# **ORIGINAL RESEARCH**

Circulating Soluble CD163, Associations With Cardiovascular Outcomes and Mortality, and Identification of Genetic Variants in Older Individuals: The Cardiovascular Health Study

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**BACKGROUND:** Monocytes/macrophages participate in cardiovascular disease. CD163 (cluster of differentiation 163) is a monocyte/macrophage receptor, and the shed sCD163 (soluble CD163) reflects monocyte/macrophage activation. We examined the association of sCD163 with incident cardiovascular disease events and performed a genome-wide association study to identify sCD163-associated variants.

**METHODS AND RESULTS:** We measured plasma sCD163 in 5214 adults (aged  $\geq$ 65 years, 58.7% women, 16.2% Black) of the CHS (Cardiovascular Health Study). We used Cox regression models (associations of sCD163 with incident events and mortality); median follow-up was 26 years. Genome-wide association study analyses were stratified on race. Adjusted for age, sex, and race and ethnicity, sCD163 levels were associated with all-cause mortality (hazard ratio [HR], 1.08 [95% Cl, 1.04–1.12] per SD increase), cardiovascular disease mortality (HR, 1.15 [95% Cl, 1.09–1.21]), incident coronary heart disease (HR, 1.10 [95% Cl, 1.04–1.16]), and incident heart failure (HR, 1.18 [95% Cl, 1.12–1.25]). When further adjusted (eg, cardiovascular disease risk factors), only incident coronary heart disease lost significance. In European American individuals, genome-wide association studies identified 38 variants on chromosome 2 near *MGAT5* (top result rs62165726, *P*=3.3×10<sup>-18</sup>),19 variants near chromosome 17 gene *ASGR1* (rs55714927, *P*=1.5×10<sup>-14</sup>), and 18 variants near chromosome 11 gene *ST3GAL4*. These regions replicated in the European ancestry ADDITION-PRO cohort, a longitudinal cohort study nested in the Danish arm of the Anglo-Danish-Dutch study of Intensive Treatment Intensive Treatment In peOple with screeNdetcted Diabetes in Primary Care. In Black individuals, we identified 9 variants on chromosome 6 (rs3129781 *P*=7.1×10<sup>-9</sup>) in the *HLA* region, and 3 variants (rs115391969 *P*=4.3×10<sup>-8</sup>) near the chromosome 16 gene *MYLK3* 

**CONCLUSIONS:** Monocyte function, as measured by sCD163, may be predictive of overall and cardiovascular-specific mortality and incident heart failure.

Key Words: cardiovascular diseases CD163 antigen genome-wide association study humans monocytes risk factors

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# **CLINICAL PERSPECTIVE**

#### What Is New?

- This was the first study to confirm that serum sCD163 (soluble cluster of differentiation 163) is predictive of incident all-cause and cardiovascularspecific mortality, and the first to identify a significant association with incident heart failure.
- We confirmed and identified genetic variants in 3 distinct genetic loci for sCD163 in European American individuals, and we identified variants for 1 genetic locus in Black individuals.

# What Are the Clinical Implications?

• Serum sCD163 may be a useful inflammatory biomarker for overall and cardiovascularspecific mortality and incident heart failure.

# Nonstandard Abbreviations and Acronyms

CD163	cluster of differentiation 163
CHS	Cardiovascular Health Study
EA	European American
eQTL	expression quantitative trait loci
SBP	systolic blood pressure
sCD163	soluble cluster of differentiation 163

therosclerosis is an inflammatory disease<sup>1</sup> characterized by an influx of monocytes and lymphocytes Lin the arterial wall, resulting from infiltration of circulating low-density lipoprotein cholesterol and its subsequent oxidation. In the subendothelial space, monocytes differentiate into macrophages and infiltrate the atherosclerotic lesion, ultimately becoming cholesterol-laden foam cells. M1 and M2 macrophages, defined by their pattern of expression of CD14 (cluster of differentiation 14) and CD16 (cluster of differentiation 16) surface receptors,<sup>2</sup> play a role in atherosclerotic plaque formation and in the repair of cardiovascular injury. M1 classically activated macrophages are considered proinflammatory because of their production of tumor necrosis factor- $\alpha$ , interleukin (IL)-6, and IL-12, as well as their phagocytic activity.<sup>3</sup> M2 (alternatively activated) macrophages produce IL-10 and play a role in fibrosis and immunomodulation.<sup>4</sup> Discovered in 1987,<sup>5</sup> CD163 (cluster of differentiation 163) is a 130 kDa type 1 transmembrane protein of the cysteine-rich scavenger receptor family<sup>6</sup> expressed on M2 macrophages. A hemoglobin scavenger receptor, CD163 is responsible for the clearance of hemoglobin-haptoglobin complexes in the liver, spleen, and plasma.<sup>7</sup> By removing the proinflammatory hemoglobin-haptoglobin complex as well as unbound hemoglobin,<sup>8</sup> CD163 contributes to the anti-inflammatory response. Both in vitro and in vivo studies have shown that CD163-hemoglobin-haptoglobin complexes binding triggers release of IL-10 and carbon monoxide, both of which exhibit substantial anti-inflammatory effects.<sup>9</sup>

