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The role of mannose-binding lectin in pneumococcal infection

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ABSTRACT: **The role of mannose-binding lectin (MBL) deficiency (*MBL2*; *XA*/*O* and *O*/*O* genotypes) in host defences remains controversial. The surfactant proteins (SP)-A1, -A2 and -D, other collectins whose genes are located near *MBL2*, are part of the first-line lung defence against infection. We analysed the role of MBL on susceptibility to pneumococcal infection and the existence of linkage disequilibrium (LD) among the four genes.**

We studied 348 patients with pneumococcal community-acquired pneumonia (P-CAP) and 2,110 controls. A meta-analysis of *MBL2* genotypes in susceptibility to P-CAP and to invasive pneumococcal disease (IPD) was also performed. The extent of LD of *MBL2* with *SFTPA1*, *SFTPA2* and *SFTPD* was analysed.

***MBL2* genotypes did not associate with either P-CAP or bacteraemic P-CAP in the case–control study. The MBL-deficient *O*/*O* genotype was significantly associated with higher risk of IPD in a meta-analysis, whereas the other MBL-deficient genotype (*XA*/*O*) showed a trend towards a protective role. We showed the existence of LD between *MBL2* and SP genes.**

The data do not support a role of MBL deficiency on susceptibility to P-CAP or to IPD. LD among *MBL2* and SP genes must be considered in studies on the role of MBL in infectious diseases.

KEYWORDS: **Mannose-binding lectin, pneumococcus, polymorphism, sepsis, *Streptococcus pneumoniae*, surfactant protein**

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Community-acquired pneumonia (CAP) remains the leading cause of death from infection in developed countries [1]. Several microorganisms may be causative agents of CAP, but *Streptococcus pneumoniae* is the most common cause [1].

Mannose-binding lectin (MBL) is a serum collectin that promotes phagocytosis of microorganisms and initiates the lectin pathway of complement activation [2]. Deficient and low MBL serum levels are mainly due to the presence of three common point mutations in the exon 1 of the *MBL2* gene (10q11.2-q21): alleles *B*, *C* and *D* are termed *O* alleles, and *A* is the wild-type allele. Heterozygous individuals for *O* alleles have reduced serum MBL levels and MBL-dependent lectin pathway activity, whereas these values are very low or absent in homozygous individuals for *O* alleles. The presence of the promoter allele *X* has an important downregulating effect, and *O/O* together with *XA/O* genotypes are considered MBL deficient genotypes, which are common in most populations [2, 3].

MBL deficiency has been considered a common primary immunodeficiency [4]. However, its role in host defence remains a matter of debate [5, 6]. An initial study suggested that *O/O* genotypes predispose to invasive pneumococcal disease (IPD) [7], but these results were not replicated in two other populations [8, 9]. The data from several studies argue against a role of MBL in host defences, particularly to pneumococcus [5, 10–13]. We have previously observed that MBL plays a redundant role in human defences against primary infection, at least in adults with CAP, but also that MBL insufficiency predisposes to higher severity and fatal outcome in CAP [14].

Surfactant proteins (SP)-A1, -A2 and -D, and other collectins, also promote phagocytosis of microorganisms and play a pivotal role in the

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regulation of the inflammatory response, as well as in clearance of apoptotic cells [15, 16]. SPs, but not MBL, take part in the first-line host defence in healthy lung. Genetic variability at genes coding for these SPs was associated with higher susceptibility and poor outcome of CAP [17]. The human SP-A locus consists of two similar genes, *SFTPA1* and *SFTPA2*, localised within a cluster (10q21-24) that includes the SP-D gene (*SFTPD*) [15]. *MBL2* was reported not to be in physical linkage with the genes of these SPs [18], but no studies of linkage disequilibrium (LD) of *MBL2* with *SFTPA1*, *SFTPA2* or *SFTPD* have been performed to date.

In the present study, we assessed the role of *MBL2* genotypes in the susceptibility to and the severity of pneumococcal CAP (P-CAP). We also performed a meta-analysis aimed to analyse the role of *MBL2* genotypes in susceptibility to P-CAP and to IPD. Lastly, we analysed the extent of LD of the most frequently studied single nucleotide polymorphisms (SNPs) of *MBL2* with missense SNPs at *SFTPA1*, *SFTPA2* and *SFTPD*.

