Determinants of vascular complications in type 2 diabetic South Asians

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3

Low Mannose-Binding Lectin (MBL) genotype is associated with future cardiovascular events in type 2 diabetic South Asians

A prospective cohort study

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Abstract

Objective

South Asians have a high burden of type 2 diabetes and vascular complications. Vascular inflammation is considered central in the pathophysiology of atherosclerosis, and the complement system is thought to play an important role. Mannose-Binding Lectin (MBL), which activates the lectin pathway of complement activation, has been introduced as a risk marker of vascular damage. The present study explores the association of MBL levels, genotype and cardiovascular events in type 2 diabetic South Asians.

Research design and methods

We conducted a prospective observational study. A cohort consisting of 168 type 2 diabetic South Asians was followed for a median duration of 7.66 years. At baseline, MBL levels and genotype were determined. The association with future cardiovascular events was assessed by Cox proportional hazard regression.

Results

During follow-up, 31 cardiovascular events occurred in 22 subjects (11 men, 11 women). Compared to wild-type, the O/O genotype was significantly associated with the occurrence of cardiovascular events (hazard ratio 3.42, 95%CI 1.24-9.49, P = 0.018). However, MBL levels were not associated with the occurrence of cardiovascular events (hazard ratio 0.93, 95% CI 0.50-1.73).

Conclusion

In type 2 diabetic South Asians, the O/O MBL genotype is associated with cardiovascular events , although single serum MBL levels are not.

Introduction

South Asian immigrants in Western societies have a high burden of diabetes and vascular complications [1]. Traditional cardiovascular risk factors only partially explain this increased risk [2]. Hence other factors must be involved.
Atherosclerosis, the pathologic substrate of macrovascular disease, is recognized to be an inflammatory process [3]. As a player in the inflammatory response, the complement system is thought to be involved in this vascular inflammation [4] Indeed, complement activation products have been demonstrated in atherosclerotic plaques [5].
The complement system can be activated via the classical, alternative or lectin pathway, which is activated when Mannose-Binding Lectin (MBL) binds to its target molecule. MBL binds carbohydrate moieties on microorganisms. However, endogenous MBL ligands, such as glycosylated immunoglobulins or cells exposed to oxidative stress, have also been identified [6]. MBL serum levels are primarily determined by 3 polymorphisms (B,C and D genotypes, commonly referred to as O-alleles) in the MBL gene (*mbl2*). Subjects with wild type MBL genotype (A/A) have the highest serum MBL levels, subjects with 1 variant allele (A/O) have intermediate levels and subjects with 2 variant alleles (O/O) have the lowest levels. In addition, polymorphisms in the promoter region influence the MBL level [7]. MBL is synthesized in the liver. Although intraindividual levels are relatively stable over time [8], a two- to threefold increase occurs during acute phase reactions [9].
It has recently been suggested that MBL is involved in the pathophysiology of cardiovascular damage in high-risk populations [10]. In a group of type 1 diabetic Caucasians, MBL levels were significantly higher in subjects with either a history of cardiovascular disease or diabetic nephropathy compared to subjects without these vascular complications [10]. In type 2 diabetic Caucasians high MBL levels were associated with increased mortality [11]. However, others found an association between low MBL levels and cardiovascular events [8,12]. Data on MBL in South Asians are lacking.
We hypothesized that MBL might be involved in the high incidence of cardiovascular complications in type 2 diabetic South Asians. The current study aims to explore the association of MBL levels and genotype with cardiovascular complications in type 2 diabetic South Asians.

Research design and methods

Design of the follow-up study

We conducted a prospective cohort study. All studied subjects were recruited from a previously published study [13]. The original study population comprised 465 South Asians. At baseline, subjects that were not known with diabetes underwent a 75 g oral glucose tolerance test. Diabetes was diagnosed based on the ADA 2003 criteria. Out of 465 subjects, 168 subjects had type 2 diabetes at baseline (122 already known with diabetes, 46 newly diagnosed), and from these subjects follow-up data were collected. The study protocol was approved by the Institutional Medical Ethics Committee. All subjects provided informed consent.

Study-patients were followed up by letter and subsequently by phone. When subjects could not be traced by address or phone number in our database, general practitioners or participating family members were involved.