A soluble form of CD163 (sCD163) is present in plasma<sup>10,11</sup> and contains 94% of the membrane bound form. The soluble form of CD163 results from cleavage of CD163 at the cell surface by a tumor necrosis factor- $\alpha$ -converting enzyme/ADAM metallopeptidase domain 17.<sup>12,13,14</sup> The shedding of CD163 is upregulated by inflammatory factors including lipopolysaccharide,<sup>12,15</sup> phorbol12-myristate 13-acetate,<sup>16</sup> and Fc $\gamma$  receptor cross-linking.<sup>17</sup> Elevated levels of sCD163 are associated with several inflammatory conditions involving macrophage proliferation and activation, including rheumatoid arthritis, multiple sclerosis, certain cancers, sepsis, and atherosclerosis.<sup>18–21</sup>

Although macrophages are intimately involved in the development of atherosclerosis and fibrosis associated with heart failure, only a few studies have examined the relationship between sCD163 levels and subclinical cardiovascular disease (CVD) (atherosclerosis on imaging),<sup>18,22,23</sup> and none have related it to the risk of future CVD events. We examined sCD163 levels in the CHS (Cardiovascular Health Study), a cohort of older adults with follow-up for incident CVD and mortality for a median of 26 years. We assessed the associations of sCD163 with known CVD risk factors and inflammatory biomarkers measured at baseline, and with incident events (all-cause mortality, CVD-related mortality, coronary heart disease, stroke, and congestive heart failure). We also conducted a race-stratified (European American [EA] and Black individuals) genome-wide association study (GWAS) for genetic associations with sCD163 levels. This article is based in part on results generated for a dissertation.<sup>24</sup>

# **METHODS**

# **Data Disclosure Statement**

The data that support the findings of this study are available from the CHS coordinating center upon reasonable request.

### **Subjects**

The CHS is a prospective, population-based cohort study of men and women recruited at age 65 years or older at baseline. The original predominantly EA cohort of 5201 participants was recruited between 1988 and 1989 at 4 field centers: Forsyth County, North Carolina; Sacramento County, California; Washington County, Maryland; and Pittsburgh, Pennsylvania. Between 1992 and 1993, an additional 687 mostly Black participants were recruited for a total cohort of 5888.<sup>25</sup> The baseline examination included a medical history, demographic

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and lifestyle history, physical exam, fasting blood collection, and an assessment of vascular disease by carotid ultrasound and ankle-brachial index. Carotid ultrasound was evaluated using high-resolution B-mode ultrasonography (model SSA-270A ultrasound machine; Toshiba, Tustin, CA). The scanning protocol has been previously described in detail.<sup>26</sup> Participants were followed with exams through 1999, and continue to be followed with telephone calls. Potential events were identified by multiple mechanisms, including self-report and Medicare hospitalization data. All events were adjudicated by an expert review panel.<sup>27–29</sup> Analyses were limited to those participants with available DNA who consented to genetic studies. The institutional review committee of each field center approved the study, and all participants provided informed written consent. Approval to use deidentified data for the analyses proposed in this study was obtained from the University of Vermont Institutional Review Board.

#### **Biomarker and Genotype Measurement**

sCD163 was measured in plasma by a commercial ELISA assay (R&D Systems) with a detectable range of 17 to 2000 ng/mL. The coefficient of variation was 3.05%. Participants were genotyped on the Illumina 370CNV BeadChip system. Genetic quality control analyses were performed using the software PLINK.<sup>30</sup> In ancestryspecific quality control analyses, single nucleotide polymorphisms (SNPs) were excluded from consideration for any of the following: (1) heterozygote frequency=0, (2) missing rate across subjects >3%, (3) Hardy-Weinberg equilibrium P value  $<1.0\times10^{-5}$ , (4) >2 duplicate errors or Mendelian inconsistencies (for reference HapMap Centre d'Etude du Polymorphisme Humain trios), or (5) an SNP not found in HapMap. A final set of 306655 autosomal SNPs remained after exclusions. Genotype imputation was subsequently 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 quality control. Imputation to the Haplotype Reference Consortium r1.1 2016 panel was performed on the Michigan imputation server.<sup>31</sup> Statistical analyses were restricted to common and uncommon imputed SNPs with minor allele frequency >0.01.

#### **Statistical Analysis**

Associations between untransformed sCD163 and quantitative traits systolic blood pressure (SBP), low-density lipoprotein cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, fasting glucose, fasting insulin, body mass index, waist circumference, C-reactive protein, IL-6, fibrinogen, and carotid intima media thickness and binary traits (diabetes and hypertension) were analyzed using multiple linear regression.

Statistical assumptions about modeled residuals were verified. Hypertension was defined as current use of antihypertensive medication and/or SBP >140 mm Hg or diastolic blood pressure >90 mm Hg.