TABLE 1 Demographic and clinical characteristics of pneumococcal community-acquired pneumonia patients

Characteristics[#]	Available data	Subjects
Age yrs	348	59.50±17.62
Sex	348	
Male		227 (65.2)
Female		121 (34.8)
ICU admission	348	133 (38.2)
MODS	348	74 (21.3)
ARDS	348	26 (7.5)
Septic shock	348	79 (22.7)
ARF	348	250 (71.8)
Acute renal failure	346	114 (32.9)
Bacteraemia	347	96 (27.7)
90-day exitus	348	23 (6.6)
28-day exitus	348	16 (4.6)
Comorbidity[#]		
No	338	122 (36.1)
COPD	343	86 (25.1)
Asthma	322	13 (4.0)
Neoplasy	344	35 (10.2)
Ischaemic cardiopathy	343	30 (8.8)
Diabetes	343	76 (22.2)
Renal insufficiency	322	13 (4.0)
Hepatic insufficiency	343	31 (9.0)
Neurological pathology	343	38 (11.1)
Autoimmune pathology	343	7 (2.1)
Pneumonia severity index	335	
I–III (low)		148 (44.2)
IV–V (moderate-high)		187 (55.8)

Data are presented as n, mean±SD or n (%). ICU: intensive care unit; MODS: multi-organ dysfunction syndrome; ARDS: acute respiratory distress syndrome; ARF: acute respiratory failure; COPD: chronic obstructive pulmonary disease. [#]:: some patients had more than one comorbidity.

METHODS

Patients and controls

In the present study, 1,398 white Spanish patients hospitalised with CAP (59.50±17.62 yrs, 34.8% females) from five Spanish hospitals (Hospital Universitario de Gran Canaria Doctor Negrín, Las Palmas de Gran Canaria; Hospital Clínico y Universitario de Valencia, Valencia; Hospital San Jorge de Huesca, Huesca; Hospital Universitario de la Princesa, Madrid; and Hospital Vall DHebron, Barcelona), were prospectively included. A total of 348 patients had P-CAP. The control group consisted of 2,110 unrelated healthy volunteers (blood and bone marrow donors as well as hospital staff) and patients without signs of relevant infectious diseases (47.27±17.40 yrs, 48.2% females) from the same origin as CAP patients. Foreigners and individuals with ancestors other than Spanish were previously excluded. Exclusion criteria and clinical definitions are shown in the Methods section of the online supplementary material. For susceptibility to P-CAP, a sex- and age-matched case–control study was performed; 340 patients and 1,736 controls were finally compared. Severity and outcome were evaluated in a prospective study of the 348 P-CAP patients.

In addition, we included a group of 84 patients with P-CAP (62.00±16.53 yrs, 32.1% females) from another Spanish population. These patients were included in a published study [19], but several patients were excluded on the basis of our inclusion/exclusion criteria. A group of 91 healthy controls from the same origin were also used (64.95±18.61 yrs, 72.5% females).

Informed consent was obtained from either the patients or their relatives. The protocol was approved by the local ethics committee of all hospitals. All steps were performed in complete accordance to the Helsinki declaration.

Genotyping

Genomic DNA was isolated as previously described [17]. The following *MBL2* polymorphisms were analysed as described elsewhere [20]: codon 52 *C/T* (rs5030737, allele *D*), codon 54 *A/G* (rs1800450, allele *B*), codon 57 *A/G* (rs1800451, allele *C*) and codon -221 *G/C* (rs7096206, alleles *X/Y*). Haplotypes were simplified as *YA, XA* and *O*.

We have previously genotyped polymorphisms in *SFTPA1* (aa19 *T/C*, rs1059047; aa50 *G/C*, rs1136450; aa219 *C/T*, 4253527), *SFTPA2* (aa9 *A/C*, rs1059046; aa91 *G/C*, rs17886395; aa223 *C/A*, rs4253527) and *SFTPD* (aa11 *T/C*, rs721917) genes in part of both patient and control groups [17]. Haplotypes were named as $6A^n$ for *SFTPA1* and $1A^n$ for *SFTPA2* based on previous nomenclature [21]. For each individual, haplotypes were inferred using PHASE statistical software (version 2.1; available at www.stat.washington.edu/stephens/phase).

LD was measured by means of Arlequin (version 3.11; available at http://cmpg.unibe.ch/software/arlequin3) and Haploview (version 4.2; available at www.broadinstitute.org/haploview/haploview) softwares. Pairwise LD between *MBL2* haplotypes and SP genes was characterised using Arlequin 3.11. The existence of LD was considered if $D' > 0.3$.

