Mannose-Binding Lectin and Risk of Cardiovascular Events and Mortality in Type 2 Diabetes: A Danish Cohort Study

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OBJECTIVE

Mannose-binding lectin (MBL) is linked to risk of cardiovascular disease (CVD) in diabetes, but the nature of the association is unclear. We investigated the association between MBL and the risk of cardiovascular events (CVE) and all-cause mortality in type 2 diabetes.

RESEARCH DESIGN AND METHODS

In a cohort study of 7,588 patients with type 2 diabetes, we measured serum MBL in 7,305 patients and performed MBL expression genotyping in 3,043 patients. We grouped serum MBL and MBL expression genotypes into three categories: low, intermediate, and high. Outcomes were CVE (myocardial infarction, stroke, coronary revascularization, unstable angina, or cardiovascular death) and all-cause mortality. The association with outcomes was examined by spline and Cox regression analyses.

RESULTS

Serum MBL and CVE showed a U-shaped association. Compared with the intermediate serum MBL category, the adjusted hazard ratio (HR) for CVE was 1.82 (95% CI 1.34–2.46) for the low-MBL category and 1.48 (95% CI 1.14–1.92) for the high-MBL category. We found a similar U-shaped association for all-cause mortality, but with lower risk estimates. Compared with the intermediate MBL expression genotype, the adjusted HR for CVE was 1.40 (95% CI 0.87–2.25) for the low-expression genotype and 1.44 (95% CI 1.01–2.06) for the high-expression genotype. MBL expression genotype was not associated with all-cause mortality.

CONCLUSIONS

Both serum MBL and MBL expression genotype showed a U-shaped association with CVE risk in individuals with type 2 diabetes. Our findings suggest that serum MBL is a risk factor for CVD in this population.

Mannose-binding lectin (MBL), also known as mannan-binding lectin, is a multifunctional serum protein primarily produced in the liver (1). It belongs to the lectin family of blood proteins and is instrumental in innate immunity (1), initiating the complement cascade via the lectin pathway and promoting pathogen clearance. In contrast to the classical complement pathway, which recognizes an antibody bound to its target, the lectin pathway starts with MBL binding to carbohydrate structures (e.g., patterns of mannose) on the surface of pathogens (1,2). Functional serum MBL levels

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are relatively stable over time (3) and largely determined genetically by six common single nucleotide polymorphisms (SNPs) (4). These SNPs can be categorized into three MBL expression genotypes, namely, low, intermediate, and high (1), with corresponding serum MBL levels (4).

The association of MBL with cardiovascular disease (CVD) is mixed. Some studies have linked low levels and other studies high levels of serum MBL and MBL expression genotypes with increased risk of CVD (1,5–11) and all-cause mortality (6,12,13) in individuals with diabetes and in the general population. MBL, thus, may play two roles in the development of CVD. Low levels (as in MBL deficiency) could impair pathogen clearance and reduce removal of atherogenic lipoproteins (10,14). High levels may amplify a low-grade immune response through complement activation, in particular if hyperglycemia is present (15,16). In support of this potential dual role, three studies (17–19), with two in patients with diabetes (17,18), have reported a U-shaped association of serum MBL levels with carotid intima-media thickness and low-grade inflammation. If both low and high serum MBL levels are directly involved in development of CVD and all-cause mortality, then both low and high MBL expression genotypes also would be expected to show a similar association (20). The potential dual role of both serum MBL and MBL expression genotypes in CVD and all-cause mortality in type 2 diabetes has not been formally investigated.

We hypothesized that compared with intermediate levels, both low and high serum MBL levels are directly associated with increased risk of cardiovascular events (CVE) and all-cause mortality in patients with early type 2 diabetes. To test this hypothesis, we conducted a Danish cohort study of 7,588 patients with type 2 diabetes followed for up to 8 years. We first investigated the link between serum MBL levels and risk of CVE and all-cause mortality. Second, we examined the association between MBL expression genotype and serum MBL levels. Third, we investigated whether MBL expression genotype was associated with the risk of CVE and all-cause mortality. According to the Mendelian randomization study design, this third step aids in substantiating a causal association between serum MBL and CVE and all-cause mortality (20).

RESEARCH DESIGN AND METHODS

Study Cohort

We accessed information from the Danish Centre for Strategic Research in Type 2 Diabetes (DD2) cohort, an ongoing cohort of patients recently diagnosed with type 2 diabetes (21). Enrollment in the cohort has been continuous from general practitioners' offices and hospital specialist outpatient clinics since 1 November 2010 (21). In brief, general practitioners or hospital physicians identify newly/recently diagnosed individuals with type 2 diabetes and complete an online questionnaire (22) eliciting lifestyle (e.g., physical activity) and clinical examination data for each entered participant at the time of enrollment. Urine and fasting blood samples are collected from each patient and stored in the DD2 biobank (23). The study cohort was all DD2 participants enrolled by December 2016 and with a stored blood sample in the DD2 biobank.

