2017

The Cardiovascular Epidemiology and Genome-Wide Associations of Biomarkers of Innate and Adaptive Immunity: sCD163 and sIL2RA

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THE CARDIOVASCULAR EPIDEMIOLOGY AND GENOME-WIDE ASSOCIATIONS
OF BIOMARKERS OF INNATE AND ADAPTIVE IMMUNITY: SCD163 AND SIL2RA

A Dissertation Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Clinical and Translational Science

October, 2017

Defense Date: June 19, 2017
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ABSTRACT

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the U.S. and worldwide. Atherosclerosis, the buildup of plaque in the arteries, is a common cause of CVD. For many years, research in atherosclerosis was focused on lipid metabolism and the accumulation of low-density lipoprotein in the arteries. While this research set public health guidelines for lipid management, lipid concentration was not the only factor influencing atherosclerosis and CVD events. Many scientists, as far back as the 1850's recognized the role of inflammation in the progression of atherosclerotic disease. The continuous low levels of immune activation in the body contribute to atherosclerosis. Research in animal models and epidemiologic studies have shown the involvement of both the innate and the adaptive immune systems in plaque development and to elucidate the roles of monocytes and T cells. In addition to animal studies and epidemiologic research, CVD and atherosclerotic research has extended to genetic analysis in the search for associations with risk factors and outcomes.

The first chapter is a review of the literature studying the immune system's involvement in atherosclerosis. Beginning with an examination of the impact of CVD and atherosclerosis, the basic pathophysiology, and the involvement of the innate and adaptive immune systems through animal models and epidemiology. Some of the significant cohort studies in CVD and genome wide association studies are also discussed.

Chapter 2 examines the associations of soluble interleukin 2 receptor alpha (sIL-2Rα) with clinical events in the Cardiovascular Health Study and genetic variants. Interleukin 2 (IL-2) and its receptor regulate both tolerance and immunity, IL-2 induces the proliferation and differentiation of T cells, part of the adaptive immune system. The results showed an association between sIL-2Rα and CVD events. The genome-wide association study found 52 variants to be significantly associated with sIL-2Rα in European Americans.

Chapter 3 assesses the involvement of the innate immune system in atherosclerosis through the associations of soluble CD163 (sCD163). CD163 is a marker of macrophage activation, specifically associated with M2 macrophages. In CHS, sCD163 levels were analyzed for associations with cardiovascular events and genetic variants. sCD163 was found to be associated with CVD risk factors and with cardiovascular events. In a genome-wide association study six variants in European Americans and three variants in African Americans were found to be significant.

Chapter 4 summarizes the results and discusses some bench to bedside translational science already seen in atherosclerosis treatment and prevention. Continued investigation of markers of T-cell and monocyte differentiation in animal models and cohort studies may lead to opportunities for the prevention of atherosclerosis and/or treatment through an increased understanding of the biology and genetics of the innate and adaptive immune.

CITATIONS

Material from this thesis has been published in the following forms:

Durda, P., Sabourin, J., Lange, E. M., Nalls, M. A., Mychaleckyj, J. C., Jenny, N. S., . . . Lange, L. A.. (2015). Plasma Levels of Soluble Interleukin-2 Receptor alpha: Associations With Clinical Cardiovascular Events and Genome-Wide Association Scan. *Arterioscler Thromb Vasc Biol, 35*(10), 2246-2253. doi:10.1161/atvbaha.115.305289

DEDICATION

To my wife, Pam, thank you for your never-ending support.

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<u>Cardiovascular Disease is a major cause of morbidity and mortality in the U.S. and Worldwide.</u>

Cardiovascular disease (CVD) is a broad term covering a family of diseases linked by common risk factors, many of which are caused by atherosclerosis. Atherosclerosis plays a major role in coronary heart disease, peripheral artery disease, and myocardial infarction, some forms of heart failure, stroke, and hypertension, and contributes to poor outcomes in diabetes, chronic kidney disease, vascular dementia, and others included in ICD10 codes 100-199. CVD is the leading cause of death throughout the world, accounting for more than 17.5 million deaths in 2012; 31% of all global deaths [1]; more deaths than all forms of cancer combined. In the United States in 2013, CVD was the underlying cause of death for over 800,900 deaths and is the leading cause of death in both women and men over the age of 65 [2].

CVD, is both a huge burden on the health care system and a huge economic burden. From 2011 to 2012, the average annual direct and indirect cost of CVD in the United States was estimated at $316.6 billion [2]. These costs included direct costs of physicians, hospitals, medication and health care, estimated at $193.1 billion, as well as the indirect costs of lost productivity and mortality, estimated at $123.5 billion [2]. Projections for 2030 indicate that 43.9% of the US population

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will have some form of CVD and total costs associated with CVD will increase to more than $1.2 trillion [2].

From 1979 to 2011 the US saw a large decline in deaths attributable to CVD; mortality rates dropped 52% in men and 49% in women between 1980 and 2002[3]. Ford *et al.* found that that 47% of the decrease was due to improved treatment and therapies, including secondary preventive treatments, initial treatment of myocardial infarctions, heart failure treatments and others [4]. Forty-four percent of the decline was due to changes in risk factors; reductions in total cholesterol, systolic blood pressure and smoking, along with increased physical activity [4]. The most consistent declines were in adults ≥65 years of age between 1979 and 1989. Despite this success, the three subsequent decades showed little change in the mortality rates. This was especially true in both men and women between the ages of 35-54. While there are fewer studies of younger people, and younger women are particularly understudied, it is possible that one of the mechanisms contributing to the sluggish decline of CVD mortality in this group is due to increased risk factors.

Atherosclerosis is an underlying cause of CVD; preventing atherosclerosis by reducing risk factors may prevent 90% of all CVD. Exercise, weight loss, healthy eating, limited alcohol consumption, the avoidance of smoking, treatment of hypertension and diabetes are all beneficial in the prevention of atherosclerosis, and have the potential to mitigate a major portion of the morbidity, mortality, and health

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care burden of atherosclerotic CVD. However, much remains to be understood about the underlying pathophysiology of CVD in general and atherosclerosis in particular if complete eradication is our goal.

The General Pathophysiology of Atherosclerosis.

The term atherosclerosis, from the Greek words *athero* meaning gruel or porridge and *sclerosis* meaning hardening specific to an artery, was first introduced in 1829 by the French surgeon, Jean Lobstein [5]. Over the years, our understanding of atherosclerosis, its development, and complications has emerged from studies of animal models, plus basic research, clinical research and epidemiology. Although until recently viewed as solely a cholesterol storage problem with lipid accumulation clogging the arteries and culminating in a heart attack, it was Virchow who first noted inflammation as the beginning of atherosclerosis in 1859 [6]. 'I have therefore felt no hesitation in siding with the old view in this matter, and in admitting an inflammation of the inner arterial coat to be the starting point of the so-called atheromatous degeneration…'[7]. Initially, Virchow's hypothesis of atherosclerosis as an inflammatory disease was considered baseless, while his notion of cellular proliferation in the progression of atheromas (blister-like bulges in the arterial wall with central lipid pools) was acknowledged. Duguid [8], in the late 1940's along with Mustard and Packham [9], French[10] in the mid 1960's, and Ross[11, 12] in the mid 1970's modified and extended those early observations of an association between inflammation and atherosclerosis.

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The beginning of an atherosclerotic lesion can be found in infants as fatty streaks, consisting of lipid filled macrophages and T lymphocytes in the intima of arteries [13]. These fatty streaks may not progress and in some cases may even regress. Autopsies of young soldiers from the Korean War (average age 22) found that greater than 70% had some atherosclerosis in their coronary arteries [14]. Over one hundred years ago, the cholesterol hypothesis of atherosclerosis was born when Anitschokow published his work on arterial lesions seen in rabbits fed a high cholesterol diet [15, 16]. Anitschkow continued his work with rabbits documenting the cholesterol accumulation in the arteries, foam cell development, white cell involvement, and the conversion of fatty streaks to the mature form of atherosclerotic lesion, the atherosclerotic plaque. While other laboratories confirmed Anitschokow's results with further experiments in rabbits [17], many scientists at the time were working with other animals including dogs and rats, and were unable to substantiate the cholesterol hypothesis with the prevailing assumption being that atherosclerosis was considered a disease of aging, a chronic, inevitable progression. Although Anitschokow believed that there could be no atherosclerosis without the deposition of cholesterol, his understanding evolved when he noted that blood pressure and arterial changes were significant factors in atheroma development, leading to his 'combination theory' of atherosclerosis [15]. Today, the importance of cholesterol and hypertension in atherosclerosis are no longer questioned and the controls of hypercholesterolemia and blood pressure have been shown to reduce morbidity and mortality of CVD. Since the early 1980's

animal experiments, clinical studies and epidemiological research have focused on lipid metabolism and cholesterol deposition, and what we now recognize as the inflammatory response to such deposition, in the development and progression of atherosclerosis.

Basic and Clinical Research: Atherosclerosis as an Inflammatory Disorder.

While atherosclerotic plaques or atheromas may develop in the intima, or innermost layer, of many large arteries from the aorta to the coronaries, the arterial sites located near branch points with low shear stress [18, 19] (Figure 1) are the most common sites.
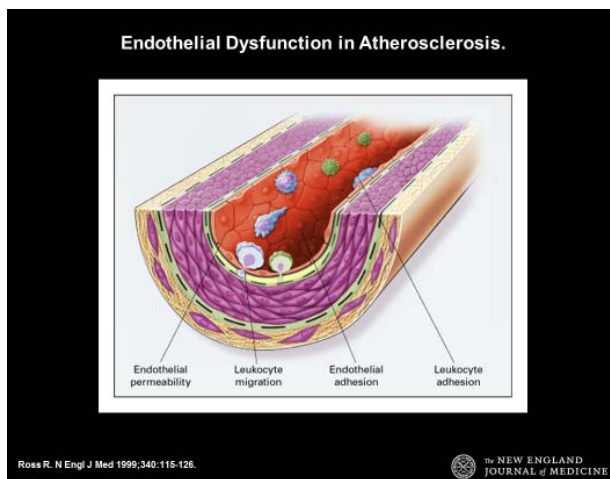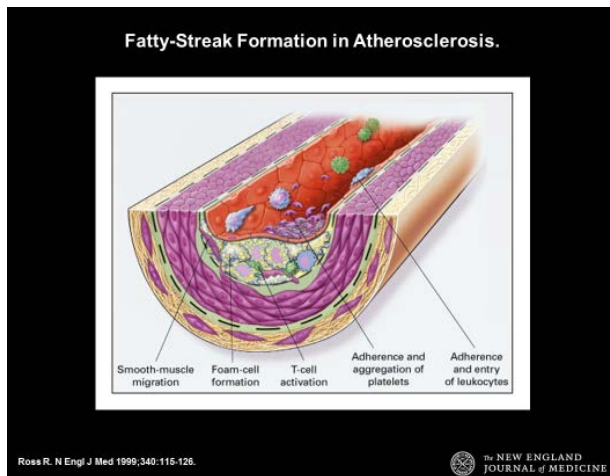
Figure 1: Fatty streak development Ross, R. NEJM 1999; 340:115-126

Plaque Development

Plaque development and progression begins in adolescence and can last > 40 years [20, 21]. The development of fatty streaks begins with the accumulation of lipid particles, primarily low-density lipoproteins (LDL), in the arterial intima. When the concentration of LDL particles exceeds the capacity to clear them from

the intima, they incorporate into the extracellular matrix and initiate activation of

the innate immune system and the atherosclerotic process begins (Figure 2).

Modified from: Libby,P. Scientific American 286: 46-55,2002
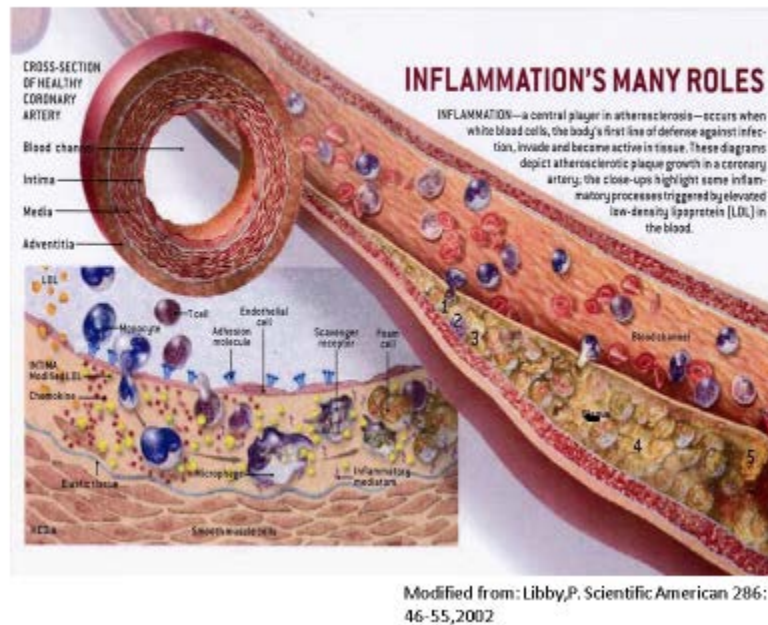
Figure 2: Inflammation and Atherosclerosis

Modification of these LDL particles to oxidized LDL (oxLDL) by activated

endothelial cells [22] leads to the expression of leukocyte adhesion molecules,

vascular cell adhesion molecule-1 and P-selectin, enabling lymphocytes and

monocytes to preferentially adhere.

The Key Role of Monocytes/Macrophages.

The adherent monocytes respond to chemokines, monocyte chemoattractant

proteins (MCP-1 and CCL2), and migrate into the intima [23]. Once exposed to

macrophage-stimulating factor, the monocytes differentiate into macrophages. We currently recognize three subsets of macrophages found in human circulation, differentiated by their expression of CD14 and CD16 receptors. M1 macrophages, or "classically" activated macrophages, constitute the majority of macrophages (~90%) and are $CD14^{++}CD16^-$; $CD14^{++}CD16^+$ macrophages are classified as intermediate macrophages, and "alternatively" activated or M2 macrophages ($CD14^+CD16^{++}$) [24-26]. Pro-inflammatory M1 macrophages are phagocytic and secrete interleukin-1β, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and IL-12[27] among other mediators of inflammation. M2 macrophages produce the regulatory cytokine IL-10 and modulate inflammation. Both types of macrophages bind and internalize oxLDL and are capable of forming foam cells. If the transport of the lipids or macrophage egress from the intima is impaired there is excess lipid accumulation which results in cell death and the release of cholesterol and inflammatory cytokines; further perpetuating the recruitment of monocytes, continued chronic inflammation, and plaque formation [28]. Macrophages have the ability to switch their phenotype from M1 to M2 and *vice versa* depending on specific cytokine signals [29, 30]. M1 macrophages have long been implicated in the atherosclerotic process and have been identified in plaques as well [31]. However, it wasn't until 2007 when Amine Bouhel *et al.* examined M2 macrophage marker RNA in the plaques of 27 patients that M2 cells were identified in plaques [29]. Histological analysis of plaques from more than 80 patients found that M2 macrophages were localized to more stable areas of the lesions while M1

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macrophages were predominant in symptomatic plaques [32]. Additionally, the

presence of CD163, a macrophage specific receptor found on M2 macrophages

[33], was inversely related to the progression of atherosclerosis [32]. Macrophage

plasticity and the switch from pro-inflammatory M1 macrophages to immune

modulating M2 macrophages illustrates the complex nature of atherosclerosis.

While atherosclerosis is caused by continued inflammation, it may be that impaired

immune regulation is also a contributing factor.

The Emerging Role of the Adaptive Immune System.

The initial inflammatory response in atherosclerosis begins with the innate

immune system, followed by the involvement of the adaptive immune system. The

cells that comprise the innate or non-specific immune system include macrophages,

neutrophils, dendritic cells, and natural killer cells, among others. These cells are

always present and mobilize when an infection occurs. Adaptive immunity is

composed of humoral immunity mediated by B cells, plasma cells, and antibodies;

and cell mediated immunity driven by T lymphocytes. Dendritic cells take up

oxLDL, other LDL particles, and heat shock proteins, present these antigens to

naïve T cells in peripheral lymphoid organs, and thereby generate an adaptive

immune response[34-38]. T cell recruitment is similar to monocyte recruitment and

involves both adhesion molecules and chemokines [39]. The naïve T cells

differentiate to T helper cells (Th CD4$^+$) and cytotoxic T cells (Th CD8$^+$). T helper

cells differentiate further becoming Th1, Th2, Th17, and T regulatory cells (Treg)

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[40, 41] (Figure 3). T cells are not as prominent in the atheroma as macrophages; the macrophage/T cell ratio ranges from 4:1 to 10:1 [40].
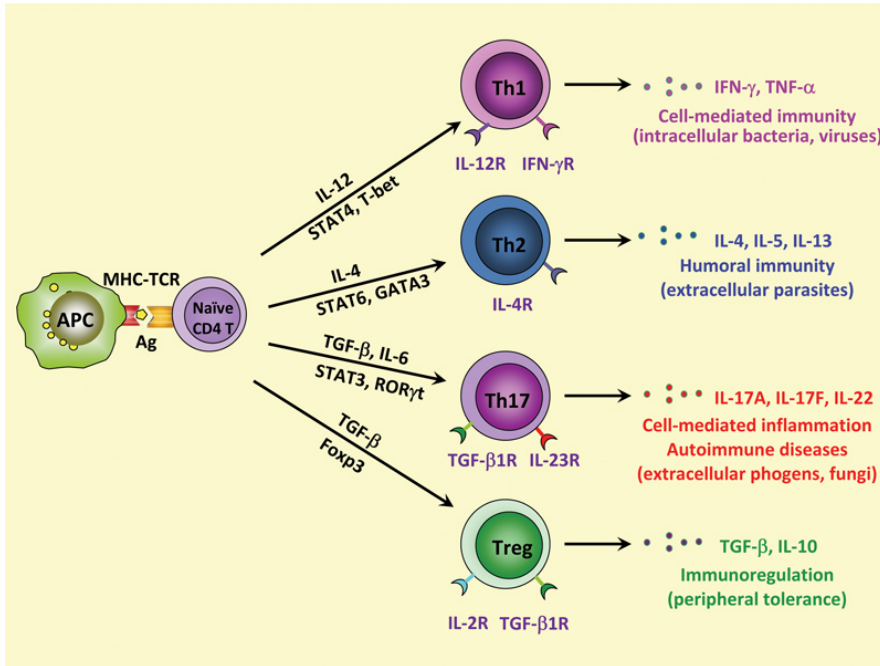
Figure 3: T cell Differentiation: Cell Mol Immunol. 2010 May;7(3):182-9.