Study selection for the meta-analysis

Eligible studies were identified by searching in PubMed using the search terms ''mannose-binding lectin'' or ''mannose

TABLE 2 Susceptibility and severity of patients with pneumococcal community-acquired pneumonia (P-CAP) related to mannose-binding lectin 2 gene (*MBL2*) genotypes: genotypic frequencies

	Genotypes							
	YA/YA	*XA/YA*	*XA/XA*	*YA/O*	*XA/O*	*O/O*	*XA/O+O/O*	*A/O+O/O*
Controls[#]	558 (32.1)	412 (23.7)	62 (3.6)	443 (25.5)	172 (9.9)	89 (5.1)	261 (15.0)	705 (40.6)
P-CAP[¶]	133 (39.1)	63 (18.5)	15 (4.4)	86 (25.3)	28 (8.2)	15 (4.4)	43 (12.6)	129 (37.9)
Severity of P-CAP patients[+]								
ICU	46 (34.6)	26 (19.5)	3 (2.3)	35 (26.3)	15 (11.3)	8 (6.0)	23 (17.3)	58 (43.6)
General ward	90 (41.9)	38 (17.7)	12 (5.6)	54 (25.1)	13 (6.0)	8 (3.7)	21 (9.8)	75 (34.9)
Bacteraemia	34 (35.4)	20 (20.8)	4 (4.2)	26 (27.1)	7 (7.3)	5 (5.2)	12 (12.5)	38 (39.6)
No bacteraemia	102 (40.6)	44 (17.5)	11 (4.4)	62 (24.7)	21 (8.4)	11 (4.4)	32 (12.7)	94 (37.5)
SSh	24 (30.4)	16 (20.3)	1 (1.3)	22 (27.8)	12 (15.2)	4 (5.1)	16 (20.3)	38 (48.1)
No SSh	112 (41.6)	48 (17.8)	14 (5.2)	67 (24.9)	16 (5.9)	12 (4.5)	28 (10.4)	95 (35.3)
SSh+SS	55 (36.2)	28 (18.4)	2 (1.3)	43 (28.3)	15 (9.9)	9 (5.9)	24 (15.8)	67 (44.1)
NSS	81 (41.3)	36 (18.4)	13 (6.6)	46 (23.5)	13 (6.6)	7 (3.6)	20 (10.2)	66 (33.7)
Acute renal failure	38 (33.3)	22 (19.3)	2 (1.8)	36 (31.6)	10 (8.8)	6 (5.3)	16 (14.0)	52 (45.6)
No acute renal failure	98 (42.2)	42 (18.1)	13 (5.6)	52 (22.4)	17 (7.3)	10 (4.3)	27 (11.6)	79 (34.1)
ARF	97 (38.8)	48 (19.2)	4 (1.6)	64 (25.6)	24 (9.6)	13 (5.2)	37 (14.8)	101 (40.4)
No ARF	39 (39.8)	16 (16.3)	11 (11.2)	25 (25.5)	4 (4.1)	3 (3.1)	7 (7.1)	32 (32.7)
ARDS	8 (30.8)	7 (26.9)	1 (3.8)	4 (15.4)	5 (19.2)	1 (3.8)	6 (23.1)	10 (38.5)
No ARDS	128 (39.8)	57 (17.7)	14 (4.3)	85 (26.4)	23 (7.1)	15 (4.7)	38 (11.8)	123 (38.2)
MODS	18 (24.3)	15 (20.3)	2 (2.7)	24 (32.4)	10 (13.5)	5 (6.8)	15 (20.3)	39 (52.7)
No MODS	118 (43.1)	49 (17.9)	13 (4.7)	65 (23.7)	18 (6.6)	11 (4.0)	29 (10.6)	94 (34.3)
PSI I–III	60 (40.5)	27 (18.2)	12 (8.1)	37 (25.0)	8 (5.4)	4 (2.7)	12 (8.1)	49 (33.1)
PSI IV–V	67 (35.8)	35 (18.7)	2 (1.1)	51 (27.3)	20 (10.7)	12 (6.4)	32 (17.1)	83 (44.4)

Data are presented as n (%). For susceptibility analysis, only sex- and age-matched P-CAP patients and controls are included. ICU: intensive care unit; SSh: septic shock; SS: severe sepsis; NSS: non-severe sepsis, includes patients without either severe sepsis or septic shock; ARF: acute respiratory failure; ARDS: acute respiratory distress syndrome; MODS: multi-organ dysfunction syndrome; PSI: pneumonia severity index. [#]: n=1736; [¶]: n=340; [+]: n=348.

binding protein'' and ''pneumococcal'' or ''pneumonia'', and abstracts and references were reviewed for relevance. Full text of the relevant articles was reviewed to ensure that they met pre-set inclusion criteria. Data were extracted independently by two investigators, and the duplicate results were compared.

