

ORIGINAL ARTICLE

Mannose-Binding Lectin Variant Alleles and the Risk of Arterial Thrombosis in Systemic Lupus Erythematosus

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ABSTRACT

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BACKGROUND

Cardiovascular disease is an important complication in patients with systemic lupus erythematosus (SLE). Variant alleles of the mannose-binding lectin gene are associated with SLE as well as with severe atherosclerosis. We determined whether mannose-binding lectin variant alleles were associated with an increased risk of arterial thrombosis among patients with SLE.

METHODS

Mannose-binding lectin alleles were genotyped by means of a polymerase-chain-reaction assay in 91 Danish patients with SLE. Arterial and venous thromboses occurring after the diagnosis of SLE were assessed in a prospective study. Arterial and venous thromboses were confirmed by appropriate diagnostic methods.

RESULTS

Fifty-four patients had no mannose-binding lectin variant alleles (*A/A* genotype), 30 were heterozygous (*A/O* genotype), and 7 were homozygous (*O/O* genotype). During a median follow-up of 9.1 years, arterial thromboses (cerebral or myocardial infarction or leg embolus) developed in 6 of the 7 patients with the *O/O* genotype, as compared with 18 of the 84 patients with the other two genotypes (hazard ratio, 5.8; 95 percent confidence interval, 2.2 to 15.2; overall incidence, 26 percent). After correction for other known risk factors, the hazard ratio was 7.0 (95 percent confidence interval, 1.9 to 25.4). Venous thromboses, which occurred in 14 patients, were statistically unrelated to the mannose-binding lectin genotype.

CONCLUSIONS

Among patients with SLE, homozygosity for mannose-binding lectin variant alleles is associated with an increased risk of arterial thrombosis. The risk of venous thrombosis is not increased, indicating that mannose-binding lectin has a specific role in providing protection against arterial thrombosis.

CARDIOVASCULAR DISEASE IS A MAJOR cause of illness and death in patients with systemic lupus erythematosus (SLE).^{1,2} Young patients with SLE are at substantially increased risk for coronary artery disease.³⁻⁵ The increased risk of cardiovascular disease in these patients is not fully accounted for by traditional atherosclerotic risk factors.⁶ However, several studies indicate that atherosclerosis is an active inflammatory and immune-mediated process⁷ and that this dyslipoproteinemia, which is characterized by high serum triglyceride levels and low serum levels of high-density lipoprotein, correlates with increased disease activity in patients with SLE.⁸ Factors modulating the inflammatory response in patients with SLE are therefore likely to be of interest with regard to the pathogenesis of cardiovascular disease in these patients.

Mannose-binding lectin is a liver-derived serum protein involved in innate immune defense. The ligands for mannose-binding lectin are mannose and *N*-acetyl glucosamine oligosaccharides, expressed by a wide range of microorganisms. Mannose-binding lectin may activate complement by means of the lectin pathway when interacting with mannose-binding lectin-associated serine proteases and can directly opsonize pathogens and enhance the activity of phagocytes by means of novel receptors.⁹ Serum mannose-binding lectin levels vary widely from person to person because of three variant alleles (*B*, *C*, and *D*, denoting the substitution of aspartic acid for glycine at codon 54, the substitution of glutamic acid for glycine at codon 57, and the substitution of cysteine for arginine at codon 52, respectively) in the structural moiety of the functional mannose-binding lectin gene, *MBL2*, located on chromosome 10 in humans.¹⁰ The normal allele is named *A*, and the common designation for the variant alleles is *O*. Each of the three variant alleles influences the stability of the final protein product, resulting in reduced serum levels and a dysfunctional mannose-binding lectin variant with a lower molecular weight than the normal protein.¹¹ Moreover, base substitutions in the promoter region of the *MBL2* gene have been shown to regulate the serum level of mannose-binding lectin.¹² Specifically, in an otherwise structurally normal *MBL2* gene, the substitution of *C* for *G* at position -221 (termed promoter allele *X* and resulting in haplotype *XA*) is associated with a low serum level of mannose-binding lectin.

Variant alleles causing low serum levels of func-

tional mannose-binding lectin have been shown to be associated with an increased risk of infections^{13,14} and to alter the phenotypic expression of autoimmune diseases such as SLE¹⁵ and rheumatoid arthritis.¹⁶ However, patients with defects in mannose-binding lectin may also have atherosclerotic disease that has an earlier onset and is more severe than that in their counterparts with normal mannose-binding lectin.¹⁷ On the basis of our previous findings, we hypothesized that homozygosity for mannose-binding lectin variant alleles is associated with an increased risk of clinically detectable arterial thrombotic events in patients with SLE.

