Elevated MBL Concentrations Are Not an Indication of Association Between the *MBL2* Gene and Type 1 Diabetes or Diabetic Nephropathy

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OBJECTIVE—Mannose-binding lectin (MBL) is an essential component of the acute-phase immune response and may thus play a role in the pathogenesis of type 1 diabetes and diabetic nephropathy. The serum concentration of MBL is mainly genetically determined, and elevated concentrations have been associated with both type 1 diabetes and diabetic nephropathy. Previous genetic studies have not been conclusive due to the small number of patients and polymorphisms studied. We investigated whether *MBL2* polymorphisms are associated with type 1 diabetes or diabetic nephropathy and whether patients with nephropathy have elevated MBL concentrations as indicated previously. Furthermore, we studied the association between *MBL2* polymorphisms and MBL concentration.

RESEARCH DESIGN AND METHODS—We genotyped 20 *MBL2* single nucleotide polymorphisms (SNPs) in a large, well-characterized Finnish case-control sample consisting of 1,297 patients with type 1 diabetes with or without nephropathy and 701 nondiabetic individuals. The serum concentration of MBL was available for 1,064 patients.

RESULTS—We found that 19 SNPs were associated with the MBL concentration ($P = 3 \times 10^{-81}$–7×10^{-4}). MBL concentrations were higher in patients with macroalbuminuria compared with patients without nephropathy even when the patients were stratified by the *MBL2* genotypic background in accordance with previous studies. However, no evidence of association between any of the SNPs or their haplotype combinations and type 1 diabetes or diabetic nephropathy was observed.

CONCLUSIONS—Although most of the *MBL2* SNPs studied were associated with the MBL concentration, no common variations (neither single SNPs nor their haplotype combinations) confer risk of type 1 diabetes or diabetic nephropathy. ***Diabetes*** **57:1710–1714, 2009**

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Diabetic nephropathy is a common and devastating long-term complication of diabetes, but its pathogenesis is poorly understood. Low-grade inflammation and complement activation may, however, play a role in the pathogenesis of both type 1 diabetes and its complications (1–3). Mannose-binding lectin (MBL) is a C-type lectin secreted by the liver as a component of the acute-phase immune response, and its binding to carbohydrate structures on microorganisms activates the MBL complement pathway (4,5).

Serum concentrations of MBL are significantly elevated in patients with type 1 diabetes (1,2) and even more elevated in those patients with micro- and macrovascular complications (6,7). Moreover, high MBL concentrations early in the course of type 1 diabetes predict later development of micro- or macroalbuminuria (8), and MBL deficiency attenuates renal changes in mice with experimentally induced diabetes (9).

The *MBL2* gene (OMIM# +154545) on chromosome 10q21 consists of five exons, four of which are protein coding (10,11). The serum concentration of MBL is largely genetically determined (estimated heritability 0.96), and substantial interindividual variation exists (12). Three nonsynonymous variants in exon 1 named alleles B (G54D/rs1800450), C (G57E/rs1800451), and D (R52C/rs5030737) decrease MBL concentration considerably due to incorrect assembly of the mature MBL protein (13). Furthermore, several single nucleotide polymorphisms (SNPs), especially promoter variants H/L (rs11003125), P/Q (rs7095891), and X/Y (rs7096206), modify the MBL concentration (13). Based on linkage disequilibrium (LD) between these six promoter and exon 1 variants, seven common *MBL2* haplotypes can be identified (13).

The low-expression *MBL2* variant carriers have an increased infection risk in situations of impaired immunity (14), and the role of *MBL2* in various autoimmune diseases has been actively studied (15,16). Previous studies on the relationship between *MBL2* and type 1 diabetes show contradictory results (1,6,17–19). This may be due to the fact that only a few polymorphisms were studied in relatively small samples, except in the Finnish study of 470 patients (17). Only the Danish study had nephropathy status available and reported association between the high-expression *MBL2* genotypes and diabetic nephropathy (6). Thus, the question whether genetic variation in the *MBL2* gene confers susceptibility to type 1 diabetes or diabetic nephropathy is still warranted.