We identified 93 publications related to MBL and pneumonia or pneumococcus. After reviewing, 89 articles were excluded because they were reviews, met our exclusion criteria, or because they provided only serum data, data from patients with or without pneumococcus could not be separated, or they were irrelevant to the focus of this meta-analysis. Four studies remained after the selection process [7–9, 22]. Studies were separated into those focused on P-CAP or IPD. Three genotypes were studied in our meta-analysis: *O/O*, *A/O+O/O* and *XA/O*.

Statistical analysis

Quantitative variables are presented using arithmetic mean±SEM. The comparison of *MBL2* genotypes distribution based on the susceptibility, severity and outcome were performed using the Chi-squared test or Fisher's exact test when needed, and odds ratios with 95% of confidence intervals were calculated. In addition, for the study of susceptibility, cases and controls were sex- and age-matched (considering intervals of 5 yrs), and the strata created were used for the conditional logistic regression analysis. The relationship between severity or outcome and genotypes was evaluated by binary logistic regression models, and hospital of origin and pneumonia severity index (PSI) were included as independent variables. The Hardy–Weinberg equilibrium for the genotypic frequencies was tested in the control groups by Chi-squared analysis. Assuming a frequency in our population of 0.15 for *XA/O+O/O* genotypes, our study (1,591 controls, 348 P-CAP patients and 96 bacteraemic P-CAP patients; incidence for bacteraemia of 0.28) had 80% power to detect an odds ratio of 1.53 and 2.03 for susceptibility to P-CAP and bacteraemic P-CAP, respectively. All tests were two-tailed. Statistical significance was taken as $p<0.05$. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Meta-analyses were performed using DerSimonian and Laird random-effects models. For individual studies and pooled estimation, odds ratios and 95% confidence intervals are given. Heterogeneity was evaluated and it is showed in the forest plot. Analysis was performed using the Metafor package (available at www.jstatsoft.org/v36/i03/).

RESULTS

Patients admitted at five Spanish Hospitals were evaluated for the diagnosis of CAP. After excluding those patients without informed consent, ethnicity other than white Spanish and those

TABLE 3 Statistical analysis of the main differences of the observed genotypic frequencies from table 2

	Genotypes					
	YA/YA	*XA/YA*	*XA/XA*	*XA/O*	*XA/O+O/O*	*A/O+O/O*
P-CAP *versus* controls	1.43 (1.11–1.84); 0.006	0.62 (0.47–0.87); 0.005				
ICU *versus* general ward					1.93 (1.02–3.65); 0.040	
SSh *versus* no SSh				2.83 (1.28–6.27); 0.008	2.19 (1.11–4.29); 0.021	1.70 (1.02–2.82); 0.040
SSh+SS *versus* NSS			0.19 (0.04–0.85); 0.015			1.55 (1.00–2.40); 0.048
Acute renal failure *versus* no acute renal failure						1.62 (1.03–2.58); 0.037
ARF *versus* no ARF			0.43 (0.24–0.76); 0.0003			
MODS *versus* no MODS	0.43 (0.24–0.76); 0.003				2.15 (1.08–4.26); 0.026	2.13 (1.27–3.59); 0.004
PSI IV-V *versus* I–III			0.12 (0.03–0.56); 0.001		2.34 (1.16–4.72); 0.015	1.61 (1.03–2.52); 0.036

Data are presented as OR (95% CI); p-value. Values are uncorrected p-value for the bivariate comparison, except for the susceptibility analysis, where values correspond to conditional estimates. P-CAP: pneumococcal community-acquired pneumonia; ICU: intensive care unit; SSh: septic shock; SS: severe sepsis; NSS: non-severe sepsis, includes patients without either severe sepsis or septic shock; ARF: acute respiratory failure; MODS: multi-organ dysfunction syndrome; PSI: pneumonia severity index.

that fulfilled the exclusion criteria, a total of 1,398 CAP patients were finally studied. *S. pneumoniae* was detected in 348 (24.89%) of these patients (57.43% of the patients with known causative microorganism). The main clinical characteristics of the P-CAP patients are shown in table 1.

Susceptibility to P-CAP related to MBL2 *genotypes*

No significant deviation from Hardy–Weinberg equilibrium of the studied *MBL2* variants was found in our control population.

When *MBL2* genotypes encompassing exon 1 wild-type (*A*) and mutated alleles (*O*), as well as promoter *X/Y* alleles, were analysed, no differences in *A/O+ O/O* or *XA/O+O/O* genotypes between sex- and age-matched P-CAP patients and controls were observed. However, the high-MBL genotype *YA/YA* was found to be over-represented in these patients, and genotype *XA/YA* was under-represented when compared with controls (tables 2 and 3).

