The association of mannose binding lectin genotype and immune response to *Chlamydia pneumoniae*: The Strong Heart Study

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Abstract

Cardiovascular disease (CVD) is an important contributor to morbidity and mortality in American Indian communities. The Strong Heart Study (SHS) was initiated in response to the need for population based estimates of cardiovascular disease in American Indians. Previous studies within SHS have identified correlations between heart disease and deficiencies in mannose binding lectin (MBL), a motif recognition molecule of the innate immune system. MBL mediates the immune response to invading pathogens including *Chlamydia pneumoniae* (*Cp*), which has also been associated with the development and progression of CVD. However, a link between *MBL2* genotype and *Cp* in contributing to heart disease has not been established. To address this, SHS collected baseline *Cp* antibody titers (IgA and IgG) and *MBL2* genotypes for common functional variants from 553 individuals among twelve participating tribes. A single nucleotide polymorphism (SNP) in the promoter, designated X/Y, correlated significantly with increased *Cp* IgG titer levels, whereas another promoter SNP (H/L) did not significantly influence antibody levels to *Cp*. Two variants within exon 1 of *MBL2*, the A and B alleles, also displayed significant association with *Cp* antibody titers. Some *MBL2* genotypes were absent from the population, suggesting linkage disequilibrium may be operating within the SHS cohort. Additional factors, such as increasing age and socioeconomic status, were also associated with increased *Cp* IgG antibody titers. This study demonstrates that *MBL2* genotype associates with immune reactivity to *C. pneumoniae* in the SHS cohort. Thus, *MBL2* may contribute to the progression of cardiovascular disease (CVD) among American Indians indirectly through pathogen interactions in addition to its previously defined roles.

Introduction

Cardiovascular disease contributes substantially to poor global health. In the United States, cardiovascular disease (CVD) is the leading cause of death, responsible for 1 in 4 deaths [1].
Cardiovascular disease risk is largely composed of two primary sources, environmental contributions and genetic predisposition. Environmental contributors to CVD include diet, lifestyle, air quality, and infectious agents. The relative contributions of these factors vary across time, geography, and socioeconomic status. Consequently, environmental factors shape the impact of CVD on specific populations differently. Some factors such as infectious disease outbreaks and air quality are communal and require large-scale concerted efforts to combat while diet and lifestyle include individualized components. Preventative therapies exist that alleviate the impact of personalized risks such as smoking cessation programs and lifestyle/dietary intervention but do not address factors that impact whole populations. For example, previous work has linked locally endemic infectious agents to CVD risk including HIV, Trypanosoma cruzi, and Chlamydia pneumoniae, which are highly prevalent within particular global regions.

Cardiovascular disease is a complex genetic disease influenced by the contribution of thousands of variant positions across hundreds of loci. Immunomodulatory molecules comprise a significant portion of genes implicated in CVD and include genes such as C-reactive protein (CRP), apolipoprotein E (apoE), and mannose binding lectin (MBL2). MBL is a major circulating C-type lectin of the innate immune system involved in self versus non-self recognition. As such, MBL plays a significant role in identification of dead, dying, or cancerous cells for clearance by professional phagocytes. Similarly, MBL binds to a variety of pathogen-associated molecular patterns (PAMPs) leading to pathogen recognition and destruction. MBL2 dysregulation is associated with coronary heart disease (CHD) and increased susceptibility to microbial infection.

One of the pathogens most consistently associated with CVD onset and progression is Chlamydia pneumoniae (Cp), an obligate intracellular bacterium that infects humans, and specifically targets endothelial cells. C. pneumoniae is capable of producing both active, acute disease as well as latent infections that persist throughout the life of the host with the potential to re-emerge. During active infection, accumulation of cellular debris during rupture of infected cells as well as attraction of circulating phagocytes can lead to plaque formation on blood vessel walls, inducing vascular complications such as coronary heart disease (CHD). MBL2 operates as a recognition receptor for mannose and N-acetylglocusamine, which are major components of the Cp cell membrane. Importantly, reduced MBL function has been linked to CVD severity and progression in patients infected with Chlamydia pneumoniae.

Variants within the MBL2 coding sequence and promoter region, have significant consequences on its molecular function. Several well characterized MBL2 promoter and coding missense SNPs significantly alter circulating levels of MBL and are associated with clinical disease. The frequency of these coding alleles within groups varies depending on population origin. For example, the B allele frequency is approximately 45% in South American Mapuche and Chiriguanos, whereas it is present in less than 1% of a Mozambican population. Such population-based variation in allele frequency may contribute to differences in disease risk among specific demographics.

The Strong Heart Study (SHS) is the largest epidemiological study within American Indian populations and is focused on cardiovascular health. Twelve tribes have participated in the Strong Heart Study to develop reliable, population-based data related to cardiovascular...
health among American Indians. Previous studies within the SHS cohort identified an association between MBL2 genotypes and coronary artery disease [27]. However, the relationship between the immune response to Cp and MBL2 genotypes associated with CVD has not yet been established.