T Helper Type 1 Cells Play a Major Role in Atherosclerosis.

Analysis of human atherosclerotic plaques has shown that CD4$^+$ T cells are present in higher numbers than CD8$^+$ T cells [42]. The T cells are usually found in the shoulder region of a plaque near major histocompatibility complex (MHC) class II expressing macrophages and dendritic cells, suggesting a continuing inflammatory response in the atheroma [43-46]. Th1 and Th2 cells have been the most investigated and well characterized of the T cell subsets. Differentiation of naïve CD4$^+$ T helper cells requires three sequential signals; first, the T cell receptor

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is stimulated, next dendritic cells produce cytokines, and last, co-stimulatory molecules on the cell surface of dendritic cells are activated [47]. Differentiation of naïve T cells depends upon the cytokines released. IL-12 is an important connection between the innate and adaptive immune systems. Produced by monocytes, macrophages, and dendritic cells during infection, IL-12 upregulates the production of IFN-γ and biases the differentiation of naïve CD4+ T cells to proinflammatory Th1 cells while reducing the production of anti-inflammatory cytokines IL-4, IL-5, and IL-13 [48, 49]. IL-12 and interferon γ (INF-γ) work by activating signal transducer and activator of transcription (STAT)-4 and T-box transcription factor TBX21 [47, 49]. The activation of TBX21 allows the continued expression of INF-γ, the predominate pro-inflammatory cytokine produced by Th1 cells, further activation of macrophages and endothelial cells with increased expression of pro-inflammatory cytokines and adhesion molecules, and the down regulation of Th2 cytokines IL-4 and IL-5 [39, 50]. Differentiation of Th2 cells initiated by IL-4, activates the expression of STAT-6, in turn promoting T-cell specific transcription factor GATA3. GATA3 upregulates the expression of both IL-4 and IL-5 while inhibiting the expression of IFN-γ thus promoting the differentiation of Th2 cells [47].

Both human and animal studies have identified Th1 cells as the primary driver of atherosclerosis with Th1 cells seen as the dominate cell type in lesions [51]. In 1999 de Boer *et al.* reported that T cell clones isolated from a human aortic plaque contained 17% Th1 cells but only 2% Th2 cells. Mouse studies have provided

compelling evidence for the pro-atherogenic function of Th1 cell, work by Huber *et al.* showed the importance of Th1 cells in the development of atherosclerosis [52, 53]. When Huber *et al.* injected both atherosclerosis-susceptible and resistant strains of mice with weekly doses of IL-6 they found a 2 to 5 fold increase in atherosclerotic lesion size [53]. Class II major histocompatibility complex (MHC) molecules regulate T cell responses; the mouse has two different MHC molecules, IA and IE. The atherosclerotic–susceptible mouse strain only expresses the IA molecules with Th1 cells being the predominant cell type, suggesting that atherosclerotic susceptibility may be due to T cell differentiation biased toward the Th1 cell type, while IE expression leads to a Th2 cell bias. The proatherogenic cytokine IFN-γ is produced by Th1 cells, while Th2 cells produce the antiatherogenic cytokine IL-4 suggesting that pro-inflammatory cytokine expression is actively involved in plaque development. Studies in the early 1990's on sections of human atherosclerotic lesions found that T cells formed complexes with macrophages and that these interactions between the innate and adaptive immune systems are integral in the pathogenesis of atherosclerosis [54]. Although lipoprotein metabolism and immune function differ between mice and humans, atherosclerosis prone mice have provided information furthering the understanding of the atherosclerotic process. Mice lacking either the LDL receptor (LDLR) or Apoliportotein E (ApoE), an integral component of lipoprotein metabolism, have been commonly used [55]. When fed a high fat diet, mice deficient in the LDLR (LDLR$^{-/-}$) develop large atherosclerotic lesions associated with severe

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hypercholesterolemia. ApoE deficient (ApoE$^{-/-}$) mice also develop atherosclerotic

lesions morphologically similar to early human lesions when fed a high fat diet.

When these mice are given INF-γ, they produce larger and more numerous

atherosclerotic lesions than control mice. Conversely, mice deficient in INF-γ or the

INF-γ receptor have fewer lesions [56-59]. Lee *et al.* showed that if ApoE$^{-/-}$ mice

were injected with IL-12 they would develop plaques even when fed a normal diet

[60]. Davenport and Tipping found that the deletion of the IL-12 encoding gene in

mice prohibited early lesion development [61] as did Hauer *et al.* when they

vaccinated mice against IL-12 [62]. Further evidence of the involvement of Th1

cells in atherosclerosis comes from research done in ApoE$^{-/-}$ mice with IL-18, a Th1

promoting cytokine. Elhage *et al.* bred a mouse that was both ApoE and IL-18

deficient and found reduced atherosclerotic lesion size when compared to wild type

mice in spite of the double knockout mice having significantly higher levels of both

serum cholesterol and triglycerides [63]. When Whitman *et al.* injected ApoE$^{-/-}$

mice with IL-18 over a period of thirty days, while being fed a normal diet, they

found that atherosclerotic lesion size was increased two fold compared to animals

not administered IL-18. Buono *et al.* bred a mouse line that was deficient in both

the LDLR and *Tbx21*, which codes for the T-bet transcription factor required for

Th1 differentiation and regulation of Th1, Th2 balance in inflammation. The

amount of atherosclerosis in the *Tbx21* deleted Ldlr$^{-/-}$ mice was significantly less

than that in the mice that were only Ldlr deficient [64]. Additionally, the *Tbx21*

deficiency caused a switch of the dominant cellular phenotype to Th2 cells [51]. In

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a study of 28 patients with acute coronary syndrome, Methe *et al.* found there were

significantly more Th1 cells when compared to controls in patients with stable

angina and that elevated Th1 cell counts were predictive of cardiovascular events

[65].

Th2 Cell Involvement in Atherosclerosis is Less Clear.

While the role of Th1 cells in plaque development has been clearly defined

as pro-atherogenic with both Th1 cells and their associated cytokines having been

identified in human atherosclerotic plaques [51], the role of Th2 cells is not as well

understood. Dendritic cells stimulate the differentiation of naïve T cells to Th2

cells through the production of IL-6/IL-13 and the interaction of the OX40 receptor

on the T cell with the OX40 ligand on the antigen presenting dendritic cell [50].

The Th2 cells produce IL-5, IL-4, IL-10 and IL-13 and activate B cell dependent

responses [66, 67]. The transcription of GATA-3, the master regulator of Th2 cells,

is triggered by the IL-4 dependent stimulation of STAT-6 and suppresses the

expression of IFN-γ [50] while upregulating IL-5 expression. Therefore, the Th2

responses would seem to modulate inflammation, the pro-atherogenic effects of Th1

cells, and provide atheroprotection. However, the role of Th2 cells and their

responses has proven to be more controversial depending upon the stage of lesion

development, the site of the lesion, and the experimental model. IL-4 has been

shown to have both anti-atherogenic and pro-atherogenic affects in a number of

murine studies [53, 61, 68]. Binder *et al.* showed in a murine model that IL-5

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deficient mice had accelerated atherogenesis and that Th2 responses taken together

not only are anti-atherogenic but also reduced lesion size[69-71]. IL-5 also

promotes B-1 cell development, increasing the production of IgM antibodies which

may inhibit foam cell formation and the initiation of an atherosclerotic lesion [72].

IL-10 is an important regulator of immunity, balancing the Th1 and Th2 response

[73-75]. Murine studies of double knockout mice, ApoE^{-/-} and IL-10^{-/-}, found

increased LDL cholesterol levels, increased numbers of Th1 cells, and increased

lesion size when compared to ApoE^{-/-} mice [76]. In addition to the effect IL-10 has

on lesion size, it also seems to stabilize plaques [67]. The role of IL-13 in

atherosclerosis has not been investigated in depth; however, work by Cardil-Reis *et

al.* in LDLR^{-/-} mice showed that when injected intraperitoneally with IL-13 the mice

had decreased expression of cellular adhesion molecules and decreased numbers of

macrophages in atherosclerotic lesions (with a biasing toward M2 macrophages)

when compared to controls [77]. A deficiency in IL-13 resulted in accelerated

atherosclerosis.

Contributions of other T Cell Subsets to Atherosclerosis.

The contribution of Th17 cells to atherosclerosis is unclear. Th17 cells

promote both pro-inflammatory and anti-inflammatory responses based on the

environment. The association of Th17 cells with inflammatory autoimmune

diseases including rheumatoid arthritis and inflammatory bowel disease is well

documented [78]. However, the data on the role Th17 cells and their signature

cytokine, IL-17, is conflicting; both Th17 and Il-17 expression have been observed in human [79] and murine lesions [80]. A study by Eid *et al.* on patients with coronary atherosclerosis showed that IL-17 and INF-γ act together to promote proinflammatory responses in vascular smooth muscle cells [81]. Further research by Erbel *et al.* found that the secretion of IL-17A was associated with plaque inflammation and destabilization [82]. Conflicting research indicates that increased levels of IL-17 reduce macrophage numbers and promote fibrosis of the plaque [79, 83].

Regulatory T cells (Tregs) are crucial in maintaining the balance between Th1 and Th2 cells by suppressing immune responses [84]. IL-10 and TGFβ are the primary cytokines responsible for the antiatherogenic effects of Treg cells. Mouse models using both mice genetically altered for reduced Tregs and mice vaccinated against Tregs have both shown increased atherosclerosis [85-88]. Tregs are atheroprotective by not only inhibiting T cell activation, but also, by inhibiting foam cell formation and stimulating anti-inflammatory M2 macrophage differentiation [89, 90]. De Boer *et al.* examined atherosclerotic vessel fragments from 42 patients for the presence of Treg cells and found their presence in all stages of lesion development (0.5-5%) [91]. Il-2 plays a crucial role in T cell development and protective immunity, as well immune modulation moderated by Treg cells [92]. Treg cells, like all T cells, express the alpha receptor of IL-2 (IL-2Rα). In addition to the attached receptor, IL-2Rα circulates in a soluble form (sIL-2Rα) high levels of which have been associated with autoimmune diseases and coronary artery

disease [93], suggesting an important role for T cells in general and possibly Treg cells in particular.

Other types of T cells including T effector memory (T_{EM}) cells, CD8$^+$ T cells, and natural killer T (NKT) cells play a role in atherosclerosis, although these cell types have not been investigated as thoroughly as the other T cell types. Murine studies showed that circulating T_{EM} levels were positively correlated with the extent of artheosclerotic lesions [94]. CD8+ cytotoxic T cells are less prevalent in human lesions than CD4+ T cells and their impact on atherosclerotic development, while minor, is still pro-inflammatory. CD8+ T cells induce macrophages through the secretion of IFN-γ. Their presence in both human and murine lesions has been noted for many years [95] and advanced lesions seem to have a high concentration of cytotoxic T cells [96]. NKT cells are a minute subset of T cells involved in self and non-self-recognition. These cells secrete numerous cytokines including IL-4, IL-10, IL-13, IL-21, IFNγ, and TNFα. Their pro-atherogenic role has been studied and confirmed in murine models, additionally, NKT cells have been detected in human plaques [97, 98].

Much like T cells, the impact of B cells on the development of atherosclerosis is also complicated and has not been studied extensively. The work of Caligiuri *et al.* in mice provided the first evidence of B cell involvement in plaque development showing that the transfer of B cells, taken from the spleen, to ApoE knockout mice conferred atherosclerotic protection [99] and these mice were

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found to have fewer CD4^{+} T cells suggesting that interactions between B cells and T cells impact immune activity. More recent investigations have shown that B cell depleted *ApoE*^{-/-} and *Ldlr*^{-/-} mice have significantly reduced plaques compared to non B cell depleted mice [100, 101], suggesting B cells promote atherosclerosis. This apparent contradiction in the role of B cells in atherosclerosis may be due to the different roles of unique subsets. B2 cells which constitute the majority of B cells and are derived from the bone marrow have an as yet undefined role in atherosclerosis. B1a cells, mainly found in the pleural and peritoneal cavities, seem to be atheroprotective [102].

The Role of Population Science in the Study of Atherosclerosis and CVD.

Numerous epidemiology studies have provided much insight into risk factors for CVD. These studies have also established risk assessment algorithms that help to identify those persons who would benefit most from risk factor interventions. In the early 1880's [103] the lack of data on CVD had already been recognized, but it took until 1934 for a conference to be convened to discuss CVD in the population and to further describe the occurrence of atherosclerotic lesion across cultures, geography, socioeconomic status and occupations [104]. The 1940's saw the first attempts at using epidemiology as a tool to identify risk factors and reduce the incidence of CVD. The first prospective study of heart disease was initiated in 1947 by Ancel Keys and colleagues when they followed 281 middle

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aged men in Minnesota for 15 years [105] and found that there was a statistically significant relationship between elevated serum cholesterol and coronary heart disease. Keys followed up this work with the Seven Countries Study, one of the pioneering epidemiologic studies of heart disease, during the mid to late 1950's. The Seven Countries Study was designed to examine the variability of heart disease in populations in relation to the fat composition of the diet and the serum cholesterol levels. Over twelve thousand men age 40 to 59, without evidence of clinical CVD were recruited from the United States, Finland, Yugoslavia, the Netherlands, Italy, Greece and Japan in this a cross cultural, prospective study [106, 107]. A 1970 paper from the Seven countries Study showed that diets high in saturated fat lead to heart disease and that this association is mediated by serum cholesterol [108]. With evidence that both saturated fatty acid intake and high levels of serum cholesterol were strongly associated with risk of heart disease, the Seven Countries Study had a large impact on the prevention of heart disease, and became a model for using population science to identify risk factors and underlying pathophysiologic mechanisms.

Important NHLBI-Funded Cohort Studies.

The Framingham Heart Study (FHS), begun in 1948, is another pioneering epidemiology study [109]. The original cohort of 5,209 participants aged 30 – 62 years old from Framingham, MA received an extensive medical history, a comprehensive physical exam, and numerous laboratory measurements including

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serum cholesterol and phospholipid and glucose levels [110]. In 1957, results from FHS showed age- and sex-related differences in CHD, and demonstrated that high blood pressure, elevated serum cholesterol levels, and being overweight were predictors of heart disease [111]. The results from FHS contributed to the identification of many factors associated with an increased risk of heart disease. Cigarette smoking, hypertension, and elevated serum cholesterol were all documented as being "risk factors"; a term coined by William B. Kannel, the director of FHS [107]. The success of FHS lead to other observational studies in CVD including the ARIC (Atherosclerosis Risk in Communities) study, the CARDIA (Coronary Artery Risk Development in Young Adults) study, the CHS (Cardiovascular Health Study), and the MESA (Multiethnic Study of Atherosclerosis) [109] . While our laboratory has worked within all of these studies, we have focused predominantly on CHS and MESA.

While CARDIA focuses on younger adults and the progression of risk factors, and ARIC focuses on risk in the middle aged, CHS is a longitudinal cohort study of 5888 participants with the main objectives of identifying risk factors for coronary heart disease and stroke in the elderly [112]. Recruitment began in 1989 at four field centers for participants aged 65 years and older. CHS was the first large scale, epidemiological study to focus on an older population. In addition to a comprehensive physical exam, the baseline exam also included measures of subclinical CVD such as intimal-medial carotid artery wall thickness by ultrasound, electrocardiogram abnormalities, ankle brachial blood pressure index, and cardiac

motion abnormalities by echocardiogram [107], as well as obtaining blood for fluid

biomarkers and DNA samples for genetic studies. Since the study's inception, CHS

researchers have reported on cardiovascular morbidity [113], associated risk factors

for mortality [114], and associations among established and novel risk

factors/biomarkers and blood pressure and subclinical CVD [115] in an older

population.

MESA began in 2000 with the goal of understanding differences among

ethnic groups in both the prevalence and outcomes of subclinical atherosclerosis

[107, 116]. A population based cohort of 6814 men and women between the ages

of 45 and 84 years was recruited at six field centers; the only major exclusions

criterion was prior clinical CVD. The ethnic composition of the cohort is

approximately 38% European American, 28% African American, 23% Hispanic,

and 11% Asian [116]. The baseline exam for the participants was extensive.

Baseline measurements included coronary calcium using computed tomography,

ventricular mass and function using cardiac magnetic resonance imaging, flow-

mediated brachial artery endothelial vasodilation, carotid artery intimal medial wall

thickness (cIMT) and distensibility using ultrasonography, peripheral vascular

disease estimated by ankle and brachial blood pressures, and cardiac function by

electrocardiography. Other assessments included microalbuminuria, standard CVD

risk factors, sociodemographic factors, life habits, and psychosocial factors. Blood

samples were assayed for putative biochemical risk factors and also stored for use

in later studies. DNA was extracted and peripheral blood mononuclear cells were

cryopreserved for genetic studies. Measurement of selected subclinical CVD indicators and risk factors have been repeated at four subsequent exams with a sixth exam currently ongoing. Participants are being followed for identification and characterization of CVD events, including acute myocardial infarction and other coronary heart disease, stroke, peripheral vascular disease, and congestive heart failure; therapeutic interventions for CVD; and mortality [116]. MESA added to literature by being the first major study to assess subclinical CVD by using cardiac MRI [117] and by being the first to show coronary artery calcium differences among ethnicities as well as its association with coronary events [118, 119]. One of the largest contributions that these epidemiology studies have made to the understanding of CVD and the reduction of CVD is the development of risk markers.

