothesis-free study of hundreds of known miR species Comparative analysis of quantity of several miRs possible Can be automated, decreasing human error Improved sensitivity and specificity compared to Northern blot	Often decreased specificity compared to PCR

Note. [a]Synthesized short strands of nucleic acids (generally <20 nucleotides in length) designed to be used as probes that will bind to a specific complementary DNA or RNA sequence.

Kevil et al., 1997; Valoczi et al., 2004). Isolated RNA is loaded onto a polyacrylamide or agarose gel and placed in a buffer solution (Alwine et al., 1977; Kevil et al., 1997). An electrical current is applied to the buffer solution, causing miR molecules to migrate through the gel matrix. The rate of movement depends on the size, shape, and/or electrical charge of each miR. The miRs are then transferred from the gel to a nylon membrane and hybridized to radioactive or chemiluminescent-labeled probes. This property of the probes allows miRs to be visualized and quantified to determine the concentration of each miR species present.

A benefit of Northern blotting is that it allows for not only detection but also quantification of miR size. However, the Northern blotting method requires a large amount of RNA relative to other measurement methods and stringent hybridization of oligonucleotides to enable detection of molecules as small as miRs (Lu et al., 2005). Because of hybridization limitations, Northern blotting is less sensitive than PCR and microarray methods; however, modifications to oligonucleotides have been shown to increase the sensitivity of Northern blotting without decreasing specificity (Valoczi et al., 2004). These modifications involve the addition of a ''locked'' nucleic acid[4] to every third nucleotide position in the oligonucleotide. Nonetheless, because of its limitations, Northern blotting is less frequently used compared to alternative methods for detection and quantification or miR.

PCR

PCR is a method for identifying and amplifying a specific nucleotide sequence (Figure 3). PCR works by thermal cycling, or repeatedly heating and cooling target sequences of nucleic acids that have been combined with oligonucleotide primers, DNA polymerase enzyme, and free single nucleotides in a buffered solution (Mullis & Faloona, 1987). When heated to approximately 95 °C, double-stranded DNA will denature as the bonds holding nucleotide strands together are disrupted (Hartl & Jones, 2005). When the solution is cooled to approximately 55 °C, these single strands will bind with specially designed primers that are complementary to the sequence of interest. The solution is then heated to approximately 70 °C, and the Thermus aquaticus (*Taq*) polymerase enzyme[5] sequentially adds free nucleotides to this newly initiated double strand, elongating it until a full complementary sequence has been created, in effect doubling the number of target nucleic acid sequences present (Hartl & Jones, 2005; Holland, Abramson, Watson, & Gelfand, 1991). This process is repeated numerous times (typically 30–45 cycles), resulting in exponential amplification of the target sequence (Hartl & Jones, 2005). The exact temperatures used for a PCR (i.e., denaturation, annealing, and extension) depend on the nucleotide composition of the primers and the specific enzymes used.

A variation of PCR is reverse transcriptase PCR (RT-PCR), which is performed in order to amplify RNA sequences into a complementary strand of DNA (cDNA). In RT-PCR, 5′ primers[6] target the terminal sequence of the RNA molecule to initiate replication. The reverse transcriptase enzyme then adds complementary nucleotides to create a cDNA molecule. Primers used for amplification of miR sequences are often structured in a stem–loop formation in which a portion of the primer folds back and binds to itself, creating a double-stranded molecule (Chen et al., 2005). This method increases stability by preventing binding between two primers, improving the efficiency of the PCR.

Simultaneous amplification of several unique nucleotide sequences in one PCR is possible with multiplex techniques that include multiple primers targeted toward different sequences of interest. Multiplex PCR enables miR amplification when stem–loop structured primers are used as described above (Lao et al., 2006; Tang, Hajkova, Barton, Lao, & Surani, 2006). More recently, researchers have carried out numerous individual PCRs for multiple samples in discrete wells on

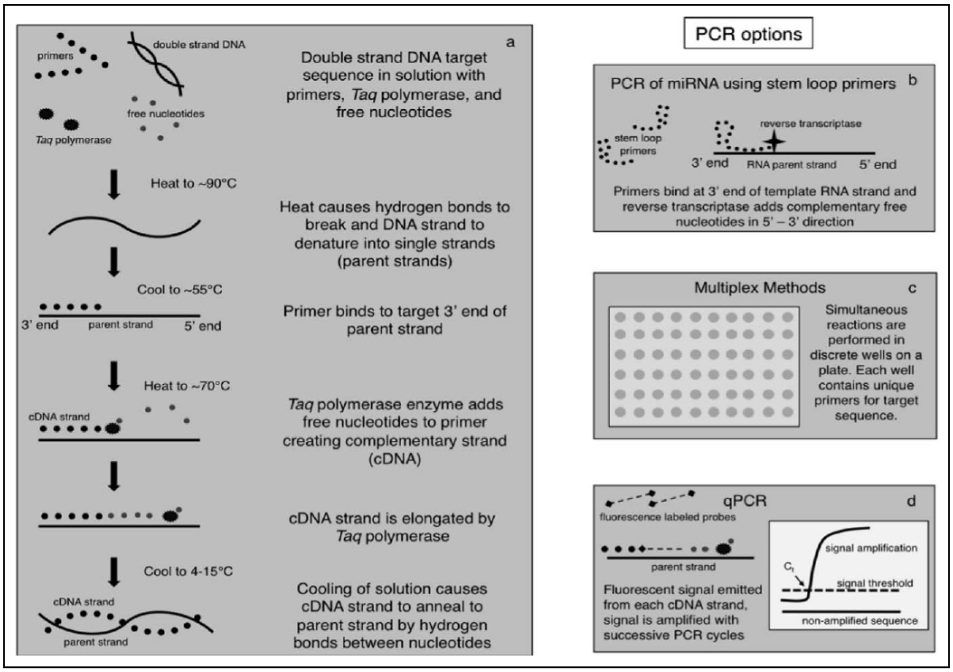

Figure 3. Polymerase chain reaction (PCR). Panel A illustrates the PCR method for amplifying nucleic acids. Panel B highlights aspects of PCR specific to measurement of microRNAs (miRs), including the use of reverse transcriptase enzyme to elongate the complementary deoxyribonucleic acid (DNA) strand and use of stem–loop primers which bind at the 3′ end of the parent strand. Panel C depicts a multiwell plate used for multiplex PCR. Panel D depicts binding of fluorescence-labeled probes used in Thermus aquaticus (*Taq*) polymerase (TaqMan assay by Applied Biosystems, Foster City, CA) quantitative PCR (qPCR) methods and graphic representation of the signal produced during qPCR quantification. Probes consist of a reporter and quencher component: the quencher silences expression of the fluorescence until the probe is digested by *Taq* polymerase during the PCR.

multiwell plates (e.g., TaqMan low-density array by Applied Biosystems, Foster City, CA). Each well is loaded with starting material (i.e., extracted and isolated RNA) for one sample along with primers, enzymes, nucleotides, and buffer. This method allows for rapid detection and quantification of numerous target sequences from multiple samples simultaneously.

A modification of PCR is real-time or quantitative PCR (qPCR), in which quantification of the number of PCR products can be made at the PCR end point, or the completion of each PCR thermal cycle (Chiang et al., 1996; Gibson, Heid, & Williams, 1996; Heid, Stevens, Livak, & Williams, 1996). qPCR requires the addition of a fluorescently labeled probe that will bind with cDNA PCR products. Measurement of the intensity of fluorescence during each PCR cycle reveals the quantity of cDNA product present. The intensity of the fluorescence signal is compared to a background signal or threshold. Intensity or quantity is quantified by a cycle threshold (C_t) value, which describes the PCR cycle at which signal intensity of the PCR product exceeds the threshold background signal. A higher concentration of starting target sequence results in a lower C_t. cDNA quantity can be reported as an absolute or relative value (Schmittgen et al., 2000). Comparative analysis of C_t levels between two PCR products can be performed in order to determine the relative values of two target sequences. The mathematical derivation of this calculation is described in detail elsewhere (Livak & Schmittgen, 2001). qPCR is the method most commonly used in comparative expression studies of miR species. A complete description and protocol for qPCR amplification of RNA sequences can be found elsewhere (Nolan, Hands, & Bustin, 2006).