TABLE 1
Clinical characteristics of the patients grouped by their nephropathy status

	Normal AER	Microalbuminuria	Macroalbuminuria	ESRD	*P*
AER Criteria	<20 μg/min or <30 mg/24 h	≥20 and <200 μg/min or ≥30 and <300 mg/24 h	≥200 μg/min or ≥300 mg/24 h	NA	
n	477	276	366	178	
Male/female	40/60	59/41*	59/41*	58/42*	<0.0001
Age (years)	42.5 ± 10.1	37.3 ± 10.9*	39.0 ± 9.0*	42.2 ± 7.5*‡‖	<0.0001
Duration of diabetes (years)	28.5 ± 7.1	25.0 ± 9.4*	27.2 ± 6.4§	30.1 ± 6.5‡‖	<0.0001
Time to DNP (years)	NA	NA	18.3 ± 6.3	17.1 ± 5.1	0.039
BMI (kg/m^2)	24.8 ± 2.9	25.7 ± 3.5†	25.7 ± 3.8†	23.9 ± 3.5†‡‖	<0.0001
SBP (mmHg)	132 ± 16	136 ± 17	144 ± 19*‡	154 ± 22*‡‖	<0.0001
DBP (mmHg)	78 ± 9	81 ± 10*	84 ± 10*§	88 ± 11*‡‖	<0.0001
Retinal laser treatment	29	48*	81*‡	98*‡‖	<0.0001
Hypertension	51	86*	98*‡	97*‡	<0.0001
Antihypertensive treatment	18	66*	95*‡	92*‡	<0.0001
A1C (%)	8.1 ± 1.2	8.8 ± 1.4*	9.0 ± 1.6*	8.7 ± 1.5*	<0.0001
AER (mg/24 h)	7 (1–85)*	59 (2–613)*	587 (4–8,348)*‡	—	<0.0001
eGFR (ml/min per 1.73 m^2)	89.8 ± 18.1	95.7 ± 25.3*	64.6 ± 31.8*‡	—	<0.0001
Serum creatinine (μmol/l)	84 (43–144)	88 (35–194)†	127 (20–1,278)*‡	—	<0.0001

Data are means ± SD, median (interquartile range), or percent unless otherwise indicated. Patients were classified into three groups based on the AER in at least two of three consecutive overnight or 24-h urine collections. A fourth patient group consisted of patients on dialysis (n = 34) and patients who had received a kidney transplant (n = 144) and were thus classified as having ESRD. The 24-h AER and serum creatinine values were derived from the last central laboratory measurements. Therefore, single values may exceed or fall behind the thresholds for the classification due to effects of treatment. Estimated glomerular filtration rate (eGFR) was calculated using the Cockcroft-Gault formula adjusted for body surface area (ref. 20). *P < 0.0001 versus normoalbuminuria. †P < 0.01 versus normoalbuminuria. ‡P < 0.0001 versus microalbuminuria. §P < 0.01 versus microalbuminuria. ‖P < 0.0001 versus macroalbuminuria. ¶P < 0.01 versus macroalbuminuria. DBP, diastolic blood pressure; DNP, diabetic nephropathy; SBP, systolic blood pressure.

Here, we studied whether the *MBL2* gene polymorphisms are associated with type 1 diabetes or diabetic nephropathy by genotyping a dense set of SNPs in a large well-characterized Finnish case-control sample and evaluated the association between MBL concentration and diabetic nephropathy.

RESEARCH DESIGN AND METHODS

We studied 1,297 patients with type 1 diabetes from the nationwide Finnish Diabetic Nephropathy Study (FinnDiane). Patients were required to have an onset of diabetes before 35 years of age and initiation of permanent insulin treatment within 1 year of diagnosis. Patients were classified into four groups based on the urinary albumin excretion rate (AER) or the presence of end-stage renal disease (ESRD) (Table 1). Patients with normal AER were required to have a duration of diabetes over 15 years. Serum concentration of MBL was determined in 1,064 patients (available in the supplemental methods at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1495/DC1). For nondiabetic control subjects, 701 Finnish blood donors (49% men; mean age 46.1 years) from all over the country were applied and compared with an age- and sex-matched subgroup of patients with type 1 diabetes (n = 701; 49% men; mean age 43.9 years). The power of our study sample was calculated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (21). Study protocol was approved by the ethics committee of the Helsinki University Central Hospital. Informed written consent was obtained from all patients.