Follow-up data consisted of medical history with regard to cardiovascular events. Subjects were sent a questionnaire and were invited for a visit to our out-patient clinic. During this visit the questionnaire was reviewed by the main investigator (M.A.S.). Subjects not willing to visit the out-patient clinic were asked permission to collect medical data from their general practitioner. For subjects who had died during the follow-up period, cause of death and cardiovascular history was retrieved from the general practitioner. All (self-) reported events were verified by contacting the hospital in which the event had occurred.

Measurements at baseline

Laboratory measurements at baseline included lipids, creatinin, fasting glucose, urinary albumin/creatinine ratio, high-sensitivity C-reactive protein (hsCRP), and plasma SC5b-9, the soluble end product of complement activation. Lipids, creatinin, glucose and urinary albumin/creatinine ratio were measured according to standard methods. High-sensitivity C-reactive protein was measured with a fully automated Cobas Integra 800, according to the manufacturers proceedings (Roche, Almere, the Netherlands). The variation coefficients (VC) were below 3 %. Plasma levels of SC5b-9 were measured with an ELISA as described earlier [14].

MBL genotyping

DNA was isolated routinely from peripheral blood leucocytes. MBL single

nucleotide polymorphisms at codons 52, 54 and 57 of the *mbl2* gene were typed by pyrosequencing. The detailed methodology has been published separately [15]. The MBL genotype of only wildtype allele carriers is designated as A/A and the presence of 1 or 2 variant alleles(s) (B, C, or D) is designated as A/O or O/O, respectively.

Serum MBL levels

At baseline, serum MBL levels were assessed by sandwich ELISA as described previously [16]. In brief, 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated with the monoclonal antibody 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml), kindly provided by Dr. T. Fujita (Fuhushima, Japan). Serum samples were diluted 1/50 and 1/500 and incubated in the coated wells. MBL was detected with Dig-conjugated 3E7. Detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated sheep anti-Dig Abs (Fab fragments, Roche, Mannheim, Germany). Enzyme activity was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO)). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL. Earlier studies indicated that this assay primarily detects wild-type MBL in serum and plasma and that there is a direct association with the MBL genotype and with MBL function [17]

Definitions and endpoint

Cardiovascular events were defined as the occurrence of either a myocardial infarction, Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary Artery Bypass Grafting (CABG), or sudden cardiac death. The latter was defined as a witnessed sudden circulatory arrest. The primary end-point was the time to the first cardiovascular event.

Statistical analysis

Normally distributed variables are expressed as arithmetic mean ± 1 standard deviation. Skewed distributed variables are expressed as median with interquartile range.

Differences between groups were assessed with the independent samples t-test or the Mann-Whitney-U test for normally and not-normally distributed variables, respectively. Comparison between multiple groups was performed

with analysis of variance. Correlations were assessed by using Pearson's correlation and Spearman's correlation as appropriate. Associations with cardiovascular events were assessed by Cox proportional hazard regression. The mean difference in log transformed MBL levels per MBL genotype (A/A, A/O,O/O) was calculated with linear regression. Hazard ratios (HR) were calculated by Cox regression analysis. The association between MBL genotype and cardiovascular events with the A/A genotype as reference genotype was calculated, and the HR for cardiovascular events per number of variant alleles. Subsequently, the association between MBL level and cardiovascular events was analyzed. In a Mendelian randomization approach following Fisher [18], we examined the estimated causative effect of MBL level on cardiovascular events using the genotype as instrumental variable (figure 2). All tests were two-sided and the level of significance was 0.05.
All analyses were performed using SPSS Statistical Software Package (version 17.0; SPSS, Chicago, IL)

Results

Baseline analysis

At baseline, serum MBL levels and MBL genotype were determined in 168 diabetic subjects (122 already known with diabetes, 46 newly diagnosed). DNA was not available in 5 diabetic patients. Baseline characteristics of the study population are shown in table 1.
The median MBL level was 476 μg/L (IQR 143-1536 μg/L). Genotype distribution in South Asians was the same as the reported genotype distribution in Caucasians [7] (table 2). MBL levels differed significantly per genotype ($P < 0.001$): subjects with the A/A genotype had the highest MBL levels (median 1300 μg/L, IQR 535-2258) with the A/O genotype had intermediate MBL levels (median 160 μg/L , IQR 75-295) and subjects with the O/O genotype had the lowest MBL levels (median 74 μg/L , IQR 38-101).
MBL levels correlated weakly with Body Mass Index ($r = -0.155$, $P = 0.014$), HbA1c ($r = 0.165$, $P = 0.034$) and hip circumference (-0.169, $P = 0.030$) , but not with sex, age, blood pressure, high-sensitivity C-reactive protein (hsCRP), smoking status, total cholesterol, fasting triglycerides, and plasma SC5b-9.