Here we investigated the relationship between Cp and MBL2 within the SHS cohort. MBL2 promoter and coding sequence genotype frequencies do not reflect majority Caucasian populations. Cp antibody titers are significantly affected by the X/Y promoter variant and the copy number of MBL2 ‘A’ and ‘B’ alleles. Several additional descriptive factors correlate with Cp antibody levels including age and education. Thus, development of CVD may be due, in part, to the interaction of the host immune system with infectious agents such as Cp.

Methods
Participants
This ancillary study was conducted between August 1, 2000, and November 30, 2000, among individuals enrolled in the Strong Heart Study (SHS) at ages 45 to 74 years between July 1, 1989, and January 31, 1992. It was approved by the Indian Health Service, MedStar Research Institute Institutional Review Boards, and by the participating tribes. All investigative methods conformed to the principles set out by the Declaration of Helsinki and involved informed consent of the participants. Since these data were collected, one tribe has declined further participation and those tribal members have been excluded from the analysis. All studies are reviewed and approved by the SHS Review Board composed of tribal representatives prior to study initiation and publication.

The original study’s design and methods have been reported [26]. An ancillary study of the relationship between infectious disease and CVD measured baseline antibodies to C pneumoniae and other pathogens on a subset of 421 CVD cases and an equal number of controls, matched by study center, sex and age (+/- 5 years). From this substudy, MBL2 genotypes were available from 553 individuals. Medical record review by trained medical record abstractors and a physician reviewer committee determined that 211 had experienced definite CVD, 161 possible CVD and 181 no CVD in subsequent follow-up. Risk factors for CVD were determined at time of entry into the SHS and at two subsequent follow-up examinations.

Blood specimen collections
Blood specimens collected during the study were separated into serum samples, frozen at -80°C, and aliquots were thawed only when performing specific tests.

MBL2 locus genotyping
Genotyping was done at the University of Pittsburgh. A total of 553 individuals were assessed for the presence of the MBL2 B allele (G54D, rs1800450), C allele (G57E, rs1800451) or D allele (R52C, rs5030737) structural variations, and two promoter polymorphisms, one a G/C transition at—550 bp (the H/L alleles, rs11003125) and the other, a G/C transition at -221 (X/Y alleles, rs7096206). The most common coding allele has been conventionally labelled “A” and the three structural variants collectively labelled “O”. MBL2 genotypes were determined by the oligonucleotide ligation assay as described by Nickerson and colleagues [28]. Quality control, duplicate, genotyping was performed by direct DNA sequencing. The structural variations were assumed to occur on opposing chromosomes. A number of promoter variants and structural alleles have been found to be in complete linkage disequilibrium and genotypes were checked against these established relationships [28].
MBL2 allele frequencies for comparisons were obtained from the following studies: Dutch [29], Inuit [30], Mozambican [25], South Korean [31], and those from the United States [32].

Haplotypes were built using previously described methods [27]. Briefly, homozygous positions could be inherently linked to alleles at other variant positions. Subsequently, unresolved haplotypes were built using previously described association including the B and C coding alleles and X promoter variant being found with the L promoter variant, the D MBL2 allele linked to the H promoter variant, etc., etc.,

**Cp antibody titer**

Serum IgG and IgA antibody titers for *C. pneumoniae* were determined by microimmunofluorescence (MIF) at the International Chlamydia Laboratory at Johns Hopkins School of Medicine. This test uses the application of purified elementary bodies (EBs) from high titer chlamydia EB antigen preparations (Washington Research Foundation, Seattle, WA). There is only one serovar of *C. pneumoniae* and the purified antigen is made from strain AR39. Antigen dots for *C. trachomatis* were also included in the series of antigen dots, so that the specificity of the anti-*C. pneumoniae* antibody could be confirmed. The highest dilution of serum demonstrating good even fluorescence of the EBs was recorded as the titer for each group. Separate assays were performed for the determination of IgG, and IgA. The laboratory has participated with others in quality assurance studies for MIF and demonstrated consistency and comparability for this assay. The *Cp* antibody titers could be determined for 514 individuals.

**Statistical analysis**

Statistical testing for correlational analysis were performed through SPSS and R (v3.3.1) software. SPSS was developed by Microsoft, and R was developed by the R development team.

**Results**

The MBL2 gene is a key component of innate immunity whose product recognizes pathogen-associated molecular patterns of invading organisms and marks them for destruction. The functional capacity of MBL2 is associated with five polymorphic sites, two in the promoter and three within the first exon [33]. The two promoter variant positions, denoted as H/L and X/Y, are located 550 and 221 nucleotides upstream of the start codon, respectively (Fig 1A). Polymorphisms at these positions impact the level of MBL2 transcription with the H and Y variants producing more robust expression [28]. Additionally, variants of exon 1 referred to as alleles B, C, and D encode changes at amino acid 52, 54, and 57 compared to the reference A allele. The B and C alleles produce proteins with reduced function due to a decrease in oligomerization capability required for function and the D allele is especially weakened in its immunological role [28].