METHODS

PATIENTS

Consecutive, unrelated, white Danish patients with SLE were identified during 1996 and 1997 as previously described.¹⁵ The time of diagnosis of SLE was defined as the date when the American College of Rheumatology classification criteria¹⁸ for SLE were met. The study was approved by the local scientific ethics committee, and all patients provided written informed consent. Independent of mannose-binding lectin analyses, demographic and clinical data, including information on thromboses, were obtained from the clinical charts that were available from the time of diagnosis until June 2003 at the latest. The patients' race was determined by the investigators. The data-collection criteria were uniform for all patients.

CLINICAL DATA

Clinical characteristics related to SLE have been described previously.¹⁵ Hypertension was defined as a blood-pressure measurement above 140/90 mm Hg alone or in combination with the need for antihypertensive medication sometime during the period of observation, but before the occurrence of any clinical outcomes. Patients were classified as never having smoked or as being former smokers or current smokers.

The primary clinical outcome in this study was arterial thrombosis, but data on venous thrombotic events were also collected to serve as thrombotic control events. Patients were considered to have arterial or venous thrombosis only when its presence was confirmed by appropriate diagnostic methods. The major clinical arterial manifestations included myocardial infarction (fatal and nonfatal), which was confirmed on the basis of elevated cardiac en-

zyme levels and electrocardiographic findings; cerebrovascular accident, which was confirmed on the basis of computed tomography, magnetic resonance imaging, or both; and peripheral arterial thrombosis, which was confirmed on the basis of arteriography. Deep-vein thrombosis was confirmed by means of Doppler studies, phlebography, or both, and pulmonary embolism was confirmed by means of ventilation–perfusion pulmonary scintigraphy.

ANTIPHOSPHOLIPID ASSAYS

Anticardiolipin antibodies were measured in serum samples at a dilution of 1:100. IgG and IgM anticardiolipin antibodies were quantitated by means of an enzyme-linked immunosorbent assay on polystyrene microtiter plates with the use of purified cardiolipin (Sigma) and bovine serum for blocking as described previously.^{19,20} Cutoff values for the anticardiolipin-antibody tests were 30 U per milliliter for IgM and 35 U per milliliter for IgG.¹⁹ Patients with a spontaneously prolonged partial-thromboplastin time were evaluated for lupus anticoagulant by mixing their plasma with normal plasma in a 1:1 ratio.²¹

GENOTYPING OF MANNOSE-BINDING LECTIN

Genomic DNA was isolated from EDTA-treated blood cells and stored at -20°C .²² The mannose-binding lectin alleles were genotyped by means of the polymerase chain reaction with the use of sequence-specific priming.^{14,23} In exon 1 of the *MBL2* gene, the presence of three single-base substitutions was investigated — at codon 54 (the *B* allele), codon 57 (the *C* allele), and codon 52 (the *D* allele).^{23–25} We also looked for down-regulating polymorphisms in the promoter region of the *MBL2* gene: the substitution of the *L* allele for the *H* allele at position -550 and the substitution of the *X* allele for the *Y* allele at position -221 .¹² In addition, we determined the molecular structure of mannose-binding lectin in serum from six patients with SLE and different *MBL2* genotypes through the use of immunologic affinity purification and subsequent sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (3 to 8 percent Novex NuPAGE gradient gel, Invitrogen) and Western blotting as previously described.¹¹

STATISTICAL ANALYSIS

First, we performed an unadjusted Cox proportional-hazards regression analysis to examine the