Ethics

This study was approved by the Danish Data Protection Agency (record number 2008-58-0035) and by the Regional Committees on Health Research Ethics for Southern Denmark (record number S-20100082). All cohort participants gave written informed consent.

Outcomes

Outcomes were a composite of CVE (first occurrence of myocardial infarction, ischemic stroke, unstable angina pectoris, coronary revascularization, or cardiovascular death), individual subtypes of CVE, and all-cause mortality. We obtained all diagnoses (primary and secondary discharge diagnoses) from the Danish National Patient Registry, which covers all hospitals in Denmark and contains records of discharge diagnoses from all inpatient hospitalizations since 1977 and all emergency department visits and hospital outpatient clinic since 1995 (24). Exact dates of death were obtained from the Danish Civil Registration System (25), and information on cardiovascular death (both as the immediate or underlying cause of death) was accessed from the Danish Registry of Causes of Death (26,27). Diagnoses and procedure codes are shown in Supplementary Table 1.

Serum MBL Levels

Functional serum MBL levels were measured using an in-house time-resolved immuno-fluorometric assay, as described elsewhere (2). Mannan-coated microtiter wells were incubated with serum samples, and bound MBL was detected with biotin-labeled monoclonal anti-MBL antibody followed by europium-labeled streptavidin and detection by time-resolved fluorometry. The limit of quantification was 10 μg/L at the dilution used, and MBL deficiency (n = 849; 12%) was set to 10 μg/L. The intra- and interassay coefficients of variation were <10%. In accordance with our hypothesis of a U-shaped association, we examined a potential nonlinear association between serum MBL levels, as a continuous variable, and the risk of outcomes using spline models. Splines allow for a precise description of the actual exposure-outcome association without categorization. In addition, serum MBL levels were categorized as low (≤100 μg/L), intermediate (101–1,000 μg/L), or high (>1,000 μg/L). These cut points were selected based on previous MBL research (28), the splines, and matching with the three MBL expression genotype categories, suggesting that these categories are close to the natural categorization. Consistent with previous studies indicating a nonlinear association between serum MBL and cardiovascular outcomes (17–19), the intermediate serum MBL category (101–1,000 μg/L) was used as the reference.

MBL Expression Genotypes

Six SNPs located within the promoter region (rs11003125, rs7096206, and rs7095891) and exon 1 (rs5030737, rs1800451, and rs1800450) of the *MBL2* gene were genotyped on the first 3,043 consecutive individuals in the DD2 cohort, using TaqMan genotyping assays as previously described (4). Because of linkage disequilibrium, the six SNPs give rise to seven major haplotypes: HYPA, LYQA, LYPA, LXPA, LYPB, LYQC, and HYPD. These MBL haplotypes were ranked according to increasing serum MBL concentrations and were categorized into three MBL expression genotypes, i.e., low, intermediate, and high (1,4), that have previously been correlated with serum MBL levels (28). To compare genotype frequencies (for the six SNPs in the *MBL2* gene) between the DD2 cohort and other European cohorts, we performed a search in the Exome Aggregation Consortium (ExAC, https://exac.broadinstitute.org/) and the Genome Aggregation Database (gnomAD,

https://gnomad.broadinstitute.org/). ExAC spans 60,706 exome sequences and gnomAD spans 125,748 exome sequences and 15,708 whole-genome sequences from unrelated individuals sequenced as part of various disease-specific and population genetic studies (https://exac.broadinstitute.org/ and https://gnomad.broadinstitute.org/). Because of differences in allele frequencies across different populations, we present information only for the European non-Finnish population from the ExAC and gnomAD databases, comprising approximately half of the total sequenced population (Supplementary Table 2). To examine possible confounding by variation in nearby genes, we searched for SNPs in linkage disequilibrium with the six SNPs used in this study. The search was performed on LDlink (https://ldlink.nci.nih.gov/), a suite of web-based applications designed to interrogate linkage disequilibrium in population groups.

Covariates

From the DD2 cohort questionnaire (22) and linked medical and administrative registries, we extracted baseline information on covariates present at the time of DD2 enrollment that could be associated with subsequent cardiovascular risk. Covariates, definitions, and codes are listed in Supplementary Table 1.