**MBL2 genotypes and distributions**

Previous investigations within the Strong Heart Study cohort identified links between heart disease and low functioning MBL2 genotypes [17, 27]. To determine the distribution of MBL2 alleles within the SHS cohort using an expanded panel of polymorphic positions, individuals were genotyped at both promoter polymorphic sites and exon 1. The majority of individuals encoded the functional A/A MBL2 genotype (69.6%) with a smaller subset either heterozygous or homozygous for a “non-A” (O) allele (27.5% and 2.9%, respectively) (Table 1). The B variant was the most common O allele (14.3%) followed by the D and C alleles (1.6% and 0.7%, respectively).
Similar exon 1 allele frequencies are observed among distinct populations from different geographic regions for the A allele (Fig 1B). In contrast, the distribution of non-A alleles within exon 1 differed widely among distinct populations. The SHS cohort, Inuit, and South Korean populations harboured high frequencies of allele B, the Mozambican population primarily

Table 1. Frequency of MBL2 genotypes within the SHS cohort.

| SNP position | Homozygous (Major) | Heterozygous | Homozygous (Minor) |
|--------------|--------------------|--------------|--------------------|
| H/L          | 0.467 (HH)         | 0.389 (HL)   | 0.14 (LL)          |
| X/Y          | 0.888 (YY)         | 0.11 (XY)    | 0.003 (XX)         |
| A            | 0.696 (AA)         | 0.275 (AO)   | 0.029 (OO)         |
| B            | 0.252 (B(A/O))     | 0.029 (BB)   |                    |
| C            | 0.007 (C(A/O))     | 0.00 (CC)    |                    |
| D            | 0.016 (D(A/O))     | 0.00 (DD)    |                    |

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contained C allele variants, and the United States population had an even distribution among all three non-A alleles (Fig 1C). Furthermore, promoter alleles frequencies varied considerably among representative populations (Fig 1D). In the SHS cohort population the H and Y variants comprised 66.2% and 94.3% of the population, respectively (Table 1). Alleles within all genotyped variant positions conformed to Hardy-Weinberg equilibrium (S2 and S3 Tables). The SHS cohort was most similar to the Inuit and South Korean populations, suggesting that the frequency of MBL2 genotypes among American Indians significantly differs from Americans of European descent.

MBL2 haplotypes were constructed from allelic genotypes to assess association of promoter and coding variants. Haplotype construction revealed a majority of HYA haplotypes among SHS participants associated with robust Mbl2 function (Table 2). Thus, relatively few haplotypes containing L variants emerged from construction and X variant haplotypes were very rare. Consequently, all assembled haplotypes included at least one high function allele from either the promoter or exon 1.

Comparison to MBL2 haplotypes from other global populations most resembled the Inuit and South Korean populations, similar to analysis of individual variant positions.

Association of MBL2 genotype with C. pneumoniae

To test for an association between MBL2 genotype and immune reactivity to C. pneumoniae, antibody titers were measured for systemic IgG and mucosa-enriched IgA. C. pneumoniae IgG titers followed a Gaussian distribution centered on 1:128, indicating relatively strong systemic reactivity (Fig 2A). Participants expressed lower levels of IgA to C. pneumoniae, however, with more than half exhibiting no detectable antibody (Fig 2B). A positive linear correlation exists between Cp IgG and IgA within the SHS cohort (Spearman, rs = 0.47, p<0.01, Fig 2C). Consequently, the majority of our subsequent analysis relied upon IgG antibody titers as an indicator of immune reactivity.

To determine the influence of MBL2 polymorphism on Cp antibody titers, we assessed the distribution of antibody levels for individual SNP positions. Analysis of covariance revealed additional copies of the X allele are significantly correlated with elevated Cp IgG titers (F(1,544) = 3.895, p = 0.049, Fig 3A, Table 3).

The H/L polymorphic site trended towards increased Cp antibody titers with increased dosage of the L allele but did not reach significance (F(1, 544) = 1.934, p = 0.165, Fig 3A).

The coding variants of exon 1 also impacted antibody levels to C. pneumoniae, with Cp antibody titer increasing with each additional A allele (F(1,544) = 4.39, p = 0.037, Fig 3B, Table 4). The B allele was also predictive of Cp IgG antibody titers (F(1,544) = 4.48, p = 0.035, Table 4). Neither the C nor D allele displayed any association with Cp titers although this may be due, in part, to their low prevalence and hence, lack of power. Taken together, both promoter and coding sequence alleles correlate with Cp antibody titers in the SHS cohort.

### Table 2. Frequency of MBL2 haplotypes.

| Population       | HYA | LYA | LXA |
|------------------|-----|-----|-----|
| Dutch [29]       | 0.36| 0.36| 0.28|
| Inuit [30]       | 0.89| 0.06| 0.05|
| Mozambican [25]  | 0.08| 0.75| 0.17|
| SHS cohort       | 0.75| 0.16| 0.09|
| South Korean [31]| 0.66| 0.21| 0.13|
| United States [32]| 0.39| 0.29| 0.33|