<u>Molecular Epidemiology.</u>

A main objective of these CVD epidemiology studies has been (and remains) the detection of persons of high-risk through screening measures. At first, research focused on tests having the greatest predictive power. However, these tests were associated with very small, high-risk populations. Toward the 1970's, the approach for a reduction of CVD began to focus on prevention and control programs centered on the entire population. In essence, CVD became a public health issue. Rose's 1981 publication defined the 'mass strategy' of prevention, which brought the focus of CVD reduction to not only those high risk patients, but

all members of the population [120]. The FHS helped to identify blood pressure, cigarette smoking, blood lipids and adiposity as conventional risk factors for CVD and a risk prediction equation was developed.   The Framingham risk score, calculated using age, sex, systolic blood pressure (treated or untreated status), total cholesterol, HDL cholesterol, smoking behavior, and diabetes status, proved to be a reliable measure of CVD potential not only in European American men and women but also for African American men and women [121, 122]. While a good predictor of CHD risk, these conventional risk factors only explained part of the risk [123, 124]. Researchers have looked to novel biomarkers, independently associated with CHD, to explain not only the remaining risk, but also to provide insight into the biology of CHD development. A number of these biomarkers are associated with the inflammatory response including IL-6, C-reactive protein (CRP), and fibrinogen among others [124-126]. Mouse studies and population studies both linked these inflammation markers to CHD development, progression and outcomes, while adding valuable knowledge of CHD biology and pathology. While they do not significantly improve risk score prediction models in the general population, recommendations suggest usefulness of CRP in certain settings [127]. Yeboah *et al.* compared novel risk markers in MESA and showed that including coronary artery calcium in the risk score improved risk classification for people previously classified as at intermediate risk by the Framingham score [128]. A novel, easily measured biomarker proven both a reliable diagnostic and prognostic tool would be beneficial in clinical practice and in treatment. One area where these biomarkers

may prove useful is in specific subpopulations such as the elderly [126]. 'If the markers reflect different aspects of the disease process at different points in the natural history of the disease, this has implications for the interpretation of marker levels and the timing of future events.' [126]

Genetic Epidemiology.

With the advent of genotyping, the quest for biomarkers, which elucidate the biological pathways of CHD and which present targets for intervention, took on a genetic focus. For decades, scientists have recognized that there is a link between a family history of CHD and an increased risk of heart disease. In 2004, Lloyd-Jones *et al.* showed that when a parent has a history of atherosclerotic CVD, the child will have ~3-fold increased risk of CVD [129]. Beginning with candidate gene studies, moving to genome wide association studies (GWAS) and now whole genome sequencing, researchers are examining associations between genotype and outcomes, genotype and risk factors, and genotype and biomarkers. Some types of CVD have a single causal gene, which has a large effect on a particular phenotype. One example of this was identified in 1985 by Lehrman *et al.* when they found a 5 kilo base deletion in the low-density lipoprotein receptor gene of a patient with a family history of hypercholesterolemia [130]. However, most CVD risk factors seem to result from complex interactions between numerous genes and non-genetic factors. The completion of the Human Genome Project in 2003 [131, 132], followed by the completion of the HapMap Project in 2005 [133, 134], gave scientists the

ability to analyze common genetic variants for their association with CVD phenotypes with a near complete catalog of genes and new technologies. GWAS genotyped common variants, mostly single nucleotide polymorphisms (SNPs) throughout the genome and analyzed these for association with CVD events, risk factors or biomarkers. GWAS results reported in 2007 identified an association between heart disease and SNPs located in the p21 region of chromosome 9 [135-137]. A recent search for results in the GWAS catalogue [138] with the search term 'cardiovascular disease' reveals that there have been 324 publications citing 2224 genetic associations with 183 traits related to CVD. Since GWAS uses tag-SNPs which "tag" functional SNPs scattered across the genome, most of these associated variants are in non-coding regions of the genome and are not causal. However, the associations may point to casual areas of the genome, which need further investigation. Mapping studies and expression profiling as well as work in human cell lines and animal models are necessary to identify the functional variant.

The Molecular Epidemiology of Innate and Adaptive Immunity & CVD.

Through many decades of observation and experimentation, it has become clear that atherosclerosis is a complex disease process involving cellular proliferation, biochemical processes, genetics and environmental influences. While, as noted above, there is ample basic research to support the critical role of innate and adaptive immunity in atherosclerosis and CVD, there is only a small

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body of work that directly addresses these roles through blood measures in epidemiologic cohorts. Regarding $CD4^+$ T helper cells, Tracy *et al.* have shown a strong, independent association of Th1 bias with both coronary calcification and cIMT [139]. They went on to show that the single greatest association of an environmental variable with Th1 bias involved cytomegalovirus response. Work by Engelbertsen *et al.* in the Malmo Diet and Cancer Study showed an association between T cells and atherosclerosis; an analysis of seven hundred participants found that a high number of Th2 cells was associated with a decreased mean cIMT [77]. This was confirmed by Tracy *et al.,* who showed that Th2 cells were associated with lower cIMT in MESA [139]. Increased numbers of Th2 cells were associated with a reduced risk of myocardial infraction in women and serum IL-4 was associated with a reduced risk of CVD in both men and women [78]. Regarding the activation of adaptive immunity as assessed by measuring Effector/Memory cells vs. Naïve cells, Ammirati *et al.* analyzed data from over 400 patients and found that T_{EM} cells were strongly related to increased IMT. Olson *et al.* analyzed T_{EM} levels from more than 900 participants in the Multi-Ethnic Study of Atherosclerosis and found that T_{EM} levels were positively associated with cIMT and the inflammatory cytokine IL-6 [41].

An analysis of the Framingham Heart Study which combined genome-wide association studies, gene expression and phenotypes found that B cells contributed to coronary artery disease [140]. This work linked phenotypic observations with molecular networks and genetic results supporting the theory that B cells are pro-

atherogenic and substantiating murine results that B cells are involved in lipid dysregulation [141].

<u>Research Reported in this Dissertation.</u>

The research reported here studied aspects of both the innate and the adaptive immune system in CVD from both molecular and genetic epidemiological standpoints in an effort to increase our understanding of the CVD disease process. '*Plasma Levels of Soluble Interleukin-2 Receptor α Associations with Clinical Cardiovascular Events and Genome-Wide Association Scan*' examines the relationship of cell-mediated immunity to CVD through the association of soluble interleukin-2 receptor α (sIL-2Rα; a biomarker of T cell activation) with CVD risk factors, CVD events and with common genetic polymorphisms. Both interleukin-2 and interleukin-2 receptor play significant roles in the proliferation and differentiation of T cells.

The second paper '*Circulating Soluble CD163, Genetic Associations, and Risk of Cardiovascular Disease and All-Cause Mortality in Older Persons: the Cardiovascular Health Study*', examines the innate immune systems through the association of soluble CD163 (sCD163), a marker for M2 macrophages, with CVD risk factors, outcomes, and common polymorphisms.

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Chapter 2:

Plasma levels of sIL-2Rα: associations with clinical cardiovascular events and genome- wide association scan

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sIL-2Rα with CVD events and genome-wide scan

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Word count: 5662

Total number of figures and tables: 5 figures and 4

tables TOC category: Clinical population studies

TOC subcategory: Vascular biology

ABSTRACT

Objective: Interleukin-2 receptor subunit alpha (IL-2Rα) regulates lymphocyte activation, which plays an important role in atherosclerosis. Associations between soluble IL-2Rα and cardiovascular disease (CVD) have not been widely studied and little is known about the genetic determinants of sIL-2Rα levels. **Approach and Results:** We measured baseline levels of sIL-2Rα in 4408 European-American (EA) and 766 African-American (AA) adults from the Cardiovascular Health Study (CHS) and examined associations with baseline CVD risk factors, subclinical CVD and incident CVD events. We also performed a genome-wide association study (GWAS) for sIL-2Rα in CHS (2964 EAs and 683 AAs) and further combined CHS EA results with those from two other EA cohorts in a meta-analysis (N=4464 EAs). In age, sex- and race- adjusted models, sIL-2Rα was positively associated with current smoking, type 2 diabetes, hypertension, insulin, waist circumference, C-reactive protein, interleukin-6, fibrinogen, internal carotid wall thickness, all-cause mortality, CVD mortality, and incident CVD, stroke and heart failure. When adjusted for baseline CVD risk factors and subclinical CVD, associations with all- cause mortality, CVD mortality and heart failure remained significant in both EAs and AAs. In the EA GWAS analysis, we observed 52 single nucleotide polymorphisms (SNPs) in the chromosome 10p15-14 region, which contains *IL2RA*, *IL15RA* and *RMB17*, that reached genome-wide significance ($p<5\times10^{-8}$). The most significant SNP was rs7911500 ($p=1.31\times10^{-75}$). The EA meta-analysis results were highly consistent with CHS-only

results. No SNPs reached statistical significance in the AAs. **Conclusions:** These results support a role for sIL-2Rα in atherosclerosis and provide evidence for multiple associated SNPs at chromosome 10p15-14.

Abbreviations

Interleukin-2 (IL-2)

Soluble interleukin-2 receptor alpha (sIL-2Rα)

Cardiovascular disease (CVD)

European-American (EA)

African-American (AA)

Cardiovascular Health Study (CHS)

Genome-wide association study (GWAS)

Single nucleotide polymorphism (SNP)

Health, Aging and Body Composition Study (Health ABC)

Quality control (QC)

Intima media thickness (IMT)

Coronary heart disease (CHD)

Congestive heart failure (CHF)

Systolic blood pressure (SBP)

Low density lipoprotein (LDL)

C-reactive protein (CRP)

Standard deviation (SD)

High density lipoprotein (HDL)

Coronary artery disease (CAD)

Body mass index (BMI)

Odds ratio (OR)

Interleukin-6 (IL-6)

Principle components (PCs)

INTRODUCTION

Interleukin (IL)-2 and IL-2 receptor (IL-2R) signaling play an important role in regulating both tolerance and immunity. IL-2 is a T cell growth factor, inducing the proliferation and differentiation of antigen-activated T cells[1,2], and is particularly important in the development of regulatory T cells in the thymus[3]. The IL-2R is a trimeric receptor composed of the IL-2Rα subunit (CD25), the IL-2Rβ subunit (CD122), and the IL-2γc subunit (CD132). IL-2Rα is specific for IL-2R, while IL-2Rβ and IL-2Rγc are shared components of other cytokine receptors (e.g., IL-15)[4,5]. sIL-2Rα results from the proteolytic cleavage of IL-2Rα at the cell surface by a membrane metalloproteinase (ectodomain shedding)[6]; which is encoded by *IL2RA* on human chromosome 10. The function of sIL-2Rα has not been fully elucidated. Since the sIL-2Rα has IL-2 binding kinetics similar to the membrane form, sIL-2Rα may serve to mitigate the immune responses by binding and sequestering IL-2[7].

High plasma levels of sIL-2Rα have been associated with autoimmune diseases including Crohn's disease[8], rheumatoid arthritis[9], and multiple sclerosis[10] and higher levels have been observed in patients with coronary artery disease[11]. Murine models have shown that IL-2 increases regulatory T cell numbers in atherosclerotic plaques and also reduces the size of those plaques[12]. When the IL-2 receptor is blocked in the same model, the plaque reduction is negated.

Despite its potential importance in the immune system and cardiovascular disease (CVD), sIL-2Rα has not been widely investigated in large prospective population-

based studies of CVD. A 2003 study in the Health, Aging and Body Composition (Health ABC) study did not result in evidence for a significant association between sIL-2Rα and CVD; however, sIL-2Rα measurements were only available in a subset of N=499 participants. In addition, little is known about the genetic determinants for sIL-2Rα levels. While genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in the *IL-2RA* gene for several autoimmune diseases[10], there have been no published reports for GWAS of serum levels of sIL-2Rα.

In the current study, we examined sIL-2Rα levels in the Cardiovascular Health Study (CHS), a cohort of older adults with follow-up for incident clinical CVD and mortality for up to 20 years. We examined the relationships between sIL-2Rα at baseline and incident events as well as cross-sectionally with other CVD and inflammatory markers (fibrinogen, C-reactive protein [CRP], and IL-6). We then conducted a GWAS and region-specific conditional analyses to identify genetic variants associated with sIL-2Rα levels. Finally, we performed a GWAS meta-analysis, including results from two additional studies: the Health ABC study and the Multi-Ethnic Study of Atherosclerosis (MESA), to increase our power to detect associated variants not detected in CHS alone.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Associations between sIL2Rα and baseline CVD risk factors and other inflammation biomarkers

The characteristics of the 5174 CHS participants with sIL-2Rα measurements at the baseline exam are summarized in Table 1, and Spearman correlation coefficients for sIL-2Ra with each continuous CVD risk factor and IMT are given in Supplemental Table 1. sIL-2Rα levels were on average higher in older individuals, higher in men, and higher in EAs. At baseline, mean sIL-2Rα levels were 1146.4 pg/mL (standard deviation (SD)=507.5 pg/mL) and 1101.6 pg/mL (SD=556.4 pg/mL) in EA men and women, respectively; and 873.1 pg/mL (SD=505.5 pg/mL) and 910.9 pg/mL (SD=581.2 pg/mL) in AA men and women, respectively. In age-, race- and sex-adjusted models sIL-2Rα was additionally associated with current smoking, type 2 diabetes, hypertension, fasting insulin, waist circumference, CRP, IL-6, fibrinogen, and internal carotid wall IMT and negatively associated with LDL and high density lipoprotein (HDL) cholesterol. After further adjustment, sIL2Ra levels remained associated with age, race, smoking, hypertension, lipids, and inflammation.

Incident events analysis

We performed survival analysis in 4406 EAs and 768 AAs. There were 2985(EA)/451(AA) all- cause deaths; including 1202/186 cases of cardiovascular mortality. There were 1234/195 incident cases of CHD, 762/117 incident strokes, and 1246/199 incident cases of heart failure (fatal and non-fatal events). When minimally adjusted for age, sex, race, and study site, baseline sIL-2Rα was significantly

associated with increased risk for all outcomes in both EAs and AAs (Table 2).

Results were slight attenuated in EAs after additional adjustment for baseline risk

factors except for stroke, and not attenuated at all in AAs. When further adjustment

was made for inflammation status and measures of subclinical CVD, all-cause

mortality and heart failure remained significant for both the EAs and AAs;

cardiovascular mortality remained significant only for EAs; and stroke remained

significant only for AAs. Effect estimates were mostly similar between EAs and

AAs (sIL-2Rα as a continuous predictor). In analyses where sIL-2Rα was modeled in

quartiles, the risk of increased sIL-2Rα for stroke in AAs appears to be driven by the

highest quartile of sIL-2Rα as compared to a more graded effect in EAs. We

estimated a 63%(EA)/67%(AA) increased risk for all-cause mortality, and a

57%/71% increased risk of heart failure for individuals in the fourth quartile versus

those in the first quartile, after adjustment for both established CVD risk factors,

inflammation biomarkers and subclinical measures of CVD. For cardiovascular

mortality, this estimated increased risk was 64% in EAs and not significant in AAs;

and 28% in EAs and 130% in AAs for stroke.

Genome-wide association study of sIL-2Rα in CHS EA and AA

We conducted a race-stratified GWAS in 2964 EAs and 683 AAs from CHS that had

both sIL-2Rα measurement and GWAS data available. A total of 52 SNPs in the

chromosome 10p15- p14 region (containing *IL2RA, IL15RA,* and *RBM17*) reached

genomewide significance ($p<5x10^{-8}$) in the EA analysis. The most significant SNP

was rs7911500 ($p=1.31x10^{-75}$), which is located between *IL2RA* and *IL15RA*. No

other regions reached genome-wide significance in the EA analysis. No SNPs reached genome-wide significance in the AA analysis. The top findings in AAs were for an intergenic SNP between *BRE* and *FOSL2* on chromosome 2 (rs7602568, p=5.8x10^{-6}) and an intronic SNP in *ADK* (rs12220238, p=8.3x10^{-6}), nearly 70Mb from *IL2RA* on chromosome 10. *IL2RA* SNP rs7911500 (p=0.52) demonstrated no evidence for association in AAs, though the minor allele frequency for this variant in AAs was only 2.5% (compared to 13.4% in EAs). Several chromosome 10p15-p14 SNPs between *IL15RA* and *IL2RA* (lead SNP rs8177607, p=3.2x10^{-4}) provided nominal evidence for an association in AAs. rs8177607 showed no evidence for association in EAs (p=0.65).

Conditional and multiple variant analysis of *IL2RA* region in CHS EA

In the CHS EAs, as described in Methods, we performed an iterative, forward-selection conditional analysis of the chromosome 10p14-15 region (approximately a 200 Kb span), beginning with conditioning on the rs7911500 SNP (Figure 1). The order of additional SNP conditioning was rs791590 (pcond=7.0x10^{-35}; an intronic SNP in *IL2RA*), rs8177757 (pcond=2.3x10^{-10}; located between *IL15RA* and *IL2RA*), rs10905716 (pcond=3.3x10^{-9}; located between *IL2RA* and *RBM17*), and finally rs7924005 (pcond=4.4x10^{-10}; located in *LOC101928080* downstream from *RBM17*). There was still nominal evidence for further association of SNPs in the region after adjusting for these five, although none reached genome-wide significance. The multiple variant penalized regression method LLARRMA identified six SNPs

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(Resample Model Inclusion Probability (RMIP) > 0.8; namely, rs2104286 (RMIP =1.00), rs7924005 (RMIP=0.995), rs10905716 (RMIP=0.995), rs4749955 (RMIP=0.911), rs11256497 (RMIP=0.899), and rs7898880 (RMIP=0.871)) that were consistently associated with sIL-2Rα levels across alternative resamplings of the data. Our top SNP in our initial GWAS, rs7911500 (RMIP = 0.002) was not predicted to be important in the multi-SNP LLARRMA model. However, LLARRMA did include the top variant, rs791590 (RMIP = 0.592), from the conditional analysis after conditioning on rs7911500, more often than not in the final multi-SNP model across different resamplings of the data.

The five index SNPs identified in the conditional analysis, in total explain approximately 14% of the variation in sIL-2Rα levels after adjusting for age, sex and PCs to account for population admixture. When we further examined these five SNPs individually for association w i t h i n c i d e n t cardiovascular events in CHS, none of them was significant. We also observed no evidence for an association between a genetic risk score (equal to the sum of the alleles individually associated with increased sIL-2Rα for these five SNPs) and clinical events.

Meta-analysis of CHS, MESA and Health ABC EA

We conducted a meta-analysis combining GWAS results for CHS (N=2964), MESA (N=714) and Health ABC (N=786) EA participants to increase power to detect loci potentially missed in the CHS-only analysis. Meta-analysis results were highly consistent with those observed in the CHS-only analysis, where only variants in the chromosome 10p15-p14 region (*IL15RA/IL2RA/ RBM17)* reached statistical

significance (Figure 2). A total of 95 SNPs in this region were significant in the meta-analysis, and the most significant SNP remained rs7911500 ($p = 1.1 \times 10^{-100}$).