Severity and outcome of P-CAP patients related to MBL2 *genotypes*

The relevance of *MBL2* variants in the severity of P-CAP was analysed in our main cohort (tables 2 and 3). The high-MBL genotype *YA/YA* was under-represented in P-CAP patients with multi-organ dysfunctions (MODS). Likewise, the *XA/XA* genotype was under-represented in P-CAP patients with the most severe forms of sepsis (septic shock (SSh) and severe sepsis), as well as in those with acute respiratory failure (ARF) or moderate-to-high PSI. Conversely, the frequency of *XA/O* genotypes was found to be higher in P-CAP patients with SSh. In addition, when MBL-deficient genotypes (*XA/O+O/O*) were analysed, we found them to be associated with a need of intensive care unit admission, development of SSh and MODS; and with a moderate-to-high PSI at admission. Six of these associations remained significant in multivariate analysis including the variables hospital of origin and PSI (except for the analysis of the PSI, which only included hospital of origin): p=0.029 (OR 0.49, 95% CI 0.26–0.93) for *YA/YA* genotype in patients with MODS; p=0.005 (OR=0.12, 95% CI 0.03–0.53) and p=0.005 (OR=0.11, 95% CI 0.02–0.52) for *XA/XA* in patients with ARF and PSI IV–V, respectively; p=0.041 (OR=2.43, 95% CI 1.04–5.70) for *XA/O* in patients with SSh; and p=0.019 (OR=2.37, 95% CI 1.16–4.88) and p=0.038 (OR=1.63, 95% CI 1.03–2.58) in patients with PSI IV–V for *XA/O+O/O* and *A/O+O/O* genotypes, respectively. No significant differences were observed when acute respiratory distress syndrome, bacteraemia and fatal outcome were analysed (data not shown).

Data from another Spanish population

We also analysed data from another Spanish population included in a previously published study [19]. A total of 84 P-CAP patients (51.19% with bacteraemic P-CAP), and a group of 91 healthy controls were compared for *MBL2* genotypes. This control population was in Hardy–Weinberg equilibrium. No relevant differences between patients and controls were observed. Data about severity were not available.

Association between MBL2 *and pneumococcal infection: meta-analysis*

The characteristics of previous studies included in our meta-analyses are shown in table 4. The role of *MBL2* genotypes on

TABLE 4 Characteristics of other published studies included in meta-analysis of mannose-binding lectin 2 gene (*MBL2*) polymorphisms and susceptibility to pneumococcal infection

First author [Ref.]	Origin	Ethnicity	Age yrs	Patients n	Controls n	Polymorphisms	HWE
KRONBORG [9]	Denmark	97.9% white	>18	140 pneumococcal bacteraemia	250 healthy blood donors and laboratory staff	Exon 1 and promoter -221	Yes
ROY [7]	UK	White	0–94	229 invasive pneumococcal disease	353 blood and transplant donors	Exon 1 and promoter -221	Yes
ROY# [7]	UK	White	NA	108 invasive pneumococcal disease	679 healthy neonates	Exon 1 and promoter -221	Yes
MOENS [8]	Belgium	Caucasian	0–92	63 invasive pneumococcal disease	162 healthy hospital staff and nonrelated children	Exon 1, promoter -221 and -550	Yes
ENDEMAN [22]	The Netherlands	NA	>18	100 pneumococcal CAP¶	223 blood donors	Exon 1 and promoter -221	Yes

HWE: Hardy–Weinberg equilibrium; NA: not available; CAP: community-acquired pneumonia. #: confirmatory study in the same paper; ¶: a total of 199 patients with CAP were included in this study, 100 had pneumococcal CAP.

susceptibility to P-CAP was analysed in a meta-analysis including our two case–control studies and data from a previous study [22]. No differences of *MBL2* genotypes between patients and controls were observed (fig. 1). There was no statistically significant heterogeneity among the included studies for any of the three meta-analysis.

Three previous studies analysed the role of *MBL2* genotypes in the susceptibility to IPD. ROY *et al*. [7] reported in two independent case–control studies that *O/O* homozygous patients have an increased risk of IPD, but these results were not replicated in other populations [8, 9]. Data from patients with bacteraemic P-CAP and healthy controls from our two case–control studies were independently included with those previous data in a meta-analysis. Figure 2a shows the *O/O versus A/A+A/O* forest plot. This analysis showed that the MBL-deficient genotype *O/O* was significantly associated with a risk of acquiring IPD ($p<0.0001$; pooled OR 2.16, 95% CI 1.52–3.09). No significant associations were found for the *AO+O/O* genotypes (fig. 2b). Nevertheless, the other MBL-deficient genotype, *XA/O*, showed a trend towards a protective role (pooled OR 0.73, 95% CI 0.51–1.04) (fig. 2c). There was no statistically significant heterogeneity among the included studies for any of the three meta-analysis. As expected, when *XA/O+O/O* genotypes were analysed, no significant differences were found (data not shown).