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The aggregate contributions of individual polymorphic sites to \( Cp \) antibody titers can either be additive or show more complex interactions. To investigate this, the average \( Cp \) IgG titers were plotted for each represented genotype in the SHS cohort (Fig 3C, S1 Fig). No specific position determined the relative antibody levels within the cohort, suggesting that the combined effects of each variant contribute to the overall immune reactivity. For example, the \( MBL2 \) A and L alleles were enriched among higher \( Cp \) antibody titer groups and co-occurred in individuals with the highest average antibody titers. Additionally, the X promoter variant clustered with genotypes displaying higher Ab titers. Interestingly, the reported strength of \( MBL2 \) function for variant positions did not seem consistent with \( Cp \) antibody titers; the high-functioning \( MBL2 \) A allele corresponded to increased antibody levels, while promoter variants with reduced function (X and L) also correlated with higher \( Cp \) antibody titers. Across variant positions, the \( MBL2 \) A allele displayed the strongest association with \( Cp \) antibody titers (S2 Fig). Furthermore, analysis of constructed haplotypes including promoter and coding variants also failed to identify a significant association with \( Cp \) antibody titer (\( F(6, 1094) = 1.575, p = 0.151 \)). Thus, individual promoter and coding sequence variants but not complete genotypes correlate with antibody production to \( Cp \).

**Associations of covariates**

Changes in \( Cp \) antibody titers can be influenced by multiple non-genetic factors that are produced by lifestyle and the environment [34]. We tested the association of \( Cp \) IgG titers against 14 variables including age, sex, education, smoking, alcohol use, ACR, and prior diagnoses of cardiovascular disease and diabetes (S4 Table). Three features showed significant associations with \( Cp \) IgG antibody levels, age (Spearman’s correlation; \( r_s = 0.09 \) \( p < 0.041 \)), smoking (Kruskal–Wallis; \( H(2) = 7.185, p = 0.028 \)), and sex (Mann Whitney; \( U = 3.27, p = 0.001 \)) (Fig 4). Additionally, the presence of the Y or H variants in the \( MBL2 \) promoter strongly correlated with a prior diagnoses of diabetes (\( r_s = -0.106, .111; p = <0.01 \) for both) (S4 Table).

**Association of \( Cp \) with CVD**

\( Cp \) infection has been previously associated with autoimmune disorders including CVD [17, 20]. To test for this association, participant genotypes were separated based on CVD diagnosis as either present, possible, or absent. No relationship between CVD and \( Cp \) antibody titers existed among SHS participants with diagnosed CVD versus healthy individuals (Mann-
Whitney U-test (MW); $F(1,369) = 1.135, p = 0.256$, $S4$ Table). Furthermore, a test for association between $C_p$ antibody titers and mortality due to CVD failed to identify a relationship (MW; $F(1,369) = 0.879, p = 0.379$). However, the $H/L$ MBL2 promoter variant associated with CVD in which increased copies of low expression $L$ alleles associated with increased CVD diagnosis as would be expected ($S4$ Table). Thus, no clear relationship between immune reactivity to $C_p$ and CVD within this SHS population can be established.

Discussion

Here, we have shown that MBL2 genotypes within an American Indian cohort correlated with the immunological response to the intracellular bacteria $C. pneumoniae$. MBL2 promoter

Table 3. ANCOVA of $C_p$ IgG versus MBL2 promoter SNP positions.

| Source | dF | $F$   | MSE   | Partial Eta Squared | p-value |
|--------|----|-------|-------|---------------------|---------|
| L allele | 1  | 1.934 | 647188.321 | 0.003       | 0.165   |
| Y allele | 1  | 3.895 | 134039.451 | 0.006       | 0.049   |

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variants within the SHS cohort were not found at similar frequencies compared to majority Caucasian populations. Both \( \text{MBL2} \) promoter and coding variants were associated with the prevalence of antibody following exposure to \( \text{C. pneumoniae} \), suggesting differences in immunological response although constructed haplotypes diminished the associations of individual variants with \( \text{Cp} \) antibody titers. The degree of immunogenicity was also correlated with more complex social and biological processes such as aging and education. These factors may contribute to the elevated risk of CVD within Native American communities.

Participants reported here had unique \( \text{MBL2} \) genotype frequencies consistent with another Indigenous population in North America and similar to East Asian populations [25, 30, 31, 35, 36]. While the SHS prevalence of the majority A allele of \( \text{MBL2} \) is similar to but slightly elevated compared most global populations, the B allele was much more common compared to populations of majority European or African ancestry [25, 32, 37]. Furthermore, the distribution of promoter variants and haplotypes in the SHS cohort differed markedly from the majority Caucasian population in the US [32, 38] and most closely resembled an Inuit and Korean population [30, 31]. Increased prevalence of high expression H and Y promoter variants in the SHS cohort could significantly influence the response to pathogens through \( \text{MBL2} \), as well as impact other, intrinsic immunological functions that may contribute to chronic inflammation and hence influence CVD among American Indian populations.