Statistical Analysis

We used restricted cubic spline models with 5 df to examine the association between serum MBL levels, as a continuous variable, and the risks of CVE and all-cause mortality. The cumulative incidence of CVE, with risk of noncardiovascular death as a competing risk, was plotted using Stata's stcompet command, and we plotted cumulative incidence of all-cause mortality using the Kaplan-Meier method. Incidence and mortality rates were calculated using Stata's stptime command, and to calculate hazard ratios (HRs) and 95% CIs, we used Cox regression analysis. We performed extensive adjustments to ensure robustness of the potential associations. In model 1, HRs were adjusted for sex and age. In model 2, HRs were adjusted for sex, age, diabetes duration, and levels of hs-CRP. In model 3, HRs were adjusted for sex, age, diabetes duration, hs-CRP, waist circumference, waist-to-hip ratio, BMI, physical activity, smoking, systolic and diastolic blood pressure, comorbidities, fasting blood glucose, HbA_{1c}, C-peptide, albumin-to-creatinine ratio, estimated glomerular filtration rate (eGFR), total cholesterol, LDL, HDL, triglycerides, and use of antidiabetes, lipid-lowering, antihypertensive, or antithrombotic drugs. Missing covariates (n = 5–3,966; 0.1–54%) (Supplementary Table 3) were treated with multiple imputation to maximize power and avoid selection bias. We used multivariate normal imputation (MVNI) to impute 20 complete data sets. Missing values were sampled from the predictive distribution based on the observed data. MVNI assumes that all variables in the imputation model follow a multivariate normal distribution and that missing data are missing at random, meaning that the probability of a variable being missing depends only on the observed values. Continuous variables (fasting blood glucose, C-peptide, systolic and diastolic blood pressure, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, HbA_{1c}, and albumin-to-creatinine ratio and eGFR) with clearly nonnormal (skewed) distributions were zero-skewness log-transformed, i.e., transformed to approximate normality before imputation. Then, the imputed values were transformed back to the original scale before analysis. Smoking (categorical variable) was also imputed using MVNI, which has been shown to perform well even in the presence of binary and ordinal variables. The binary variable smoking (1, smoking [former/current], versus 0, never smoking) was imputed on a continuous scale and rounded to 0 or 1 by simple rounding. Each variable in the data set was characterized as being imputed or regular. Imputed variables contain missing values, and those values are imputed. Regular variables usually do not contain missing values, or if they do, the missing values are not imputed. All covariates used in the analysis model, as well as the outcomes, were included in the imputation model to ensure maximum recovery of information about the association of interest. The imputed models were validated by comparing the mean, median, and interquartile range (IQR) of the first and last imputed data set with the complete data set. We did not impute MBL expression genotype where this information was missing (n = 4,262; 58%).

For the Cox regression analysis, we graphically verified the proportional hazards assumption by plotting −ln(survival probability) against ln(analysis time) and detected no violations. Individuals with type 2 diabetes were followed from the enrollment date until an event, death, or 10 August 2018, whichever came first. All-cause and cardiovascular death data were available up to 22 August 2018 and 28 December 2016, respectively. Individuals with an event prior to enrollment (myocardial infarction, n = 434; ischemic stroke, n = 248; coronary revascularization, n = 602; unstable angina pectoris, n = 163; any CVE, n = 924) were excluded from the analyses for the relevant outcome. We did not consider recurrent events.

To assess the risk of genotype misclassification, we used Hardy-Weinberg equilibrium and the χ^2 test. We performed a Cuzick nonparametric test for trend and calculated R^2 by simple linear regression to evaluate the association between MBL expression genotype and serum MBL levels.

Sensitivity Analyses

In addition to the main analyses, we also performed a number of sensitivity analyses. First, to decrease risk of misclassification of serum MBL related to acute infection and/or inflammation, we excluded individuals with serum CRP levels >10 mg/L (n = 641; 9%) (29). Second, to focus exclusively on individuals newly diagnosed with type 2 diabetes, we excluded anyone with a registered diabetes duration >1 year (n = 4,042; 55%). Third, we excluded individuals with any previous record of CVD (including diagnoses not included in the composite CVE outcome, such as any atherosclerotic disease or heart failure) (n = 1,451; 20%). For statistical analyses, we used Stata version 14.2.

RESULTS

Baseline Characteristics

The study included 7,588 participants with type 2 diabetes, of whom 7,305 (96%) had a serum MBL measurement available and 3,043 (42%) had been genotyped for the six SNPs in the *MBL2* gene (Supplementary Fig. 1). The cohort was followed for a median period of 4.7 years (IQR 3.3–5.7 years) for CVE and 4.8 years (IQR 3.6–5.8 years) for all-cause mortality. Between 2010 and 2018, 324 patients

developed the composite outcome of CVE, including 106 with myocardial infarction, 124 with ischemic stroke, 73 with cardiovascular death, 157 with coronary revascularization, and 45 with unstable angina pectoris. More than one outcome was possible. There were 439 all-cause deaths.

Table 1 shows baseline characteristics according to serum MBL categories (≤100, 101–1,000, or >1,000 μg/L), and Supplementary Table 4 shows them according to MBL expression genotypes (low, intermediate, and high). Serum MBL and MBL expression genotype categories showed no clear associations with any baseline characteristics. No MBL categories were associated with hs-CRP levels.