With the first application of PCR in miR analysis, researchers described amplification of an miR precursor called pre-miR, finding that detection of these molecules using a fluorescence qPCR method was identical to comparative detection of mature miR using Northern blot analysis (Schmittgen, Jiang, Liu, & Yang, 2004). This method was validated and also

shown to work for multiplex PCR performed on plates containing up to 384 wells in which discrete experiments for individual miR species are performed (Jiang, Lee, Gusev, & Schmittgen, 2005). In this setting, qPCR amplification of mature miR is highly sensitive and easily performed using widely available instruments (Lu et al., 2005; Raymond, Roberts, Garrett-Engele, Lim, & Johnson, 2005; Shi & Chiang, 2005). qPCR is a useful method for detecting the presence of computationally predicted or validating the presence of previously observed miR. PCR is often considered the gold standard for detection and quantification of miR. Benefits of the PCR method are that creation and amplification of cDNA allows for comprehensive analysis even when the starting quantity of cellular genetic material is limited, as is often the case with miR, and increased sensitivity and specificity compared to Northern blot. Quantification is a particularly important aspect of this method of miR detection, allowing for analysis of comparative expression between tissue types and during various physiologic states.

Microarray

Microarray is a multiplex method for simultaneous detection of numerous target nucleotide sequences. As opposed to PCR, which is often used to identify specific miR sequences of interest, microarray is used to simultaneously detect a large number of miR species present in a sample. The precursor to microarray was the dot-blot method, in which antisense oligonucleotide probes for sequences of interest were bound to a nylon membrane. Researchers used this method in early miR analysis to study miR expression from mouse brain tissue during development (Krichevsky, King, Donahue, Khrapko, & Kosik, 2003). However, the dot-blot method is limited by poor sensitivity. Improvement on the dot-blot method came with robotic spotting of oligonucleotide probes onto glass slides, often called ''chips,'' creating a microarray platform with improved precision of placement of antisense oligonucleotides[7] (Liu et al., 2004; Zhao et al., 2006). In order to perform microarray, cDNA are created for all miR species in a sample using nonspecific primers in a PCR and are fluorescently labeled (Liu et al., 2004; Shingara et al., 2005; Sun et al., 2004). The cDNA is washed over the probe-spotted chips. When a nucleotide sequence match between the probe and an miR sequence is present, the cDNA will hybridize to the probe. Detection of the strength of the fluorescence signal from the hybridized cDNA describes the quantity of cDNA present in the sample, which reflects the number of copies of miR in the original sample (Liu et al., 2004; Sun et al., 2004).

Microarray chips for miR analysis most often contain several hundred probes. ''Housekeeping'' probes are included for quality control. Until recently, the housekeeping probes selected for miR microarray were small noncoding RNAs that are ubiquitously and abundantly expressed across species and tissues. More recently, however, tissue- and species-specific miR have been identified for use as housekeeping probes in microarray analysis (Carlsson et al., 2010; Neville, Collins, Gloyn, McCarthy, & Karpe, 2011). The remaining probes are chosen based on known commonly occurring and pathway-specific miR and computationally predicted target miR for a specific physiologic process of interest. Microarray chips for miR analysis containing prespecified miR probes are commercially available. Similarly to PCR, both absolute and relative quantities of miR can be calculated. For absolute detection, the intensity of each individual miR probe signal is compared to signals for oligonucleotides of known abundance (Dudley, Aach, Steffen, & Church, 2002). In order to compare relative miR quantities between two samples, each is labeled with different color fluorescent dye and washed over the same chip, then signal intensity for each probe location is compared. Until recently, microarray has been limited by decreased sensitivity for absolute quantification compared to PCR. One technique to enhance the capabilities of this method is the use of a universal reference containing oligonucleotides of known concentration in addition to test samples in a microarray (Bissels et al., 2009). The strength of the microarray method is high-throughput parallel measurement of known miR in comparative analysis in order to generate hypotheses about specific miR species for further study. However, high-throughput also comes with a cost: it limits the sensitivity of the parallel measurements. Targets of interest revealed by microarray analyses should be verified with target-specific and more sensitive methods of quantitation (i.e., qPCR).

Bioinformatics

Comparative Analysis

Typically, miR expression levels are compared between two sources (e.g., cases and controls). In order to compare expression levels of single miR species, which are measured on a continuous scale, between two sources, the Student's *t* test or two-sample *t* test can be used (Pagano & Gauvreau, 2000). This test can be performed using mean expression values for an individual case compared to an individual control or averaged expression level for a group of cases compared to a group of controls. This type of analysis is useful for targeted investigation of a specific miR species that appears to be associated with the physiologic function of interest. However, studies of miR expression are often exploratory, and measurement of numerous miR species is performed simultaneously, requiring more complex analytic methods.

Quantitative comparison of relative expression of a large number of miR species requires statistical methods that account for multiple comparisons and can accommodate a large amount of data. When many hypothesis tests are performed simultaneously, as is the case with comparative microarray analysis, a type I error of finding significant differences by chance alone is likely. In order to conclude that statistically meaningful differences in expression between two samples exist, it is necessary to make adjustments to the significance threshold to minimize the likelihood of false positives without increasing the probability of type II errors.[8] For microarray analysis, this

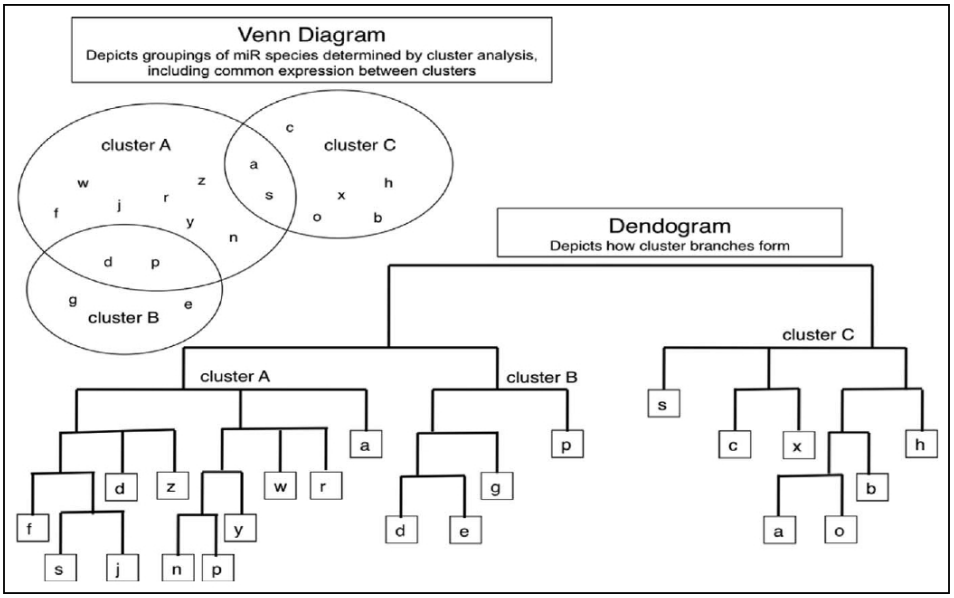

Figure 4. Venn diagram and dendogram. Graphic representation of clusters, or families, of microRNA species determined by clustering algorithms. Individual microRNA species are represented by letters, which either uniquely characterize clusters or are common to multiple clusters.

adjustment is most often accomplished with the establishment of a false discovery rate, a prespecified threshold defining an acceptable proportion of false positives from the total number of significant differences detected. There are several widely used methods for establishing a false discovery rate, including the Benjamini–Hochberg procedure (Hochberg & Benjamini, 1990) and the Bayesian statistics–based Efron–Tibshirani–Storey procedure (Efron & Tibshirani, 2002). Mathematical derivation and application of these methods is described in detail elsewhere (Efron & Tibshirani, 2002; Hochberg & Benjamini, 1990).