relation between the presence of mannose-binding lectin genotype *O/O* and the risk of arterial thrombosis, venous thrombosis, or death among the 91 patients with SLE. Second, using multivariate Cox proportional-hazards regression analysis, we calculated the individual hazard ratios for arterial thrombosis with respect to the presence of mannose-binding lectin genotype *O/O*, lupus anticoagulant, IgG and IgM anticardiolipin antibodies, and hypertension; current or former smoking; male sex; and age at diagnosis (per decade). In these analyses, we examined the time from the diagnosis of SLE to a particular type of thrombotic event (arterial or venous), with data censored at the time of death or the end of follow-up, whichever came first. When we examined the time from diagnosis to death, data were censored only at the end of follow-up. Cumulative incidence estimates were plotted as a graphic representation of the risk of arterial thrombosis in the three genotype subgroups. All reported P values are two-sided, and P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Ninety-one patients with SLE were included in the study.¹⁵ The total duration of follow-up was 1007 patient-years, and the median duration of disease was 9.1 years. The basic demographic, clinical, and serologic characteristics of the patients are shown in Table 1. Fifty-four (59 percent) of the patients had genotype *A/A*, 30 (33 percent) had genotype *A/O*, and 7 (8 percent) had genotype *O/O*. The distribution of the specific variant alleles in the heterozygous (*A/O*) patients was as follows: 16 were *A/B*, 3 were *A/C*, and 11 were *A/D*. The distribution of the specific variant alleles in the patients with the *O/O* genotype was as follows: one was *B/C*, five were *B/D*, and one was *D/D*, as previously reported.¹⁵ Of the 30 patients who were heterozygous, 8 (27 percent) carried the *MBL2* promoter allele *X*, and 26 (87 percent) carried the promoter allele *L* on a functional *A* background. Figure 1 shows that carriers of the *O/O* genotype lacked stable, high-molecular-weight forms of mannose-binding lectin in the blood, as compared with carriers of the *A/O* and *A/A* genotypes.

During follow-up, arterial thrombosis occurred in 24 of the 91 patients (26 percent), and venous thrombosis occurred in 14 (15 percent) (Table 2). Ten patients died during follow-up; two of them had the *O/O* genotype and died after a myocardial

infarction. During follow-up, there were 17 myocardial infarctions in 12 patients and 12 cerebral infarctions in 11 patients. This was equal to a yearly event rate of myocardial infarction and cerebral infarction of 2.9 percent (95 percent confidence interval, 1.9 to 4.1 percent). Venous thrombosis developed in 14 patients (15 percent).

The risk of arterial thrombosis was significantly greater among patients with the *O/O* genotype than among those with the *A/A* or *A/O* genotype (hazard ratio, 5.8; 95 percent confidence interval, 2.2 to 15.2) (Table 2), mainly because of the strong association between the *O/O* genotype and myocardial infarction (hazard ratio, 9.4; 95 percent confidence interval, 2.6 to 33.7). The corresponding hazard ratio for cerebral infarction, 3.7, was not significant ($P=0.10$). There was no significant association between mannose-binding lectin variant alleles and venous thrombosis. The risk of death was greater among patients with the *O/O* genotype than among patients with one of the other two genotypes, but not significantly so. Among patients with the *A/O* or *A/A* genotype, the presence of the mannose-binding lectin promoter allele *X* or *L* was not significantly associated with arterial or venous thrombosis. Immunosuppressive therapy was not related to mannose-binding lectin genotypes¹⁵ or any of the thrombotic outcomes (data not shown).

Figure 2 shows the cumulative incidence of arterial thrombosis in each of the three genotype subgroups and reveals a recessive effect of mannose-binding lectin variant alleles on the risk of arterial thrombosis. Cox regression analysis that included the *O/O* genotype, the age at diagnosis, male sex, the presence of lupus anticoagulant, current or former smoking, IgG anticardiolipin antibodies, IgM anticardiolipin antibodies, and hypertension showed that only the *O/O* genotype (hazard ratio as compared with the other two genotypes, 7.0; 95 percent confidence interval, 1.9 to 25.4; $P=0.003$) and the age at diagnosis ($P=0.01$) were associated with a significantly increased risk of arterial thrombosis (Table 3).

DISCUSSION

We found that homozygosity for mannose-binding lectin variant alleles is a major risk factor for arterial thrombosis in patients with SLE. Moreover, the increased thrombotic risk was specific for the arterial side of the circulation, since there was no significant association between mannose-binding lectin

Table 1. Demographic, Clinical, and Serologic Characteristics of 91 Patients with Systemic Lupus Erythematosus.