Serum MBL Levels and Risk for CVE/All-Cause Mortality

Serum MBL levels and CVE showed a U-shaped association (Fig. 1*A*). Compared with intermediate serum MBL levels, both low and high serum MBL levels were associated with increased CVE risk (Figs. 1*A* and 2*A*). All-cause mortality had a similar but attenuated association (Figs. 1*B* and 2*C*), suggesting a similar event rate nadir around MBL of 500 μg/L for both CVE and all-cause mortality (Fig. 1). Supplementary Table 5 lists incidence rates.

Figure 3 shows the HRs for CVE and all-cause mortality by serum MBL categories and MBL expression genotypes. Compared with the intermediate serum MBL category, the adjusted HR (model 3) for CVE was 1.82 (95% CI 1.34–2.46) for the low serum MBL category and 1.48 (95% CI 1.14–1.92) for the high serum MBL category. Results for the CVE subtypes were consistent with the analysis of the composite outcome of CVE (Supplementary Figs. 2–16): there was a U-shaped association between serum MBL levels and all individual subtypes of CVE, but with limited statistical precision because of fewer events. The spline for all-cause mortality was similar to the spline for cardiovascular mortality, but attenuated. Compared with the intermediate serum MBL category, the adjusted HR (model 3) for all-cause mortality was 1.20 (95% CI 0.89–1.61) for the low serum MBL category and 1.19 (95% CI 0.94–1.50) for the high category.

Table 1—Characteristics of DD2 cohort members at baseline by serum MBL category

	Low serum MBL (≤100 μg/L)	Intermediate serum MBL (101–1,000 μg/L)	High serum MBL (>1,000 μg/L)
Total patients	1,295 (17.7)	2,975 (40.7)	3,035 (41.6)
Male sex	727 (56.1)	1,612 (54.2)	1,939 (63.9)
Age, years	61.6 (52.7–69.0)	61.9 (53.1–68.7)	62.3 (53.0–68.8)
Diabetes duration, years	1.3 (0.3–2.9)	1.4 (0.4–2.9)	1.2 (0.3–2.9)
Waist circumference, cm	106 (97–117)	107 (97–117)	105 (96–115)
Waist-to-hip ratio	0.98 (0.92–1.04)	0.98 (0.92–1.04)	0.98 (0.93–1.04)
BMI, kg/m^2	30.5 (27.1–34.5)	30.7 (27.4–34.7)	29.7 (26.4–33.7)
Physical activity, days/week*	3 (2–7)	3 (2–7)	4 (2–7)
Smoking			
Never	434 (45.5)	1,039 (47.7)	1,052 (46.3)
Former	351 (36.8)	749 (34.4)	750 (33.0)
Current	170 (17.8)	389 (17.9)	471 (20.7)
Systolic blood pressure, mmHg	130 (124–140)	130 (123–140)	130 (124–140)
Diastolic blood pressure, mmHg	80 (74–85)	80 (74–85)	80 (75–86)
CCI score†			
0	882 (68.1)	2,034 (68.4)	2,109 (69.5)
1–2	339 (26.2)	783 (26.3)	763 (25.1)
3	74 (5.7)	158 (5.3)	163 (5.4)
Antidiabetes drug use	1,080 (83.4)	2,547 (85.6)	2,582 (85.1)
Lipid-lowering drug use	932 (72.0)	2,176 (73.1)	2,037 (67.1)
Antihypertensive drug use	926 (71.5)	2,175 (73.1)	2,143 (70.6)
Antithrombotic drug use	372 (28.7)	873 (29.3)	855 (28.2)
Fasting blood glucose, mmol/L	7.1 (6.3–8.1)	7.1 (6.3–8.2)	7.2 (6.4–8.3)
HbA_{1c}, %	6.6 (6.2–7.2)	6.6 (6.1–7.2)	6.6 (6.2–7.3)
HbA_{1c}, mmol/mol	49 (44–55)	49 (43–55)	49 (44–56)
C-peptide, pmol/L	1,151 (865–1,579)	1,197 (890–1,605)	1,131 (838–1,528)
Albumin-to-creatinine ratio, mg/g	9 (4–22)	9 (4–22)	9 (4–22)
eGFR, mL/min/1.73 m^2	89 (75–99)	89 (74–98)	88 (75–98)
Total cholesterol, mmol/L	4.4 (3.8–5.2)	4.3 (3.7–5.1)	4.3 (3.7–5.1)
LDL cholesterol, mmol/L	2.1 (1.7–2.7)	2.2 (1.7–2.8)	2.2 (1.7–2.9)
HDL cholesterol, mmol/L	1.2 (1.0–1.4)	1.2 (1.0–1.4)	1.2 (1.0–1.5)
Triglycerides, mmol/L	1.7 (1.2–2.5)	1.7 (1.2–2.4)	1.6 (1.1–2.3)
hs-CRP, mg/L	2.0 (0.8–4.7)	2.0 (0.9–4.5)	1.9 (0.8–4.3)

Data are median (IQR) or *n* (%). Number of participants varied depending on availability of data (missing covariates are listed in Supplementary Table 2). *Days per week with a minimum of 30 min of physical activity. †Charlson comorbidity index (CCI) score excluding diabetes.