Within the SHS cohort, all allelic variants were present but some specific genotypes were missing. Combinations of \( \text{MBL2} \) “C” and “D” alleles were present only with “A” or “B” despite being found together in similarly sized cohorts within the US [32]. Additionally, certain promoter genotypes were absent from the population despite being present in combination with other alleles (i.e., HHXX). Initial studies of \( \text{MBL2} \) haplotypes also reported an absence of the HX haplotype [39]; however, recent studies have described its presence among a number of distinct global populations [40, 41]. Interestingly, none of these reports include Indigenous peoples of the Americas, suggesting that these genotypes may be absent because of maintained haplotype blocks that do not recombine or are selected against when present. It is tempting to speculate that selective pressures have modulated MBL activity by altering genotypic frequencies, reflecting the dual roles of controlling infectious agents and promoting cardiovascular function.

\( \text{MBL2} \) functions as a “first responder” within the innate immune system in recognizing pathogens such as \( \text{C. pneumoniae} \), which have been implicated in promoting CVD. Two particular \( \text{MBL2} \) variant positions correlated with antibody production to \( \text{Cp} \), the X/Y promoter variant and exon 1. Surprisingly, the high functioning A allele of \( \text{MBL2} \) correlated with elevated \( \text{Cp} \) antibody titers; in a consistent manner, the lower functioning B allele correlated with reduced \( \text{Cp} \) antibody titers. The high prevalence of the \( \text{MBL2} \) B allele and its corresponding reduced function may play a role in the development of CVD within American Indian populations specifically [14]. Within the promoter, both low expression X and L alleles were associated with increased antibody production. Similarly, immunization of mice lacking \( \text{MBL2} \) led to increased IgG production compared to wildtype mice [42]. It is unclear how \( \text{MBL2} \) variants that promote opposing levels of expression and/or function both increase \( \text{Cp} \) antibody titers but suggests that production of antibody through MBL regulation is complex, which is further

### Table 4. ANCOVA of \( \text{Cp} \) IgG versus \( \text{MBL2} \) exon 1 alleles.

| Source | dF | F   | MSE      | Partial Eta Squared | p-value |
|--------|----|-----|----------|---------------------|---------|
| A allele | 1  | 4.389 | 30623.570 | 0.000 | 0.037 |
| B allele | 1  | 4.479 | 309858.252 | 0.007 | 0.035 |
| C allele | 1  | 2.802 | 193877.528 | 0.004 | 0.095 |
| D allele | 1  | 1.863 | 129062.322 | 0.003 | 0.173 |

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\( \text{MBL2} \) genotype alters \( \text{Cp} \) immune reactivity in American Indians

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Fig 4. Age, gender, and education are associated with *C. pneumoniae* antibody titer. A. The mean *C. pneumoniae* antibody titer increased for each represented age within the SHS cohort (Spearman’s correlation; $r_s = 0.09 \ p < 0.041$).
supported by the non-additive nature of individual alleles in haplotype construction with immune reactivity. Additionally, a large prospective study demonstrated the unexpected relationship between high prevalence of \textit{MBL2} genotypes associated with high expression and increased risk of CVD [43]. Thus, additional factors such as Interleukin-6 (IL-6) and C-reactive protein (CRP), which contribute to \textit{Cp} antibody production, and other host-specific factors may contribute to these discrepancies [44].

Other environmental factors contributed significantly to \textit{Cp} antibody titers among the SHS cohort. Our data indicated a linear relationship between age and higher amounts of circulating \textit{Cp} antibody. This trend is consistent with previous studies that found an increase in seroprevalence transitioning from childhood into adulthood [34, 45, 46] and extends those studies to suggest that immune response to \textit{Cp} continues to accumulate later in life as well. Association between \textit{Cp} antibody titers and smoking have also been noted previously although the mechanism is unclear [46]. The impact of gender on \textit{Cp} immune reactivity is less well defined as studies have found both higher antibody titers in males as we observed [47] or no difference between sexes [46], suggesting this may vary depending on the sampled population.

The previously observed association between \textit{MBL2} genotype and CVD may include both the direct action of MBL in regulation of host cellular maintenance and its additional role in immune surveillance of pathogens [27]. For example, some \textit{MBL2} variants correlated with increased levels of \textit{Cp} antibodies were also shown to associate with CVD risk [27]. Thus, \textit{MBL2} genotyping could be used in the clinic to screen individuals for host- and microbe-associated risk factors in CVD development. As a result of screening, physicians could introduce preventative strategies earlier to reduce the likelihood of disease onset and progression within American Indians populations.

**Supporting information**

**S1 Fig.** \textit{C. pneumoniae} antibody titers for SHS cohort genotypes. The \textit{C. pneumoniae} antibody titer for IgG (A) or IgA (B) are plotted for all genotypes among the 553 individuals found within the SHS cohort.

**S2 Fig.** Association of promoter and coding allelic variants are associated in \textit{MBL2}. The relationship between \textit{MBL2} genetic variants and \textit{Cp} antibody titers was constructed using hierarchical clustering and Euclidean distances.

**S1 Table.** Nomenclature of studied \textit{MBL2} variant positions.

**S2 Table.** \textit{MBL2} allele frequencies in the SHS cohort conformed to the Hardy-Weinberg equilibrium.

**S3 Table.** \textit{MBL2} Exon 1 frequencies in the SHS cohort conformed to the Hardy-Weinberg equilibrium.
S4 Table. Correlation analysis of genotypic and phenotypic traits. Pairwise associations of traits were performed using a Spearman’s correlation analysis for all genetic polymorphisms assayed and a number of important covariates. Pink indicates p<0.05 and red indicates p<0.01.