SNP selection and genotyping. We selected 17 SNPs using the HapMap database release 21a (CEU) (http://www.hapmap.org/) and the Haploview Tagger program (http://www.broad.mit.edu/mpg/haploview/index.php) (22) from a 23-kb region covering the 6.3-kb *MBL2* gene and the 5′ untranslated region and 3′ untranslated region. SNPs were chosen to capture all known HapMap SNPs having a minor allele frequency (MAF) >0.01 in the CEU population with pairwise $r^2 \geq 0.8$. Additionally, three functional SNPs (allele C, allele D, and variant H/L) were genotyped (supplementary Table 1). For genotyping, see the supplemental methods in the online appendix.

Statistical analyses of MBL concentrations. Comparisons between groups were performed using the Mann-Whitney *U* test because MBL concentrations were not normally distributed. These analyses were done using the SPSS v. 15.0 software (SPSS, Chicago, IL).

Association analyses. Allele frequencies and genotype distributions for single SNPs were compared between the control subjects and the matched subset of patients with type 1 diabetes as well as between patients with macroalbuminuria and patients with type 1 diabetes but normal AER. We also compared patients having ESRD with patients with normal AER and compared a combined group of patients with macroalbuminuria or ESRD with patients with normal AER. An allelic association χ^2 test and three genotypic tests (general, dominant, and recessive models) were performed for each SNP. Association of the SNPs with MBL concentration was studied using the asymptotic Wald test. Additionally, analyses with adjustment for potential confounders (age, sex, A1C, diabetes duration, and history of smoking) were performed. The PLINK analysis program, version 1.00, was applied in all analyses (http://pngu.mgh.harvard.edu/~purcell/plink/) (23). For haplotype association analyses, see the supplemental methods in the online appendix.

RESULTS

SNP genotyping. The 17 tag SNPs genotyped captured the 68 HapMap SNPs having an MAF>0.01 with a mean pairwise r^2 of 0.97. The average distance between all of the 20 studied SNPs is 1.2 kb. All SNPs were in Hardy-Weinberg equilibrium (P > 0.01) both in the healthy control subjects (supplementary Table 1) and in type 1 diabetic patients (data not shown). Pairwise LD values (r^2) between the SNPs are shown in supplementary Fig. 1. The frequencies of the exon 1 variants were 0.13 (B), 0.06 (C), and 0.006 (D) in the healthy subjects, which is in line with the previously published frequencies in the Finnish population (17). Of the patients with diabetes, 30.6% were heterozygous carriers of one of these variants (A/O genotype) and 5.8% were homozygotes or compound heterozygotes (O/O genotype).

MBL concentrations. The median serum concentration of MBL was higher in patients with macroalbuminuria (1,881 μg/l [interquartile range 608–3,124]) than in patients with normal AER (1,548 μg/l [514–2,635]); P = 0.019 (Fig. 1). Supplementary Table 2 shows the distribution of the *MBL2* diplotypes and the corresponding median MBL concentrations. As expected, the carriers of the exon 1 variants and the individuals homozygous for the X promoter polymorphism had clearly reduced MBL concentrations, with a median of 397 μg/l. When the patients were stratified both by nephropathy status and *MBL2* diplotype