Cox proportional hazards models were used to test for association between sCD163 and the risk of all-cause mortality, CVD mortality, incident coronary heart disease (CHD), incident stroke, and incident congestive heart failure (CHF). Incident CHD included non-procedure-related fatal or nonfatal myocardial infarction (MI). CVD mortality included fatal events where death was adjudicated as caused by atherosclerotic CHD or cerebrovascular disease, including definite fatal MI, definite fatal stroke, and definite or probable fatal CHD.<sup>28</sup> Participants with adjudicated baseline-prevalent disease for the corresponding incident disease were excluded from analyses (eq, individuals with a history of MI 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. Model A was minimally adjusted for baseline age, sex, race, and clinic site. Model B was additionally adjusted for established CVD risk factors (baseline smoking status, total cholesterol, HDL cholesterol, SBP, and blood pressure medication), and baseline CVD (for mortality outcomes). Model C was additionally adjusted for C-reactive protein. For the Cox models, sCD163 was standardized to aid interpretation. A second analysis, using quartiles of the sCD163 distribution (comparing the 3 highest quartiles of sCD163 to the lowest [reference] quartile) was also performed. The Cox proportional hazards assumption was verified using Schoenfeld residuals.<sup>32</sup>

For the genetic analyses, the associations between untransformed sCD163 and individual imputed SNPs, scored as dosage values (expected number of copies of the minor alleles), were tested in linear regression models implemented in Mach2qtl.<sup>33,34</sup> Analyses were stratified by self-reported race (EA, Black). Covariates in the regression models included age, sex, clinic site, and the first 10 principal components, used to control for potential population substructure. Principal components were calculated separately for Black and EA individuals using EIGENSOFT.<sup>35,36</sup> The statistical significance threshold was set to  $5 \times 10^{-8}$ . Regional association plots showing results were constructed using LocusZoom.<sup>32</sup>

# Expression Quantitative Trait Loci Look-Ups

For our lead variants, we identified linkage disequilibrium proxies using 1000G phase 3 version 5 (European and Black populations, for EA and Black CHS analyses)

using the LDlink LDproxy function.<sup>37</sup> Lists of conditionally independent expression quantitative trait loci (eQTLs) and splice quantitative trait loci mapped using stepwise regression for Genotype-Tissue Expression version 8 were downloaded from https://www.gtexp ortal.org/home/datasets, and overlap was assessed. We also downloaded a full list of cis eQTLs from the blood based eQTLGen<sup>38</sup> consortium (SNP-gene pairs with false discovery rate <0.05, with distance <1 Mb from the center of the gene and tested in at least 2 cohorts, limited to pairs with Bonferroni corrected P value <0.05). Conditionally distinct signals were not specified in this meta-analysis effort, so for identified eQTLs we assessed if 1 of our proxy variants was the lead cis eQTL for a given gene to assess colocalization (colocalization at secondary eQTL signals was difficult to assess with publicly available data). No colocalized eQTLs were found in eQTLGen using this method. We did not attempt eQTL look-ups for the Black-specific signal in the *HLA* region.

# Replication of Significant SNPs in EA Individuals

Loci with  $P < 5 \times 10^{-8}$  in the EA cohort were validated in the ADDITION-PRO cohort for association with sCD163 levels. The ADDITION-PRO cohort, a longitudinal cohort study nested in the Danish arm of the Anglo-Danish-Dutch study of Intensive Treatment Intensive Treatment In peOple with screeNdetcted Diabetes in Primary Care is a population-based, longitudinal study of Danish participants at high risk for diabetes.<sup>39</sup> sCD163 was measured with an in-house ELISA assay,<sup>40</sup> which correlates well with the assay used in the CHS ( $r^2$ =0.97). Genotyping was performed with the Illumnia Cardio-Metabochip and Illumina Human Exome 12v1 arrays. Analyses were

 Table 1.
 Association Results for sCD163 With Cardiovascular Risk Factors, Inflammation Biomarkers, and Measures of

 Subclinical Cardiovascular Disease at the Cardiovascular Health Study Baseline Examination

Baseline characteristics	Mean±SD or %	Model A, sCD163, ng/mL, β±SE	Model B, sCD163, ng/mL, β±SE
Age, y	72.5±5.4	3.2±0.6*	2.3±0.6*
Male sex	41.4%	-38.5±6.3*	-75.8±6.9*
Black race	15.8%	-69.5±8.5*	-49.8±14.9*
Current smoking	13.6%	-57.2±9.5*	-60.5±9.6*
Type 2 diabetes	16.4%	40.1±3.16*	62.6±8.8.5 <sup>†</sup>
Hypertension	66.3%	50.6±6.6*	11.4±9.7
BMI	26.7±4.7 kg/m <sup>2</sup>	8.3±0.7*	4.9±0.7*
Waist circumference	94±13 cm	2.9±0.3*	1.7±0.3*
Systolic blood pressure	139±20mmHg	0.7±0.2*	0.5±0.2 <sup>†</sup>
LDL cholesterol	130±36 mg/dL	-0.1±0.1	-0.4±0.3
HDL cholesterol	54±16 mg/dL	-3.2±0.2*	-3.0±0.2*
Triglycerides	141±78 mg/dL	0.3±0.0*	0.5±0.5
C-reactive protein	4.8±8.3 mg/L	3.4±0.4*	2.6±0.4*
Interleukin-6	2.2±1.9 pg/mL	15.7±1.7*	12.9±1.7*
Fibrinogen	324±67 mg/dL	0.3±0.1*	0.2±0.1*
Internal carotid wall thickness	1.4±0.6mm	27.7±6.1*	20.1±6.1 <sup>‡</sup>
Diastolic blood pressure	73±12 mm Hg	0.2±0.3	-0.1±0.3
Minimum ankle–arm index	1.07±0.18	-66.5±18.0*	-74.2±19.7*
Fasting glucose cohort, n=5201	mg/dL 111±37.3	1.0±0.1*	0.8±0.1*
Fasting glucose, no diabetes, n=4349	Mg/dL 99±9.8	1.5±0.3*	0.4±0.4
Fasting insulin cohort, n=5162	IU/mL 17.2±27.4	0.9±0.1*	0.5±0.1*
Fasting insulin, no diabetes, n=4331	IU/mL 13.8±10.1	2.8±0.3*	2.7±0.4*
HOMA-IR cohort, n=5162	5.5±15.4	1.4±0.2*	0.9±0.2*
HOMA-IR, no diabetes, n=4331	3.5±2.9	9.7±1.1*	8.8±1.5*