We assessed the evidence for association between sIL2r and 1093 variants reported as significant in prior GWAS studies, according to the NHGRI GWAS catalogue (https://www.genome.gov) for the traits listed in Table 1. Only our top two SNPs, rs7911500 and rs12722606, which were previously reported to be significantly associated with an inflammatory phenotype based on the IL-6 – CRP pattern, were statistically significant with the sIL2rα levels in this candidate variant analysis after Bonferroni correction for 1093 test ($p < 4.6 \times 10^{-5}$). Additionally, we searched the CARDIoGRAM+C4D database containing data from multiple GWAS (63,746 case and 130681 controls) combined to determine variants associated with coronary artery disease and myocardial infarction (http://www.cardiogramplusc4d.org[14,15,16]). No significant associations (all $p > 0.05$) between our SNPs and CVD were identified.

DISCUSSION

We report the first large-scale assessment of sIL-2Rα for association with CVD related traits and events in a prospective cohort and the first GWAS for SNPs associated with sIL-2Rα levels. The major findings from this study are: A) sIL-2Rα levels are associated with a number of established CVD risk factors and carotid IMT, a measure of subclinical CVD. B) Plasma sIL-2Rα predicted all-cause mortality and cardiovascular mortality independently of CVD risk factors and baseline subclinical CVD. C) In CHS alone (N=2961) we identified 52 SNPs in the chromosome 10p15-p14 region with genome-wide significance for association with plasma sIL-2Rα levels;

most significant was rs7911500, intergenic to *IL15RA* and *IL2RA*. D) Conditional analysis indicated that there are multiple SNPs independently associated in this region; the five most significant loci, in total explain approximately 14% of the variation in plasma sIL-2Rα levels in CHS EAs. E) Combining results from EAs in CHS and two additional cohort studies, MESA and Health ABC (n=4464), did not result in any additional significantly associated loci. F) We did not identify any significant associations in the CHS AAs, although we did observe nominal evidence for association in the *IL15RA*/*IL2RA* region. G) There was no evidence that sIL-2Rα-associated SNPs were associated with incident clinical events in CHS; we also observed no evidence of association with coronary artery disease and myocardial infarction in a search of the CARDIoGRAM+C4D database results for these SNPs[14,15,16].

Activated T lymphocytes play an important role in atherosclerosis promoting chemokine secretion, inflammation, and eventually, the formation of atherosclerotic plaques. IL-2, produced by T helper 1 cells, has been found in plaques and contributes to the development of atherosclerosis by its interaction with the IL-2 receptor increasing lymphocyte activation[13]. IL-2 stimulates the synthesis of interferon gamma thereby promoting an increased immune response and atherosclerotic progression. However, IL-2 also promotes regulatory T cells, and may have an atheroprotective role as well[12].

While sIL-2Rα is a strong biological candidate for use as a biomarker for CVD morbidity and mortality, epidemiologic studies have been limited. Analysis in the

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Health ABC study did not identify evidence for an association between sIL-2Rα and either subclinical (p=0.27) or clinical CVD (p=0.27), but measured sIL-2Rα levels were only available on a subset of 499 of the 3045 participants with incident event data. Although it was not statistically significant, median sIL-2Rα level was slightly higher in those with incident clinical CVD as compared to those with no CVD (1.4 mg/mL versus 1.2 mg/mL)[14]. Investigators from another study of 286 Japanese patients that underwent angiography (167 coronary artery disease [CAD] cases and 119 controls) reported a significant positive association of sIL-2Rα and cross-sectional CAD case status based on extreme quartiles of sIL-2Rα (p=0.005 for minimally adjusted model and p=0.035 for model with additional adjustment for CVD risk factors)[11]. The current study represents the first well-powered effort examining sIL-2Rα level prospectively with clinical CVD events and all-cause mortality. We observed statistically significant evidence for all incident events examined (all-cause mortality, CVD mortality, incident CHD, stroke and heart failure) in minimally adjusted models, and for all-cause mortality, CVD mortality and incident heart failure in fully adjusted models. We found sIL-2Rα levels to be significantly associated with carotid intima-media thickness in the minimally adjusted model; although this did not remain significant when other cardiovascular risk factors were added to the model.

Fifty-two chromosome 10p15-p14 SNPs were significantly associated ($p<5\times10^{-8}$) with plasma sIL-2Rα levels in CHS EAs; no other regions reached genome-wide significance. The most significant SNP, rs7911500, was located between *IL15RA* and *IL2RA*. Iterative conditional analyses identified a total of five significant "independent"

SNPs across the region. LLARRMA identified six SNPs that were consistently associated with sIL-2Rα levels across alternative resamplings of the data. Both iterative conditional analyses and LLARRMA provide compelling evidence for the existence of multiple important causal variants in the region, though they did not agree with respect to the importance of our most significant SNP, rs7911500. Higher density genotype data, including both common haplotype-tagging variants and less-common putative functional variants, will be necessary to fine map the association signals in this region. Two of our significant SNPs in the region, rs2104286 (p=4.9x10^{-59}; the top SNP identified by LLARRMA) and rs11594656 (p=1.5x10^{-41}), have been shown to function in transcription factor binding. These SNPs have also been reported to be associated with sIL2Rα levels and type 1 diabetes and multiple sclerosis[15, 16].

No regions reached genome-wide significance in the smaller cohort of CHS AAs. Nominal evidence for association in AAs was detected between *IL15RA* and *IL2RA* (best result: rs8177607, p=3.2x10^{-4}). The lead SNP in EAs, rs7911500, was less polymorphic in AAs and demonstrated no evidence for association. Similarly, no evidence for association was found for rs791590 (p=0.31) or rs10905716 (p=0.43), two significant variants in EAs in the conditional analyses. The two other significant SNPs in the conditional analyses, rs8177757 and rs7924005, were not successfully imputed in the AAs. The difference in findings between EAs and AAs could suggest different risk variants in the two populations, be reflective of different LD structures in the region that mask common underlying causal variants, or be the result of lower power in AAs. There are strong allele frequency differences between the two

populations for many of the EA SNPs in the region (see Supplemental Table 2 for

frequencies in HapMap CEU and YRI populations) and the AA sample size is

considerably smaller than for EAs.

Interestingly, the top SNP from LLARRMA, rs2104286, in EAs was nominally

significant in AAs (p=0.011) despite the lower estimated frequency of the minor allele

in AAs (MAF=0.065) compared to EAs (MAF=0.27). The effect estimates for the

SNP were similar in AAs (β= -0.17) and EAs β=-0.15), where carriers of the minor

allele were predicted to have lower sIL-2Rα levels.

Elevated sIL-2Rα levels have been shown to be associated with a number of

autoimmune diseases and may predict a relapse of those diseases[7]. We found a

number of *IL2RA* SNPs previously associated with autoimmune-related diseases to be

significantly associated with sIL-2Rα levels. A number of our significant SNPs have

also been observed to be associated in GWAS, fine mapping studies and SNP specific

genotyping studies for autoimmune diseases including Graves' disease (rs11594656,

Odds Ratio (OR)=1.54, p=0.0053)[17] , vitiligo (rs706779 OR=1.27, p=3X10^{-9})[18],

Crohn's disease (rs12722489, OR=1.11, p=3X10^{-9}) [19] , type 1 diabetes (rs7090530,

OR=1.23, p=0.003)[20] and multiple sclerosis (rs2104286, OR=0.81, p=0.017)[20] . Our

two most significant SNPs, rs7911500 and rs12722605, were found to be

significantly associated with an inflammatory phenotype derived from the high-

sensitivity CRP-interleukin-6 (IL-6) pattern in a G W A S o f the Genetics of Lipid

Lowering Drugs and Diet Network (p=5x10^{-9} and p=5x10^{-8})[21]. The nature of this

association is uncertain; it is possible that these variants or others in linkage

disequilibrium with them are directly increasing the sIL-2Rα levels which in turn results in downstream increases in both IL-6 and CRP.

There are several limitations in the current study which should be noted. We only analyzed common variants; rare polymorphisms may account for much of the variability in the sIL-2Rα levels. Also, we had weak statistical power to detect associations in AAs. Finally, our study was focused on older adults and the results may not be generalizable to other populations.

Our findings suggest that serum sIL-2Rα, a surrogate marker of T lymphocyte activation, may be a valuable novel biomarker for all-cause mortality, cardiovascular morality, stroke and heart failure in older adults. Additional studies are needed to assess whether sIL-2Rα levels predict mortality in younger populations. Also, further studies are needed a) to identify the causal variants in the chromosomal region harboring *IL15RA* and *IL2RA* influencing sIL-2rα, b) to provide very large and multi-ethnic samples to identify additional genetic loci for this trait, and c) to determine the complex biology of the genetic control of IL-2/IL-2R interactions with respect to regulatory T cell promotion and pro-inflammatory cytokine production.

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Disclosures:

None.

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Significance

This study found that sIL-2Rα, a regulator of white blood cells, is associated with a number of cardiovascular disease risk factors, as well as with all-cause mortality, cardiovascular disease   mortality, and heart failure in the Cardiovascular Health Study. Analysis of genetic variants in European Americans found a number of variants in the chromosome 10 region containing the genes *IL2RA, IL15RA, and RMB17* to be significantly associated with sIL-2Rα. These results provide support for a role of sIL-2Rα in atherosclerosis and cardiovascular disease.

Table 1: Associations between Soluble IL-2Rα and Other Cardiovascular Risk Factors and Atherosclerosis at the CHS Baseline Examination

Each variable was examined for association with sIL-2Ra in a separate model, adjusting for the variables listed in each model; the exception is that a variable is not adjusted for itself when it is the variable being tested. β for all measures except sex, race, diabetes, and hypertension are for a 1–SD change in the predictor; sIL-2Rα ln-transformed p values;
*P<0.01; **P<0.001; ***P<0.0001.
Model A: adjusted for age, race, and sex.
Model B: adjusted for age, race, sex, smoking, diabetes mellitus, hypertension, systolic blood pressure, and BMI.
Model C: adjusted for age, race, sex, smoking, diabetes mellitus, hypertension, systolic blood pressure, BMI, LDL cholesterol, HDL cholesterol, C-reactive protein, interleukin-6, and fibrinogen.

Baseline Characteristics (Mean±SD or %)	Model A β±SE	Model B β±SE	Model C β±SE
Age, y (5.6)	0.074±0.005***	0.078±0.006***	0.067±0.006***
Female sex (57.2 %)	-0.029±0.011*	-0.031±0.011*	0.017±0.012
Black race (14.8%)	-0.120±0.007***	-0.133±0.008***	-0.134±0.008***
Current smoking (54.0 %)	0.100±0.164***	0.108±0.017***	0.079±0.017***
Type 2 diabetes (16.2 %)	0.056±0.014**	0.042±0.015**	-0.002±0.015
Hypertension (44.5 %)	0.042±0.006***	0.046±0.007***	0.030±0.007***
Systolic blood pressure, mm Hg (136.6±21.8)	0.013±0.005	-0.011±0.026	0.003±0.007
LDL cholesterol, mg/dL (129.8±35.6)	-0.030±0.005***	-0.030±0.005***	-0.032±0.007***
HDL cholesterol, mg/dL (54.2±15.7)	-0.055±0.006***	-0.050±0.006***	-0.046±0.006***
Triglycerides, mg/dL (139.8±76.7)	0.007±0.005	-0.002±0.006	-0.025±0.008*
Glucose, mg/dL (111.1±35.9)	0.013±0.005	-0.009±0.008	-0.013±0.007

Insulin, IU/mL (17.4±27.4)	0.023±0.005***	0.017±0.005*	0.011±0.005
BMI, kg/m^2 (26.6±4.7)	0.009±0.006	0.005±.006	-0.016±0.006*
Waist circumference, cm (94.4±13.1)	0.014±0.005*	0.016±0.010	0.003±0.010
C-reactive protein, mg/L (4.8±8.0)	0.083±0.005***	0.080±0.005***	0.043±0.008***
Interleukin-6, pg/mL (2.2±1.8)	0.059±0.005***	0.051±0.005***	0.024±0.006***
Fibrinogen, mg/dL (323.8±67.3)	0.067±0.005***	0.063±0.054***	0.032±0.006***
Internal carotid wall thickness, mm (1.5±0.7)	0.027±0.006***	0.014±0.006	0.009±0.006

Table 2: Hazard ratios (HR) between sIL-2Rα and incident events in CHS

A: European Americans

Model 1: Adjusted for age, sex, and study site

Model 2: Model 1 + smoking, diabetes, hypertension, SBP, LDL, baseline CVD

Model 3: Model 2 + C-reactive protein, interleukin-6, fibrinogen, carotid intima-media thickness
‡ Hazard ratios for a 1-SD unit increase in soluble IL-2R.
‡‡Hazard ratios comparing quartiles to first quartile of soluble IL-2R. P values; *p<0.05; **p<0.005;
***p<0.0001.

	All-Cause Mortality (n=2985 events)	Cardiovascular Mortality (n=1202 events)	Coronary Heart Disease (n=1234 events)	Stroke (n=762 events)	Heart Failure (n=1246 events)
Model	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)
Minimal‡ (1)	1.17 (1.14-1.19)***	1.16 (1.11-1.20)***	1.11 (1.05-1.15)***	1.08 (1.01-1.14)*	1.16 (1.12-1.21)***
Multivariable ‡ (2)	1.16 (1.13-1.19)***	1.15 (1.10-1.20)***	1.10 (1.05-1.15)***	1.06 (0.99-1.14)	1.17 (1.12-1.22)***
Subclinical ‡ (3)	1.14 (1.11-1.18)***	1.13 (1.07-1.19)***	1.05 (0.99-1.11)	1.03 (0.95-1.12)	1.14 (1.08-1.19)***
2nd Q vs 1st Q ‡‡ (3)	1.17 (1.04-1.32)*	1.19 (0.98-1.45)	1.16 (0.97-1.39)	1.46 (1.16-1.84)**	1.17 (0.97-1.40)
3rd Q vs 1st Q ‡‡ (3)	1.25 (1.11-1.41)***	1.38 (1.10-1.62)**	1.23 (1.03-1.46)*	1.38 (1.09-1.74)*	1.16 (0.97-1.40)
4th Q vs 1st Q ‡‡ (3)	1.63 (1.45-1.83)***	1.64 (1.36-1.99)***	1.16 (0.97-1.402)	1.28(1.01-1.64)*	1.57 (1.31-1.88)***

Table 2: Hazard ratios (HR) between sIL-2Rα and incident events in CHS

B: African Americans

Model 1: Adjusted for age, sex, and study site
Model 2: Model 1 + smoking, diabetes, hypertension, SBP, LDL, baseline CVD
Model 3: Model 2 + C-reactive protein, interleukin-6, fibrinogen, carotid intima-media thickness
‡ Hazard ratios for a 1-SD unit increase in soluble IL-2R.
‡‡Hazard ratios comparing quartiles to first quartile of soluble IL-2R. P values; *p<0.05; **p<0.005;
***p<0.0001.