LD of MBL2, SFTPA1, SFTPA2 *and* SFTPD *genes*

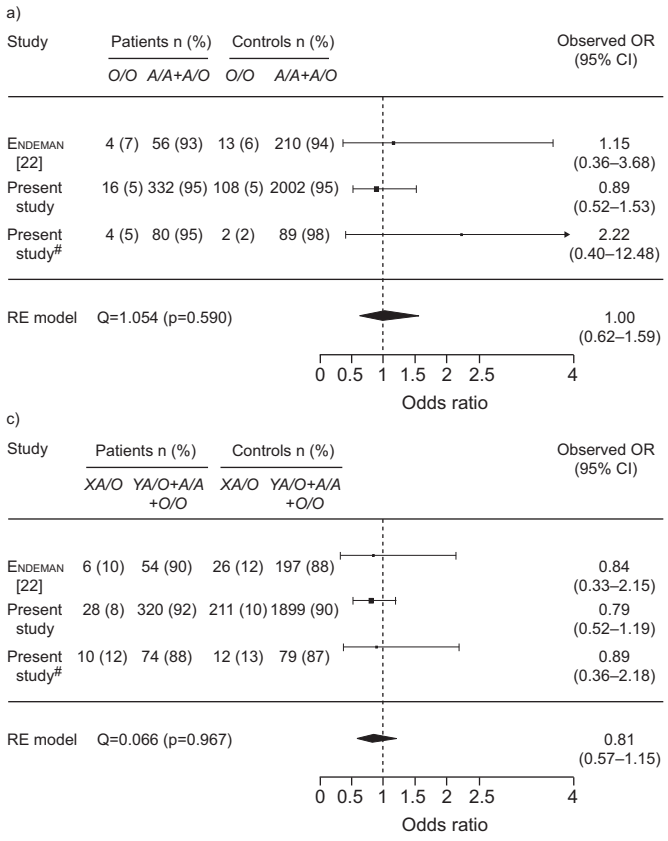
As we have previously shown in our population, there is LD among several SNPs at *SFTPA1* and *SFTPA2*, whereas *SFTPD aa11* was only observed in LD with *SFTPA1 aa19* (fig. S1) [17]. As expected, pairwise LD (D') confirmed the existence of a very strong LD within *MBL2* SNPs. Several SNPs of *SFTPA1* and *SFTPA2*, but not the *SFTPD aa11* SNP, were found to be in LD with *MBL2* SNPs (fig. S1). The value of LD measured as r^2 was very low for every pair of SNPs (data not shown), and none of the studied SNPs could be used as haplotype-tagging SNP to infer the observed haplotypes. In addition, when pairwise LD was measured among haplotypes instead of among SNPs; some haplotypes were found to be in LD with *MBL2* variants (table 5).

Susceptibility to P-CAP related to haplotypes encompassing SFTPD, SFTPA1, SFTPA2 *and* MBL2

We also intended to analyse whether phased variants encompassing the four genes were involved in susceptibility to P-CAP. Due to the existence of LD, only 177 of the 2,048 expected haplotypes encompassing *SFTPD, SFTPA1, SFTPA2* and *MBL2* were observed, and only 18 had frequencies higher than 1% (data not shown). We previously reported a protective effect of the $6A^2$, $1A^0$, $6A^2$-$1A^0$ and *C*-$6A^2$-$1A^0$ haplotypes on susceptibility to CAP [17]. When susceptibility to P-CAP was studied in the present study, the protective effect of these haplotypes was even higher when they co-segregate with the *MBL2 XA* variant (table 6). However, these results did not remain significant after a conservative Bonferroni correction for the number of observed haplotypes.

DISCUSSION

Previous meta-analysis based on genetic association studies concluded that the *MBL2 O/O* genotype predisposes to infection by *S. pneumoniae*. We herein provide new data