Characteristic	Value
Sex — no. (%)	
Male	9 (10)
Female	82 (90)
Age at diagnosis — yr	
Median	28.2
Range	13.0–72.5
Current or former smoker — no. (%)	50 (55)
Hypertension — no. (%)	22 (24)
Lupus anticoagulant — no. (%)	27 (30)
Anticardiolipin antibodies — no. (%)	
IgG	26 (29)
IgM	35 (38)
Lupus anticoagulant and IgG anticardiolipin antibodies — no. (%)	11 (12)
Lupus anticoagulant and IgM anticardiolipin antibodies — no. (%)	12 (13)
Outcome events — no. (%)	
Arterial thrombosis	24 (26)
Venous thrombosis	14 (15)
Arterial and venous thrombosis	5 (5)
Death	10 (11)
Death without previous arterial thrombosis	4 (4)

deficiency and venous thrombosis. The increased risk of thrombosis was particularly pronounced for myocardial infarction. This finding is in line with previous findings among patients with severe atherosclerotic coronary disease, who were four times as likely as control subjects to have the *O/O* genotype.¹⁷ In a large study of clinically healthy subjects, mannose-binding lectin variant alleles were associated with increased formation of atherosclerotic plaque in the carotid arteries,²⁶ a finding that parallels our finding of an increased risk of stroke (albeit not significant) among patients with variant alleles. The relative weakness of the association between the *O/O* genotype and cerebral infarction may indicate that factors other than atherosclerosis are more important causes of this type of thrombotic event in patients with SLE.

We found no significant association between the risk of thrombosis and heterozygosity for mannose-binding lectin variant alleles (*A/O*), with or without reduced expression of a promoter allele on

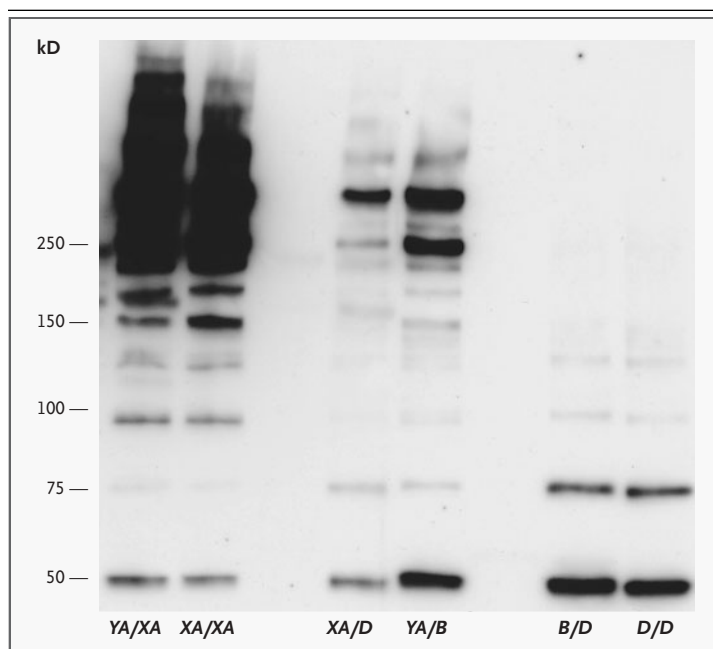


Figure 1. Variations in Mannose-Binding Lectin Oligomerization, According to the Mannose-Binding Lectin (MBL2) Genotype.

Serum from patients with SLE and various *MBL2* genotypes was processed as described previously and subjected to Western blotting after sodium dodecyl sulfate–polyacrylamide-gel electrophoresis under nonreducing conditions.¹¹ YA denotes the normal *MBL2* gene (without a structural variation), and XA the *MBL2* gene with a base substitution (from G to C) in the promoter region at position –221, resulting in the down-regulation of serum levels of mannose-binding lectin.¹² D denotes an allele with a single-base substitution at codon 52 (resulting in the substitution of cysteine for arginine in the collagenous part of mannose-binding lectin), and B an allele with a single-base substitution at codon 54 (resulting in the substitution of aspartic acid for glycine).¹⁰ The common designation for the *MBL2* structural variant alleles is O.

the functional chromosome (XA/O). Patients with the XA/O genotype have serum levels of mannose-binding lectin that are almost indistinguishable from those of patients with the O/O genotype.¹² Using alternative detection systems, we recently showed that variant alleles give rise to detectable levels of circulating mannose-binding lectin.¹¹ As we confirmed in the present study, however, variant mannose-binding lectin has less oligomerization and a lower molecular weight than normal mannose-binding lectin.¹¹ Moreover, variant mannose-binding lectin has a reduced capacity to bind ligands and is dysfunctional. Thus, patients who are heterozygous for mannose-binding lectin variant alleles — even those with reduced expression of promoters — still have some normal mannose-binding

lectin, whereas patients with the O/O genotype lack functional mannose-binding lectin, which probably explains the recessive phenotype we observed.