Table 1 baseline characteristics of the type 2 diabetic South Asian study population

	Follow-up (n=134)	Lost to follow-up (n=34)	P-value
Age (years)	50.7 ± 11.2	48.9 ± 11.2	0.392
% male sex	46	45	0.886
Diabetes duration (years)	7.0 (0-13)	5.0 (0-11)	0.519
HbA1c (%)	7.7 ± 1.8	7.7 ± 2.0	0.976
Systolic blood pressure (mm Hg)	138 ± 24	140 ± 27	0.638
Diastolic blood pressure (mm Hg)	84 ± 11	84 ± 11	0.937
Urinary albumin/creatinine ratio (mg/mmol)	1.0 (0.4-4.6)	1.0 (0.4-5.0)	0.991
High-sensitivity C-reactive protein (mg/L)	3.5 (1.8-8.0)	4.8 (1.7-8.4)	0.851
Cockroft-Gault creatinine clearance (ml/min)	86 ± 27	93 ± 20	0.205
Total cholesterol (mmol/L)	5.1 ± 1.0	5.0 ± 0.9	0.666
Fasting triglycerides (mmol/L)	1.59 (1.16-2.32)	1.46 (1.24-2.13)	0.869
HDL-cholesterol (mmol/L)	1.23 ± 0.3	1.28 ± 0.3	0.954
Ratio total cholesterol: HDL-cholesterol	4.14 (3.45-5.05)	4.20 (3.10-5.0)	0.432
Body Mass Index	28.0 ± 4.7	28.4 ± 4.0	0.551
Waist circumference (cm)	96.4 ± 15.4	100 ± 10.1	0.154
Waist-to hip ratio	0.97 (0.93-1.03)	0.99 (0.95-1.04)	0.223
% previous cardiovascular event	14	11	0.663
% current or previous smoker	45	39	0.528

normally distributed variables are expressed as mean +/- SD, skewed distributed variables as median and interquartile range

Longitudinal analysis

Out of 168 type 2 diabetic subjects at baseline, 21 could not be traced and 13 subjects refused to participate and thus were excluded from analysis. Eighty-six subjects visited the out-patient clinic, 31 subjects did not visit the out-patient clinic but medical information was retrieved from the general practitioner, and 17 subjects had died (see below). The median duration of follow-up was 7.66

Table 2 median MBL level (interquartile range in brackets), genotype distribution and cardiovascular events

	Median MBL level	MBL genotype (%)		
		A/A	A/O	O/O
Cross sectional				
South Asians (n=168)	476 µg/L (143-1536)	57	35	8*
Caucasians [7]		60	36	4
Longitudinal				
cardiovascular event† (n=22)	390 µg/L (77-1348)	50	20	30‡
no cardiovascular event (112)	466 µg/L (139-1545)	56	37	7

† Cardiovascular event: see text for definition
* P = 0.226 (% O/O in South Asians compared to Caucasians)
‡ P = 0.005 (% O/O in subjects with cardiovascular event compared to subjects without cardiovascular event)

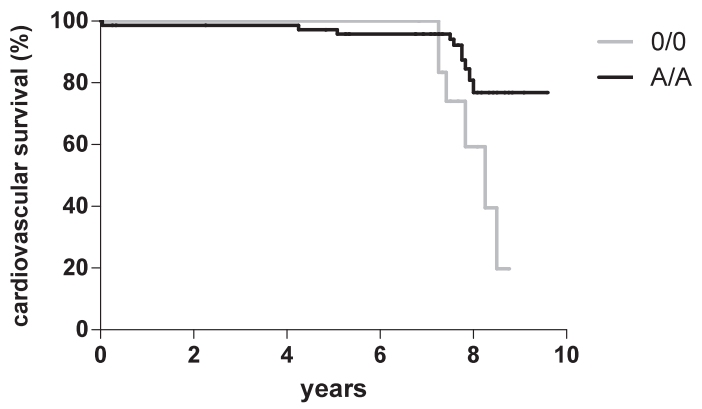
Table 3 MBL and risk for cardiovascular events