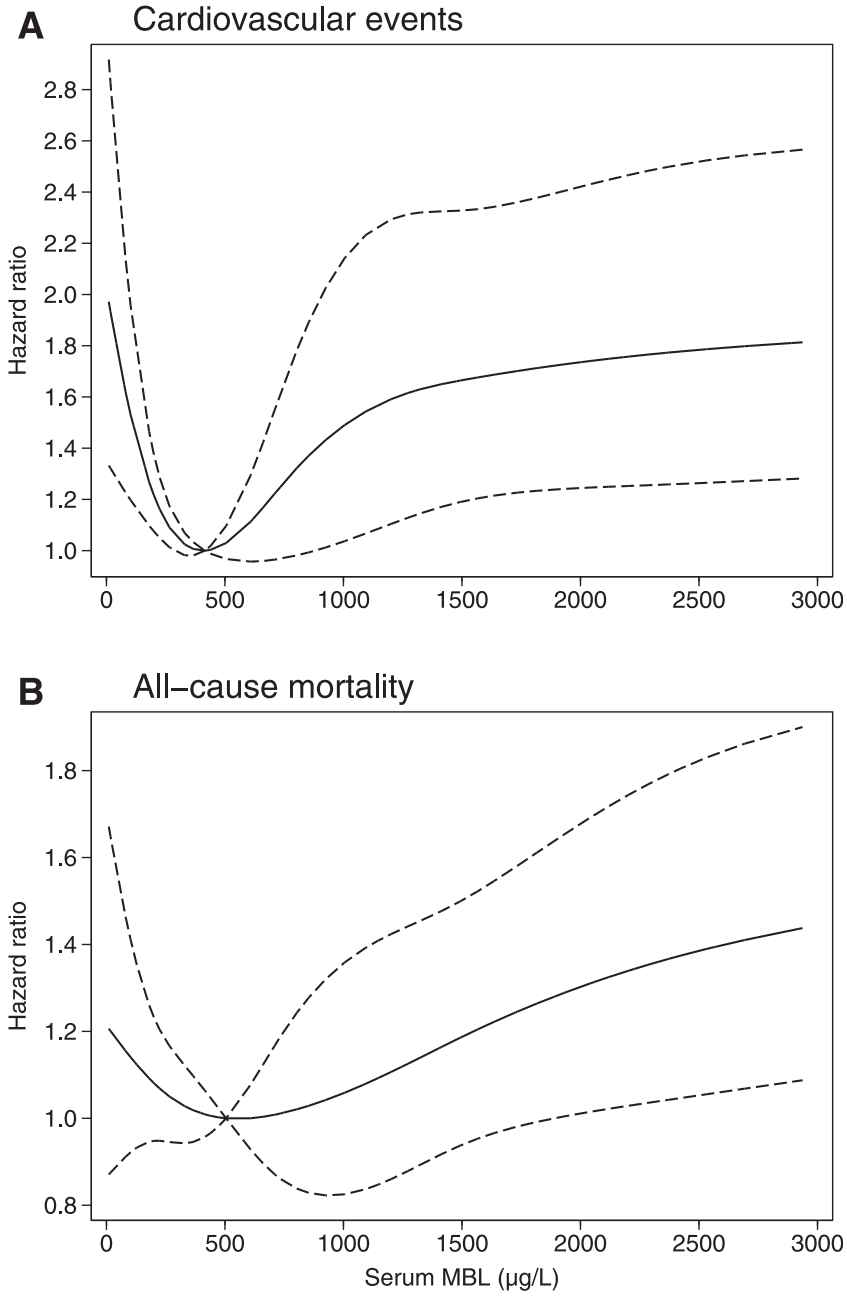

Figure 1—Risk of CVE (*A*) and all-cause mortality (*B*) by serum MBL levels. The solid lines indicate the HRs, and the dotted lines indicate 95% CIs. The continuous variable serum MBL was modeled with five restricted cubic splines.

MBL Expression Genotypes and Serum MBL Levels

The distributions of all MBL haplotypes with corresponding median serum MBL levels are shown in Supplementary Table 6. We identified no major deviations from Hardy-Weinberg equilibrium (χ^2 test for rs11003125, $P = 0.97$; for rs7095891, $P = 0.10$; for rs7096206, $P = 0.66$; for rs1800451, $P = 0.91$; for rs1800450, $P = 0.78$; and for rs5030737, $P = 0.01$) (Supplementary Table 2). Median serum MBL levels for individuals with low, intermediate, and high MBL expression genotypes were 10 μg/L (IQR 10–26 μg/L), 321 μg/L (IQR 199–545 μg/L), and 1,527 μg/L (IQR 974–2,394 μg/L), respectively (Supplementary Table 6). Serum MBL levels were strongly associated with MBL expression genotypes ($R^2 = 0.31$, P for trend $< 1 \times 10^{-300}$) (Supplementary Fig. 17).