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, sex and clinic site. Model B: adjusted for age, race, sex, clinic site, smoking, blood pressure medication, systolic blood pressure, total cholesterol and HDL cholesterol. Diabetes was defined as having diabetes by the American Diabetes Association criteria. β for all measures except sex, race, diabetes, and hypertension are for a 1-unit change in the predictor. BMI indicates body mass index; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; and sCD163, soluble cluster of differentiation 163.

<sup>\*</sup>*P*<0.0001. †*P*<0.01. ‡*P*<0.001.

performed on 939 participants with both sCD163 measurements and genotype data adjusting for age, sex, and principal components 1 to 4, with a significance threshold (P<0.05) when corrected for multiple testing at 3 loci.

# RESULTS

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

sCD163 levels were approximately normally distributed with a mean of 787 ng/mL (SD, 221 ng/mL) and a range of 145 to 1633 ng/mL. In EA individuals, the mean baseline level of sCD163 was 780 ng/mL (SD, 216 ng/mL) in men and 810 ng/mL (SD, 214 ng/mL) in women. In Black individuals, the mean sCD163 level

was 688 ng/mL (SD, 232 ng/mL) in men and 756 ng/ mL (SD, 242 ng/mL) in women. sCD163 level was significantly higher in older individuals, in EA compared with Black individuals, and in women compared with men (Table 1; all P<0.0001). In Model A, adjusted for age, race, sex, and clinic site, sCD163 levels were positively associated with type 2 diabetes and hypertension, and with higher SBP, triglycerides, fasting glucose, fasting insulin, body mass index, waist circumference, C-reactive protein, IL-6, fibrinogen, carotid intima media thickness, minimum ankle-arm index, and homeostatic model assessment for insulin resistance; sCD163 levels were negatively associated with current smoking (versus former or never smokers) and HDL cholesterol (Table 1). There was no evidence for association (P>0.05) of sCD163 with low-density lipoprotein cholesterol or diastolic blood pressure.

Table 2.	Hazard Ratios for sCD163 and Incident Events in the C	Cardiovascular	Health Study
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		HR (95% CI) comparin	HR (95% CI) comparing each quartile to first quartile					
	HR (95% CI) for 1-SD unit increase in sCD163	First quartile (reference) 145.9≤626.8ng/mL	Second quartile >626.8≤770.3 ng/mL	Third quartile >770.3≤932.7 g/mL	Fourth quartile >932.7–1633.0 ng/mL			
All-cause mortality								
No. of events	3392	792	828	854	918			
Model A: HR (95% Cl)	1.08 (1.04–1.12)*	1	1.05 (0.95–1.16)	1.07 (0.97–1.18)	1.22 (1.10–1.34)*			
Model B: HR (95% Cl)	1.08 (1.03–1.12)*	1	1.03 (0.92–1.14)	1.07 (0.96–1.19)	1.19 (1.07–1.32)†			
Model C: HR (95% Cl)	1.06 (1.02–1.01)†	1	1.01 (0.91–1.13)	1.05 (0.95–1.17)	1.16 (1.04–1.29)‡			
Cardiovascular mortality								
No. of events	1360	278	341	353	388			
Model A: HR (95% Cl)	1.15 (1.09–1.21)*	1	1.24 (1.06–1.45) <sup>‡</sup>	1.27 (1.08–1.49)†	1.47 (1.26–1.72)*			
Model B: HR (95% CI)	1.09 (1.03–1.16)†	1	1.12 (0.94–1.33)	1.17 (0.98–1.39)‡	1.25 (1.05–1.49) <sup>‡</sup>			
Model C: HR (95% Cl)	1.08 (1.01–1.15)‡	1	1.11 (0.93–1.32)	1.15 (0.97–1.36)	1.22 (1.03–1.46)‡			
Incident CHD								
No. of events	1367	316	362	348	341			
Model A: HR (95% Cl)	1.10 (1.04–1.16)*	1	1.24 (1.06–1.44)†	1.23 (1.06–1.44)‡	1.32 (1.13–1.55)*			
Model B: HR (95% Cl)	1.03 (0.97–1.09)	1	1.10 (0.93–1.30)	1.09 (0.92–1.29)	1.09 (0.92–1.30)			
Model C: HR (95% Cl)	1.02 (0.96–1.08)	1	1.09 (0.92–1.28)	1.08 (0.91–1.27)	1.07 (0.90–1.27)			
Incident stroke								
No. of events	861	189	224	225	223			
Model A: HR (95% Cl)	1.08 (1.01–1.16)‡	1	1.18 (0.97–1.43)	1.16 (0.96–1.41)	1.23 (1.01–1.49)‡			
Model B: HR (95% Cl)	1.05 (0.97–1.13)	1	1.09 (0.88–1.35)	1.11 (0.90–1.38)	1.11 (0.89–1.38)			
Model C: HR (95% Cl)	1.04 (0.96–1.12)	1	1.08 (0.87–1.33)	1.11 (0.89–1.37)	1.09 (0.87–1.36)			
Incident CHF		·						
No. of events	1421	323	323	364	411			
Model A: HR (95% Cl)	1.18 (1.12–1.25)*	1	1.04 (0.89–1.22)	1.19 (1.02–1.38)‡	1.46 (1.26–1.70)*			
Model B: HR (95% Cl)	1.11 (1.05–1.18)*	1	0.95 (0.80–1.13)	1.08 (0.91–1.27)	1.24 (1.05–1.47)‡			
Model C: HR (95% CI)	1.10 (1.03–1.16)†	1	0.93 (0.79–1.11)	1.06 (0.90–1.25)	1.20 (1.01–1.42)‡			