Data analysis methods that organize a large number of data points, known generally as hierarchical clustering, are commonly used in gene expression studies, including miR analyses. Clustering methods have the important feature of pattern detection and creation of data-based structures to accommodate a large number of data points. A widely used method for hierarchical clustering of expression data is the quality cluster (QT) algorithm. A full description of this analytic method is available elsewhere (Heyer, Kruglyak, & Yooseph, 1999). Briefly, the QT algorithm clusters miRs into an unspecified number of groups based on pair-wise jackknife correlations[9] between individual miRs. The QT clustering algorithm begins with a single miR signal and iteratively adds additional signals while minimizing the cluster diameter until no additional signals can be added without exceeding a predetermined cluster size. This process is repeated to develop additional clusters. A unique feature of QT clustering that makes it particularly appropriate for expression studies is the allowance of full iterative resampling of the data during the initial cluster assembly, in which signals already added to a cluster are still considered for successive clusters (Heyer et al., 1999). At the completion of the initial clustering process, the largest cluster is removed from the data set, and the iterative process begins again with the remaining data to determine subsequent clusters.

At the completion of the analysis, a dendogram[10] can be created to depict the clustering patterns of miR, and Venn diagrams[11] can be used to describe how clusters compare between samples from comparative studies (Figure 4). Depiction of miR clustering patterns in Venn diagrams permit visualization of patterns of miR expression within phenotype groups. For example, Ikeda et al. (2007) performed a microarray experiment measuring miR expression in cardiomyocytes of individuals with discrete mechanisms of heart failure (i.e., ischemic, dilated, aortic stenosis, and control). The aim of the study was to determine how miR expression clusters within phenotype groups and the extent to which miR expression differs between groups. The researchers created a dendogram clustering miR expression by phenotype from the heat map[12] depicting up- and downregulation of each individual miR species. The dendogram shows which miRs are characteristic of each phenotype group and how individual miR species are coexpressed within

phenotype groups, allowing for pattern detection. Similarly, the researcher created a Venn diagram in order to depict differential miR expression between phenotype groups and the extent to which individual miRs characterize discrete phenotypes.

Quantitative Prediction of miR Targets

MiRs are an abundant class of regulatory molecules arising from the genome. The number of miRs present for an individual species is poorly established, but most predictions for humans estimate that approximately 1,000 miRs are present. MiRs are nonspecific for their mRNA targets, and an individual miR species may bind with numerous mRNA species in order to regulate transcription. Determining which mRNA are bound by each miR has been the primary challenge to understanding the full cadre of functions of discrete miR species. Methods of computational prediction of mRNA targets based on miR nucleotide sequences have been successfully employed. Typically, these methods of computational prediction are experimentally validated for a subset of miR and mRNA pairs. Experimental validation studies yield estimates of specificity ranging from 50% to 80% (John et al., 2004; Krek et al., 2005; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003; Rajewsky & Socci, 2004; Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004). Several important features of both miR and mRNA have improved the capability of these computational prediction methods. The miR "seed," composed of the nucleotides in positions 2–8 of the 5′ end of the miR, appears to be the most important miR region for target recognition (Lewis et al., 2003). Binding sites on mRNA are often rich in the guanine and cytosine nucleotides, and mRNA regions rich in these nucleotides are more likely to contain miR binding sites (Rajewsky & Socci, 2004). Many genes appear to contain multiple miR binding sites for multiple miRs, and recent advances in computational prediction include identification of miR binding site clusters (Krek et al., 2005; Rehmsmeier et al., 2004). A comprehensive review of computational prediction of miR binding sites and methods for experimental validation is available elsewhere (Chaudhuri & Chatterjee, 2007).

Conclusions

Common, complex diseases arise in the setting of combined genetic and environmental risk, including gene–environment interactions. MiRs are an important form of epigenetic regulation of gene expression and function in animals, acting by altering protein synthesis in response to environmental changes. Measurement of miRs requires extraction of tissue from organs involved in the physiologic pathway of interest, preservation of tissue, and isolation of miRs from other genetic material. MiRs can be detected using a number of methods. Northern blot capitalizes on differences in size, shape, and/or charge of miRs to detect individual species hypothesized to be present in a tissue sample. Improving on the sensitivity and specificity of this method is PCR, which is an automated method for detecting miR species. Additional advantages of PCR are the ability to also quantify the number of miRs present in a sample and to perform multiplex analysis in which numerous experiments are run simultaneously. PCR experiments are performed in the setting of an a priori hypothesis for the presence of specific miR species in a tissue. Microarray has similar functions and advantages as PCR and is often used in hypothesis-free experiments to determine which miR species are present in a tissue.

Recent studies of miR activity in multiple disease conditions have yielded promising evidence for a regulatory role of miRs in the development and progression of disease. Clinical applications of miR measurement may enhance nursing practice by providing real time, personalized evaluation of individual health status, facilitating individualized treatment recommendations and assessment of the impact of nursing practice on individual and population health. However, implementation of this utility in humans using current techniques is largely limited by access to the organs involved in specific disease processes. However, preliminary data from studies of miR activity in blood plasma indicate that expression differences exist between diseased and healthy individuals. Accessing blood as a tissue source presents far fewer feasibility and ethical limitations and is routinely performed in clinical care and during health risk assessments. Additional studies are needed in order to determine the extent to which miR expression patterns in blood characterize specific disease processes. The dynamic nature of miR expression makes it an appealing target for early detection of an organism's gene expression response to changes in its environment, and miR quantification holds promise not only as a target of therapeutic interventions but also as a measurement of response to interventions. Whether miR expression in blood from individuals with risk factors for disease is responsive to interventions targeted toward reducing risk remains to be explored.

Declaration of Conflicting Interests

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Notes

1. Involving changes in the phenotype or appearance of an organism that do not result from underlying changes to the genetic sequence.
2. Heat-catalyzed activation of a chemical reaction that emits a light signal.
3. Synthesized short strands of nucleic acids (generally <20 nucleotides in length) designed to be used as probes that will bind to a specific cDNA or RNA sequence.

4. Nucleotide analogues that have undergone a conformational change to their sugar–phosphate backbone, making them extremely stable when bound to complementary strands.
5. A DNA polymerase originally cloned from the thermophilic bacteria *Taq*; stable at the high temperatures required for PCRs and functions by adding single nucleotides to a cDNA strand.
6. Primers that bind at the 5′ end of an RNA sequence, which is structurally the end of an RNA sequence compared to the 3′ beginning.
7. Short strands of nucleic acids that bind to mRNA to prevent translation.
8. Failing to detect a difference when one exists.
9. Calculated by a Pearson's correlation coefficient between all data points except one, with iterative calculation of the coefficient after leaving out each data point in a data set. Jackknife correlations are robust to outliers and decrease the number of false-positive correlations.
10. Tree diagram depicting hierarchical relationships among clusters.
11. Circle diagram depicting theoretical relationships between clusters.
12. Graphical representation of data utilizing color to depict strength and magnitude of each variable.

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CHAPTER 8

MicroRNA ASSOCIATED WITH ATHEROGENIC DYSLIPIDEMIA IN SOUTH ASIAN MEN

Introduction

MicroRNA are structural components of an epigenetic mechanism of post-transcriptional regulation of messenger RNA (mRNA). MicroRNA function by binding to a complementary 18-24 nucleotide precursor region on mRNA known as the “seed” sequence, thereby preventing initiation of translation of mRNA to amino acids. MicroRNA regulation can be temporary, when the microRNA release the mRNA, or permanent, causing degradation of the mRNA strand. Currently, there are several hundred discrete microRNA species identified in humans, with approximately 85 currently known to be detectable in human plasma and serum.