MBL Expression Genotypes and Risk for CVEs/All-Cause Mortality

Both low and high MBL expression genotypes were associated with increased risk of CVE (Fig. 2*B*). There was no clear association for all-cause mortality (Fig. 2*D*). Supplementary Table 5 shows incidence rates.

Compared with the intermediate MBL expression genotype, the adjusted HR (model 3) for CVE was 1.40 (95% CI 0.87–2.25) for the low MBL expression genotype and 1.44 (95% CI 1.01–2.06) for the high MBL expression genotype (Fig. 3). Individual subtypes of CVE showed results consistent with those from the analysis of the composite outcome of CVE (Supplementary Figs. 2–16), with limited precision because of fewer events.

Compared with the intermediate MBL expression genotype, the adjusted HR (model 3) for all-cause mortality was 1.13 (95% CI, 0.73–1.77) for the low MBL expression genotype and 1.12 (95% CI, 0.81–1.55) for the high MBL expression genotype (Fig. 3).

Sensitivity Analyses

Overall, the sensitivity analyses were restricted to individuals with CRP below 10 mg/L, newly diagnosed type 2 diabetes, and no previous CVD yielded results similar to analyses of the composite CVE and all-cause mortality (Supplementary Figs. 18–32).

CONCLUSIONS

In this prospective study of 7,305 patients with type 2 diabetes, compared with the intermediate expression genotype, both low and high MBL expression genotypes were associated with a 40% and 44% increased risk of CVE. The consistency of this association for serum levels and MBL expression genotypes suggests a causal role for serum MBL in the development of CVD in type 2 diabetes.

Previous studies of the association between MBL and CVD have yielded contradictory findings regarding the direction of the associations. Some studies have shown links only for low serum MBL levels (30,31) or low MBL expression genotype (5,6,10,11) and increased risk of CVD. Other groups have reported that only high serum MBL levels (1,7,32,33) or high MBL expression genotype were associated with increased risk. A possible explanation for these