Model	All-Cause Mortality (n=451 events) HR (95%CI)	Cardiovascular Mortality (n=186 events) HR (95%CI)	Coronary Heart Disease (n=195 events) HR (95%CI)	Stroke (n=117 events) HR (95%CI)	Heart Failure (n=199 events) HR (95%CI)
Minimal‡ (1)	1.17 (1.10-1.25)***	1.14 (1.02-1.28)*	1.15 (1.04-1.28)**	1.16 (1.02-1.33)*	1.18 (1.07-1.29)**
Multivariable ‡ (2)	1.17 (1.04-1.26)***	1.15 (1.02-1.31)*	1.19 (1.06-1.33)**	1.21 (1.04-1.40)*	1.19 (1.07-1.32)**
Subclinical ‡ (3)	1.16 (1.07-1.18)***	1.07 (0.89-1.29)	1.12 (0.96-1.31)	1.22 (1.06-1.41)*	1.21 (1.09-1.35)***
2nd Q vs 1st Q ‡‡ (3)	0.99 (0.76-1.29)	1.02 (0.67-1.55)	1.42 (0.98-2.06)	0.94 (0.55-1.61)	1.37 (0.93-2.01)
3rd Q vs 1st Q ‡‡ (3)	1.31 (0.99-1.73)	1.48 (0.94-2.23)	1.50 (0.99-2.29)	1.20 (0.67-2.14)	1.84 (1.23-2.77)**
4th Q vs 1st Q ‡‡ (3)	1.67 (1.22-2.28)**	1.33 (0.79-2.25)	1.09 (0.61-1.94)	2.30 (1.34-3.95)***	1.71 (1.05-2.80)*
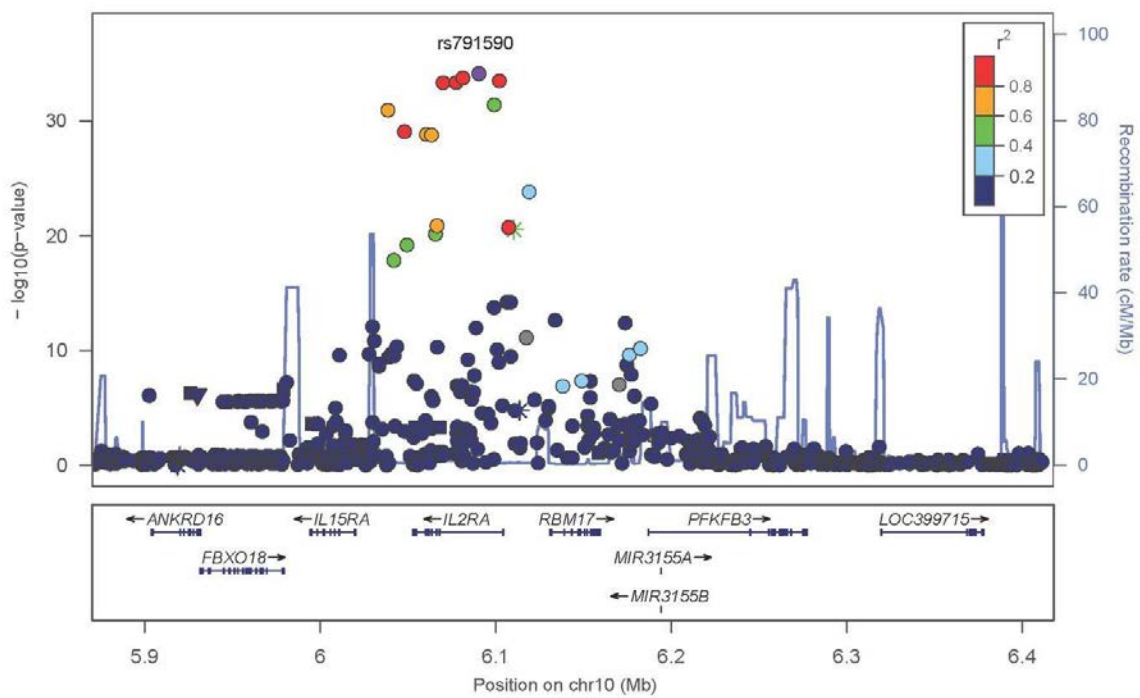
Figure 1a: Conditional Analysis - CHS IL2sR alpha adjusted by rs7911500
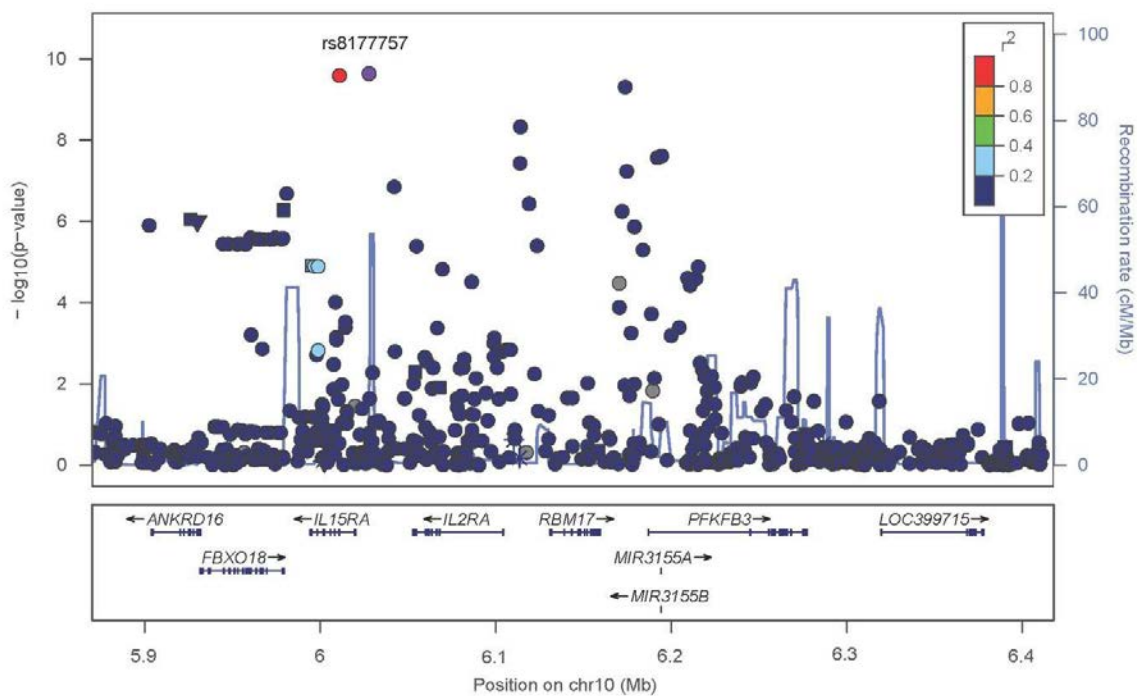
Figure 1b: Conditional Analysis - CHS IL2sR alpha adjusted by rs7911500 and rs791590
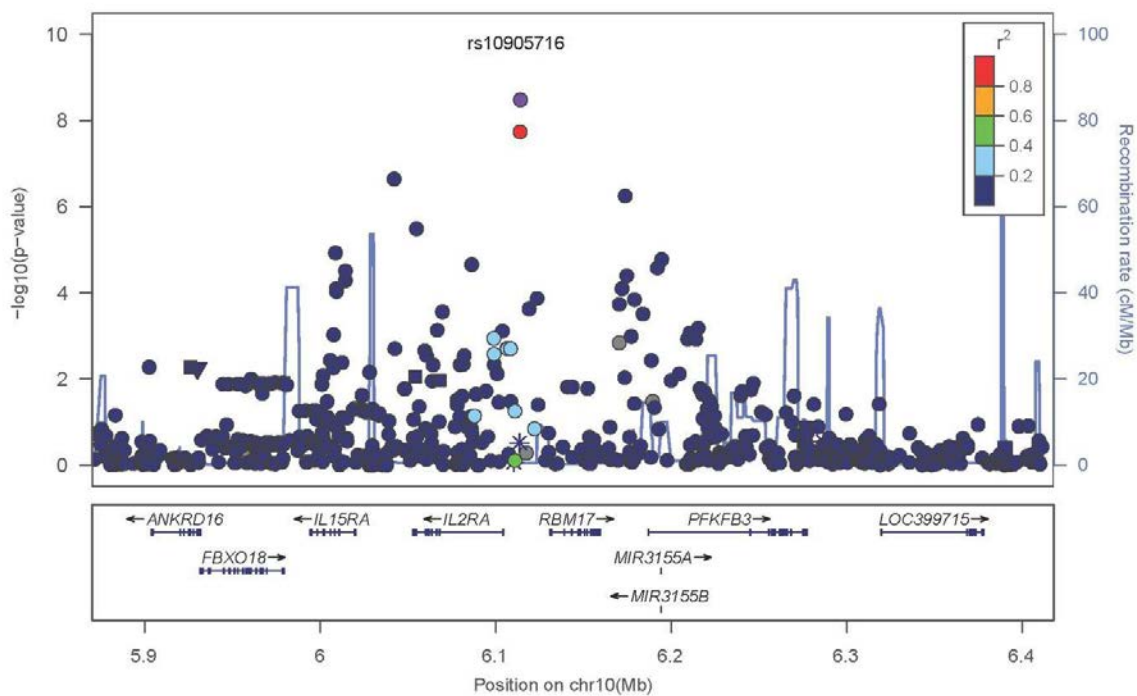
Figure 1c: Conditional Analysis - CHS IL2sR alpha adjusted by rs7911500, rs791590 and rs8177757
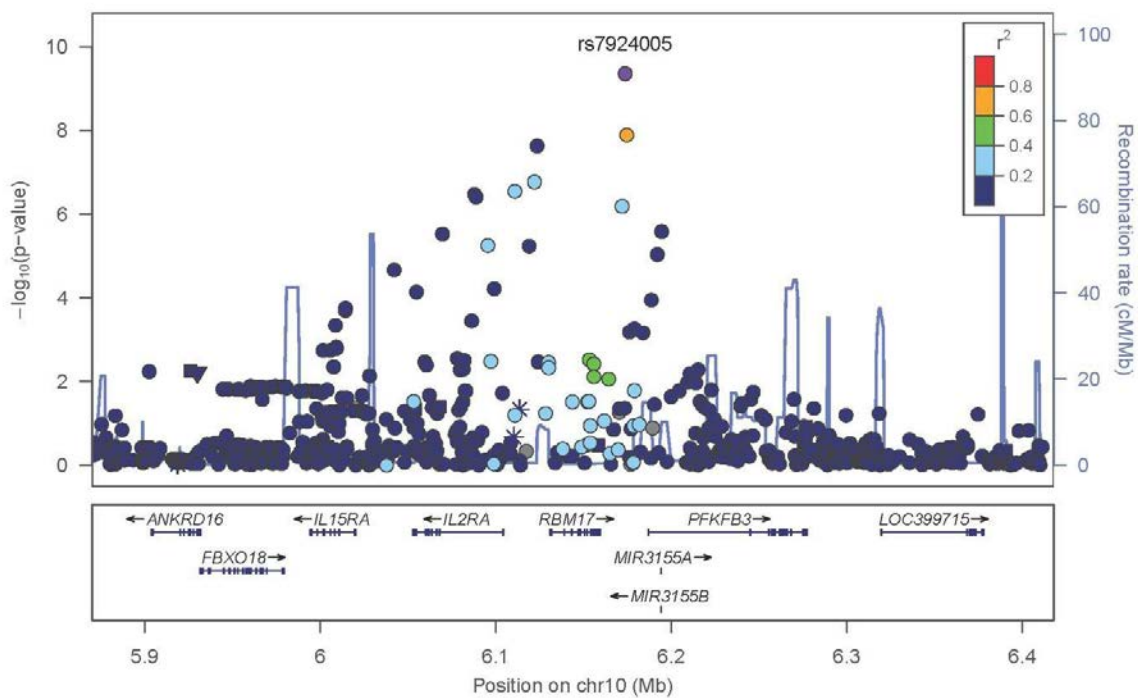
Figure 1d: Conditional Analysis - CHS IL2sR alpha adjusted by rs7911500, rs791590, rs8177757, and rs10905716
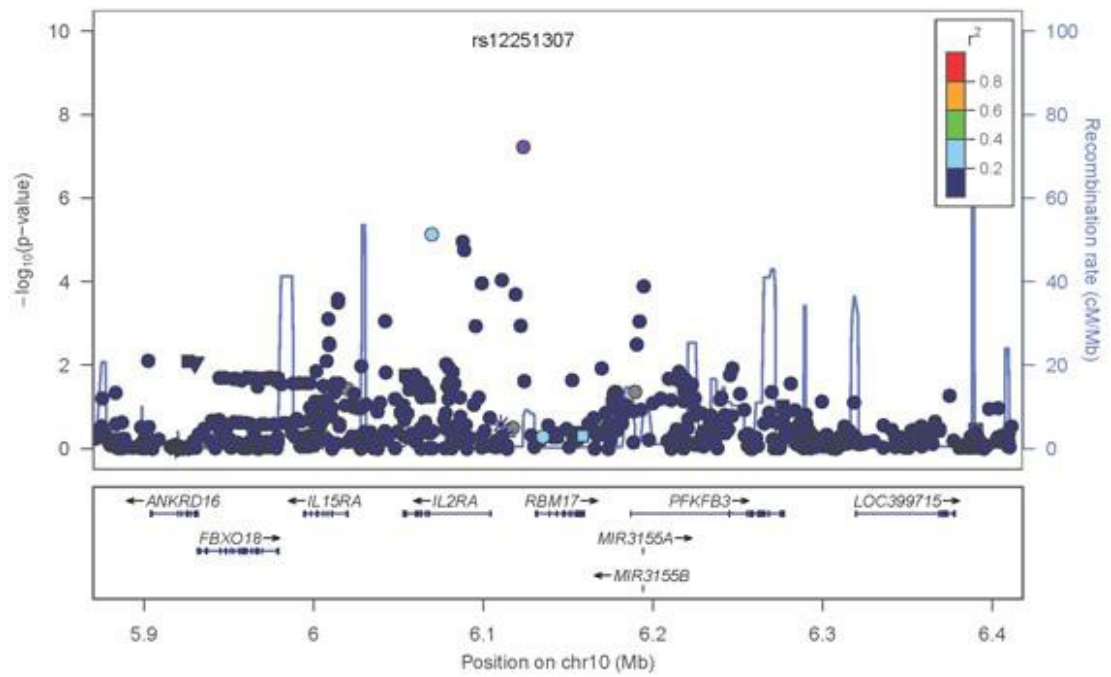
Figure 1e: Conditional Analysis - CHS IL2sR alpha adjusted by rs7911500, rs791590, rs8177757, rs10905716, and rs7924005
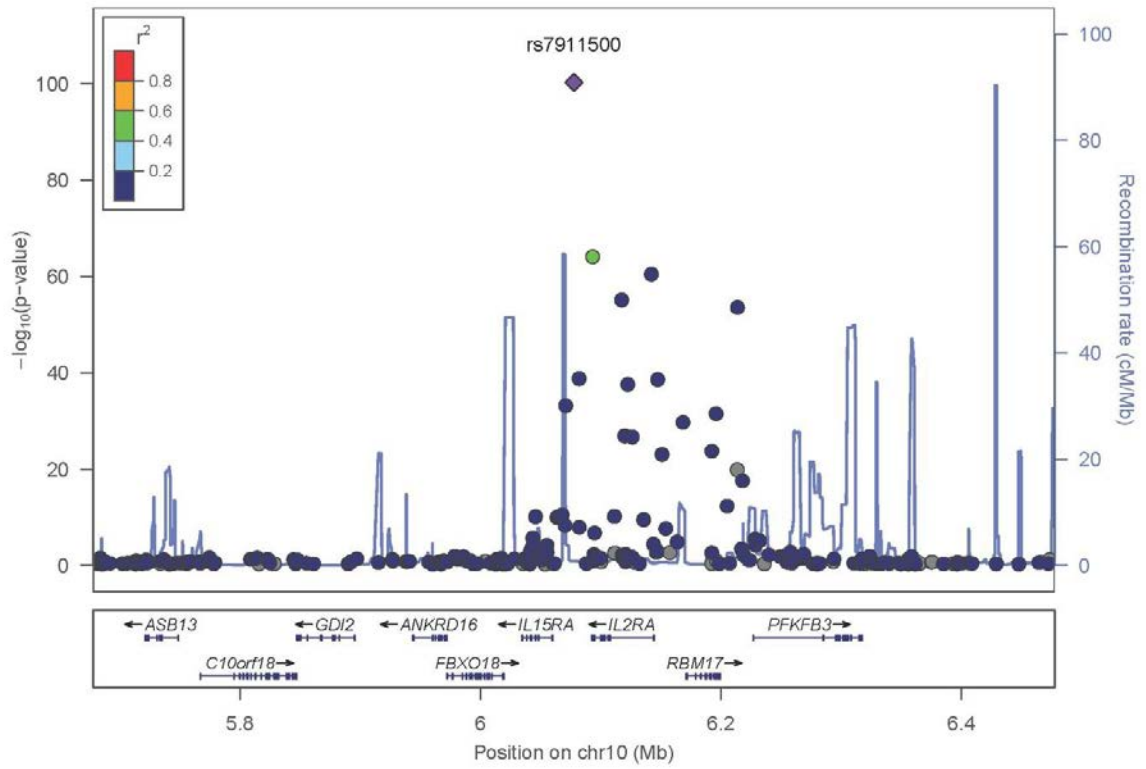
Figure 2:   **Meta Analysis of European Americans**

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

Plasma Levels of Soluble Interleukin-2 Receptor α: Associations With Clinical Cardiovascular Events and Genome-Wide Association Scan

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Study samples

The Cardiovascular Health Study (CHS) is a prospective population-based cohort study of men and women recruited at age 65 or older at baseline. The original cohort of 5201 participants was recruited between 1988 and 1989 at four field centers: Forsyth County, NC; Sacramento County, CA; Washington County, MD; and Pittsburgh, PA. Between 1992 and 1993, an additional 687 mostly African-American (AA) participants were recruited for a total cohort of 5888. The baseline examination for CHS participants included a medical history, demographic and lifestyle history, physical exam, fasting blood collection and an assessment of vascular disease by carotid ultrasound and ankle-brachial index.

The Multi-Ethnic Study of Atherosclerosis (MESA) is a prospective cohort study comprised of 6814 European American (EA), African-American, Hispanic, and Asian participants between the ages of 45 and 84 recruited at six sites from 2000 to 2002. The six study sites were: Wake Forest University, University of Minnesota, Northwestern University, University of California at Los Angeles, Columbia University, and Johns Hopkins University. At baseline, participants had no clinical CVD or atrial fibrillation. Baseline examinations included medical, demographic and lifestyle history, measurement of coronary calcium, ventricular mass, carotid intimal-medial wall thickness, ankle and brachial blood pressures, and standard CVD risk factors. Fasting blood was also collected. Our study used data from 699 EA participants and

647AA participants.

The Health, Aging and Body Composition Study (Health ABC) is a cohort study of 3075 participants age 70– 79 residing in Memphis, TN or Pittsburgh, PA who were enrolled between 1997 and 1998.   Participants were interviewed for medical and social history. The baseline clinical exam included a general physical, tests of physical performance and body composition as well as a blood collection. This analysis used data from 786   EA participants and 561 AA participants.

Biomarker and Genotype Measurement

sIL-2Rα was measured in plasma by ELISA (R&D Systems) with a detectable range of 312 – 20,000pg/mL. The coefficients of variation in the current study ranged from 5.11% to 7.59%.

A total of 3388 EA and 607 AA CHS samples were genotyped using the Illumina 370CNV platform. In ancestry specific quality control (QC) analyses, SNPs were excluded from consideration if any of the following applied: 1) minor allele frequency < 0.005, 2) missing rate across subjects > 5%, or 3) Hardy-Weinberg equilibrium p-value < 1.0×10^{-5}. Genotype imputation was performed to expand the coverage of common variants in our GWAS to SNPs that were not included on the genotype panel or that were included but were lost during QC. Ancestry-specific imputation was performed using the software package MaCH[1,2]. Genotype data for 314,364 SNPs in EAs and 311,324 SNPs in AAs, after QC SNP removal, were used to impute 2.2 million SNPs from HapMap Phase 2 and HapMap Phase 3 reference samples. For EAs, HapMap CEU

(Phases 2 and 3) and TSI (Phase 3) reference samples were included. For AAs, CEU
(Phases 2 and 3), YRI (Phases 2 and 3), TSI (Phase 3), LWK (Phase 3), ASW (Phase 3)
reference samples were used. Finally, sets of unrelated subjects for analyses (n=3232 EA
and n=594 AA) were identified by iteratively removing one subject at a time from
subject-pairings with a global identity-by-descent (IBD) estimate > 0.10 until no subject
pairs had a global IBD estimated greater than that threshold. IBD estimation was
performed using a linkage- disequilibrium-pruned set of SNPs that had similar
frequencies in EAs and AAs (to minimize confounding of IBD with background ancestry
similarity). QC analyses and IBD estimation were performed using the software
PLINK[3].

MESA participants were genotyped using the Affymetrix Human SNP array 6.0
(Affymetrix Inc. Santa Clara, CA). Ancestry-specific imputation was performed using
IMPUTE v2[4] using HapMap Phase 2 CEU reference samples for the European
American (EA) participants.

In the Health ABC study, genotyping was performed using the Illumina Human1M-
DuoBeadChip system.

Imputation in the EAs was performed using Mach version 1.0.16 using HapMap Phase 2
CEU reference samples.