Data are presented as HR with 95% Cl. Model A: age, sex, race, clinic site. Model B: Model A+smoking, total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, blood pressure medication. Model C: Model B+C-reactive protein. CHD indicates coronary heart disease; CHF, congestive heart failure; HR, hazard ratio; and sCD163, soluble cluster of differentiation 163.

\**P*<0.0001.

†*P*<0.005.

<sup>‡</sup>P<0.05.



**Figure 1.** Manhattan plot of CHS (Cardiovascular Health Study) association results for European American individuals. Significance line at 5×10<sup>-8</sup>.

When further adjusted for smoking, blood pressure medication, SBP, total cholesterol, and HDL cholesterol, all associations remained significant (Table 1).

#### **Incident Events Analysis**

Incident events adjudicated through June 2015 were used for the analyses. There were 3392 all-cause deaths, including 1360 CVD-related deaths. There were 1367 incident cases of CHD, 861 cases of incident stroke, and 1421 cases of incident CHF (fatal and nonfatal events). In survival Model A, minimally adjusted for age, sex, race, and clinic site, higher baseline sCD163 levels were significantly associated with increased risk for all-cause mortality, CVD mortality, incident CHD, incident stroke, and incident CHF (Table 2). In Model B, with further adjustment for smoking, blood pressure medication, SBP, total cholesterol, and HDL cholesterol, the association between baseline sCD163 and all-cause mortality, CVD mortality, and incident CHF remained significant. In Model C, C-reactive protein was added to the adjustments from Model B; the association between baseline sCD163 and all-cause mortality, CVD mortality, and incident



Figure 2. Manhattan plot of CHS (Cardiovascular Health Study) association results for Black individuals. Significance line at  $5 \times 10^{-8}$ .

Nearest gene	Chromosome	Lead variant	Base pair position, GRCh38	Annotation	P value	β	Effect allele frequency	Effect allele	No. of significant variants	Replication <i>P</i> value
MGAT5	2	rs62165726	134208991	Intronic	3.3E-18	-123.5	0.04	А	38	4.7E-04
ASGR1	17	rs55714927	7 176 997	Synonymous variant	1.5E-14	58.3	0.19	Т	19	2.4E-08
ST3GAL4	11	rs11220463	126378316	Intronic	1.4E-8	-44.9	0.14	Т	18	8.2E-07

Table 3.	Lead Variants and Number of Signals in the European American Genome-Wide Association Study Analy	sis

Replication analyses were conducted in the European ancestry ADDITION-PRO cohort, a nested study in the Danish arm of the Anglo-Danish-Dutch study of Intensive Treatment In peOple with screeNdetected Diabetes in Primary Care, (n=939); all effect estimates were directionally concordant with those reported in the Cardiovascular Health Study.

CHF remained significant. If we shortened the time period to events to 10 years from baseline, we found all-cause mortality to be significant for both Models A and B, cardiovascular mortality, and incident CHF to be significant for Model A.

#### GWAS of sCD163

We conducted race-stratified GWAS analyses in 2838 EA individuals and 763 Black individuals from the CHS who had both sCD163 measurements and GWAS data available (Figure 1 and Figure 2). The EA GWAS identified a total of 75 significant ( $P<5\times10^{-8}$ ) SNPs from 3 regions (Table 3). Thirty-eight were near the *MGAT5* gene on chromosome 2 (Figure 3), with the most significant variant being

rs62165726 (P=3.3×10<sup>-18</sup>), previously reported as associated with sCD163.<sup>41</sup> Nineteen novel significant SNPs were identified on chromosome 17 near a tight cluster of genes, namely *ASGR1*, *DLG4*, and *ACADVL* (Figure 4), with rs55714927 being the most significant (P=1.5×10<sup>-14</sup>). Eighteen novel significant SNPs were identified near the *ST3GAL4* gene on chromosome 11 (Figure 5), with the most significant SNP being rs11220463 (P=1.4×10<sup>-8</sup>).