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References

1. CDC N. Underlying Cause of Death 1999–2013 on CDC WONDER Online Database, released 2015. Data are from the Multiple Cause of Death Files, 1999–2013, as compiled from data provided by the 57 vital statistics jurisdictions through the Vital Statistics Cooperative Program. 2015. Epub Feb. 3, 2015.
2. Heron MaA, R.H. Changes in the Leading Cause of Death: Recent Patterns in Heart Disease and Cancer Mortality. NCHS Data Brief. 2016;(254):1–8. Epub August 2016. PMID: 27598767
3. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012; 380(9859):2224–60. https://doi.org/10.1016/S0140-6736(12)61766-8 PMID: 23245609; PubMed Central PMCID: PMCPMC4156511.
4. de Waure C, Lauret GJ, Ricciardi W, Ferket B, Tejink J, Spronk S, et al. Lifestyle interventions in patients with coronary heart disease: a systematic review. Am J Prev Med. 2013; 45(2):207–16. https://doi.org/10.1016/j.amepre.2013.03.026 PMID: 23867029.
5. Triant VA. Cardiovascular disease and HIV infection. Curr HIV/AIDS Rep. 2013; 10(3):199–206. Epub 2013/06/25. https://doi.org/10.1007/s11904-013-0168-6 PMID: 23793823; PubMed Central PMCID: PMCPMC3964878.
6. Fong IW. New perspectives of infections in cardiovascular disease. Curr Cardiol Rev. 2009; 5(2):87–104. https://doi.org/10.2174/157340309788166679 PMID: 20436849; PubMed Central PMCID: PMCPMC2805819.
7. Nikpay M, Goel A, Won HH, Hall LM, Willenborg C, Kanoni S, et al. A comprehensive 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. Nat Genet. 2015; 47 (10):1121–30. https://doi.org/10.1038/ng.3396 PMID: 26343387; PubMed Central PMCID: PMC4589895.

8. Madsen HO, Videm V, Svejgaard A, Svennevig JL, Garred P. Association of mannose-binding-lectin deficiency with severe atherosclerosis. Lancet. 1998; 352(9132):595–60. https://doi.org/10.1016/S0140-6736(05)61513-9 PMID: 9752823.

9. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. J Infect Dis. 1999; 180(1):238–41. https://doi.org/10.1086/314855 PMID: 10353889.

10. O'Donnell CJ, Nabel EG. Genomics of cardiovascular disease. N Engl J Med. 2011; 365(22):2098–109. https://doi.org/10.1056/NEJMra1105239 PMID: 22129254.

11. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med. 2001; 194(6):781–95. PMID: 11560994; PubMed Central PMCID: PMCPMC2195958.

12. Ali YM, Lynch NJ, Haleem KS, Fujita T, Endo Y, Hansen S, et al. The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. PLoS Pathog. 2012; 8(7):e1002793. https://doi.org/10.1371/journal.ppat.1002793 PMID: 22792067; PubMed Central PMCID: PMCPMC3390405.

13. Stuart LM, Ezekowitz RA. Phagocytosis: elegant complexity. Immunity. 2005; 22(5):539–50. https://doi.org/10.1016/j.immuni.2005.05.002 PMID: 15894272.

14. Rantala A, Lajunen T, Juvonen R, Bloigu A, Paldanius M, Silvennoinen-Kassinen S, et al. Low mannose-binding lectin levels and MBL2 gene polymorphisms associate with Chlamydia pneumoniae antibodies. Innate Immun. 2011; 17(1):35–40. https://doi.org/10.1177/1753425909349759 PMID: 19969625.

15. Pesonen E, Hallman M, Sarna S, Andsberg E, Haataja R, Meri S, et al. Mannose-binding lectin as a risk factor for acute coronary syndromes. Ann Med. 2009; 41(8):591–8. https://doi.org/10.1080/07853890903110994 PMID: 19711212.

16. Asnar O, Nejatizadeh A, Dehghan F, Kargar M, Zolghadri N. Association of Chlamydia pneumoniae Infection With Atherosclerotic Plaque Formation. Glob J Health Sci. 2015; 8(4):260–7. https://doi.org/10.5539/gjhs.v8n4p260 PMID: 26573036; PubMed Central PMCID: PMCPMC4673590.

17. Rugonfalvi-Kiss S, Endresz V, Madsen HO, Burian K, Duba J, Prohaszka Z, et al. Association of Chlamydia pneumoniae with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. Circulation. 2002; 106(9):1071–6. PMID: 12196331.

18. Fong IW, Chiu B, Viira E, Tucker W, Wood H, Peeling RW. Chlamydial heat-shock protein-60 antibody and correlation with Chlamydia pneumoniae in atherosclerotic plaques. J Infect Dis. 2002; 186(10):1469–73. Epub 2002/10/31. https://doi.org/10.1086/344730 PMID: 12404163.