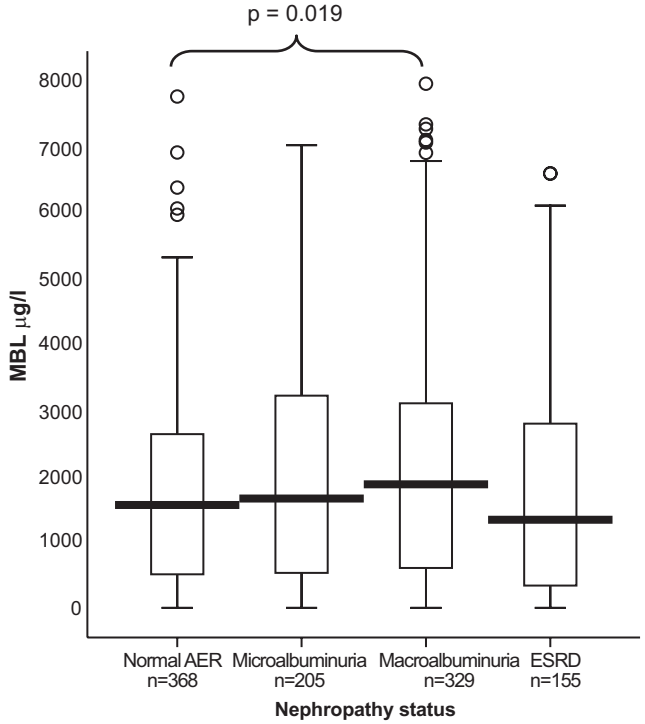

FIG. 1. Box plot diagram showing the distribution of serum MBL concentrations, for patients with type 1 diabetes, stratified by nephropathy status. Black horizontal lines, median values; □, interquartile ranges; ○, outliers. The median serum concentration of MBL was higher in patients with macroalbuminuria (median 1,881 µg/l [interquartile range 608–3,124]) than in patients with normal AER (1,548 µg/l [514–2,635]), $P = 0.019$. The median concentration was 1,645 µg/l (531–3,220) in patients with microalbuminuria and 1,359 µg/l (343–2798) in patients with ESRD, $P = 0.17$ and $P = 0.45$, respectively, compared with patients with normal AER.

category, it was evident that patients with macroalbuminuria had significantly higher MBL concentrations than patients with normal AER for two of the six (YA/YA: $P = 0.0002$; YA/YO: $P = 0.003$) diplotype categories (supplementary Table 2 and Fig. 2).

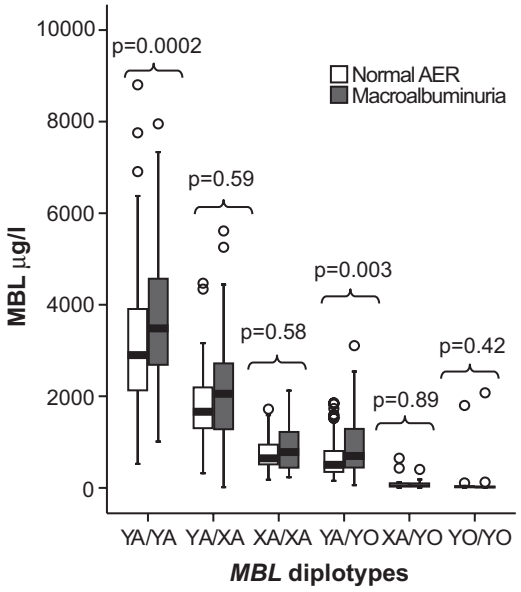

FIG. 2. Distribution of serum MBL concentrations in patients with normal AER and patients with macroalbuminuria, stratified by the *MBL2* diplotype.

Association analyses between single SNP and type 1 diabetes. There were no significant differences in allele frequencies or genotype distributions between the patients with type 1 diabetes and nondiabetic control subjects (supplementary Table 3). Although nominal evidence of allelic association ($P = 0.01–0.04$) was seen for four SNPs (rs930507, rs2384045, rs11003132, and rs11003137), the P values did not remain significant after correction for the number of tests performed. Men and women were also tested separately, but no significant P values were achieved (data not shown).