There is mounting evidence to support a role for microRNA in regulation of lipoprotein metabolism by influencing lipoprotein synthesis, reverse cholesterol transport, and insulin signaling (1). MicroRNA inhibition studies in animals show very promising results, where inhibition of microRNA-33 in large primates resulted in significant increases in high-density lipoprotein cholesterol (HDL-c) and decrease in triglyceride levels with no apparent short-term side effects. (2) The majority of studies to date have been performed *in vitro*, and little is known about the function of microRNA and lipoprotein metabolism in humans. While, *in vitro* studies of human hepatocytes and other cell types are useful for elucidating molecular events, they have limited potential for translational biomarker applications, as the model tissues (e.g., hepatocytes) studies are rarely accessed in routine clinical care. Moreover, the majority of *in vitro* models utilize cell lines that differ considerably from primary cells.

Currently, microRNA have two obvious translational biomarker applications: biomarkers of underlying pathology (e.g. dyslipidemia), and biomarkers for response to interventions. However, in order for microRNA expression to be clinically practical in humans, detection must

be feasible in a readily accessible tissue such as blood. Measurement of microRNA expression in blood for a wide array of conditions including myocardial infarction and injury (3, 4) and stroke (5) have shown distinct patterns of expression in disease states compared to controls. These findings suggest that measurement of microRNA in peripheral blood of individuals with dyslipidemia could provide clinical biomarkers. To our knowledge, no studies of blood-based microRNA expression in humans with dyslipidemia have been reported.

In order to identify potential microRNA biomarkers for pro-atherogenic dyslipidemia, we selected a population with a markedly increased prevalence of this condition. South Asians are disproportionately afflicted with early, severe atherosclerotic cardiovascular disease. (6, 7) Dyslipidemia is highly prevalent and appears to be a primary contributor to disease in this population (8), and is often characterized by normal levels of total cholesterol and low-density lipoprotein cholesterol (LDL-c), but low HDL-c and elevated triglycerides. (6, 9) Because this phenotype is common in not only South Asians but also in other ethnic groups (10), it was selected as the initial focus for studying blood-based microRNA expression associated with abnormal cholesterol. The aims of this study were to measure microRNA species known to be prevalent in human plasma and serum, to determine their expression in South Asian men with and without atherogenic dyslipidemia, and to validate those microRNA with plausible biologic function in atherogenic dyslipidemia via quantitative polymerase chain reaction (qPCR).

Methods

Participants

The design of this study was a nested case-control study of South Asian men with and without atherogenic dyslipidemia. Participants were recruited from the South Asian Heart Center at El Camino Hospital (Mountain View, CA), a not-for-profit cardiovascular risk reduction program. Adult men who self-identified as South Asian and were not taking medication to alter cholesterol were approached to participate. In order to limit potential variability in microRNA

expression resulting from physiological phenomena (i.e. hormonal fluxuation) other than dyslipidemia, we excluded women. Additionally, individuals with a body mass index <20kg/m^2 or >35kg/m^2, current smokers, heavy alcohol users, and those with previously diagnosed type 1 or type 2 diabetes were excluded. Men with HDL-c < 40mg/dL and triglycerides > 150mg/dL were defined as cases, and the comparison controls had neither of these conditions.

Demographic and medical history data were collected by scripted telephone interview administered by trained personnel during enrollment into the risk reduction program. (9) Anthropometrics and blood pressure were obtained during the initial enrollment visit by trained personnel. Venipuncture was performed following 10-hour fast, and lipoprotein quantification was performed using calorimetric methods; HDL-c, triglycerides, and glucose were measured using reagents from Roche Diagnostics (Indianapolis, IN) and performed on the Roche Modular PPP Analyzer (11). Potential participants who expressed interest in the study provided a sample of their blood for microRNA analysis during the same visit in which anthropometric, blood pressure, and lipoprotein measures were performed. This study was approved by the Committee on Human Research at the University of California, San Francisco, and the Institutional Review Board at El Camino Hospital.

microRNA Isolation

Blood for microRNA analysis was collected into PAXgene tubes (PreAnalytiX, Switzerland), which contain a reagent to lyse cells and stabilize RNA, per the manufacturer's protocol, and stored at -80°C until accrual targets were reached. The PAXgene Blood microRNA Kit (PreAnalytix, Switzerland) was used to isolate microRNA from whole blood following the manufacturer's instructions. Briefly, solid contents from PAXgene tubes were isolated and treated with guanidine thiocyanate, proteinase K, 99.9% ispropanol, and DNase, then suspended in an RNase free hydration buffer. Total RNA quantity was measured by spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE), and total RNA and small RNA quantity and

quality were measured by gel electrophoresis (Bioanalyzer, Agilent Technologies, Santa Clara, CA).

Case and Control Pools

For array analysis, pools of RNA representing cases (20 samples composed the case pool) and controls (20 samples composed the control pool) were created. Input quantity for each sample was based on the microRNA concentration (defined as 18-24 nucleotide size range) determined by gel electrophoresis. Four participants (2 cases, 2 controls) were excluded from pools due to unacceptably low microRNA concentration, making inclusion in each pool untenable (i.e., requiring lyophilization to reduce fluid volume). Parenthetically, while total RNA concentration as measured by spectrophotometry and gel electrophoresis was highly correlated ($\rho = 0.81$, $p < 0.05$) microRNA concentration was less well correlated with either total RNA measure ($\rho = 0.08$, $p = 0.6$ (nanodrop), $\rho = 0.18$, $p = 0.26$ (bioanalyzer)).

Reverse Transcription

Total RNA from individual samples and pools was reverse transcribed into complementary copy DNA (cDNA) using the miScript II RT Kit according to manufacturer's standard protocol (Qiagen, Valenica, CA). Approximately 250ng total RNA was reverse transcribed in 20µl reaction volumes. The resulting 20µl cDNA was diluted to 220µl using RNAse free water per standard protocol.

MicroRNA Array Experiment

Simultaneous detection of 85 microRNA and small nuclear RNA U6 (RNU6) was performed in 10ul reaction volumes on a Bio-Rad CFX Connect (Hercules, CA) using the miScript microRNANA PCR Array for human serum and plasma (Catalog # MIHS-106Z) in 384 well plate format (Qiagen, Valencia, CA). The miScript SYBR Green PCR Kit (Qiagen, Valencia, CA) was used for the experiment according to the manufacturer's standard protocol. The microRNA targets included in this array are a subset of microRNA previously observed to be detectable in human plasma and serum. Pooled sample measurements (1 case pool sample, 1

control pool sample) were performed in sextuplicate (n = 6) for each pool with equal distribution of pools on each plate (i.e., two replicates each of the case and control pools). Melt curve results were visually inspected, and any wells that did not appear to denature at a temperature consistent with the pattern for each individual microRNA were excluded from analysis. Additional quality control methods for data from array experiments are described in the Statistical Analysis section below.

Quantitative PCR

Quantitative PCR was performed on a Bio-Rad CFX Connect (Hercules, CA) using the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA) with 20µl reaction volumes according to the manufacturer's standard protocol. Cases and controls were randomly batched in equal numbers on each 384 well plate. A minimum of three replicates were done for each sample, and >50% of replicates were required to meet quality control criteria (described below) in order for the sample to be included in downstream statistical analysis. RNU6 was amplified in tandem with each target for each sample and used to normalize input quantity between samples. A standard curve for each microRNA target and the RNU6 normalizer was constructed using a series of five 10-fold or five 4-fold dilutions in order to capture the linear range within which the samples amplified. Melt curve results were visually inspected, and any wells that did not appear to denature at a temperature consistent within the replicate group for each sample were excluded from analysis. Additional quality control procedures for data from qPCR experiments are described in the Statistical Analysis section below.