19. Fong IW, Chiu B, Viira E, Jang D, Mahony JB. De Novo induction of atherosclerosis by Chlamydia pneumoniae in a rabbit model. Infect Immun. 1999; 67(11):6048–55. Epub 1999/10/26. PMID: 10531266; PubMed Central PMCID: PMCPMC969992.

20. Nagy A, Kozma GT, Keszei M, Treszl A, Falus A, Szalai C. The development of asthma in children infected with Chlamydia pneumoniae is dependent on the modifying effect of mannose-binding lectin. J Allergy Clin Immunol. 2003; 112(4):729–34. Epub 2003/10/18. https://doi.org/10.1067/SCIM.14564351.

21. Asner SA, Morre SA, Bochud PY, Greub G. Host factors and genetic susceptibility to infections due to intracellular bacteria and fastidious organisms. Clin Microbiol Infect. 2014; 20(12):1246–53. Epub 2014/11/05. https://doi.org/10.1111/1469-0691.12806 PMID: 25366416.

22. Babovic-Vuksanovic D, Snow K, Ten RM. Mannose-binding lectin (MBL) deficiency. Variant alleles in a midwestern population of the United States. Ann Allergy Asthma Immunol. 1999; 82(2):134–8, quiz 42–3. https://doi.org/10.1016/S1081-1206(99)00123-9 PMID: 10071515.

23. Jakab L, Laki J, Sallai K, Temesszentandras G, Pozsonyi T, Kalabay L, et al. Association between early onset and organ manifestations of systemic lupus erythematosus (SLE) and a down-regulating promotor polymorphism in the MBL2 gene. Clin Immunol. 2007; 125(3):230–6. https://doi.org/10.1016/j.clim.2007.08.020 PMID: 17942372.

24. Madsen HO, Satz ML, Hogh B, Sveigaard A, Garred P. Different molecular events result in low protein levels of mannose-binding lectin in populations from southeast Africa and South America. J Immunol. 1998; 161(6):3169–75. PMID: 9743385.
25. Valles X, Sarrias MR, Casals F, Farnos M, Piner R, Suarez B, et al. Genetic and structural analysis of MBL2 and MASP2 polymorphisms in south-eastern African children. Tissue Antigens. 2009; 74(4):298–307. Epub 2009/09/25. https://doi.org/10.1111/j.1399-0039.2009.01328.x PMID: 19775369.

26. Lee ET, Welty TK, Fabrisz R, Cowan LD, Le NA, Oopik AJ, et al. The Strong Heart Study. A study of cardiovascular disease in American Indians: design and methods. Am J Epidemiol. 1990; 132(6):1141–55. PMID: 2260546.

27. Best LG, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET, et al. Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. Circulation. 2004; 109(4):471–5. https://doi.org/10.1161/01.CIR.0000109757.95461.10 PMID: 14732744.

28. Madsen HO, Garred P, Thiell S, Kurtzhals JA, Lamm LU, Ryder LP, et al. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J Immunol. 1995; 155(6):3013–20. PMID: 7673719.

29. Bernig T, Breunis W, Brouwer N, Hutchinson A, Welch R, Roos D, et al. An analysis of genetic variation across the MBL2 locus in Dutch Caucasians indicates that 3 haplotypes could modify circulating levels of mannan-binding lectin. Hum Genet. 2005; 118(3–4):404–15. Epub 2005/10/07. https://doi.org/10.1007/s00439-005-0053-5 PMID: 16208516.

30. Hegele RA, Busch CP, Young TK, Connelly PW, Cao H. Mannose-binding lectin gene variation and cardiovascular disease in Canadian Inuit. Clin Chem. 1999; 45(8 Pt 1):1283–5. Epub 1999/08/03. PMID: 10430797.

31. Lee SG, Yum JS, Moon HM, Kim HJ, Yang YJ, Kim HL, et al. Analysis of mannose-binding lectin 2 (MBL2) genotype and the serum protein levels in the Korean population. Mol Immunol. 2005; 42(8):969–77. https://doi.org/10.1016/j.molimm.2004.09.036 PMID: 15829288.

32. Stevenson HL, Amador A, McCue J, Weppeler D, Tryphopoulos P, Roth D, et al. Mannose binding lectin (mbll) haplotype frequencies in solid organ transplant patients and correlation with MBL protein levels—evaluation of complement-mediated effector pathway deficiency. Transpl Immunol. 2013; 28(2–3):73–80. Epub 2013/02/27. https://doi.org/10.1016/j.trim.2013.02.002 PMID: 23439277.

33. Garred P, Larsen F, Seyfarth J, Fujita R, Madsen HO. Mannose-binding lectin and its genetic variants. Genes Immun. 2006; 7(2):85–94. https://doi.org/10.1038/sj.gene.6364283 PMID: 16395391.

34. Paldanius M, Bloigu A, Alho M, Leinonen M, Saikku P. Prevalence and persistence of Chlamydia pneumoniae antibodies in healthy laboratory personnel in Finland. Clin Diag Lab Immunol. 2005; 12(5):654–9. https://doi.org/10.1128/CDLI.12.5.654-659.2005 PMID: 15879028; PubMed Central PMCID: PMCPMC1112086.