Association analyses between single SNPs and nephropathy. Association analyses showed no significant differences in allele frequencies or genotype distributions between patients with macroalbuminuria and patients with type 1 diabetes but normal AER (supplementary Table 4). When patients with ESRD were compared with patients with normal AER, significant evidence of association was observed for rs920727. The MAF of this SNP was 0.19 in patients with normal AER and 0.29 in patients with ESRD ($P = 0.00009$). The genotype distribution of this SNP was also significantly different between these groups ($P = 0.0002$), with homozygous individuals being more common among ESRD patients (10 vs. 3%). The same SNP had a P value of 0.006 in the genotypic test when a combined group of patients with macroalbuminuria or ESRD was compared with patients with normal AER. No other SNP provided evidence of association in these analyses. The analyses were also performed with adjustment for potential confounders (supplementary Table 4). None of the SNPs showed evidence of association with nephropathy after the adjustment procedure.

Association analyses between single SNPs and MBL concentrations. All SNPs, except rs10824793, were associated with MBL concentration (supplementary Table 5). The SNPs showing the strongest associations were rs7899547 ($P = 3.0 \times 10^{-81}$), rs1031101 (tagging the B allele; $P = 8.0 \times 10^{-76}$), rs2384045 ($P = 2.2 \times 10^{-59}$), and rs920727 ($P = 7.6 \times 10^{-45}$). The promoter variants H/L (rs11003125) and P/Q (tagged by rs920724) had P values of 8.8×10^{-25} and 6.1×10^{-10}, respectively, whereas the alleles C and D had P values of 1.5×10^{-7} and 9.0×10^{-19}, respectively. The analyses were also performed with adjustment for potential confounders (supplementary Table 5).

Haplotype and diplotype analyses. The haplotypes constructed using the Haploview program were studied for association with type 1 diabetes and diabetic nephropathy. The results did not improve compared with the single SNP results. The distribution of the *MBL2* diplotypes, diplotype categories, and the high- and low-MBL genotypes in each patient group is presented in Table 2. There were no differences in the frequency of the high-MBL genotypes between the patients with normal AER (0.60) and macroalbuminuria (0.61; $P = 0.39$). Similarly, when the distribution of the six diplotype categories was analyzed, no differences were seen ($P = 0.63$).

DISCUSSION

Our study showed no evidence of association between the *MBL2* SNPs and type 1 diabetes, although weak association signals (uncorrected $P = 0.01–0.04$) were seen for four SNPs. This is in accordance with most of the previous studies (1,6,17,18). Importantly, using tag SNPs, we have

TABLE 2
Distribution of the *MBL2* genotypes within the different patient groups

MBL2 diplotype			Normal AER	Microalbuminuria	Macroalbuminuria	ESRD
High-MBL genotypes	YA/YA	HYPA/HYPA	63	37	58	19
		HYPA/LYQA	70	28	46	16
		HYPA/LYPA	16	13	12	13
		LYQA/LYQA	19	8	13	5
		LYPA/LYQA	8	6	7	5
		LYPA/LYPA	2	2	0	1
		Total	178 (37.6)	94 (34.2)	136 (37.3)	59 (33.9)
	YA/XA	HYPA/LXPA	70	41	54	24
		LYQA/LXPA	29	23	27	9
		LYPA/LXPA	7	10	6	2
		Total	106 (22.4)	74 (26.9)	87 (23.8)	35 (20.1)
	Total		284 (59.9)	168 (61.1)	223 (61.1)	94 (54.0)
Low-MBL genotypes	XA/XA	LXPA/LXPA	27 (5.7)	12 (4.4)	12 (3.3)	8 (4.6)
	YA/YO	HYPA/LYPB	46	24	27	19
		LYQA/LYPB	20	11	18	7
		LYPA/LYPB	3	4	2	4
		HYPA/LYQC	3	3	1	1
		LYQA/LYQC	3	1	1	1
		LYPA/LYQC	1	1	1	0
		HYPA/HYPD	21	10	18	8
		LYQA/HYPD	9	8	13	4
		LYPA/HYPD	3	2	2	0
		Total	109 (23.0)	64 (23.3)	83 (22.7)	44 (25.3)
	XA/YO	LXPA/LYPB	24	6	18	8
		LXPA/LYQC	2	0	1	2
		LXPA/HYPD	8	9	8	3
		Total	34 (7.2)	15 (5.5)	27 (7.4)	13 (7.5)
	YO/YO	LYPB/LYPB	8	4	9	6
		LYPB/LYQC	1	0	0	3
		LYPB/HYPD	9	8	5	2
		HYPD/HYPD	2	4	1	4
		LYQC/HYPD	0	0	5	0
		Total	20 (4.2)	16 (5.8)	20 (5.5)	15 (8.6)
	Total		190 (40.1)	107 (38.9)	142 (38.9)	80 (46.0)
All			474 (100)	275 (100)	365 (100)	174 (100)