	Hazard Ratio	95% CI
MBL genotype		
A/A (n=74)	1	
A/O (n=42)	0.65	0.20-2.07
O/O (n=13)	3.43	1.24-9.49
Combined A/O and O/O	1.26	0.52-3.04
Log MBL level (per log MBL increase)		
Crude	0.93	0.50-1.73
Adjusted[a]	1.19	0.61-2.30

[a] Adjusted for urinary albumin-to-creatinin ratio, log high sensitivity CRP, waist-to-hip ratio, smoking status, ratio total cholesterol: HDL-cholesterol, systolic blood pressure, age, sex, HbA1c, diabetes duration

(IQR 7.48-8.10) years. Participants lost to follow-up did not differ in baseline characteristics from participants for whom follow-up data were available (table 1).
During follow-up, 31 cardiovascular events occurred in 22 subjects (11 men, 11 women): 3 sudden cardiac deaths, 2 fatal and 5 non-fatal myocardial infarction, 13 percutaneous coronary interventions, and 8 coronary artery bypass graft procedures. Eight of these 22 subjects had already experienced a cardiovascular event at baseline.
Twelve subjects died due to non-cardiovascular causes. These patients were censored, none of them reached the primary end-point before dying.
Compared to the wild-type genotype, the O/O genotype was significantly associated with the occurrence of a cardiovascular event (hazard ratio 3.42, 95%CI 1.24-9.48, P = 0.018) (figure 1). Subjects with the O/O genotype did not differ in lipid parameters or blood pressure compared to subjects with the A/A or A/O genotype. The A/O genotype was not associated with cardiovascular events (HR 0.65, 95% CI 0.20-2.07, P = 0.456). Cardiovascular events were also associated with a previous cardiovascular events (HR 4.3, 95% CI 1.2-10.3) and log urinary albumin/creatinine ratio (HR 1.58, 95% CI 1.0-2.48).

Figure 1



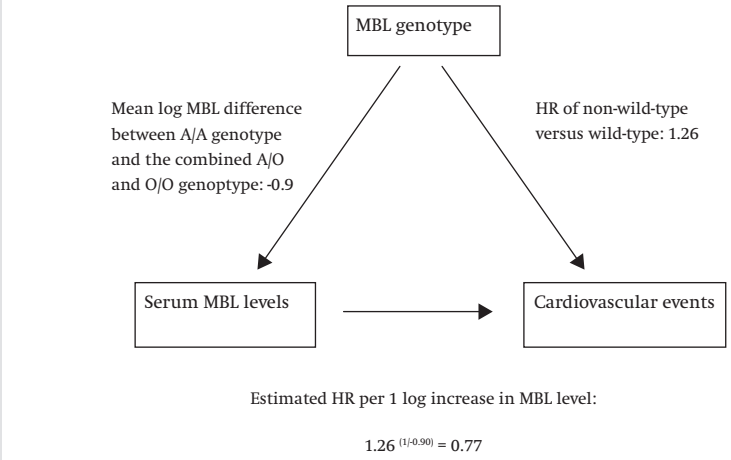
Unadjusted Kaplan-Meier survival curves according to MBL genotype (black line = A/A (wild type) genotype, grey line = O/O genotype. The O/O genotype has a worse event-free survival compared to the A/A genotype (Log rank test P = 0.011).

There was no statistically significant difference in baseline median MBL level between subjects experiencing a cardiovascular event during follow-up and subjects without a cardiovascular event (390 μg/L (IQR 77-1348 μg/L) versus 466 μg/L (IQR 139-1545 μg/L), P = 0.674). Log-transformed MBL levels were not associated with the occurrence of cardiovascular events (hazard ratio 0.93, 95% CI 0.50-1.73). MBL levels above the median were not associated with cardiovascular events (hazard ratio 0.94, 95% CI 0.40-2.20). Using other MBL cut-off levels also failed to show an association with cardiovascular events (data not shown).