The Black GWAS identified a total of 12 significant SNPs (Table 4), all novel. Nine were on chromosome 6 in the *HLA* region (Figure 6), with the most significant being rs3129781 (P=7.1×10<sup>-9</sup>), and the other 3 were on chromosome 16 in the *MYKL3* region (Figure 7), with the most significant being rs115391969 (P=4.3×10<sup>-8</sup>).



# Figure 3. LocusZoom plot of CHS (Cardiovascular Health Study) association results for European American individuals for *MGAT5* locus 1000 Genomes Project November 2014 release European Population National Center for Biotechnology Information genome build 37 (hg19/1000Genomes Nov2014 EUR).

chr2 indicates chromosome 2.



Figure 4. LocusZoom plot of CHS (Cardiovascular Health Study) association results for European American individuals for *ASGR1* locus 1000 Genomes Project November 2014 release European Population National Center for Biotechnology Information genome build 37 (hg19/1000Genomes Nov2014 EUR). chr17 indicates chromosome 17.

Our 75 significant SNPs in the EA individuals were analyzed in the ADDITION-PRO cohort for replication. All 18 significant variants on chromosome 11 in the *ST3GAL4* region replicated when adjusted for age, sex, and principal components 1 to 4 at *P*<0.003, with a consistent direction of effect when corrected for multiple testing. The 19 significant SNPs on chromosome 17 in the *ASGR1* region replicated, and the 5 most significant SNPs on chromosome 2 in the *MGAT5* region replicated in the ADDITION-PRO cohort. Results for the lead variant at each locus are included in Table 3.

### DISCUSSION

In this study of sCD163 we observed that: (1) sCD163 levels were associated with established CVD risk factors and with carotid intima media thickness, a measure of subclinical atherosclerosis, as well as minimum ankle–arm index, an indicator of peripheral artery disease; (2) higher sCD163 was significantly associated with all-cause mortality, CVD-related mortality, and incident CHF (but not with incident CHD or stroke), independent of established CVD risk factors; (3) genetic variants near *MGAT5*, the *ASGR1/DLG4/ACADVL* gene cluster, and *ST3GAL4* were associated with sCD163 in EA individuals, and variants near *HLA-DQB1* and

*MYLK3* were associated with sCD163 levels in Black individuals.

It has been postulated that sCD163 contributes to innate immunity by binding hemoglobin-iron in the circulatory system, thus making the iron unavailable to pathogens.<sup>14,15,42</sup> Interestingly, a similar hypothesis has been raised about haptoglobin, centered on haptoglobin's role distal to CD163 in clearing hemoglobin and ameliorating its toxic effects.<sup>43</sup> sCD163 also modulates the immune system by inhibiting T-cell proliferation.<sup>44,45</sup> Macrophages scavenge hemoglobin-haptoglobin complexes via membrane bound CD163, resulting in the upregulation of IL-10 and heme oxygenase-1 levels having an anti-inflammatory, atheroprotective effect.<sup>46</sup>

There is some uncertainty as to the origin of circulating sCD163. Although sCD163 may directly reflect the level of M2 macrophages,<sup>47</sup> sCD163 might also reflect the transitioning between M1 and M2 macrophages during tissue repair, including cardiac injury repair. Upon initial cardiac injury, the presence of M1 macrophages increases.<sup>48</sup> Once the acute phase of the injury has passed, there is a transition to a population of relatively anti-inflammatory CD163expressing 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



Figure 5. LocusZoom plot of CHS (Cardiovascular Health Study) association results for European American individuals for *ST3GAL4* locus 1000 Genomes Project November 2014 release European Population National Center for Biotechnology Information genome build 37 (hg19/1000Genomes Nov2014 EUR).

chr11 indicates chromosome 11.

of monocytes to M2 macrophages and as a result of M1 macrophages transitioning to M2 macrophages.<sup>48</sup> As the tissue remodeling continues, there is a return to the M1 and M2 macrophage balance; this may result in the shedding of the CD163 receptor and an increase in the level of circulating sCD163. The transition of macrophages from the inflammatory M1 type to the anti-inflammatory M2 type may be a means of protection following a cardiac event. However, the continued expression of M2 macrophages may also lead to fibrosis.<sup>49</sup> Other studies have shown that sCD163 is a more general marker of macrophage activation,<sup>50,51</sup> associated with noncalcified coronary plaque,<sup>50</sup> and is over-expressed in symptomatic carotid plaques.<sup>52</sup>

After accounting for established CVD risk factors, sCD163 was a modest, but significant, predictor of incident CHF (hazard ratio [HR] comparing the top to the bottom quartile of the distribution, 1.24 [95% CI, 1.05–1.47]), CVD mortality (HR, 1.25 [95% CI, 1.05–1.49]), and all-cause mortality (HR, 1.19 [95% CI, 1.07–1.32]). A proteomics study with fewer participants (n=1737) in the Malmo Preventative Project found significant associations of CD163 with both all-cause mortality (HR, 1.19 [95% CI, 1.09–1.30]) and cardiovascular mortality (HR, 1.21 [95% CI, 1.09–1.36]) but not incident heart failure when analyses were adjusted for cardiovascular

risk factors.<sup>53</sup> An analysis of sCD163 in the Norwegian Trondelag Health Study (HUNT) study (n=3513) found that higher levels of sCD163 were associated with increased risk of MI in minimally adjusted models (odds ratio [OR], 1.27 [95% CI, 1.07–1.50]), but this association was attenuated when the adjustments included cardiovascular risk factors (OR, 1.02 [95% CI, 0.84– 1.24]).<sup>54</sup> By way of confirmation, when the CHS data were analyzed with similar adjustments to those in the HUNT study, there also was no significant association of sCD163 with MI.