Data are *n* or *n* (percent). The *MBL2* haplotypes were determined based on the co-occurrence of the three promoter variants (H/L, rs11003125; X/Y, rs7096206; and P/Q, rs7095891) and the three exon 1 variants (B, rs1800450; C, rs1800451; and D, rs5030737). Diplotypes were grouped into six categories (YA/YA, YA/XA, XA/XA, YA/YO, XA/YO, and YO/YO) based on the X/Y polymorphism and the presence of any of the exon 1 variants (B, C, and D), collectively designated with O, and further into low- and high-MBL genotypes. There were no differences in the frequency of the high-MBL genotypes between the patients with normal AER and macroalbuminuria. However, the frequency of the high-MBL genotypes within the group of ESRD patients (0.54) was lower than in patients with normal AER (0.60), although this difference was not significant ($P = 0.10$).

captured information on the whole gene and its surroundings, whereas the other studies addressed only the functional exon 1 variants.

In a Danish study (6), genotypes producing high MBL concentrations were more common in patients having diabetic nephropathy than in patients with normal AER. We found no evidence of such an association, although our study sample was about double the size (6). Furthermore, we thoroughly studied association between both single *MBL2* SNPs and haplotypes and nephropathy, but all the results were negative.

In accordance with previous studies (6,7), we showed that the median serum MBL concentration was significantly higher in patients with macroalbuminuria compared with the patients with normal AER. This result persisted even when the patients were stratified by the *MBL* diplotype categories, although the difference was significant in only two of them. Thus, it seems that the high serum MBL concentration in patients with nephropathy is likely to reflect some still unknown pathogenic event such as chronic low-grade inflammation. Further studies are needed to resolve whether elevated MBL concentrations are a marker associated with some other contributing factor or a consequence of nephropathy or diabetic microvascular complications in general.

The minor allele of the SNP rs920727 was more common in ESRD patients than in the other patients 0.29 vs. 0.19–0.20). Furthermore, the frequency of the high-MBL genotypes was somewhat lower in the ESRD patients than in the other groups (0.54 vs. 0.60–0.61), potentially explaining the relatively low MBL concentration in the ESRD patients. However, we do not consider these differences as an indication of a causal association with ESRD phenotype. Most likely they are due to chance or even signs of selective mortality. Supporting this hypothesis, evidence exists that mortality of patients with type 2 diabetes is higher among individuals with an MBL concentration $\geq$1,000 μg/l (24). Moreover, most of the ESRD patients in our study have received a kidney transplant, and graft

rejection is less common for the group of patients with low MBL concentrations (25).

Many of the SNPs showing strong association with MBL concentration (including the ESRD-associated rs920727) did not belong to the group of SNPs forming the common *MBL2* haplotypes. Some of these variants may have an independent effect on the MBL concentration or tag other still unknown functional variants. However, this somewhat surprising association pattern can mainly be explained by LD between the associated and haplotype-forming SNPs (supplementary Table 6).

Our study sample has a reasonably high power to detect associations with relatively common SNPs (population frequency >20%) with modest effects. The previously described higher median MBL concentration in patients with macroalbuminuria (6,7) was evident also in our sample. We have thoroughly covered the common variation within the *MBL2* gene in carefully characterized Finnish type 1 diabetic patients. We conclude that although most of the *MBL2* SNPs studied were associated with the MBL concentration, neither any single SNP nor any of their haplotype combinations confer risk of type 1 diabetes or diabetic nephropathy.

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