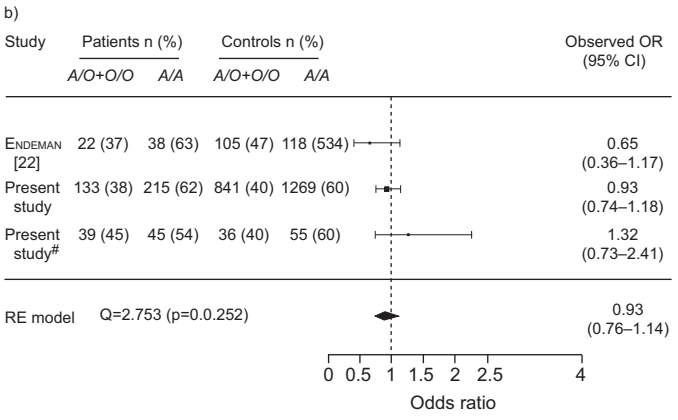
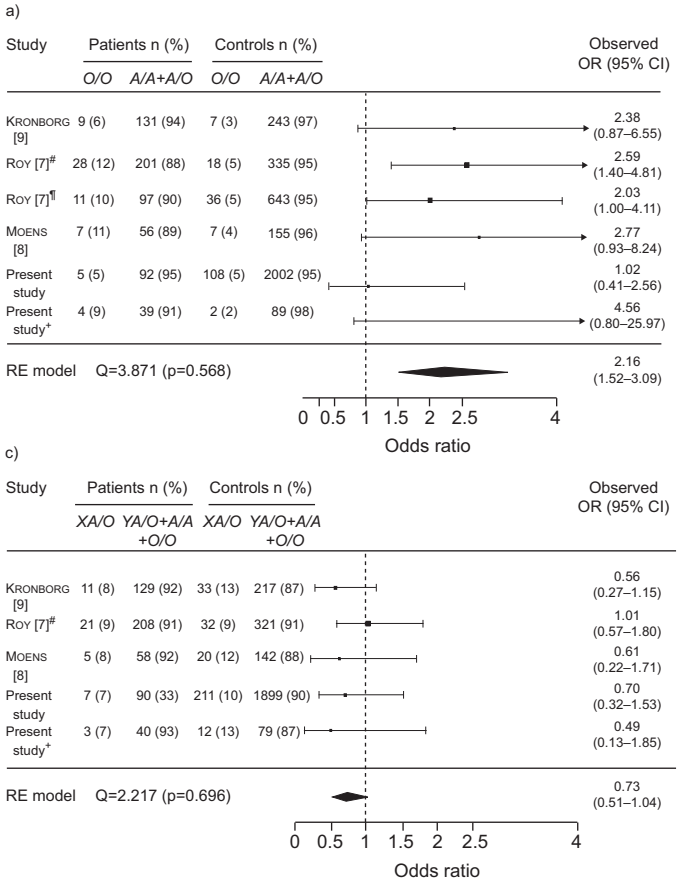


FIGURE 1. Meta-analysis of association between *MBL2* and pneumococcal pneumonia. RE: random effects. #: additional group of patients.



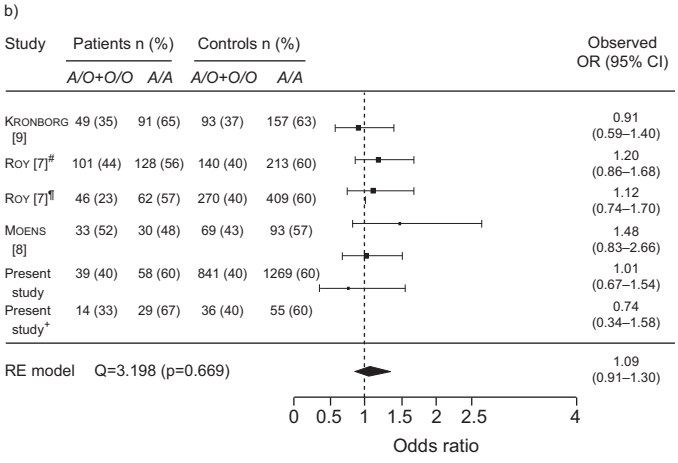


FIGURE 2. Meta-analysis of association between the mannose-binding lectin 2 gene (*MBL2*) and invasive pneumococcal disease. RE: random-effects. #: initial group of patients; ¶: confirmatory study in the same paper; +: additional group of patients.

TABLE 5 Pairwise linkage disequilibrium measure (D') for surfactant proteins A1, A2 and D alleles with regard to mannose-binding lectin (MBL) alleles from 748 healthy controls

	MBL2				
	YB	***YC***	***YD***	***XA***	***YA***
SFTPA1					
6A					
$6A^2$	0.53 (<0.0001)	0.55 (0.033)	0.74 (<0.001)	0.31 (0.001)	
$6A^3$	0.37 (0.039)		0.78 (0.006)		
$6A^4$	1 (0.003)				
SFTPA2					
$1A^0$	0.45 (<0.001)	0.71 (0.003)	0.76 (<0.001)	0.37 (<0.001)	
$1A^1$		1 (0.037)		0.42 (0.026)	
SP-D					

Data are presented as D' (p-value). D' values <0.3 or with a corresponding p-value >0.05 have not been considered. Linkage disequilibrium was measured using Arlequin 3.11 software. Only relevant haplotypes are shown. *MBL2*: mannose-binding lectin 2 gene.