We used the MBL genotype as an instrumental variable to estimate the causal effect of MBL levels on cardiovascular events (Mendelian Randomization [19], figure 2). The rationale in Mendelian randomization is that, if an exposure is causally related to an outcome, a genetic variant, which is associated with the exposure, should have a similar relation to the outcome as the supposedly causal exposure itself. If the association with the outcome differs between genetic variant and genetic product, residual confounding or reverse causation (the outcome influencing the exposure) is present.

Because of the small number in the O/O genotype and the relatively small difference in mean log MBL level between the A/O and O/O genotype, we combined the A/O and the O/O genotype and compared this to the A/A genotype. Given the mean log MBL difference of 0.9 μg/L between the A/A and the combined A/O and O/O genotype and the HR for cardiovascular events of 1.26 for the combined A/O and O/O genotype with the A/A genotype as reference, the causative effect of MBL level on cardiovascular events was estimated, resulting in an HR of 0.77 per log MBL increase (Figure 2)

Figure 2



Calculation of the estimated causative hazard ratio of MBL levels on cardiovascular events, given the observed mean difference in log MBL level (between the A/A genotype and the combined A/O and O/O genotype) and the observed association between the genotype and cardiovascular events

Discussion

The main finding of the present study is that in type 2 diabetic South Asians, the O/O MBL genotype was significantly associated with the occurrence of cardiovascular events compared to wild-type.

The association between low MBL genotype and cardiovascular events was previously reported in the Strong Heart Study [12]. This study included American Indians, which - like South Asians- have a high burden of diabetes and subsequent vascular complications. A low MBL genotype was associated with a threefold increased risk for coronary heart disease.

With respect to serum MBL levels and cardiovascular events, data are more controversial. Cross sectional studies found higher MBL levels in type 1 and type 2 diabetic Caucasians with a previous cardiovascular event compared to

diabetic subjects without cardiovascular disease [10,11]. In non-diabetic Caucasian males but not in females, high MBL levels were associated with future cardiovascular events [20]. In type 2 diabetic Caucasians high MBL levels were associated with increased all cause mortality, although data with respect to cardiovascular events were not reported [11]. In contrast, the prospective Reykjavik study found that in type 2 diabetic subjects low rather than high MBL levels were associated with increased incidence of myocardial infarction [8]. Recently, the Strong Heart Study provided data on MBL levels, confirming that low baseline MBL levels indeed were associated with future cardiovascular events [21]. In our study, MBL levels were not associated with future cardiovascular events.

A possible explanation for an association between low MBL levels and cardiovascular events might be a defective clearance of atherogenic particles. MBL binds N-acetylglucosamine moieties, which are expressed on several lipoproteins and oxidized LDL [22], and this may facilitate their phagocytic clearance. This hypothesis is supported by a recently published study showing that MBL deficient subjects have impaired clearance of triglyceride-rich lipoproteins [23]. Additionally, MBL deficiency might influence the susceptibility and course of infection with *Chlamydia pneumoniae*, which is associated with coronary artery disease [24]. On the other hand, MBL levels may increase in the setting of an inflammatory response [9]. Experimental studies show that in the setting of ischemia/reperfusion injury high MBL levels are detrimental rather than protective [25]. Oxidative stress induces a change on the cellular surface [26], which results in binding of MBL leading to enhanced complement mediated injury. However, since MBL levels were not correlated with plasma SC5b-9 levels in our study, we found no evidence that high MBL levels result in increased complement activation.

Summarizing the above, whereas most cross-sectional studies found an association between cardiovascular disease and high MBL levels, most prospective studies show an association between low MBL levels and cardiovascular events.