Statistical Analysis

To satisfy model assumptions, sIL-2Rα was natural log-transformed for association

analyses with CVD risk factors and genetic variants. Associations between sIL-2Rα and

quantitative traits (systolic blood pressure [SBP], LDL cholesterol, HDL cholesterol, triglycerides, fasting glucose, fasting insulin, BMI, waist circumference, CRP, IL-6, fibrinogen, and carotid intima media thickness [IMT]) and binary traits (diabetes mellitus and hypertension) were analyzed using multiple linear regression and logistic regression, respectively. Hypertension was defined as current use of antihypertensive medication or SBP>140 and DBP>90.

Cox proportional hazards models were used to test for association between sIL-2Rα and the risk of incident coronary heart disease (CHD), incident stroke, congestive heart failure (CHF), CVD mortality and all-cause mortality, separately for EAs and AAs. All events were adjudicated by an expert review panel. Incident CHD included non-procedure-related fatal or nonfatal MI. CVD mortality included fatal events where death was adjudicated as due to atherosclerotic CHD or cerebrovascular disease, including definite fatal MI, definite fatal stroke and definite or probable fatal CHD[5]. Participants with adjudicated baseline prevalent disease for the corresponding incident disease were excluded from analysis (e.g. individuals with a history of myocardial infarction at first visit were excluded from incident CHD analysis). Three progressive levels of covariate adjustments were used to assess risk of incident events associated with sIL-2Rα levels. The first model was minimally adjusted for the potential confounders baseline age, sex and study site. The second model was additionally adjusted for CVD risk factors (baseline measures of current smoking status, type 2 diabetes, hypertension, systolic blood pressure (SBP), and low density lipoprotein (LDL) cholesterol) and baseline CVD (for the mortality outcomes). The third model added adjustments for baseline measures of

inflammation (C- reactive protein (CRP), interleukin-6 (IL-6), fibrinogen), and carotid IMT.

For the genetic analyses of CHS data, the associations between sIL-2Rα and individual genotyped and imputed SNPs, scored as dosage values (expected number of copies of the minor alleles), were tested in linear regression models implemented in Mach2qtl[1, 2]. Covariates in the regression models included age, sex, study site, and the first two principal components (PCs), used to control for potential population substructure. PCs were calculated using the program EIGENSOFT[6, 7]. The statistical significance threshold used for defining significance was set to $5x10^{-8}$.

Targeted conditional analysis was performed in regions where multiple SNPs achieved statistical significance to ascertain how many sIL-2Rα-associated SNPs provided independent evidence for association in the region of interest. The conditional analysis was performed by iteratively adding the most significant genotyped or imputed SNP in with other model covariates and re-assessing the region for any SNP meeting genome-wide significance using forward-stepwise linear regression. A series of regional association plots showing results after each successive model iteration were constructed using the software LocusZoom[8]. Given the rigidity of the forward step wise conditional analysis approach with respect to order of SNP inclusion, we additionally applied the LASSO local automatic regularization resample model averaging (LLARRMA) method[9] to assess the number of important SNPs across the region. Both the conditional analyses and the LLARRMA analyses were restricted to CHS EA HapMap Phase 2 imputed data.

Estimation of sIL-2Rα phenotypic variance explained by individual SNPs was performed

using the REG procedure with PCORR2 option in SAS. Cox proportional hazards models were used to assess whether SNPs associated with sIL-2Rα were also associated with incident events, both before and after adjusting for sIL-2Rα level. The significance threshold for these analyses was set at p=0.05 and analyses were performed using STATA statistical software.

Tests of association between imputed SNP dosage and sIL-2Rα were performed using SNPTEST version

2.4.1[10] in MESA and Mach2qtl[1, 2] in Health ABC. We used fixed effects inverse-variance weighted meta- analysis implemented in Metal[11] to combine results from CHS, MESA and Health ABC EAs.

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Supplemental Table I: Spearman correlation coefficients for sIL2sR and continuous CVD risk factors and subclinical CVD. *P<0.01; **P<0.001; ***P<0.0001

Baseline characteristic	Spearman correlation coefficient
Age, y	0.19***
Systolic blood pressure	0.037*
LDL cholesterol	-0.084***
HDL cholesterol	-0.16***
Triglycerides	0.042*
Glucose	0.038*
Insulin	0.080***
BMI	-0.038**
Waist circumference	0.018
C-reactive protein	0.17***
Interleukin-6	0.24***
Fibrinogen	0.14***
Internal carotid wall thickness	0.13***

Supplemental Table II. Minor allele frequencies for 5 CHS independently associated SNPs, using HapMap data. *Not available in Hapmap, based on 1000 Genomes YRI data.

SNP	Minor allele	CEU	YRI
rs7911500	T	0.14	0
rs791590	T	0.16	0.10
rs8177757	T	0.04	0
rs10905716	T	0.24	0.31
rs7924005	C	0.19	0.18*

CHAPTER 3:

Circulating soluble CD163, genetic associations, and risk of cardiovascular disease and all-cause mortality in older persons: the Cardiovascular Health Study

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Running title

sCD163 with CVD events and genome-wide scan

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Abstract

Objective: Monocytes/macrophages play a key role in atherosclerosis and emerging evidence supports a role for the M2 lineage in fibrosis and heart failure. CD163 is a monocyte/macrophage specific receptor involved in the clearance and endocytosis of hemoglobin-haptoglobin complexes, and soluble CD163 (sCD163) has been shown to reflect macrophage activation especially of the M2 lineage. There are no large epidemiologic studies of sCD163 with incident cardiovascular disease (CVD) events or genetic studies of sCD163 levels. **Approach and Results:** We measured sCD163 in 5000 Cardiovascular Health Study (CHS) participants; 4208 European Americans (EA) and 792 African Americans (AA). At baseline, sCD163 levels were positively associated with female sex, white race, increasing age, BMI, systolic blood pressure, C-reactive protein, interleukin-6, and fibrinogen levels (all $p<0.0001$). In minimally adjusted (race, age and sex) models we observed sCD163 levels to be strongly associated ($p<0.0001$) with all-cause mortality, cardiovascular mortality, incident coronary heart disease, and incident heart failure. After adjustment for established CVD risk factors, evidence for association weakened for all outcomes but remained significant for incident heart failure ($p<0.005$), all-cause mortality ($p<0.05$), and cardiovascular mortality ($p<0.05$). A genome-wide association study (using Hapmap Phase 2 genotype imputation) of 2769 unrelated CHS EAs and 552 AAs identified ($p<5x10^{-8}$)in EAs five variants upstream of chromosome 2q gene *MGAT5* (top result rs4954118, $p=7.1x10^{-14}$) and a single variant (rs314253, $p=6.0x10^{-13}$) on chromosome 17p gene *DLG4*; and three variants in AAs in the *HLA* region of chromosome 6 (top results rs9271366, $p=1.8x10^{-8}$). **Conclusions:** Our

results implicate sCD163 levels as a potentially useful biomarker for incident heart failure, provide evidence for the association of multiple genetic variants with sCD163 levels, and support a potential role for M2 monocyte/macrophage in heart failure.

Introduction

Atherosclerosis is an inflammatory disease [1] characterized by an influx of monocytes and lymphocytes in the arterial wall, resulting from an increase in both circulating low-density lipoprotein (LDL) cholesterol and the oxidized LDL in the subendothelial space. In the subendothelium space monocytes differentiate into macrophages and infiltrate the atherosclerotic lesion, ultimately becoming cholesterol-laden foam cells. M1 and M2 macrophages, defined by their expression of CD14 and CD16 surface receptors [2], have different roles in innate immunity and express different cell surface markers and receptors. Both M1 and M2 macrophages play a role in atherosclerotic plaque formation and in the repair of cardiovascular injury. M1 classically activated macrophages are considered proinflammatory due to their production of Tumor Necrosis Factor–α (TNF-α), interleukin 6 (IL-6) and IL-12; as well as their phagocytic activity [3]. M2 alternatively activated macrophages produce IL-10 and play a role in fibrosis and immunomodulation [4].   Discovered in 1987[5], CD163 is a 130 kDa type 1 transmembrane protein of the cysteine-rich scavenger receptor family [6] expressed on M2 macrophages. A hemoglobin scavenger receptor, CD163 is responsible for the clearance of hemoglobin-haptoglobin (Hb-Hp) complexes in the liver, spleen, and plasma [7].   By removing the proinflammatory Hb-Hp complex as well as unbound hemoglobin (Hb)[8], CD163 contributes to the anti-inflammatory or immune

modulating response; lowering oxidative stress and the metabolism of the extracellular Hb. Both *in vitro* and *in vivo* studies have shown that CD163 - Hb-Bp binding triggers the release of IL-10 and carbon monoxide both of which exhibit substantial anti-inflammatory effects [9]. Increased IL-10 further up-regulates CD163 and heme-oxygenase-1 expression which protects against an inflammatory response due to the extracellular Hb [10, 11]. M2 macrophage also produce TGF-□, a major stimulant of collagen production and fibrosis [12].

A soluble form of CD163 (sCD163) is present in serum [13, 14] with a median concentration of 1.9 mg/L in healthy individuals [13]; sCD163 contains 94% of the membrane bound form, consisting of 945 amino acids [15]. The shedding of CD163 is upregulated by inflammatory factors including lipopolysaccharide [16, 17], phorbol12-myristate 13-acetate [18], and Fcγ receptor cross-linking [19]. The soluble form of CD163 results from proteolytic cleavage of CD163 at the cell surface by Matrix MetalloProteinase-9 (MMP-9) [16] and A Disintegrin and Metalloproteinase 17 (ADAM17)/TNF-α-cleaving enzyme (TACE) [20]. Elevated levels of sCD163 are associated with a number of inflammatory conditions all involving macrophage proliferation and activation including rheumatoid arthritis, multiple sclerosis, cancers, sepsis, and atherosclerosis [21-24].

Despite the fact that macrophages are intimately involved in the development of atherosclerosis as well as the fibrosis associated with heart failure, only a small number of studies have examined the relationship between sCD163 levels and cardiovascular disease (CVD) [21, 25, 26]. Aristoteli, *et al.* found an association between sCD163

levels and the extent of atherosclerotic burden in a study of 147 coronary patients [21]

Moreno, *et al*. observed an association between carotid intima-thickness and elevated

sCD163 [27], and McKibben *et al.* found an association between sCD163 levels and

coronary artery calcium in a cohort of HIV infected and HIV uninfected men [28]. We

examined sCD163 levels in the Cardiovascular Health Study (CHS), a cohort of older

adults with follow-up for incident CVD and mortality for up to 20 years. We determined

the associations of sCD163 with known CVD risk factors and inflammatory biomarkers

measured at baseline, and with incident events. We also conducted a genome-wide

association study (GWAS) to identify genetic variants associated with sCD163 levels.

Results

**Associations Between sCD163 and Baseline CVD Risk Factors and Inflammation
Biomarkers**

sCD163 levels were approximately normally distributed with a mean of 787.3

ng/mL (SD=221.6 ng/mL) and a range of 145.9 – 1633.0 ng/mL. In European Americans

(EA) the mean baseline level of sCD163 was 780.0 ng/mL (SD=216.9 ng/mL) in men

and 810.9 ng/mL (SD=214.9 ng/mL) in women. In African Americans (AA) men and

women the mean sCD163 levels were 688.3 ng/mL (SD=232.6 ng/mL) and 756.0 ng/mL

(SD=242.0 ng/mL), respectively. sCD163 levels were significantly higher in older

individuals, EA as compared to AA, and women (Tables 1 and 2; all p<0.0001). In an

age-, race-, and sex-adjusted models, sCD163 levels were positively associated with type

2 diabetes, hypertension, systolic blood pressure, triglycerides, glucose, insulin, body

mass index, waist circumference, C-reactive protein, interleukin-6, fibrinogen and internal carotid wall thickness; while negatively associated with current smoking, and high density lipoprotein (table 1). There was no statistically significant association (p>0.05) with low density lipoprotein.

Incident Events Analysis

The median follow up time for the CHS participants was 13 years. There were 3392 all-cause deaths; including 1360 cardiovascular deaths. There were 1367 incident cases of coronary heart disease, 861 cases of incident stroke, and 1421 cases of incident heart failure (fatal and nonfatal events). In survival models minimally adjusted for age, sex, race, and study site, baseline levels of sCD163 were significantly (all p<0.0001) associated with increased risk for all-cause mortality, cardiovascular mortality, coronary heart disease, and incident heart failure (Table 3). The association between baseline sCD163 and incident stroke was significant at p<0.05. With further adjustment for known CVD risk factors (smoking, diabetes, hypertension, systolic blood pressure, LDL cholesterol) and BMI the association between baseline sCD163 and all-cause mortality, cardiovascular mortality, and incident heart failure remained significant, although the signals were attenuated. The associations with coronary heart disease and incident stroke were no longer significant.

GWAS of sCD163 in CHS EA and AA

We conducted a race-stratified GWAS in 2769 EAs and 552 AAs from CHS that had both sCD163 measurements and GWAS data available. The EA GWAS identified

five significant ($p<5 \times 10^{-8}$) SNPs near the *MGAT5* gene on chromosome 2; rs4954118 ($p=7.11 \times 10^{-14}$), rs3748896 ($p=8.16 \times 10^{-14}$), rs1257169 ($p=1.68 \times 10^{-12}$), rs1879018 ($p=4.68 \times 10^{-8}$), and rs1996589 ($p=4.80 \times 10^{-8}$) and one significant SNP near the *DLG4* gene on chromosome 17; rs314253 ($p=6.03 \times 10^{-13}$) (Figures 1, 2, and 3). When we modeled sCD163 levels using the five *MGAT5* SNPs (Table 4) in a stepwise forward regression analysis, we found that four SNPs, rs314253, rs4954118, rs125169, and rs1879018, all remained significant (all $p \leq 0.001$) in a model adjusted for age, sex, and clinic site. These four SNPs explained 5.2% of the sCD163 distribution.

Three SNPs on chromosome 6 (rs9271366, $p=1.18 \times 10^{-8}$; rs3135005, $p=2.86 \times 10^{-8}$; and rs9270986, $p=3.13 \times 10^{-8}$) reached genome-wide significance in the AA analysis (Figures 4 and 5); all are in *HLA-DRB1* region and all are highly correlated with each other (all pairwise $R^2>0.90$). rs9271366 accounts for approximately 5% of the variance in sCD163 in AAs.

None of the SNPs that were identified in one race/ethnic group were significant in the other (Table 5). In AAs, the most significant *MGAT5* SNP was rs1111961 ($p=0.02218$) and the most significant *DLG4* gene SNP was rs2242449 ($p=0.18554$). In EAs, the most significant *HLA* region SNP was rs9271366 ($p=0.214$). No SNPs in the *CD163* region were statistically significant at the genome-wide level in either EAs or AAs. The most significant *CD163* SNPs were rs6488429 ($p=8.17 \times 10^{-5}$) in EAs and rs7485773 ($p=4.74 \times 10^{-4}$) in AAs.

We searched the CARDIoGRAM+C4D database, which contains meta-analyzed GWAS results from multiple case controls studies (total of 63,746 cases and 130,681 controls) for coronary heart disease and myocardial infarction (http://www.cardiogramplusc4d.org) [29-31]. None of the nine SNPs identified in the current study for sCD163 were significantly associated with case control status in CARDIoGRAM+C4D (all p>0.05).   We further examined whether any of the SNPs identified to be associated with sCD163 level were also associated with incident events in CHS. The two perfectly correlated *MGAT5* SNPs, rs4954118 and rs3748896, were significantly associated with incident heart failure in analyses adjusted for baseline age, sex, and study clinic site (p=0.030). When the model was further adjusted for sCD163 level the p-value for association was slightly smaller than for the model without adjustment for sCD163 level (p=0.006). We also conducted a Mendelian randomization analysis, using SNP rs4954118, to test whether there was evidence for a causal association between sCD163 levels and heart failure. The result for this analysis was not significant (p=0.086), although it was suggestive. We observed evidence for association between *HLA* SNPs rs9271366; p=0.048, rs3135005; p=0.038; with incident coronary heart disease in AAs. When further adjusted for sCD163 level, the *HLA* SNPs were no longer significantly associated with incident heart failure (both p>0.05).

Discussion

This represents the first large-scale study of sCD163, a marker of macrophage activation, in a population based CVD study with incident events, as well as the first GWAS for sCD163. Our major findings are: (1) sCD163 levels are associated with many

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established CVD risk factors and with carotid intima thickness; a measure of subclinical

CVD; (2) In older adults, sCD163 levels predict all-cause mortality, cardiovascular

mortality, coronary heart disease, stroke, and incident heart failure in minimally adjusted

models (P<0.0001), with a 50% greater risk of cardiovascular mortality and incident heart

failure comparing the fourth quartile to the first quartile of sCD163; (3) sCD163 levels

significantly predict all-cause mortality, cardiovascular mortality, and incident heart

failure independently of established CVD risk factors (P<0.05); (4) Genetic variants near

MGAT5 and *DLG4* are associated with sCD163 in EAs and variants near *HLA-DRB1* are

associated with sCD163 levels in AAs; (5) There is evidence that genetic variants

associated with sCD163 level (*MGAT5* in EAs and *HLA-DRB1* in AA) are also

associated with incident heart failure in older adults.

The function of sCD163 has not been well characterized but it has been postulated

that sCD163 may contribute to innate immunity by binding hemoglobin-iron in the

circulatory system thus making the iron unavailable to pathogens [17, 32]. sCD163 also

modulates the immune system by inhibiting T cell proliferation[33, 34]. Frings, *et al.*

have shown that it is only the soluble form of CD163 and not the membrane bound form

that inhibits T cells [33]. The work of Timmermann, *et al*. showed that sCD163 binds to

non-muscle myosin heavy chain in T lymphocytes causing this inhibition [35]. *In vivo*

studies have shown reduced T cell response and increased sCD163 expression in patients

with rheumatoid arthritis [36].