Statistical Analysis

Descriptive statistics and Student's t-test were performed to compare demographic and clinical characteristics of participants. Quality control procedures for the pooled sample array-based experiments were as follows: cycle threshold (Ct) values greater than 2 standard deviations from the mean Ct for each target microRNA were excluded, as were replicates with a Ct greater than 35. Only microRNA targets with at least four remaining replicates after exclusion of outliers

were retained for downstream analysis. Normalized expression was calculated as $2^{-\Delta Ct}$ where $\Delta Ct = Ct_{target} - mean(Ct_{normalizer})$, and comparison of normalized expression between the case pool replicates and the control pool replicates was determined using the Wilcoxon non-parametric test of equality for unmatched pairs. Fold change was calculated to compare difference in expression of each target microRNA between the case pool replicates and control pool replicates. Fold change was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = mean(Ct_{target, cases} - mean(Ct_{normalizer, cases})) - mean(Ct_{target, controls} - mean(Ct_{normalizer, controls}))$. (12) MicroRNA displaying at least 2-fold statistically significant ($p < 0.05$) differences in expression with biologically relevant mRNA targets were selected for qPCR validation.

For qPCR, which was performed with a minimum of three replicates for each sample, outliers, defined as any replicate > 0.5 standard deviations from the mean for all replicates, were excluded. Normalized expression was calculated as $2^{-\Delta Ct}$ and compared using student's t-test with unequal variance. Fold change between cases and controls was calculated as $(E_{target}^{-\Delta Ct\ target}) / (E_{normalizer}^{-\Delta Ct\ normalizer})$ where $\Delta Ct = mean\ Ct_{cases} - mean\ Ct_{controls}$ and E = efficiency of amplification for each qPCR experiment. (13) As multiple 384 well plate experiments were required to validate each microRNA target, fold change was determined using the efficiency for each plate, and the average fold change, weighted for the number of samples per plate, was calculated. Fold change estimates were calculated for the full set of individual samples ($n = 44$), the subset of individual samples included in the pools ($n = 40$), and the pools of cases and pools of controls. Statistical analysis was performed using Stata 11 (College Station, TX) and Microsoft Excel (Redmond, WA).

Results

Twenty-two cases with combined low HDL-c and elevated triglycerides, and 22 controls with normal lipid parameters were enrolled. The mean age in both groups was 42 ± 2 years (Table

1). By design, cases had lower HDL-c (33 mg/dL vs 47 mg/dL, $p<0.05$) and higher triglycerides (237 mg/dL vs 109 mg/dL, $p<0.05$) than controls. Cases had higher fasting blood glucose (97 mg/dL vs 88 mg/dL, $p<0.05$) and were overweight (body mass index 26.1 vs 23.9, $p<0.05$), likely due to abdominal adiposity (waist circumference 94 cm vs 82 cm, $p<0.05$). In contrast, there were no statistically significant differences between cases and controls for total cholesterol (197 mg/dL vs 194 mg/dL, $p = 0.8$) or LDL-c (116 mg/dL vs 123 mg/dL, $p = 0.3$).

Data for 11 of the 96 wells included on the array are not presented, as these wells quantified small nuclear RNA (i.e., SNORD61, SNORD68 SNORD72, SNORD95, SNORD96A) and experimental controls. Of the 85 remaining microRNA measured by PCR-based array, 74 (87%) were detected with sufficient precision to meet all of the quality control criteria. Three microRNA (miR-214, miR-885-5p, miR-205) displayed increased expression in cases compared to controls (Table 1S). Sixteen microRNA (miR-100, miR-374a, miR-7, miR-18a, miR-125b, miR-148a, miR-17, miR-221, miR-21, miR-93, miR-143, miR-17*, miR-96, miR-106b, miR-103a, miR-20a) displayed at least two-fold decreased expression ($p < 0.05$) in cases compared to controls (Table 2). Three targets (miR-106b, miR-125b, miR-21) previously shown to expression of gene pathways related to lipoprotein metabolism (14-16) were selected for further analysis.

Table 3 displays the fold change estimates calculated from the qPCR validation experiments. For miR-106b, the estimated fold change for the full set of individual samples (n = 44) was -1.55. For the set of individual samples included in pools for cases and controls (n = 40) the fold change estimate was -1.68. For the pool of cases compared to pool of controls, the fold change estimate was -1.35. For microRNA-125b, the fold change estimate for the full sample was -1.84, for the subset included in the pools the estimate was -1.86, and for the pooled cases and controls, the estimate was -1.86. For microRNA-21, the fold change estimate for the full set of individual samples was -2.12, for the subset of individual samples included in pools the estimate was -2.02, and for the pooled cases compared to pooled controls the estimate was -1.69.

Conclusion

MicroRNA appear to have regulatory functions in lipoprotein metabolism, however little is known about the possibility of microRNA as a blood-based biomarker for atherogenic dyslipidmia. We sought to measure microRNA levels in blood of South Asian men with and without dyslipidemia characterized by low HDL-c and elevated triglycerides. We found that 16 microRNA species exhibit at least two-fold statistically significant differential expression (Supplemental Table 1). Of these, three with a priori evidence for biologic relevance to dyslipidemia were validated and found to be differentially expressed.

MicroRNA-21 is expressed in endothelial cells (17) and vascular smooth muscle cells (18), and shows increased expression in vascular proliferation, cardiac hypertrophy, heart failure, and ischemic heart disease (15). MicroRNA-21 targets phosphatase and tensin homolog (PTEN) (18) as well as programmed cell death 4 (PDCD4) (19) in vascular smooth muscle cells with proliferative and anti-apoptotic effects, facilitating vascular neo-intimal growth. MicroRNA-21 is protective of ischemia-induced injury in rats, also apparently by targeting PDCD4. (20) Thus, the activity of microRNA-21 in vascular smooth muscle cells has possible implications for recovery following ischemic insult and development of collateral vasculature.

In cardiomyocytes, microRNA-21 regulates cardiac hypertrophy in mice by targeting sprouty2, a growth inhibitor, to prevent development of cell-cell connections. (21) In hypertrophic mice, microRNA-21 inhibition decreased cardiomyocyte size and heart weight (22). The aggregate effect of microRNA-21 in cardiomyocytes appears to be control of growth and proliferation, with possible implications for cardiac hypertrophy and heart failure.

In hepatic tissue, microRNA-21 is up-regulated by free unsaturated fatty acids, with increased expression in the livers of rats fed a high fat diet and human liver tissue of obese individuals (23). The apparent action of microRNA-21 in this context is down-regulation of PTEN. (23) Increased PTEN expression is positively associated with the development of insulin resistance (24), and insulin resistance is associated with changes in lipoproteins, specifically

increased triglycerides and decreased HDL-c (25). In this context, microRNA-21 appears to have a role in preventing the onset of insulin resistance and possibly concomitant development of atherogenic dyslipidemia.

In prior studies of cardiac tissues, microRNA-21 is consistently up-regulated in diseased conditions. By contrast, we found 2.12-fold decreased expression. Previous studies of microRNA-21 expression have isolated microRNA from tissues other than blood (i.e. cardiomyocytes, hepatocytes) or studied *in vitro* models, whereas this study measured the levels of microRNA circulating in blood. Additionally, the direction of effect of microRNA-21 may differ depending on the function of specific mRNA targets within discrete cell types. For example, microRNA-21 expression inhibits PTEN and PDCD4 in vascular smooth muscle cells, increasing vascular proliferation, whereas microRNA-21 inhibition of sprouty2 in cardiomyocytes decreases hypertrophy. Additional studies are needed to determine the full cadre of mRNA targets of microRNA-21, which targets are relevant in discrete cell types (e.g. vascular smooth muscle cells versus hepatocytes), and the physiologic consequence of microRNA-21 inhibition of mRNA in discrete cell types.