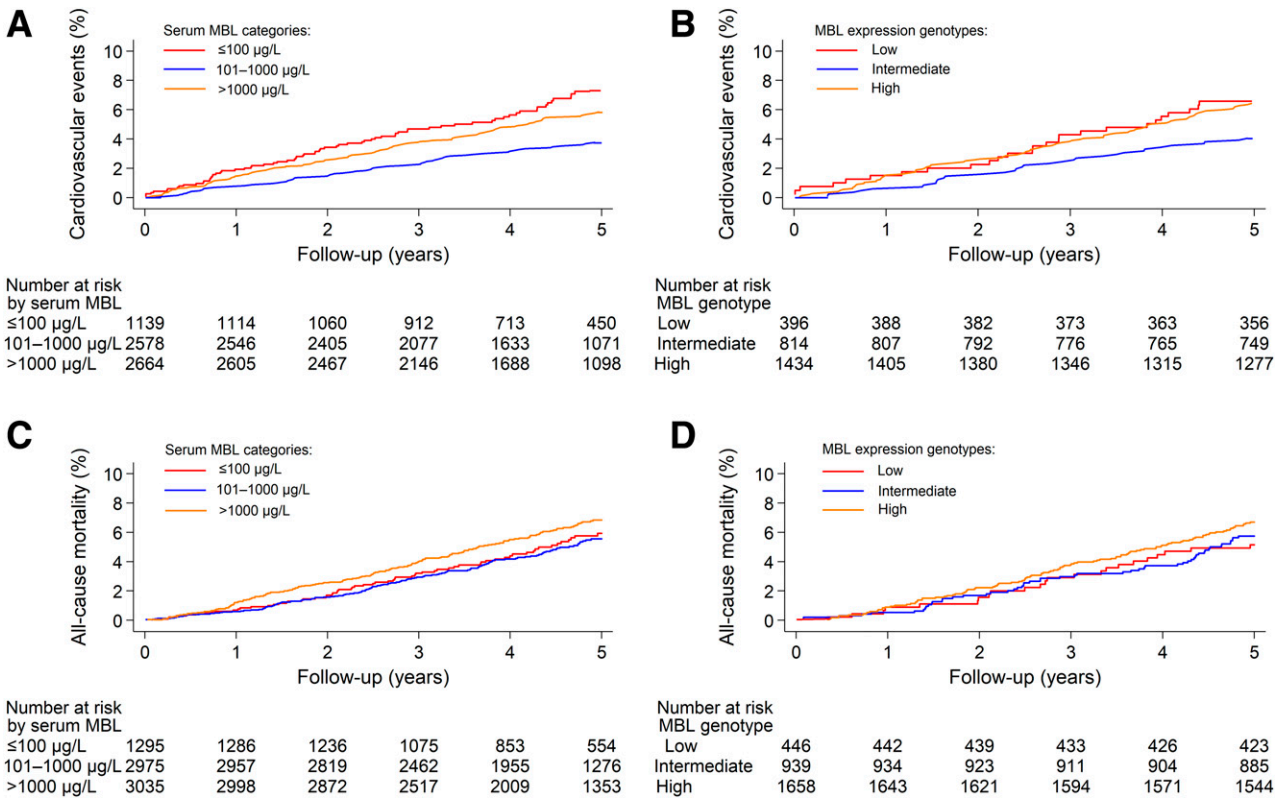

Figure 2—Time-to-event curves of CVE and all-cause mortality by serum MBL and MBL expression genotype categories. Cumulative incidence plots of CVE (*A* and *B*) and all-cause mortality (*C* and *D*) by serum MBL (*A* and *C*) and MBL expression genotype (*B* and *D*) categories. Cumulative incidence estimates are based on time from DD2 enrollment date to first event, with risk of noncardiovascular death as a competing risk (*A* and *B*).

apparent discrepancies is that the studies used different combinations of MBL categories (low and intermediate versus high, or low versus high and intermediate). They also had heterogeneous study populations and designs that precluded direct comparison with the present results. As an example, a previous cohort study of 9,245 individuals from the Danish general population (6) yielded a relative risk of 1.2 with low versus high MBL expression genotype, and 0.95 with intermediate versus high MBL expression genotype, which is compatible with a U-shaped association. If the authors had used the intermediate MBL expression genotype as the reference category, the identified risks might have been comparable with the current values. This suggests that our findings might be applicable to the general population, not only to patients with type 2 diabetes. Taken together, these previous observations support our findings that intermediate MBL levels are the most advantageous for healthy aging (34,35) and that both low and high MBL levels may raise risk of CVD.

We found no association between MBL expression genotype and risk of all-cause mortality, in agreement with the Danish general population study mentioned above (6) but in contrast with another Danish study of 372 patients with type 1 diabetes (13). These latter authors reported a 47% increased risk of all-cause mortality with a high versus low MBL expression genotype. We also found that low and high serum MBL levels were compatible with a 19–20% increased risk of all-cause mortality, although we could not rule out a null association. These findings are at the low end of previously reported values, including the results of the earlier study of patients with type 1 diabetes (13) and another Danish study of 326 individuals with type 2 diabetes (12) suggesting that high serum MBL levels were associated with a 20–79% increased risk of all-cause mortality. Our findings of a weaker association of MBL with all-cause mortality than with CVE (a composite outcome also including cardiovascular mortality) suggest that the modest association between MBL and all-cause mortality is driven by cardiovascular mortality.

It is biologically plausible that both low and high serum MBL could promote chronic inflammation and atherosclerosis in individuals with type 2 diabetes. Low MBL levels (such as in MBL deficiency) could do so by impairment of pathogen clearance (8,14,36) and reduced removal of atherogenic lipoproteins (10). High MBL levels could have this effect by amplifying a low-grade immune response through complement activation in an interplay with hyperglycemia (15,16). In support of these explanations, two cross-sectional studies (18,19) and one prospective study (17) have shown a U-shaped association of serum MBL with carotid intima-media thickness and low-grade inflammation, supporting the hypothesis of a dual role for MBL in the development of CVD.

The main strengths of this large nationwide cohort study include the assessment of detailed lifestyle and clinical factors available in the DD2 database and DD2 biobank and by linkage with high-quality population-based health registries