In our study, the annual rate of myocardial infarction and stroke was 2.9 percent overall (95 percent confidence interval, 1.9 to 4.1 percent), which overlaps the corresponding figure of 2.0 percent reported in a similar study, from Canada, of atherosclerosis in patients with SLE.⁶ The relation between premature atherosclerosis and SLE is clinically well described but cannot be fully accounted for by traditional risk factors. We found the expected association between arterial thrombosis and increasing age, but not between arterial thrombosis and hypertension — a finding that differs from that of the Canadian study.⁶ However, our use of a dichotomous classification of hypertension may have led to a loss of discriminative power. Also, we did not find a significant association between arterial thrombosis and antiphospholipid antibodies. However, the hazard ratios with respect to lupus anticoagulant and IgG anticardiolipin antibodies were higher than the hazard ratio with respect to IgM anticardiolipin antibodies, which is in line with previous findings in two separate cohorts of patients with SLE.²⁷ In previous studies of patients with SLE, smoking has been found to have a variable effect on the risk of arterial thrombosis.^{6,28,29} In our study, former or current smoking was not associated with a significantly increased risk of arterial thrombosis in patients with SLE. Unfortunately, we were unable to account for lipid status; this information was not recorded systematically because the trial was not primarily designed as a study of cardiovascular outcomes. Other factors that have been found to be associated with arterial thrombosis in patients with SLE include signs of complement activation and corticosteroid treatment.²⁸ In our cohort, none of the immunosuppressive treatments used were related to arterial thrombosis.

Women with SLE have an increased prevalence of carotid and femoral plaque that is not accounted for by other predictors of atherosclerosis, such as age, lipid status, and the cumulative dose of corticosteroids.²⁹ Many of these SLE-related atherosclerotic risk factors are related to increased disease activity; a recent study has corroborated these findings and, furthermore, has demonstrated an association between carotid plaque and disease-related damage.³⁰ Some studies have shown that mannose-binding lectin variant alleles are associated with in-

Table 2. Incidence of Arterial and Venous Thrombotic Events and Death among 91 Patients with Systemic Lupus Erythematosus, According to the Mannose-Binding Lectin Genotype.

Event	A/A Genotype (N=54)	A/O Genotype (N=30)	O/O Genotype (N=7)	Hazard Ratio (95% CI)*	P Value
	number of patients (percent)				
Arterial thrombosis	13 (24)	5 (17)	6 (86)	5.8 (2.2–15.2)	<0.001
Acute myocardial infarction	5 (9)	3 (10)	4 (57)	9.4 (2.6–33.7)	0.001
Cerebral infarction	7 (13)	2 (7)	2 (29)	3.7 (0.8–17.9)	0.10
Leg embolus	1 (2)	0	0	—	—
Venous thrombosis	9 (17)	4 (13)	1 (14)	1.1 (0.1–8.8)	0.91
Death	7 (13)	1 (3)	2 (29)	2.9 (0.6–13.8)	0.18

* A univariate Cox regression analysis was used to estimate the hazard ratios. The hazard ratios are for the comparison of the O/O genotype with the A/A and A/O genotypes. CI denotes confidence interval.

creased disease activity in patients with SLE³¹ and with signs of increased inflammation in patients with rheumatoid arthritis or giant-cell arteritis.^{16,32}

Therefore, the possibility that part of the atherosclerotic risk associated with mannose-binding lectin deficiency is attributable to the extent of disease activity cannot be ruled out. However, as previously stated in a description of this cohort of patients, the mannose-binding lectin genotypes were not associated with clinical characteristics specific for SLE or treatment regimens that reflect increased disease activity or severity.¹⁵

Several other mechanisms may underlie the atherogenic effect of mannose-binding lectin deficiency. A well-described effect of mannose-binding lectin deficiency is an increased risk of infection, which has also been described in patients with SLE.^{15,31} A number of studies have indicated an association between infections in general, and *Chlamydia pneumoniae* infection in particular, and coronary artery disease, although this remains controversial.³³ In a controlled study of 210 patients, *C. pneumoniae* infection and coronary artery disease were associated, but only in patients with mannose-binding lectin variant alleles.³⁴ Whether patients with SLE have an increased prevalence of previous infection with *C. pneumoniae* needs further exploration.