MicroRNA-106b targets Adenosine Binding Cassette Transporter A1 (ABCA1), a cholesterol efflux pump involved in HDL-c formation and reverse cholesterol efflux. (16) This study found 1.55-fold decreased expression of microRNA-106b in dyslipidemic individuals compared to healthy controls. In contrast, *in vitro* studies using HepG2 cells revealed that microRNA-106b inhibits ABCA1-mediated cholesterol efflux function and inhibits liver X mediated ABCA1 expression. (16) Thus microRNA-106b appears to inhibit reverse cholesterol transport mechanisms, which may result in decreased HDL-c. Longitudinal studies may inform whether the observed decreased microRNA-106b in dyslipidemia in this study is a compensatory action to increase ABCA1 efflux secondary to the presence of low HDL-c.

MicroRNA-125b inhibition *in vitro* causes increased total cholesterol and oxidized LDL-c uptake in human macrophages, as well as corresponding changes in inflammatory markers. (14) MicroRNA-125b is decreased in ischemic endothelial cells in rats, while corresponding increases in expression of microRNA-21 were observed. (18) We found 1.84-fold decreased expression of microRNA-125b in dyslipidemic individuals compared to healthy controls. The direction of the expression difference for cases compared to controls is consistent with prior observations from ischemic endothelial cells, but inverse of what has been seen for macrophages *in vitro*. The function of microRNA-125b appears to be regulation of the development of foam cells and subsequent atherosclerotic lesions. Importantly, the prior data about microRNA-125b support a role in dyslipidemic processes, but not specifically HDL-c and reverse cholesterol transport.

To our knowledge, this is the first study to apply findings from *in vitro* studies and animal models to pursue blood-based measurement of candidate microRNA in humans with impaired lipoprotein metabolism. We speculate several possible explanations for some of the apparently paradoxical findings between the expression of microRNA observed in this study and previous observations. First, nearly all prior studies have been performed *in vitro* or in animal models, which may not accurately represent human physiology. Second, blood functions as a signaling medium for physiologic phenomena in other tissues, and therefore it is reasonable to postulate that blood-based expression of microRNA may correspond with changes in physiologic needs and gene expression in specific tissues. However, the nature of this relationship is not known, and blood-based expression may not be a direct reflection of the expression in underlying tissues. For example, inverse expression of microRNA between an organ and blood may be observed if changes in physiologic requirements cause an organ to offload specific microRNA via exosomal secretion into the circulation, or when organ injury occurs and damaged cells are sloughed into the circulation. Further, this study measured microRNA in whole blood, which provides an aggregate measure of all sources of blood microRNA. Specific components (i.e. free microRNA in plasma, microRNA contained within exosomes, and microRNA contained within

leukocytes) may yield different findings. Thirdly, *in vitro* studies have the advantage of determining causality, whereas this study may describe upstream microRNA changes that cause the observed differences in HDL-c and triglycerides, or compensatory changes that are an attempt to ameliorate the dyslipidemic condition. Lastly, there may be genetic and or epigenetic variability between individuals causing differential microRNA transcription, possibly associated with differential co-translation of mRNA targets. Epigenetic variability is determined in part by the environmental pressures exerted on an organism, and further studies are needed to begin to tease apart physiologic consequences of interactions between the environment and epigenetic mechanisms.

Limitations

We measured microRNA expression in whole blood obtained by peripheral venipuncture. Future studies should seek to establish if differences in these microRNA exist between free microRNA in plasma, microRNA contained within exosomes, and microRNA from leukocyte cytoplasm. This was a cross-sectional study, and therefore no conclusions about whether the observed differences in microRNA are markers for the development of dyslipidemia, result from the presence of dyslipidemia, and/or characterize clinical consequences of dyslipidemia can be made. While the homogeneity (i.e., gender, age, ethnicity) of the population contributes to the internal validity of the study, generalizability (e.g., women, younger and older ages, other ethnicities) is currently limited. This study was designed to detect changes of greater than 2-fold, and larger studies may reveal more modest expression differences. Also, we focused on candidate microRNA with a priori evidence for a role in lipoprotein metabolism (n = 3); there are 13 other targets that merit analysis in subsequent studies.

This study used array-based methods to screen a large number of microRNA detectable in human serum and plasma in the blood of South Asian men with and without dyslipidemia. Nineteen percent (n = 16) of the microRNA targets measured were significantly differentially expressed at two-fold or greater magnitude in cases compared to controls. Three of these were validated by qPCR methods with confirmatory findings. Additional studies are needed to validate the remaining 13 microRNA targets that were differentially expressed, and to determine the mRNA targets for each of these microRNA. Further research is needed to differentiate between expression of free microRNA in plasma versus microRNA in exosomes versus microRNA released from the cytoplasm of leukocytes lysed during the RNA isolation process, and how this expression relates to microRNA levels in other tissues (e.g., liver, endothelial cells).

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Table 1. Clinical Characteristics of Cases and Controls

Characteristics Mean ± SD or *n* (%)	Cases (*n* = 22)	Controls (*n* = 22)	p-value
Age (years)	43 ± 2	43 ± 2	0.84
Systolic Blood Pressure (mm Hg)	129 ± 16	116 ± 29	0.06
Diastolic Blood Pressure (mm Hg)	80 ± 10	72 ± 20	0.08
Total cholesterol (mg/dL)	197 ± 28	194 ± 32	0.81
LDL-c (mg/dL)	116 ± 25	123 ± 30	0.26
HDL-c (md/dL)	33 ± 3	47 ± 7	<0.05
Triglycerides (mg/dL)	237 ± 70	109 ± 28	<0.05
Blood glucose (mg/dL)	97 ± 11	88 ± 9	<0.05
Waist circumference (cm)	94 ± 8	82 ± 19	<0.05
Body mass index (kg/m^2)	26.1 ± 3.1	23.9 ± 2.8	<0.05

Abbreviations

Low-density lipoprotein cholesterol: LDL-c
High-density lipoprotein cholesterol: HDL-c

Table 2. MicroRNA Targets with at least Two-Fold Differential Expression

microRNA	Fold Change*	p-value¥
100	0.34	0.0039
374a	0.34	0.0104
7	0.36	0.0039
18a	0.36	0.0065
125b	**0.39**	**0.0039**
148a	0.41	0.0039
17	0.44	0.0104
221	0.45	0.0065
21	**0.45**	**0.0039**
93	0.46	0.0039
143	0.48	0.0250
17*	0.49	0.0039
96	0.49	0.0250
106b	**0.49**	**0.0104**
103a	0.50	0.0163
20a	0.50	0.0039

MicroRNA targets in bold are known to regulate expression of genes in pathways that regulate lipoprotein metabolism, and were selected for qPCR validation.

*Fold change calculated using the following formula: $2^{-\Delta\Delta Ct}$ where

$\Delta\Delta Ct = \text{mean}\,(Ct_{\text{target, cases}} - \text{mean}\,(Ct_{\text{normalizer, cases}})) - \text{mean}\,(Ct_{\text{target, controls}} - \text{mean}\,(Ct_{\text{normalizer, controls}}))$

¥p-value calculated using Wilcoxon non-parametric test of equality for unmatched pairs

Table 3. qPCR Validation of Differentially Expressed microRNA

	Individual samples		Individual samples*		Pooled samples	
microRNA	Fold Change**	p-value[¥]	Fold Change**	p-value[¥]	Fold Change**	p-value[$]
106b	-1.55	0.16	-1.68	0.14	-1.35	<0.05
125b	-1.84	0.14	-1.86	0.15	-1.86	<0.01
21	-2.12	0.07	-2.02	0.12	-1.69	<0.05

*After exclusion of 4 samples not included in the case and control pools

**Fold change calculated using the following formula:

$(E_{target}^{-\Delta Ct\ target}) / (E_{normalizer}^{-\Delta Ct\ normalizer})$ where $\Delta Ct = \text{mean } Ct_{cases} - \text{mean } Ct_{controls}$

using the mean of replicates for each sample after excluding outliers. Outliers were defined as greater than 0.5 standard deviations from mean Ct.