In our study, low MBL genotype was associated with cardiovascular events and MBL genotype corresponds with MBL level. One would therefore expect low MBL levels to be associated with cardiovascular events, which however was not the case in our study. MBL genotype probably is a more accurate estimate of cumulative MBL exposure than a single serum MBL level. In addition, the contribution of MBL to vascular disease might differ according to the pathophysiologic phase: early in the course low MBL levels might promote

atherosclerosis, and once a vascular inflammatory response is established MBL levels might secondarily become increased and - perhaps - subsequently promote vascular inflammation. A recent experimental study demonstrated local MBL synthesis in early atherosclerotic plaques [27], supporting the hypothesis that MBL levels might become increased due to atherogenesis. Based on the assumption of time-dependency of the association between MBL and cardiovascular disease, our single baseline sample might have been too late in the pathophysiologic course to detect the effect of low MBL level on cardiovascular outcome. In addition, variations of the MBL assay might contribute to the discordant findings between MBL genotype and MBL level. Furthermore, because we did not include the MBL promoter genotype, the genotype-phenotype relation might be attenuated. Finally, at least theoretically, the association of low MBL genotype with cardiovascular events might be based on an association with other susceptibility genes for cardiovascular events.

Noteworthy, although the O/O genotype was associated with cardiovascular events, the A/O genotype was not, although the difference in median MBL level between the O/O and A/O genotype was relatively small. The deleterious effect of low MBL level may only become apparent below a critical MBL value.

To further assess the causative effect of low MBL levels on cardiovascular events, we estimated the effect of low MBL levels on cardiovascular events based on the relation between MBL genotype and cardiovascular events using a Mendelian Randomization approach [19]. In this approach, it is expected that the association between the genetic variant and the outcome (cardiovascular events) is equal to the association between exposure levels (MBL levels) and outcome. Differences between the expected and observed association are caused by residual confounding or reverse causation (that is, the disease influences the exposure levels). In our data, the expected HR per log MBL increase was 0.77, whereas the observed HR was 0.93. This suggests that, based on a single serum value, the association of low MBL levels and cardiovascular events is attenuated. Reverse causation (MBL levels being increased because of vascular inflammation) might account for this.

In conclusion, low MBL genotype is associated with cardiovascular events in type 2 diabetic South Asians, suggesting that MBL is involved in the pathogenesis of cardiovascular events. However, single serum MBL concentrations were not associated with cardiovascular events and therefore a single MBL level is not a clinically useful risk marker for cardiovascular events in type 2 diabetic South Asians.

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References

1. Anand SS, Yusuf S. Risk factors for cardiovascular disease in Canadians of South Asian and European origin: a pilot study of the Study of Heart Assessment and Risk in Ethnic Groups (SHARE). *Clin Invest Med*. 20:204-210, 1997
2. Forouhi NG, Sattar N, Tillin T, McKeigue PM, Chaturvedi N. Do known risk factors explain the higher coronary heart disease mortality in South Asians compared with European men? Prospective follow-up of the Southall and Brent studies, UK. *Diabetologia* 49:2580-2588, 2006
3. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 340:115-126,1999
4. Ostergaard J, Hansen TK, Thiel S, Flyvbjerg A. Complement activation and diabetic vascular complications. *Clin Chim Acta*. 361:10-19, 2005
5. Rus HG, Niculescu F, Vlaicu R. Co-localization of terminal C5b-9 complement complexes and macrophages in human atherosclerotic arterial walls. *Immunol Lett*. 19:27-32, 1988
6. Takahashi K, Ip WE, Michelow IC, Ezekowitz RA. The mannose-binding lectin: a prototypic pattern recognition molecule. *Curr Opin Immunol*. 18:16-23, 2006
7. Garred P, Larsen F, Seyfarth J, Fujita R, Madsen HO. Mannose-binding lectin and its genetic variants. *Genes Immun*. 7:85-94, 2006
8. Saevarsdottir S, Oskarsson OO, Aspelund T, Eiriksdottir G, Vikingsdottir T, Gudnason V, Valdimarsson H. Mannan binding lectin as an adjunct to risk assessment for myocardial infarction in individuals with enhanced risk. *J Exp Med*. 201:117-125, 2005
9. Thiel S, Holmskov U, Hviid L, Laursen SB, Jensenius JC. The Concentration of the C-Type Lectin, Mannan-Binding Protein, in Human Plasma Increases During An Acute Phase Response. *Clinical and Experimental Immunology*. 90:31-35, 1992
10. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG, Parving HH, Flyvbjerg A. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes*. 53:1570-1576, 2004
11. Hansen TK, Gall MA, Tarnow L, Thiel S, Stehouwer CD, Schalkwijk CG, Parving HH, Flyvbjerg A. Mannose-binding lectin and mortality in type 2 diabetes. *Arch Intern Med*. 166:2007-2013, 2006
12. Best LG, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET, Howard BV, DeCroo S, Ferrell RE. Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. *Circulation*. 109:471-475, 2004
13. Chandie Shaw PK, van Es LA, Paul LC, Rosendaal FR, Souverijn JH, Vandenbroucke JP. Renal disease in relatives of Indo-Asian Type 2 diabetic patients with end-stage diabetic nephropathy. *Diabetologia*. 46:618-624, 2003
14. Siezenga MA, Chandie Shaw PK, van der Geest RN, Mollness TE, Daha MR, Rabelink TJ, Berger SP. Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. *Clin Exp Immunol* 157:98-103, 2009
15. Roos A, Dieltjes P, Vossen RH, Daha MR, de Knijff P. Detection of three single nucleotide polymorphisms in the gene encoding mannose-binding lectin in a single pyrosequencing reaction. *J Immunol Methods*. 309:108-114, 2006
16. Berger SP, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant*. 5:1361-1366, 2005
17. Roos A, Garred P, Wildenberg ME, Lynch NJ, Munoz JR, Zuiverloon TC, Bouwman LH, Schlagwein N, Fallaux van der Houten FC, Faber-Krol MC, Madsen HO, Schwaeble WJ, Matsushita M, Fujita T, Daha MR. Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol* 34(9):2589-2598, 2004
18. Fisher E, Stefan N, Saar K, Drogan D, Schulze MB, Fritsche A, Joost HG, Häring HU, Hubner N, Boeing H, Weichert C. Association of AHSG gene polymorphisms with fetuin-A plasma levels and cardiovascular diseases in the EPIC-Potsdam study. Circ Cardiovasc Genet 2009;2:607-613