35. Sasaki K, Tsutsumi A, Wakamiya N, Ohtani K, Suzuki Y, Watanabe Y, et al. Mannose-binding lectin polymorphisms in patients with hepatitis C virus infection. Scand J Gastroenterol. 2000; 35(9):960–5. Epub 2000/11/04. PMID: 11063157.

36. Tsutsumi A, Sasaki K, Wakamiya N, Ichikawa K, Atsumi T, Ohtani K, et al. Mannose-binding lectin gene: polymorphisms in Japanese patients with systemic lupus erythematosus, rheumatoid arthritis and Sjogren's syndrome. Genes Immun. 2001; 2(2):99–104. Epub 2001/06/08. https://doi.org/10.1038/sj.gene.6363744 PMID: 11393663.

37. Garred P, Thiell S, Madsen HO, Ryder LP, Jensenius JC, Sveigaard A. Gene frequency and partial protein characterization of an allelic variant of mannan binding protein associated with low serum concentrations. Clin Exp Immunol. 1992; 90(3):517–21. PMID: 1458688; PubMed Central PMCID: PMCPM10430797.

38. Bernig T, Taylor JG, Foster CB, Staats B, Yeager M, Chanock SJ. Sequence analysis of the mannose-binding lectin (MBL) gene reveals a high degree of heterozygosity with evidence of selection. Genes Immun. 2006; 7(2):99–104. https://doi.org/10.1038/sj.gene.6364283 PMID: 16395391.

39. Stevenson HL, Amador A, McCue J, Weppeler D, Tryphopoulos P, Roth D, et al. Mannose binding lectin (mbll) haplotype frequencies in solid organ transplant patients and correlation with MBL protein levels—evaluation of complement-mediated effector pathway deficiency. Transpl Immunol. 2013; 28(2–3):73–80. Epub 2013/02/27. https://doi.org/10.1016/j.trim.2013.02.002 PMID: 23439277.

40. Lee SG, Yum JS, Moon HM, Kim HJ, Yang YJ, Kim HL, et al. Analysis of mannose-binding lectin 2 (MBL2) genotype and the serum protein levels in the Korean population. Mol Immunol. 2005; 42(8):969–77. https://doi.org/10.1016/j.molimm.2004.09.036 PMID: 15829288.

41. Stevenson HL, Amador A, McCue J, Weppeler D, Tryphopoulos P, Roth D, et al. Mannose binding lectin (mbll) haplotype frequencies in solid organ transplant patients and correlation with MBL protein levels—evaluation of complement-mediated effector pathway deficiency. Transpl Immunol. 2013; 28(2–3):73–80. Epub 2013/02/27. https://doi.org/10.1016/j.trim.2013.02.002 PMID: 23439277.

42. Garred P, Thiell S, Madsen HO, Ryder LP, Jensenius JC, Sveigaard A. Gene frequency and partial protein characterization of an allelic variant of mannan binding protein associated with low serum concentrations. Clin Exp Immunol. 1992; 90(3):517–21. PMID: 1458688; PubMed Central PMCID: PMCPM10430797.

43. Bernig T, Taylor JG, Foster CB, Staats B, Yeager M, Chanock SJ. Sequence analysis of the mannose-binding lectin (MBL) gene reveals a high degree of heterozygosity with evidence of selection. Genes Immun. 2006; 7(2):99–104. https://doi.org/10.1038/sj.gene.6364283 PMID: 16395391.
43. Keller TT, van Leuven SI, Meuwese MC, Wareham NJ, Luben R, Stroes ES, et al. Serum levels of mannose-binding lectin and the risk of future coronary artery disease in apparently healthy men and women. Arterioscler Thromb Vasc Biol. 2006; 26(10):2345–50. Epub 2006/08/12. https://doi.org/10.1161/01.ATV.0000240517.69201.77 PMID: 16902159.

44. Cortes C, Rzomp KA, Tvinnewerim A, Scidmore MA, Wizel B. Chlamydia pneumoniae inclusion membrane protein Cpn0585 interacts with multiple Rab GTPases. Infect Immun. 2007; 75(12):5586–96. https://doi.org/10.1128/IAI.01020-07 PMID: 17908815; PubMed Central PMCID: PMCPMC2168330.

45. Blasi F, Tarsia P, Arosio C, Fagetti L, Allegra L. Epidemiology of Chlamydia pneumoniae. Clin Microbiol Infect. 1998; 4 Suppl 4:S1–S6. PMID: 11869264.

46. Branden E, Koyi H, Gnarpe J, Gnarpe H, Tornling G. Chronic Chlamydia pneumoniae infection is a risk factor for the development of COPD. Respir Med. 2005; 99(1):20–6. Epub 2005/01/28. PMID: 15672844.

47. Phoon MC, Desbordes C, Howe J, Chow VT. Linoleic and linoleaidic acids differentially influence proliferation and apoptosis of MOLT-4 leukaemia cells. Cell Biol Int. 2001; 25(8):777–84. Epub 2001/08/03. https://doi.org/10.1006/cbir.2001.0733 PMID: 11482901.