Our EA GWAS identified 3 regions associated with sCD163 levels, near *MGAT5*, *ASGR1/DLG4/ACADVL*, and *ST3GAL4*. We did not observe any genome-wide significant cis protein quantitative trait loci at the CD163 locus in either the EA or Black analysis. No significant cis protein quantitative trait loci (at the CD163 gene locus) were observed in previous analyses of CD163 using the SOMAscan platform in the INTERVAL cohort, a randomized trial recruited through the National Health Service Blood and Transplant service in England,<sup>41</sup> although we note that 2 cis protein quantitative trait loci at *CD163*, relatively small effect insertion/deletion variants with no assigned reference single nucleotide polymorphism cluster identifier, were recently identified in large analyses from Iceland (n>35000) using the SOMAscan



**Figure 6.** LocusZoom plot of CHS (Cardiovascular Health Study) association results for Black individuals for chromosome 6 (chr6) 1000 Genomes Project November 2014 release African American Population National Center for Biotechnology Information genome build 37 (hg19/1000Genomes Nov2014 AFR).

platform.<sup>55</sup> Our most significant variants in each region replicated in the ADDITION-PRO study. The replication adds credibility to the genetic associations with sCD163 levels we found at these 3 genomic regions. Thirtyeight significant SNPs were in or near MGAT5, a gene on chromosome 2, which codes for a glycosyltransferase (mannosyl [α-1,6-]-glycoprotein β-1,6-N-acetylglucosaminyltransferase) and has previously been associated with CD163. In a mouse model, MGAT5 was linked to the M2 macrophage phenotype and fibrosis; MGAT5<sup>-/-</sup> mice had far fewer M2 macrophages.<sup>56</sup> Deregulation of the glycosyltransferase increases susceptibility to autoimmune diseases and is associated with the severity of multiple sclerosis.<sup>57,58</sup> 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 with MGAT5-/mice, and that glycosylated cell surface proteins cause a shift in the macrophage phenotype to M2.<sup>59</sup> The concordance of CD163 around the association of *MGAT5* in SOMAscan-based GWAS results<sup>41</sup> with our results (with an identical lead variant at *MGAT5*), strengthens this finding. We performed look-ups in Sun et al's<sup>41</sup> summary statistics for all our lead variants except the African-specific variant at *MYLK3* (rs115391969, which in GnomAD version 2.1.1 is found only in 5.5% individuals of African ancestry and <0.5% of individuals from other ancestry groups), as listed in Table 5.

Eighteen significant SNPs were in a region near *ST3GAL4*,  $\beta$ -galactoside  $\alpha$ -2,3-sialytransferase 4, 3gal4, on chromosome 11, which encodes for several enzymes involved in protein glycosylation. To date, none of these genes has been associated with sCD163 or CD163 expression. Rs11220462 and rs11220463 in *ST3GAL4* have previously been associated with both total cholesterol and low-density lipoprotein cholesterol in EA individuals.<sup>60,61</sup> All 18 of the SNPs were

Table 4.	Lead Variants and Number of Signals in the Black Genome-Wide Association	Study Analy	ysis
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Nearest gene	Chromosome	Lead variant	Base pair position, GRCh37	Annotation	P value	β	Effect allele frequency	Effect allele	No. of significant variants
HLA-DQB1	6	rs3129781	32690723	intergenic	7.1E-9	-98.9	0.87	Т	9
MYLK3	16	rs115391969	46740797	Intronic	4.3E-8	159.6	0.05	Т	3



Figure 7. LocusZoom plot of CHS (Cardiovascular Health Study) association results for Black individuals for chromosome 16 (chr16) 1000 Genomes Project November 2014 release African American Population National Center for Biotechnology Information genome build 37 (hg19/1000Genomes Nov2014 AFR).

associated with the expression of *ST3GAL4* in the genotype-tissue expression project.