19. Verduijn M, Siegerink B, Jager KJ, Zocalli C, Dekker FW. Mendelian randomization: use of genetics to enable causal inference in observational studies. Nephrol Dial Transplant 2010;25:1394-1398.
20. Keller TT, van Leuven SI, Meuwese MC, Wareham NJ, Luben R, Stroes ES, Hack E, Levi M, Khaw KT, Boekholdt SM. Serum levels of mannose-binding lectin and the risk of future coronary artery disease in apparently healthy men and women. *Arterioscler Thromb Vasc Biol*. 26:2345-2350, 2006
21. Best LG, Ferrell RE, DeCroo S, North KE, MacCluer JW, Zhang Y, Lee ET, Howard BV, Umans J, Palmieri V, Garred P. Genetic and other factors determining mannose-binding lectin levels in American Indians: the Strong Heart Study. *BMC Med Genet* 10(5), 2009
22. Tertov VV, Sobenin IA, Tonevitsky AG, Orekhov AN, Smirnov VN. Isolation of atherogenic modified (desialylated) low density lipoprotein from blood of atherosclerotic patients: separation from native lipoprotein by affinity chromatography. *Biochem Biophys Res Commun*. 167:1122-1127, 1990
23. Alipour A, van Oostrom AJHHM, van Wijk JPH, Verseyden C, PLokker HWM, Jukema JW, Rabelink AJ, Castro Cabezas M. Mannose binding lectin deficiency and triglyceride-rich lipoprotein metabolism in normolipidemic subjects. *Atherosclerosis* 206:444-450, 2009
24. Rugonfalvi-Kiss S, Endrész V, Madsen HO, Burian K, Duba J, Prohaszka Z, Karadi I, Romics L, Gönczöl E, Füst G, Garred P. Association of Chlamydia pneumonia with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. *Circulation* 106:1071-1076, 2002
25. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SC, Ezekowitz AB, Stahl GL. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol*. 175:541-546, 2005
26. Collard CD, Montalto MC, Reenstra WR, Buras JA, Stahl GL. Endothelial oxidative stress activates the lectin complement pathway: role of cytokeratin 1. *Am J Pathol*.159:1045-1054, 2001
27. Matthijsen RA, de Winther MPJ, Kuipers D, van der Made I, Weber C, Herias V, Gijbels MJJ, Buurman WA. Macrophage-specific expression of Mannose-binding lectin controls atherosclerosis in low-density lipoprotein receptor deficiënt mice. Circulation 2009;119:2188-2195