[¥]p-value calculated using Student's t-test

[$]p-value calculated using Wilcoxon rank-sum non-parametric test

Table 1S. Mean cycle threshold, standard deviation, and fold change for 85 microRNA screened by array

	Controls		Cases			
microRNA	Mean Ct	Standard Deviation Ct	Mean Ct	Standard Deviation Ct	Fold Change*	p-value¥
RNU6b	19.8	1.0	19.1	0.7	1.00	0.7488
hsa-let-7a	19.2	0.9	19.5	0.5	0.52	0.0547
hsa-let-7c	21.9	1.6	22.3	1.1	0.45	0.2002
hsa-microRNA-100	23.5	0.7	24.3	0.4	0.34	0.0039
hsa-microRNA-103a	19.2	0.7	19.5	0.4	0.50	0.0163
hsa-microRNA-106b	19.6	0.6	19.9	0.3	0.49	0.0104
hsa-microRNA-107	26.5	1.1	26.2	0.6	0.77	0.6310
hsa-microRNA-10a	28.6	1.0	28.5	0.3	0.65	0.3613
hsa-microRNA-122	29.7	0.6	29.4	0.1	0.75	0.3367
hsa-microRNA-124	32.0	2.3	31.7	1.1	0.74	0.6242
hsa-microRNA-125b	21.8	0.6	22.4	0.3	0.39	0.0039
hsa-microRNA-126	22.5	0.5	22.6	0.3	0.59	0.0250
hsa-microRNA-128	22.0	0.6	21.5	0.4	0.86	0.4233
hsa-microRNA-130b	22.6	0.7	22.6	0.5	0.60	0.1093
hsa-microRNA-133a	30.9	0.7	30.7	0.4	0.70	0.1495
hsa-microRNA-133b	32.1	1.3	31.4	1.0	0.97	0.7150
hsa-microRNA-134	30.1	0.8	29.9	1.0	0.71	0.7488
hsa-microRNA-143	28.1	0.7	28.4	0.3	0.48	0.0250
hsa-microRNA-145	22.1	0.7	22.2	0.3	0.59	0.0374
hsa-microRNA-146a	24.2	0.5	24.2	0.5	0.60	0.0547
hsa-microRNA-148a	21.9	0.5	22.5	0.3	0.41	0.0039
hsa-microRNA-150	17.5	0.5	17.3	0.3	0.68	0.1093
hsa-microRNA-155	27.5	0.4	27.1	0.4	0.84	0.1093
hsa-microRNA-15a	21.7	0.8	21.9	0.4	0.53	0.0250
hsa-microRNA-15b	18.3	0.5	18.0	0.3	0.74	0.1093
hsa-microRNA-16	17.2	0.5	17.1	0.4	0.66	0.0547
hsa-microRNA-17	20.9	0.8	21.4	0.4	0.44	0.0104
hsa-microRNA-17*	24.0	0.5	24.3	0.3	0.49	0.0039
hsa-microRNA-18a	23.2	0.8	24.0	0.3	0.36	0.0065
hsa-microRNA-191	15.5	0.6	14.9	0.4	0.91	0.4233
hsa-microRNA-192	21.9	0.6	21.8	0.3	0.67	0.0547
hsa-microRNA-193a-5p	26.6	0.7	26.3	0.7	0.73	0.5218
hsa-microRNA-195	17.5	0.7	17.4	0.4	0.63	0.1093
hsa-microRNA-196a	30.7	1.6	31.2	0.7	0.43	0.2623
hsa-microRNA-19a	18.8	0.5	18.7	0.4	0.65	0.0782
hsa-microRNA-19b	18.9	0.5	18.8	0.4	0.69	0.0782
hsa-microRNA-200b	28.2	1.1	28.2	0.7	0.62	0.3367

hsa-microRNA-200c	27.0	0.7	26.9	0.4	0.67	0.1495
hsa-microRNA-204	29.6	0.6	29.4	0.4	0.72	0.2002
hsa-microRNA-205	32.7	1.3	31.7	1.5	1.23	0.7150
hsa-microRNA-20a	19.4	0.7	19.7	0.4	0.50	0.0039
hsa-microRNA-21	21.0	0.6	21.5	0.4	0.45	0.0039
hsa-microRNA-210	25.9	0.9	25.6	0.3	0.76	0.5218
hsa-microRNA-211	31.0	0.8	30.5	0.6	0.90	0.7488
hsa-microRNA-214	32.9	1.8	32.1	1.7	1.09	0.8728
hsa-microRNA-215	29.5	0.6	29.5	0.4	0.64	0.0782
hsa-microRNA-22	18.0	0.6	17.4	0.3	0.98	0.8728
hsa-microRNA-221	22.5	0.7	23.0	0.4	0.45	0.0065
hsa-microRNA-222	22.3	0.7	22.4	0.3	0.59	0.0250
hsa-microRNA-223	15.5	0.7	15.6	0.4	0.58	0.0547
hsa-microRNA-224	30.0	0.6	29.9	0.5	0.66	0.1093
hsa-microRNA-23a	19.1	0.4	19.0	0.2	0.69	0.0374
hsa-microRNA-24	22.0	0.6	21.7	0.4	0.78	0.2623
hsa-microRNA-25	15.5	0.7	15.5	0.6	0.62	0.1093
hsa-microRNA-26a	19.2	0.4	18.8	0.4	0.86	0.4233
hsa-microRNA-26b	20.8	0.9	20.5	0.5	0.80	0.6310
hsa-microRNA-27a	23.4	0.6	23.6	0.3	0.56	0.0163
hsa-microRNA-296-5p	22.5	0.6	21.9	0.5	0.95	1.0000
hsa-microRNA-29a	22.9	0.6	22.7	0.7	0.68	0.1495
hsa-microRNA-30d	19.3	0.4	18.9	0.3	0.82	0.2623
hsa-microRNA-30e	20.0	0.7	20.0	0.4	0.64	0.0374
hsa-microRNA-31	30.3	0.8	29.8	0.5	0.88	0.5839
hsa-microRNA-34a	30.6	0.8	30.3	0.5	0.78	0.5218
hsa-microRNA-374a	25.0	0.7	25.9	0.9	0.34	0.0104
hsa-microRNA-375	29.5	1.4	29.2	1.3	0.75	0.5839
hsa-microRNA-376c	29.3	0.7	29.6	0.4	0.49	0.0542
hsa-microRNA-423-5p	20.0	0.5	19.8	0.5	0.68	0.0782
hsa-microRNA-499-5p	33.8	1.3	33.5	1.0	0.77	0.6015
hsa-microRNA-574-3p	22.5	0.8	22.3	0.5	0.69	0.4233
hsa-microRNA-7	23.5	0.5	24.3	0.4	0.36	0.0039
hsa-microRNA-885-5p	29.7	1.0	28.8	0.7	1.14	0.7488
hsa-microRNA-92a	13.3	0.8	13.0	0.8	0.79	0.3367
hsa-microRNA-93	19.2	0.4	19.6	0.4	0.46	0.0039
hsa-microRNA-96	26.8	0.8	27.1	0.4	0.49	0.0250

*Fold change calculated using the following formula: $2^{-\Delta\Delta Ct}$ where

$\Delta\Delta Ct$ = mean (Ct target, cases – mean (Ct normalizer, cases)) – mean (Ct target, controls – mean (Ct normalizer, controls))

¥p-value calculated using Wilcoxon non-parametric test of equality for unmatched pairs using normalized expression ($2^{-\Delta Ct}$)

CHAPTER 9

CONCLUSION

The purpose of this dissertation was to investigate cardiovascular risk in South Asians through the theoretical framework of gene-environment interactions. While many environmental contributors to disease are well known, the nature of how environmental factors interact with the genome in individuals or populations, and the ensuing consequences for health, are not well understood. An increasing body of knowledge is developing around a phenomenon termed epigenetics. Epigentic mechanisms are those that cause alteration in how genes are expressed without changing the underlying genome. As such, epigenetic mechanisms are the nexus of gene-environment interactions. Greater understanding of how gene-environment interactions occur through epigenetic mechanisms will enhance our understanding of the etiology of complex disease conditions like cardiovascular disease, and is essential knowledge for fulfilling the promise of individualized medicine.