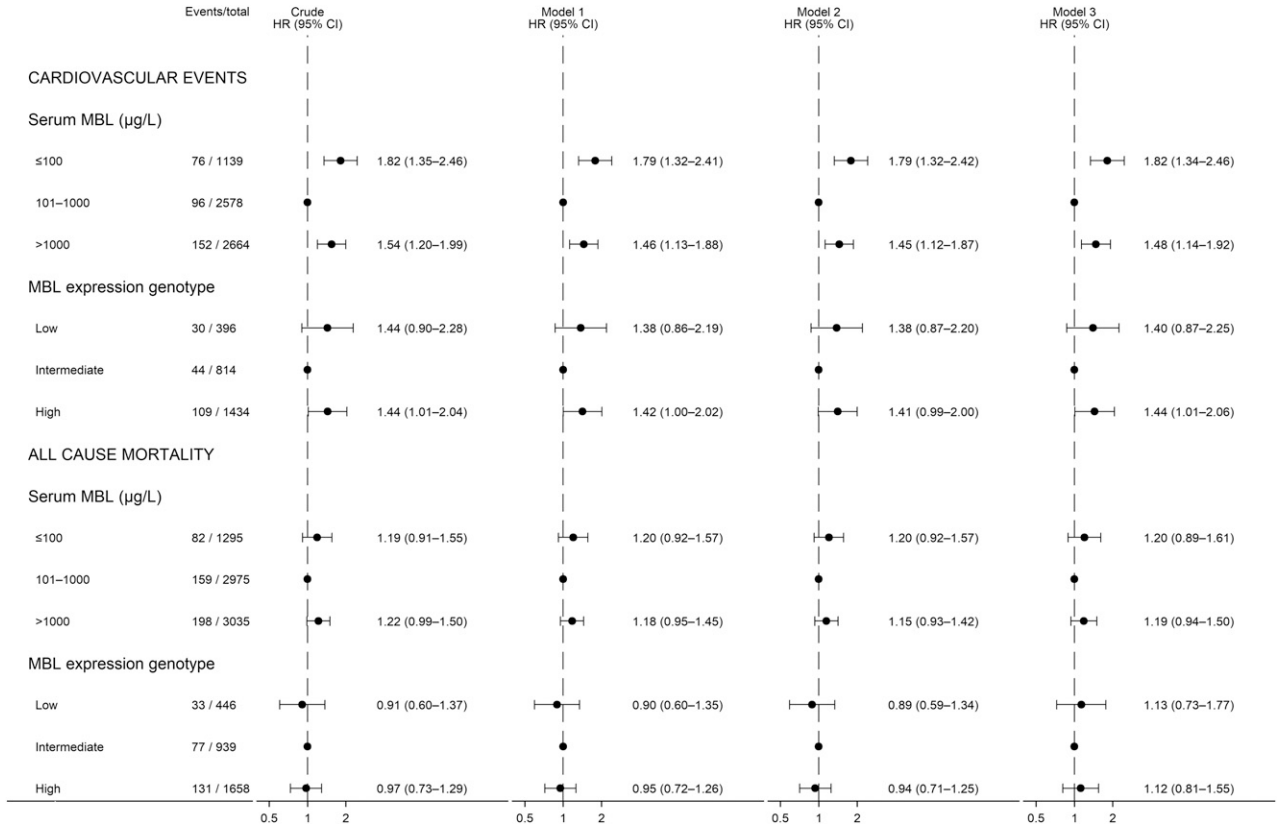

Figure 3—HRs of CVE and all-cause mortality by serum MBL and MBL expression genotype categories. Model 1 is adjusted for sex and age. Model 2 is adjusted for sex, age, diabetes duration, and hs-CRP. Model 3 is adjusted for sex, age, diabetes duration, waist circumference, waist-to-hip ratio, BMI, physical activity, smoking, systolic and diastolic blood pressure, comorbidities, fasting blood glucose, HbA_{1c}, C-peptide, albumin-to-creatinine ratio, eGFR, total cholesterol, LDL, HDL, triglycerides, hs-CRP, and use of antidiabetes, lipid-lowering, antihypertensive, and antithrombotic drugs. Missing covariates were treated with multiple imputation.

(21–27). These resources provide nearly 100% completeness for serum MBL and complete follow-up for outcome events.

Limitations of this study include the possibility of survival bias and selection bias before entering the DD2 cohort. Both situations would likely lead to decreased participation of individuals with a severe type 2 diabetes phenotype or high cardiovascular risk and likely bias results toward the null hypothesis. Moreover, severe selection bias is unlikely because the age, characteristics, and comorbidities of individuals participating in DD2 are similar to those of individuals with early treated type 2 diabetes in routine practice in Denmark (21,37). Another potential concern includes misclassification of diagnoses, which we do not believe happened to a large degree because the validity of cardiovascular diagnoses and procedures in the national Danish registries is high (38,39). In addition, misclassification of genotype is unlikely given the *MBL2* genotype frequencies (and serum levels) that were similar to those of previous studies and the lack of major deviations from Hardy-Weinberg equilibrium (1,28). Another limitation is potential pleiotropy. According to LDlink (https://ldlink.nci.nih.gov/), no SNPs in the *MBL2* gene were in linkage disequilibrium with any genetic variants outside the *MBL2* gene. Confounding by variation in nearby genes therefore cannot explain our findings. Pleiotropy is often impossible to refute completely, but it is unlikely because the six SNPs we used were located within the promoter region and exon 1 of the *MBL2* gene, were highly associated with serum MBL levels, and were not associated with any potential confounders. Imperfectly measured or unmeasured variables (e.g., ethnicity, hemoglobin, and hematocrit levels) may have led to residual confounding in our study. More than 90% of Denmark's population is Caucasian, and our results may not necessarily apply to other ethnic groups. Finally, we did not estimate the magnitude of the potential causal effect, i.e., the fourth step of a complete Mendelian randomization design (20), because of the nonlinear association between serum MBL levels and outcomes (40).

In conclusion, in this prospective study of individuals with type 2 diabetes, we found a U-shaped association for both serum MBL and MBL expression genotype with risk of CVE. This result suggests that serum MBL is directly involved in the development of CVD. Individuals with low and high serum MBL might benefit from a more aggressive preventative treatment.

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