The accumulation of lipid material is unquestionably important in atherogenesis, but why this material accumulates and is not cleared by phagocytic cells, such as macrophages, is not well understood. Defective clearance of apoptotic endothelial cells may be of importance in this context. It is therefore of particular interest that mannose-bind-

ing lectin binds to and sequesters apoptotic and damaged host cells.³⁵ A reverse effect of mannose-binding lectin has been suggested in experimental models of ischemic reperfusion injury after myocardial infarction, since mannose-binding lectin may initiate complement activation central to the pathophysiology of this condition.³⁶

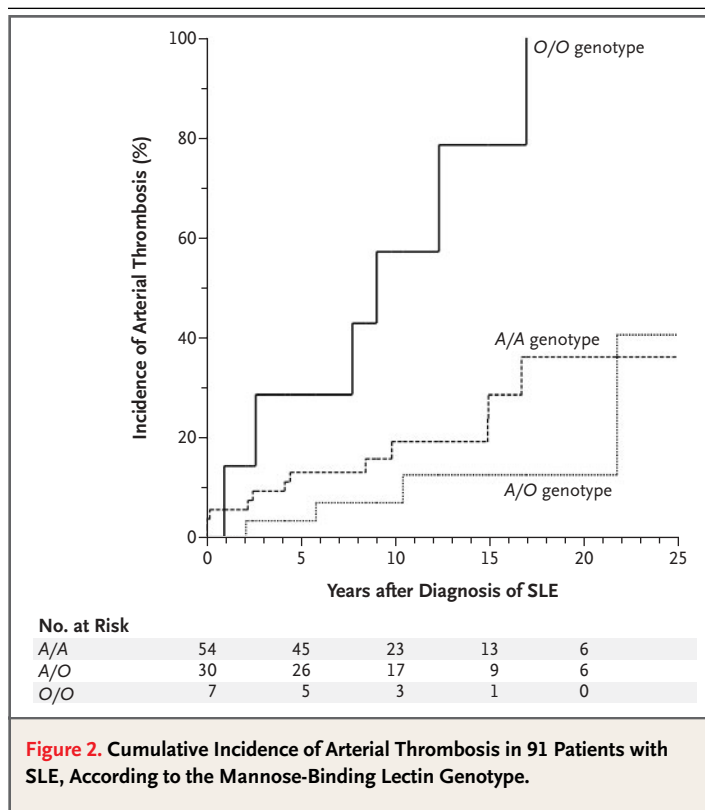


Figure 2. Cumulative Incidence of Arterial Thrombosis in 91 Patients with SLE, According to the Mannose-Binding Lectin Genotype.

Table 3. Risk of Arterial Thrombosis among 91 Patients with Systemic Lupus Erythematosus.

Risk Factor	No. of Patients	Hazard Ratio (95% CI)*	P Value
O/O genotype (vs. A/A and A/O)	7	7.0 (1.9–25.4)	0.003
Age at diagnosis (per decade)	91	1.6 (1.1–2.4)	0.01
Hypertension	22	2.4 (0.8–7.2)	0.10
Lupus anticoagulant	27	2.2 (0.8–6.0)	0.12
IgG anticardiolipin antibodies	26	2.1 (0.8–5.8)	0.14
Current or former smoker	50	2.3 (0.7–7.0)	0.16
Male sex	9	1.5 (0.3–6.5)	0.63
IgM anticardiolipin antibodies	35	0.8 (0.3–2.3)	0.68

* A multivariate Cox regression analysis was used to calculate the hazard ratios. CI denotes confidence interval.

In the general population, mannose-binding lectin deficiency is unlikely to be particularly dangerous, except during the vulnerable period of infancy, when the immune system is immature.¹³ However,

the deficiency may represent a threat to patients with complex conditions such as cystic fibrosis, which involve both genetic and acquired dysfunctions,³⁷ or SLE, in which more than one of the above-described pathogenetic mechanisms may be operative and even act synergistically with traditional atherosclerotic risk factors.

In conclusion, we found that homozygosity for mannose-binding lectin variant alleles significantly increased the risk of arterial thrombosis in a cohort of patients with SLE. This thrombotic effect, which was not seen in the venous side of the circulation, may indicate a specific role for mannose-binding lectin in protecting against atherosclerosis. The compelling and strong association between mannose-binding lectin deficiency and arterial thrombotic events in patients with SLE indicates the need for mannose-binding lectin genotyping to guide future prophylactic initiatives in these and other patients at high risk for cardiovascular disease.

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