South Asians are a population disproportionately afflicted with cardiovascular disease. Although genetic predisposition likely plays a primary role, interactions between genetic risk factors and the environment are undoubtedly an important part of the story. Given this high level of risk, prevention is of paramount importance, and interventions to reduce risk are themselves a form of gene-environment interactions. One approach to reducing risk in this population is through comprehensive risk assessment followed by individualized, culturally-tailored coaching by non-medical personnel. While coaching is a proven method for improving chronic disease risk factors, very little is known about the effect of adapting coaching strategies to be culturally specific. In a study describing a cardiovascular risk reduction program targeted towards South Asians, we found that a year-long coaching intervention is appealing to this population, and feasibly implemented. Over five years, 98% of potential participants screened were eligible candidates for the program, 87% of those elected to participate in the program, and 50% were active participants or successfully completed one-year of coaching. Additional research is needed

to determine whether this intervention is effective in reducing cardiovascular risk in this population.

While South Asians are widely recognized to have a high prevalence of cardiovascular disease, the exact causes are not fully understood. Previous studies have observed a high level of abdominal adiposity, low HDL-c, and high prevalence of type-2 diabetes and insulin resistance. In order to begin to disentangle the specific mechanisms underlying cardiovascular risk in this population, we performed two epidemiologic studies. The first measured the prevalence of the metabolic syndrome, a clustering of cardiovascular and metabolic risk factors. We found that this condition occurred in 27% of the sample, compared to estimates of 26-38% in other South Asian studies, and 34% in the US population. The occurrence of the metabolic syndrome is largely driven by the combination of low HDL-c with elevated triglycerides, whereas elevated blood pressure and elevated blood glucose were less common. Interestingly, this high prevalence was observed despite the fact that the study sample was relatively young (42 ± 10 years), smoking was rare (4%), only 4% had less than college-level education, and 93% were married.

The second study sought to determine whether abdominal adiposity is a necessary precursor to the development of cardiovascular and metabolic risk factors in this population. A commonly held belief is that abdominal adiposity is the precipitating factor leading to the development of combined insulin resistance, dyslipidemia, hypertension, and inflammation. Our results challenge this belief, showing that cardio-metabolic abnormalities were present in normal weight individuals (23% men, 7% women). Among obese individuals, 21% of men and 50% of women had fewer than two cardio-metabolic abnormalities. This study also confirmed our previous findings that low HDL-c with elevated triglycerides is by far the most common risk phenotype, occurring in 34% of the sample. This is strong evidence to dispute the notion that cardio-metabolic risk only occurs in the setting of abdominal adiposity in South Asians. Further, South Asian women appear to be able to tolerate overweight/obesity without developing cardio-metabolic risk factors to a greater extent than men.

Within the category of epigenetics, there are mechanisms of transcriptional regulation of DNA (i.e. methylation, histone modification), and translational regulation of messenger RNA (i.e. microRNA). While transcriptional regulation is semi-permanent, microRNA (miR) regulation of translation is highly dynamic, allowing an organism to rapidly adapt to a changing environment in order to maintain homeostasis. There are two possible implications for miR as a biomarker: detection of underlying pathology, and markers for response to interventions. While the combination of low HDL-c with elevated triglycerides is considered a more discrete type of dyslipidemia, it is still quite complex and is regulated by numerous biologic pathways. Differences in miR expression between affected and healthy individuals may provide some insight into the specific mechanisms underlying the causes of this cardiovascular risk factor in a single individual or population. This insight may lead to targeted treatments. Although current pharmacologic treatment options for dyslipidemia, particularly low HDL-c, are limited, modalities such as gene therapy will require enhanced understanding of the various biologic pathways underlying complex diseases. Non-medical risk reduction interventions show variable effectiveness, but application of miR as a biomarker for treatment response may help patients to focus on interventions that will be highly effective for their specific condition. A third possible clinical implication for miR are as a therapy themselves through administration of synthetic miR mimics or miR antagonists.

Previous studies of miR regulation of lipoprotein metabolism have been primarily conducted *in vitro* and in animal models. Mi-33 shows the greatest promise to date as a potential therapeutic agent, with direct effects on both HDL-c and non-HDL-c causing dramatic improvements in lipoprotein levels following exogenous miR-33 administration in African Green Monkeys. MiR-122 is the predominant miR found in liver, targeted several genes involved in cholesterol metabolism, resulting in decreased total cholesterol and triglycerides, and an increased number of LDL-c receptors in *in vitro* and animal studies. There are several additional

miR with early evidence suggesting functional roles in cholesterol metabolism, and the activity of miR appears to be responsive to changes in the environment.

In order to explore the feasibility of miR as a biomarker for dyslipidemia, we undertook a study of miR expression in South Asians with and without the common low HDL-c and elevated triglycerides phenotype. This was a case-control study with cross-sectional time frame. Cases were defined as South Asian men with low HDL-c (<40mg/dL) and elevated triglycerides (>150mg/dL), while controls had neither of these conditions. Women were excluded from this study in order to decrease likely variability in miR expression secondary to cyclic hormonal fluxuations. We collected blood and isolated RNA from 22 cases and 22 healthy controls. Of these, the equivalent of one miRome from 40 participants (equally distributed between cases and controls) was combined to create pooled samples for cases and controls. The expression of 85 miR targets was measured using array-based methods, and compared between pooled cases and controls. Of these, 16 were significantly differentially expressed ($p<0.05$) with at least two-fold difference. Three of these (miR-21, miR-106b, miR-125b) were selected for validation using qPCR methods based on high a priori biologic plausibility for regulation lipid metabolism. We found similar fold-change estimates for measurement of both individual samples and pools of cases and controls. The results of this final study are promising evidence for clinical utility of miR as a biomarker of dyslipidemia. Additional studies are needed to determine the time-sequence of miR changes and alterations in lipoprotein profiles, and to determine whether miR expression is responsive to interventions.

Gene-environment interactions likely account for gaps in knowledge about the etiology of chronic diseases, including disparities in disease conditions between populations. Epigenetic mechanisms are an important component of the machinery underlying these interactions, including the highly dynamic regulation of messenger RNA translation by miRNA. South Asians are a population severely afflicted with cardiovascular disease. We found that a common cardiovascular risk phenotype in this population is low HDL-c with elevated triglycerides, and

that cardiovascular and metabolic risk in this population does not necessitate overweight or obesity as a precursor to other risk factors. Promising evidence from *in vitro* and animal studies supports a role for miR in pathways related to regulation of cholesterol levels. We measured miR in the blood of South Asian men with and without the common low HDL-c and elevated triglyceride phenotype, finding differential expression of several miR species. To our knowledge, this is the first blood-based biomarker study of miR expression and dyslipidemia. Further research is needed to determine the specific pathways and messenger RNA targeted by these miR in order to gain an enhanced understanding of the causes of cardiovascular risk factors in South Asians. Additional studies are also needed to investigate whether miR may be plausible biomarkers for response to interventions.

Publishing Agreement

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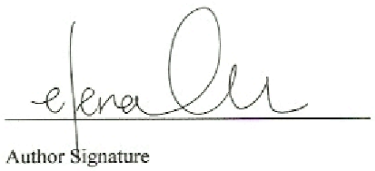

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