Nineteen significant SNPs were in or near ASGR1. DLG4, and ACADVL on chromosome 17, and none of which has a known association with CD163. ASGR1 codes for a transmembranous receptor involved in serum glycoprotein homeostasis<sup>62</sup> and has been linked to lower levels of non-HDL cholesterol and a lower risk of heart disease.<sup>62</sup> DLG4 is involved in the cellular response to oxidative stress, a key factor in cardiovascular diseases.<sup>63</sup> Rs314253 has been cited in the literature as being associated with total cholesterol<sup>64</sup> and with the concentration of liver enzymes in plasma.<sup>65</sup> DLG4 codes for postsynaptic 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 multiple sclerosis.<sup>63</sup> An increase in reactive oxygen species is a key feature of the development of cardiovascular disease. ACADVL codes for an acyl-CoA dehydrogenase, an enzyme that catalyzes the alpha, beta dehydrogenation of acyl-CoA esters in fatty acid and amino acid catabolism. The transcribed regions of DLG4 and ACADVL overlap and share common regulatory elements.<sup>66</sup> Interestingly, both MGAT5 and DLG4 have been linked to fibrotic diseases.56,63 Fibrosis is a contributing factor to CHF and cardiac senescence. It is possible that our results showing an association of higher sCD163 levels with an increased risk of heart failure are the result of increased fibrosis. Mouse studies showed that the M2 macrophages in the aging heart might contribute to cardiac senescence and heart failure.<sup>67</sup> Further research is necessary to understand the balance and role of the anti-inflammatory and the profibrotic effects

Table 5.	Look-Ups of	Our Lead	Variants in	Summary	Statistics
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Variant	Chromosome	Position	Effect allele	Other allele	β	Standard error	P value
rs62165726	2	134966562	а	с	-0.69	0.06	1.62E-30
rs11220463	11	126248211	t	а	-0.15	0.04	3.55E-05
rs3129781	6	32648500	t	g	-0.09	0.04	0.02
rs55714927	17	7080316	t	с	0.24	0.03	3.09E-14

Data derived from Sun et al (https://www.phpc.cam.ac.uk/ceu/proteins/, PMID 29875488).

of M2 macrophages in heart failure. Further research is also necessary to understand whether any of the variants we found have functional relevance and if the closest genes are the origins of these variants' effects.

The Black GWAS identified 12 significant SNPs associated with sCD163 levels, and none had been identified before. Nine significant results were on chromosome 6 in the HLA region. We did not have good guality data in additional Black populations with GWAS and sCD163 data to replicate these variants; associations in the HLA region are difficult to replicate across populations because of extremely complex linkage disequilibrium patterns and high heterogeneity between different ancestry populations, and SNPs at MYLK3 were rare in European-ancestry populations (minor allele frequency for rs115391969 6.9% African, 0.1% European in 1000 Genomes Project phase 3). Multiethnic analyses of sCD163 should be the focus of future studies to better understand the genetic variants associated with sCD163 levels. HLA-DRB1 is a linchpin of the inflammatory response and has been associated with fibrosis.<sup>68</sup> HLA-DRB1 is an major histocompatability complex class II gene that encodes for proteins that are on particular immune cells. The HLA-DRB1 protein binds to the product of HLA-DRA forming the functional HLA-DR antigen binding heterodimer which presents peptides T-helper cells triggering an immune response.

Three of the significant SNPs were located on chromosome 16 in the *MYLK3 (myosin light chain kinase 3)* region of the genome. The expression level of *MYLK3* is highly expressed in the heart and has been shown to be highly correlated with the severity of heart failure.<sup>69</sup>

To have strong instruments available for Mendelian randomization, it is necessary to identify variants that are plausibly associated with an outcome (in our case CVD) only through the exposure (in our case CD163 levels).<sup>70</sup> The genomic regions identified in this analysis also have pleiotropic relationships with other proteins plausibly associated with cardiovascular disease (for example immediate early release gene 2 protein, transmembrane protein 132C, semaphorin-3G, and soluble glycoprotein 130 with the ST3GAL4 locus,<sup>41</sup> associations with vascular cell adhesion protein 1, cluster of differentiation 5 (CD5), complement receptor 1 for the MGAT5 locus in recently published data<sup>55</sup>), and we identified no cis protein quantitative trait loci at the CD163 locus. Future work may identify more promising instruments for Mendelian randomization and help clarify the putative causal role of sCD163 with CVD.

There are several limitations to this study. Although associations of sCD163 with outcomes were highly statistically significant in some cases, effect sizes were modest, so it is possible that residual confounding is present. We adjusted for a variety of risk factors, and the positive association of sCD163 SNPs with CHF would argue against residual confounding for this outcome, although this association was only nominally significant. In our GWAS, we excluded rare (minor allele frequency <0.01) variants, for example coding variants in CD163, which may also be important in accounting for the variability in the sCD163 levels. Given that the sample size for Black individuals was considerably smaller than that in EA individuals, the statistical power to detect associations was much more limited in Black individuals, and appropriate samples were not available for replication of genetic associations in Black individuals. Because our study was focused on older adults, results may not be generalizable to younger populations. Finally, we did not stratify our heart failure cases into preserved ejection fraction versus reduced ejection fraction because of power considerations. Because the underlying pathophysiology of these 2 types of heart failure is different,<sup>71</sup> this may have diluted our results.

In summary, our findings suggest that sCD163 is a risk factor for all-cause mortality, cardiovascular mortality, and CHF in older adults, independent of established CVD risk factors. We have observed several novel genetic associations for sCD163 suggesting a possible causal role for monocyte activation, especially related to the M2 phenotype, in CHF. Additional studies are needed to assess functional variants in the genes we have identified, and whether sCD163 levels predict these outcomes in younger populations.

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