While sCD163 may directly reflect the level of M2 macrophages, sCD163 might

also result from the transition of M2 macrophages to M1 macrophages during tissue

repair, including cardiac injury repair. Upon initial cardiac injury the percentage of proinflammatory, M1 macrophages increase [37]. Once the acute phase of the injury has passed, there is a transition to an increased population of anti-inflammatory, CD163 expressing macrophages essential in the remodeling phase where fibrosis and anti-inflammatory cytokine expression is increased. This transition from M1 to M2 macrophages can happen through the differentiation of monocytes to M2 macrophages and through the plasticity of the M1 macrophages transitioning to M2 macrophages [37]. As the tissue remodeling continues there is a return to the M1 and M2 macrophage balance; it is possible that this also occurs due to macrophage plasticity and the shedding of the CD163 receptor thereby increasing the level of circulating sCD163. Work done in a mouse model of skeletal muscle repair shows that after the injury, pro-inflammatory, M1 macrophages phagocytose cellular debris and then change their phenotype to alternatively activated, anti-inflammatory, M2 macrophage promoting tissue remodeling [38]. This study went on to show that ultimately these macrophages entered an 'exhaustion-like state' with no cytokine expression; if this inflammatory response was altered muscle regeneration was negatively impacted [38]. Other studies have shown sCD163 to be a marker of macrophage activation [39, 40], associated with noncalcified coronary plaque [39], and over expressed in carotid plaques [41].

Our findings indicate that sCD163 is a predictor of heart failure (HR=1.50, p<0.0001) and cardiovascular mortality (HR=1.51, p<0.0001) when comparing the first quartile of sCD163 levels to the fourth quartile in a model adjusted for age, race, sex, and clinic.

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Our GWAS identified three genes associated with sCD163 levels, *MGAT5*, *DLG4*, and *HLA-DRB1*. *MGAT5*, has been associated with the M2 macrophage phenotype and fibrosis [42] and DLG4 is involved in the cellular response to oxidative stress, a key factor in cardiovascular diseases [43]. *HLA-DRB1* is a linchpin of the inflammatory response and has been associated with fibrosis [44]. Five of the six significant SNPs from the EA GWAS were in *MGAT5*, a gene on chromosome 2, which codes for a glycosyltransferase (mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase) and has been shown to be associated with multiple sclerosis (MS), systemic sclerosis, cancer metastasis, and liver fibrosis [45-47]. The deregulation of the glycosyltransferase increases susceptibility to autoimmune diseases and is associated with the severity of MS [48, 49]. Kato *et al.* showed in a mouse model of scleroderma, a fibrotic disease involving vascular injury and repair, with similarities to heart failure, that $MGAT5^{+/+}$ mice had higher levels of M2 macrophages compared to $MGAT5^{-/-}$ mice and that glycosylated cell surface proteins cause a shift in the macrophage phenotype to M2 [47]. The other significant SNP found in the EA GWAS was on chromosome 17 in *DLG4*, rs314253. rs314253 has been cited in the literature as being associated with total cholesterol [50] and with the concentration of liver enzymes in plasma [51]. *DLG4* codes for post-synaptic density protein 95, a protein involved in the regulation and structure of receptors and associated signaling proteins. DLG4 is an important regulator of enzyme complexes essential to the cellular response to oxidative stress and has been linked to MS, a chronic inflammatory autoimmune disease [43]. An increase in reactive oxygen species is a key feature of the development of cardiovascular

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disease. It is interesting that both MGAT5 and DLG 4 have been linked to fibrotic

diseases. Fibrosis is a contributing factor to heart failure and cardiac senescence. It is

possible that our results showing an association of higher sCD163 levels with an

increased hazards ratio for heart failure are the result of increased fibrosis. Work by

Pinto *et al*. in mice showed that the M2 macrophages in the aging heart might contribute

to cardiac senescence and heart failure [52]. Further research is necessary to understand

the balance and role of the anti-inflammatory and the pro-fibrotic effects of M2

macrophages in heart failure. Further research is also necessary to understand whether

any of the variants we found have functional relevance.

The three significant results from the AA GWAS were all on chromosome 6 in

the *HLA-DRB1* region. Two of the significant SNPs from the AA GWAS have been

cited in the literature; rs9271366 has been shown to be associated with Crohn's disease,

inflammatory bowel disease [53], multiple sclerosis [54], and immunoglobulin A

deficiency [55] and rs9270986 has been found to be associated with myasthenia gravis

[37]. *HLA-DRB1* is a MHC class II gene that encodes for proteins that are on particular

immune cells. *HLA-DRB1* protein binds to the product of *HLA-DRA* forming the

functional HLA-DR antigen binding heterodimer; involved in triggering an immune

response by presenting antigen to T helper cells.

There are several limitations to this study. In our GWAS we only analyzed

common variants (minor allele frequency>0.05); rare variants may also be important in

accounting for the variability in the sCD163 levels. Given that the sample size in AAs

was considerably smaller than that in EAs, the statistical power to detect associations was

much more limited. The Mendelian randomization analysis in EAs had limited power to detect a causal effect, as large sample sizes are required for this approach. Lastly, our study was focused on older adults and, therefore, our results may not be generalizable to other populations.

In summary, our findings suggest that sCD163 may be a novel biomarker for all-cause mortality, cardiovascular mortality, coronary heart disease, and in particular heart failure in older adults. The association of sCD163 with heart failure is independent of established CVD risk factors. We have observed several novel genetic associations for sCD163, and association and Medelian Randomization studies suggest a possible causal role monocyte activation, especially related to M2, in heart failure. Additional studies are needed to assess whether sCD163 levels predict the outcomes in younger populations.

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Table 1. Association Between Soluble CD163 and Cardiovascular Risk Factors, Inflammation Biomarkers and Measures of Subclinical Cardiovascular Disease at the CHS Baseline Examination

Each variable was examined for association with sCD163 in a separate model, adjusting for the variables listed (the exception is that a variable is not adjusted for itself when it is being tested): Model A: adjusted for age, race, and sex. Model B: adjusted for age, race, sex, smoking, diabetes mellitus, hypertension, systolic blood pressure, and BMI. β for all measures except sex, race, diabetes mellitus, and hypertension are for a 1-SD change in the predictor. P values: *P<0.01, **P<0.001, ***P<0.0001.

Baseline Characteristics (Mean ± SD or %)	**Model A,**sCD163 β±SE	**Model B,** sCD163 β±SE
Age, y (72.5 ± 5.4)	3.22±0.58***	3.15±064l***
Male sex (41.4%)	-38.51±6.30***	-38.83±6.63***
Black race (15.8%)	-69.54±8.49***	-79.29±15.96***
Current smoking (13.6%)	-57.21±9.47***	-42.36±10.24***
Type 2 diabetes (16.5%)	101.28±8.51***	77.20±9.20***
Hypertension (66.3%)	50.63±6.62***	27.64±7.56***
Systolic blood pressure, mm Hg (138.7±19.9)	0.69±0.17***	0.28±0.18
LDL cholesterol, mg/dL (130.11±35.7)	-0.11±0.09	-0.18±0.09
HDL cholesterol, mg/dL (54.3±15.8)	-3.23±0.21***	-2.74±0.23***
Triglycerides, mg/dL (140.7±77.7)	0.33±0.04***	0.14±0.04*
Glucose, mg/dL (111.4±37.2)	1.05±0.08***	0.50±0.14***
Insulin, IU/mL (17.3±27.4)	0.90±0.11***	0.34±0.14
BMI, kg/m^2 (26.7±4.7)	8.30±0.67***	6.99±0.76***
Waist circumference, cm (93.7±12.7)	2.93±0.26***	0.74±0.47
C-reactive protein, mg/L (4.8±8.3)	3.42±0.37***	3.03±0.42***
Interleukin-6, pg/mL (2.2±1.9)	15.66±1.65***	12.96±1.78***
Fibrinogen, mg/dL (324.0±67.0)	0.30±0.05***	0.27±0.05***
Internal carotid wall thickness, mm (1.4±0.6)	27.70±6.06***	19.43±6.28*

Table 2. **Spearman correlation coefficients for sCD163 and continuous CVD risk factors and subclinical CVD.**

*P<0.01; **P<0.001; ***P<0.0001

Variables are unadjusted.

Baseline characteristic	Spearman correlation coefficient
Age, y	0.071***
Systolic blood pressure	0.064***
LDL cholesterol	-0.006 (0.682)
HDL cholesterol	-0.194***
Triglycerides	0.015***
Glucose	0.136***
Insulin	0.235***
BMI	0.134***
Waist circumference	0.123***
C-reactive protein	0.176***
Interleukin-6	0.211***
Fibrinogen	0.081***
Internal carotid wall thickness	0.083***
sCD14	0.152***
sIL-2Rα	0.261***

Table 3. Hazard Ratios Between sCD163 and Incident Events in CHS

Minimal model: Age, sex, race, clinic. Model 2: Minimal Model + smoking, diabetes, hypertension, systolic BP, LDL, BMI

Hazard ratios reflect a 1-SD change in sCD163. P values: *=P<0.05 **=P<0.005 ***=P<0.0001

	All-Cause Mortality (5000 records / 3392 events)	Cardiovascular Mortality (5000 records / 1360 events)	Coronary Heart Disease (3963 records / 1367 events)	Stroke (4777 records / 861 events)	Incident Heart Failure (4758 records / 1421 events)
Model	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)
Minimal	1.08 (1.04-1.12)***	1.15 (1.09-1.21)***	1.10 (1.05-1.17)***	1.07 (1.00-1.15)*	1.18 (1.12-1.25)***
2nd Q vs 1st Q	1.06 (0.96-1.17)	1.25 (1.06-1.47)*	1.26 (1.08-1.47)**	1.18 (0.97-1.44)	1.06 (0.91-1.25)
3rd Q vs 1st Q	1.07 (0.96-1.18)	1.27 (1.08-1.50)**	1.23 (1.05-1.44)*	1.15 (0.94-1.40)	1.21 (1.03-1.41)*
4th Q vs 1st Q	1.23 (1.11-1.36)***	1.50 (1.28-1.76)***	1.36 (1.16-.160)***	1.22 (1.00-1.49)	1.51 (1.30-1.76)***
Model 2	1.05 (1.01-1.10)*	1.08 (1.01-1.14)*	1.03 (0.96-1.09)	1.02 (0.94-1.10)	1.11 (1.04-1.18)**
2nd Q vs 1st Q	1.02 (0.91-1.14)	1.11 (0.93-1.33)	1.12 (0.94-1.33)	1.04 (0.83-1.30)	0.95 (0.79-1.14)
3rd Q vs 1st Q	1.05 (0.94-1.18)	1.20 (1.00-1.43)*	1.10 (0.92-1.31)	1.09 (0.88-1.36)	1.08 (0.91-1.29)
4th Q vs 1st Q	1.14 (1.02-1.27)*	1.23 (1.03-1.47)*	1.09 (0.91-1.31)	1.04 (0.83-1.30)	1.22 (1.03-1.46)*

Table 4. Pairwise R^2 *MGAT5* SNPs EA

SNP	rs4954118	rs3748896	rs1257169	rs1879018	rs1996589
rs4954118	1	0.997	0.3747	0.0711	0.0712
rs3748896	0.997	1	0.3732	0.0705	0.0707
rs1257169	0.3747	0.3732	1	0.0268	0.0269
rs1879018	0.0711	0.0705	0.0268	1	0.9998
rs1996589	0.0712	0.0707	0.0269	0.9998	1

Table 5. Significant SNPs in EAs and AAs

Chrm	SNP	Gene	EA p value	EA MAF	EA β	AA p value	AA MAF	AA β
2	rs4954118	*MGAT5*	7.11×10^{-14}	0.7128	-48.9	0.1867	0.7453	21.2
2	rs3748896	*MGAT5*	8.16×10^{-14}	0.7123	-48.5	0.1886	0.7455	21.1
2	rs1257169	*MGAT5*	1.68×10^{-12}	0.4548	-43.2	0.0787	0.8563	36.0
2	rs1879018	*MGAT5*	4.68×10^{-8}	0.6912	-33.9	0.9774	0.7190	0.5
2	rs1996589	*MGAT5*	4.80×10^{-8}	0.691	-33.9	0.0383	0.4261	-30.4
17	rs314253	*DLG4*	6.03×10^{-13}	0.6575	43.15	0.8085	0.6128	3.7
6	rs9271366	*HLA_DRB1*	0.214	0.8592	10.4	1.81×10^{-8}	0.8612	115.1
6	rs3135005	*HLA_DRB1*	0.1994	0.8696	10.9	2.86×10^{-8}	0.8647	114.1
6	rs9270986	*HLA_DRB1*	0.2763	0.8558	8.9	3.31×10^{-8}	0.8537	113.7
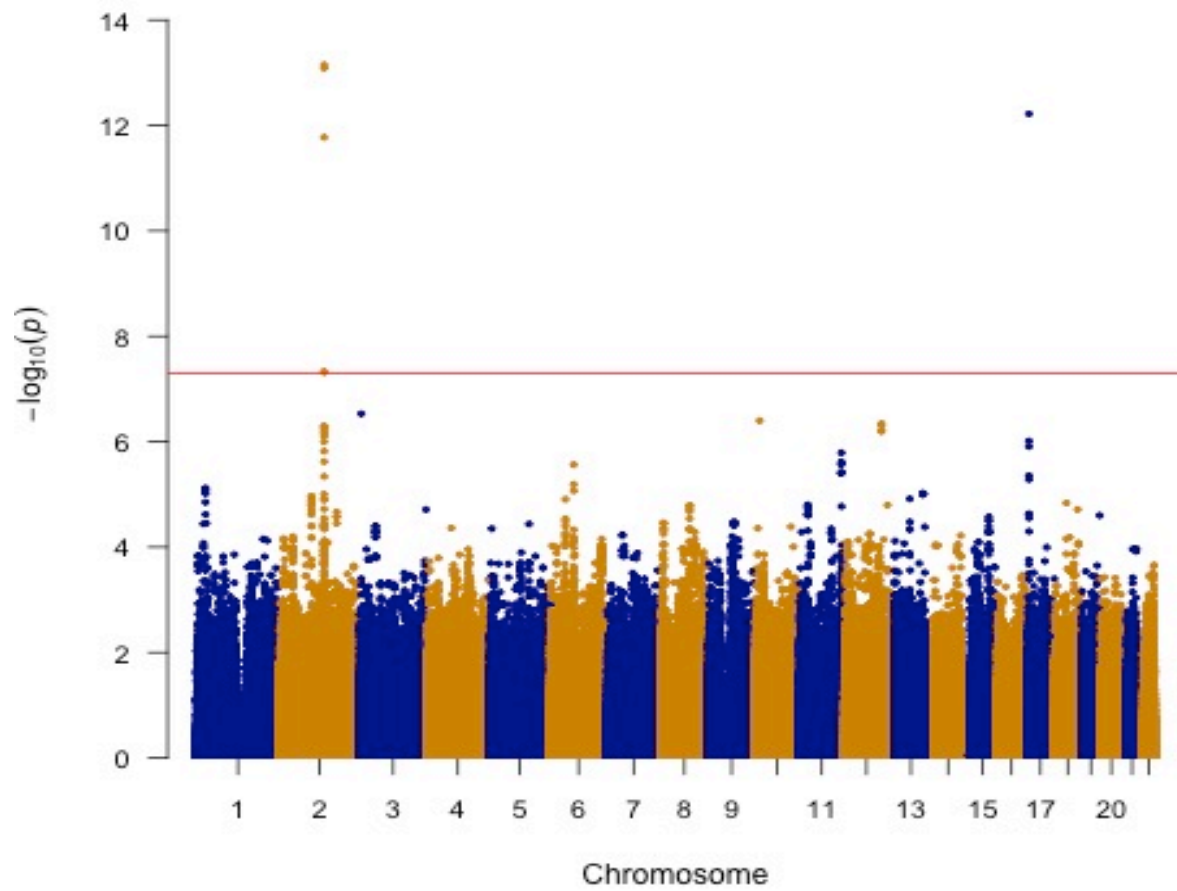
Figure 1. Manhattan Plot of CHS EA Results
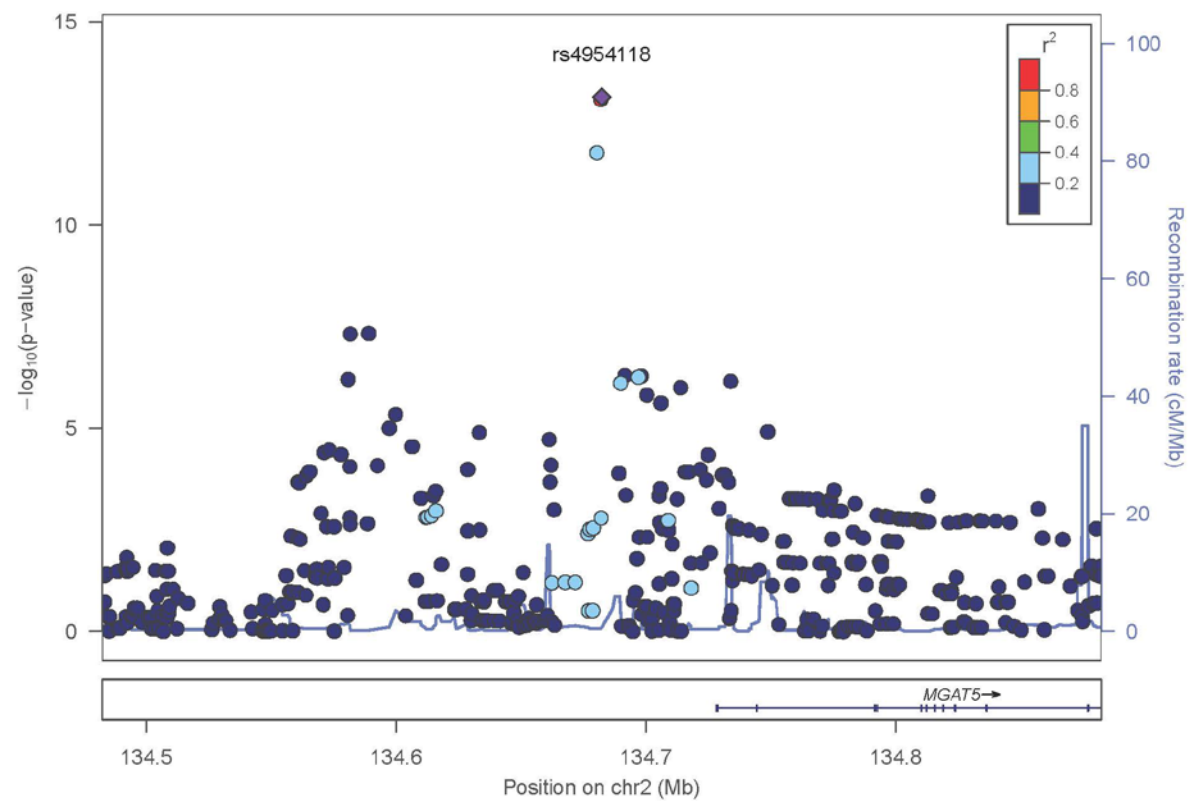
Figure 2. LocusZoom Plot of CHS EA Results Chromosome 2
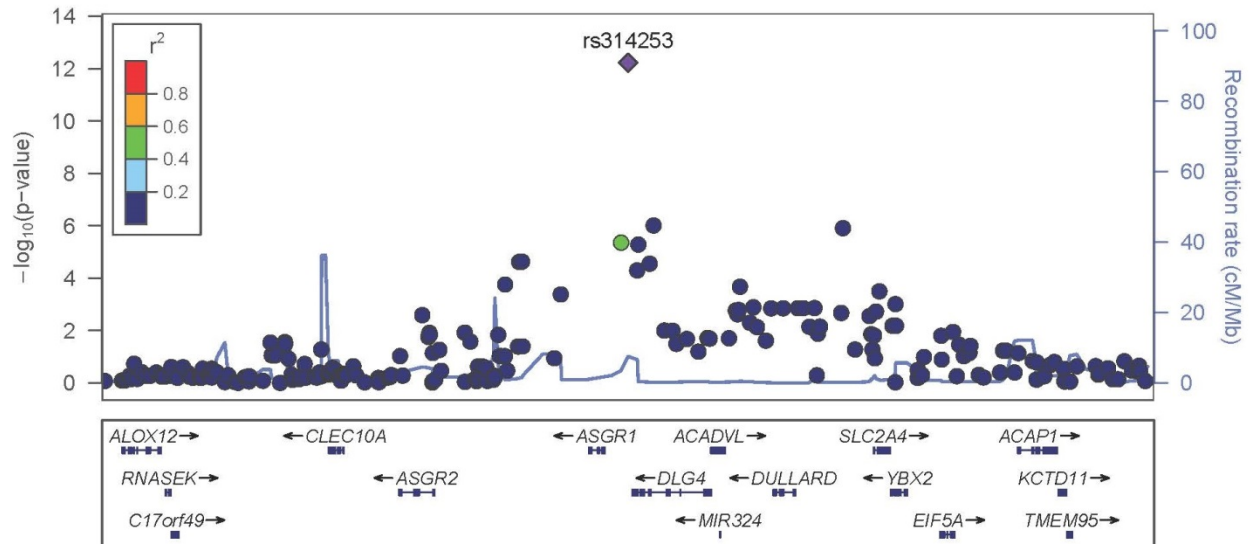
Figure 3. LocusZoom Plot of CHS EA Results Chromosome 17
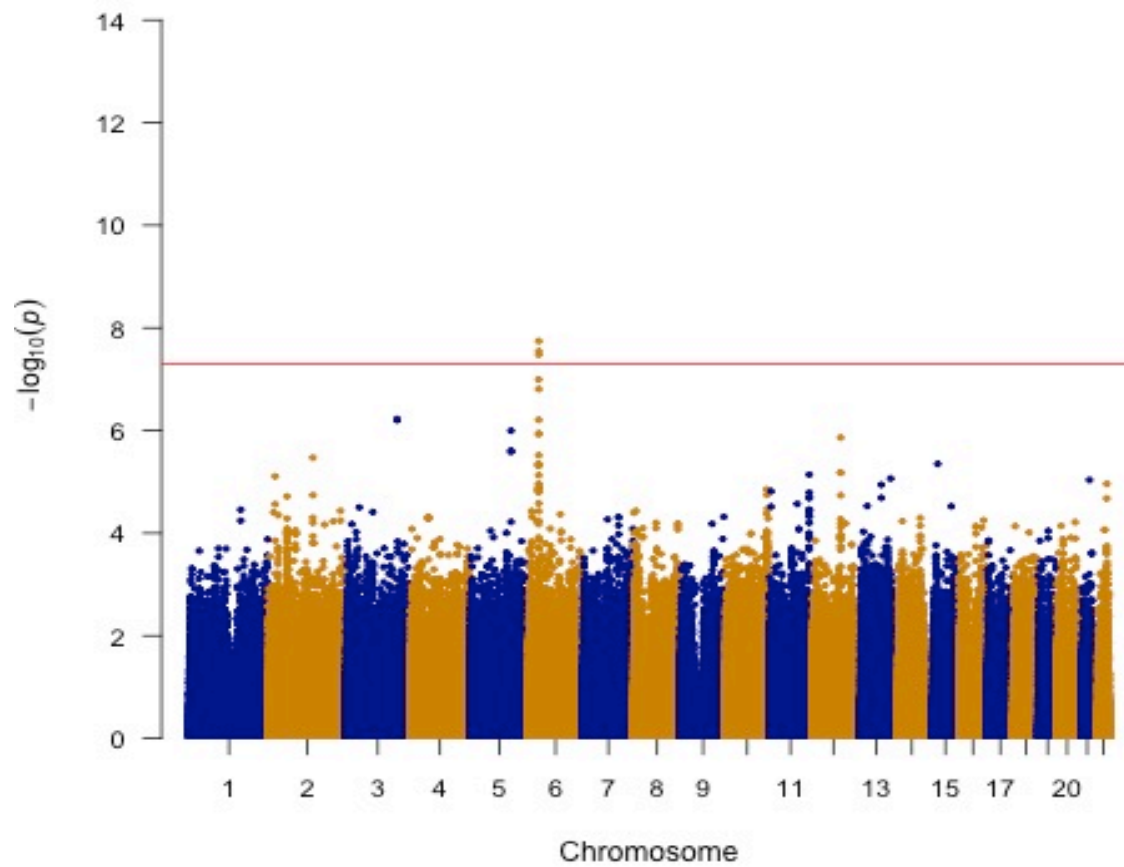
Figure 4. Manhattan Plot of CHS AA Results
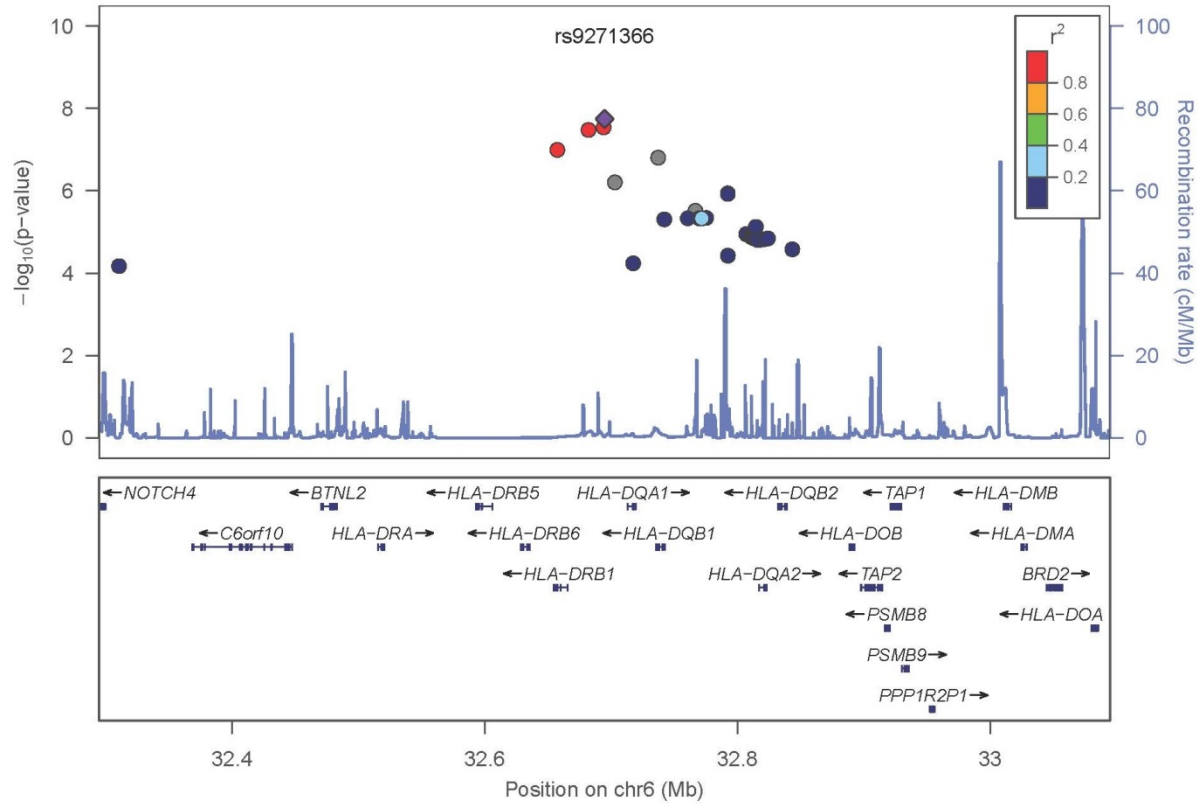
Figure 5. LocusZoom Plot of CHS AA Results Chromosome 6

METHODS   *sCD163 with CVD events and genome-wide scan*

Study samples

The Cardiovascular Health Study (CHS) is a prospective population-based cohort study of men and women recruited at age 65 or older at baseline. The original cohort of 5201 participants was recruited between 1988 and 1989 at four field centers: Forsyth County, NC; Sacramento County, CA; Washington County, MD; and Pittsburgh, PA. Between 1992 and 1993, an additional 687 mostly African-American (AA) participants were recruited for a total cohort of 5888. The baseline examination for CHS participants included a medical history, demographic and lifestyle history, physical exam, fasting blood collection and an assessment of vascular disease by carotid ultrasound and ankle-brachial index. Our analysis was carried out on 5000 CHS participants with sCD163 measurements at baseline.

Biomarker and Genotype

Measurement

sCD163 was measured in plasma by ELISA (R&D Systems) with a detectable range of 17 – 2,000 ng/mL. The coefficients of variation in the current study ranged from 2.37% to 3.72%.

A total of 4368 EA and 846 AA CHS samples were genotyped using the Illumina 370CNV platform. In ancestry specific quality control (QC) analyses, SNPs were excluded from consideration if any of the following applied: 1) minor allele frequency <

0.005, 2) missing rate across subjects > 5%, or 3) Hardy-Weinberg equilibrium p-value < 1.0×10^{-5}. Genotype imputation was performed to expand the coverage of common variants in our GWAS to SNPs that were not included on the genotype panel or that were included but were lost during QC. Ancestry-specific imputation was performed using the software package MaCH[1,2]. Genotype data for 314,364 SNPs in EAs and 311,324 SNPs in AAs, after QC SNP removal, were used to impute 2.2 million SNPs from HapMap Phase 2 and HapMap Phase 3 reference samples. For EAs, HapMap CEU (Phases 2 and 3) and TSI (Phase 3) reference samples were included. For AAs, CEU (Phases 2 and 3), YRI (Phases 2 and 3), TSI (Phase 3), LWK (Phase 3), ASW (Phase 3) reference samples were used. Finally, sets of unrelated subjects for analyses (n=3232 EA and n=594 AA) were identified by iteratively removing one subject at a time from subject-pairings with a global identity-by-descent (IBD) estimate > 0.10 until no subject pairs had a global IBD estimated greater than that threshold. IBD estimation was performed using a linkage- disequilibrium-pruned set of SNPs that had similar frequencies in EAs and AAs (to minimize confounding of IBD with background ancestry similarity). QC analyses and IBD estimation were performed using the software PLINK[3].

Statistical Analysis

Associations between sCD163 and quantitative traits (systolic blood pressure [SBP],

LDL cholesterol, HDL cholesterol, triglycerides, fasting glucose, fasting insulin, BMI, waist circumference, CRP, IL-6, fibrinogen, and carotid intima media thickness [IMT]) and binary traits (diabetes mellitus and hypertension) were analyzed using multiple linear regression and logistic regression, respectively. Hypertension was defined as current use of antihypertensive medication or SBP>140 and DBP>90.

Cox proportional hazards models were used to test for association between sCD163 and the risk of incident coronary heart disease (CHD), incident stroke, congestive heart failure (CHF), CVD mortality and all-cause mortality, separately for EAs and AAs. All events were adjudicated by an expert review panel. Incident CHD included non-procedure-related fatal or nonfatal MI. CVD mortality included fatal events where death was adjudicated as due to atherosclerotic CHD or cerebrovascular disease, including definite fatal MI, definite fatal stroke and definite or probable fatal CHD[4]. Participants with adjudicated baseline prevalent disease for the corresponding incident disease were excluded from analysis (e.g. individuals with a history of myocardial infarction at first visit were excluded from incident CHD analysis). Three progressive levels of covariate adjustments were used to assess risk of incident events associated with sCD163 levels. The first model was minimally adjusted for the potential confounders baseline age, sex and study site. The second model was additionally adjusted for CVD risk factors (baseline measures of current smoking status, type 2 diabetes, hypertension, systolic blood pressure (SBP), and low density lipoprotein (LDL) cholesterol) and baseline CVD (for the mortality outcomes). The third model added adjustments for baseline measures of inflammation (C- reactive protein (CRP), interleukin-6 (IL-6), fibrinogen), and carotid

IMT.

For the genetic analyses of CHS data, the associations between sCD163 and individual

genotyped and imputed SNPs, scored as dosage values (expected number of copies of the

minor alleles), were tested in linear regression models implemented in Mach2qtl[1, 2].

Covariates in the regression models included age, sex, study site, and the first two

principal components (PCs), used to control for potential population substructure. PCs

were calculated using the program EIGENSOFT [5,6]. The statistical significance

threshold used for defining significance was set to $5x10^{-8}$. A series of regional

association plots showing results were constructed using the software LocusZoom[7].

METHODS REFERENCES *sCD163 with CVD events and genome-wide scan*

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Chapter 4: <u>Summary and a Look to the Future.</u>

The major cause of CVD is atherosclerosis, a chronic inflammatory process involving both the innate and adaptive immune systems. Animal work and epidemiology have elucidated many of the underlying processes in the development of atherosclerotic plaques. However, CVD is complex and has other contributing factors including genetic predisposition, modifiable and non-modifiable risk factors, environmental exposures and the interactions of all of these. Our studies of both sIL-2Rα and sCD163 provide further evidence of both the adaptive and innate immune systems involvement in CVD. Our analysis of sIL-2Rα levels in CHS was the first large-scale assessment of sIL-2Rα for association with CVD traits and events; and the first GWAS for SNPs associated with sIL-2Rα levels. When we analyzed data from CHS our results found that sIL-2Rα, a surrogate for T lymphocyte activity, is a novel biomarker for all-cause mortality, cardiovascular mortality, stroke, and heart failure in older adults as well as associated with other CVD risk factors. We also identified 52 SNPs in chromosome 10 at and near the *IL2RA* gene that had genome wide significance for association with sIL-2Rα levels in European Americans. A number of these SNPs were previously associated with type 1 diabetes mellitus and multiple sclerosis. These findings combined with our results provides additional support for the position that T cell activation plays an important role in several disorders including, but not limited to, CVD.

We cannot identify the casual SNP(s) based on our current analyses; further high-density genotyping of the chromosome 10 region will be necessary. While sIL-2Rα

levels may be a novel biomarker in older adults, further studies are necessary to verify the generalizability to other populations. Continued investigation into the genetic control of IL-2/IL-2R interplay with regard to T-cell differentiation and cytokine production is also needed.

Our work on sCD163 represents the first large-scale study in a population-based cohort with incident events and GWAS data. We found sCD163, a marker of macrophage activation, to be associated with many CVD risk factors and to be an independent predictor of all-cause mortality, cardiovascular mortality, and incident heart failure in older adults. Our GWAS found genetic variants in the genes *MGAT5* and *ASGR1/DLG4* to be associated with sCD163 levels in European Americans while variants in *HLA-DRB1* were associated with sCD163 levels in African Americans; no SNPs in the region of *CD163* reached genome-wide significance. Mendelian Randomization analysis indicated that the variants in *MGAT5* in European Americans and those in *HLA-DRB1* in African Americans associated with sCD163 levels also associated with incident heart failure in older adults. As for sIL2Rα, additional genetic work is necessary to identify the functional variant for sCD163 levels.

Atherosclerosis prevention and treatment is a prime example of translational science. Basic science research in mice and rabbits combined with studies of biochemical pathways and epidemiological and genetic studies in cohorts have informed clinical practice. To date much of atherosclerosis prevention has fallen under the umbrella of Public Health with the focus being on lifestyle changes as a management of risk factors including smoking, hypertension, hyperlipidemia, diabetes mellitus, high

blood pressure, sedentary lifestyle, and diet. Research into the development and

progression of atherosclerosis led to an understanding of the importance of cholesterol in

the process. In the 1950's the work of Konrad E. Bloch, Feodor Lynen, John Cornforth,

and George Popják elaborated the biosynthetic pathway of cholesterol leading to the

search for cholesterol synthesis inhibitors [1]. With the Food and Drug Administration

approval of the Merck drug lovastatin, it became the first commercially available drug for

lowering LDL cholesterol [2] moving atherosclerosis prevention from Public Health to a

more targeted approach. Since the advent of statins, many approaches to the prevention

of CVD have been examined. In addition to cholesterol lowering medications, other

treatments include anti-platelet drugs to prevent platelet clumping, beta-blockers,

angiotensin-converting enzyme inhibitors, and calcium channel blockers. Other

treatments have targeted the inflammatory system. Work by Huber *et al.* in mice showed

that increased levels of IL-6 resulted in a large increase in size of fatty lesions [3]. Down

regulating, the immune response or biasing the immune response toward an

antithrombotic response might slow plaque progression. A recent report on the effects of

cyclodextrin in mice found that the drug increases cholesterol solubility and essentially

reprograms the macrophages to improve cholesterol efflux and reduce atherosclerotic

plaque size [4]. Genetic associations and the continued search for casual variants will

lead to targeted, personalized, therapies for atherosclerosis.   The identification of loss of

function variants and their association with a decreased risk of heart disease in genes

PCSK9, NPC1L1, APOC3, and *APOA5* [5] give encouragement to other potential

therapies and the translation of bench science to clinical practice.   Continued

investigation of both sIL-2Rα and sCD163, markers of T-cell and monocyte

differentiation, in animal models and cohort studies may lead to opportunities for the

prevention of atherosclerosis and/or treatment through an increased understanding of both

the biology and genetics of both the innate and adaptive immune responses in

atherosclerosis.

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