TABLE 6 Comparison of relevant haplotypes encompassing *SFTPD*, *SFTPA1*, *SFTPA2* and *MBL2* between pneumococcal community-acquired pneumonia (P-CAP) patients and controls

Haplotype	P-CAP[#]	Controls[¶]	OR (CI 95%)	p-value[+]
XA	48 (16.1)	388 (21.2)	0.72 (0.51–0.99)	0.053
$6A^2$	158 (53.0)	1088 (59.4)	0.60 (0.57–0.99)	0.042
$1A^0$	150 (50.3)	1069 (58.4)	0.72 (0.57–0.93)	0.010
$6A^2$-XA	23 (7.7)	205 (11.2)	0.66 (0.42–1.04)	0.085
$1A^0$-XA	21 (7.0)	207 (11.3)	0.60 (0.37–0.95)	0.033
$6A^2$-$1A^0$	131 (44.0)	940 (51.5)	0.74 (0.58–0.95)	0.021
$6A^2$-$1A^0$ -XA	18 (6.0)	185 (10.1)	0.57 (0.35–0.94)	0.033
C-$6A^2$-$1A^0$-XA	3 (1.0)	80 (4.4)	0.22 (0.07–0.71)	0.005

Data are presented as the n chromosomes (%), unless otherwise stated. Only relevant haplotypes are shown. *MBL2*: mannose-binding lectin 2 gene. [#]: n=298; [¶]: n=1832; [+]: uncorrected for the bivariate comparison of haplotypes.

showing that *MBL2* genotypes are not involved in susceptibility to either P-CAP or to IPD.

Earlier studies from our group [14] and from ENDEMAN *et al.* [22] failed to find any significant association between *MBL2* genotypes and susceptibility to P-CAP, although both studies were underpowered to test it. When susceptibility to IPD was studied, only one out of three studies found a significant association with the *O/O* genotype [7–9]. However, a previous meta-analysis of these three studies yielded a significant association [23]. We have now studied the role of *MBL2* genotypes on susceptibility to P-CAP and to invasive P-CAP in two different case–control studies, and no association was observed. We also performed a meta-analysis, including the three previous studies [7–9] and our results. Our meta-analysis also showed that *O/O* genotypes significantly associated with susceptibility to IPD. MBL deficiency is considered to result mainly from the presence of *O/O* or *XA/O* genotypes [2, 3]. However, when the genotype *XA/O* was analysed in our meta-analysis, a surprising trend towards a protective effect against IPD was observed in our meta-analysis. This data is intriguing, as the effect of *O/O* and *XA/O* genotypes on MBL levels and MBL-dependent LP activity was repeatedly found to be similar, as it has also been previously reported in our population [2, 14, 24–26]. More recently, a small study in children from Africa suggested that MBL deficiency would associate to IPD by means of low invasive serotypes [27].

MBL deficiency has been associated with infections by Gram-negative bacteria [6]. MBL binding to *Pseudomonas aeruginosa* has been documented, and susceptibility is conferred by both MBL-deficient genotypes (*O/O* and *XA/O*) [28]. However, the proposal of a putative role of MBL deficiency in susceptibility to pneumococcal infection is challenged by several functional and evolutionary studies. The lack of association between MBL deficiency and P-CAP would not be surprising, as no MBL binding and/or MBL-mediated opsonophagocytosis of *S. pneumonia*e was observed *in vitro* [10, 12]. The classical pathway of complement is the main complement pathway in host defences against pneumococcus in mice and humans, and loss of the lectin pathway does not seem to affect significantly innate immunity to pneumococcus in mice [11]. Infectious diseases have been the main selective force shaping the human genome during evolution. Pneumonia, particularly by *S. pneumoniae*, is the biggest killer worldwide of children aged <5 yrs [29]. If MBL plays a role in protective immunity against *S. pneumoniae*, then we would expect a selective removal of *MBL2*-deficient alleles. However, *MBL2*-deficient alleles are frequent in many populations worldwide. MBL deficiency was suggested to be protective against several inflammatory and infectious diseases, particularly against tuberculosis, which would positively select for low MBL genotypes. Nevertheless, the results from genetic association studies in the field of tuberculosis are controversial [6, 30], and recent studies have shown that the patterns of *MBL2* variation worldwide are compatible with neutral evolution [5, 13].

The influence of a suspected LD among *SFTPA1*, *SFTPA2* and/or *SFTPD* with *MBL2* on the results of genetic association studies in infectious diseases has been recently proposed [6, 17]. We have previously found that several haplotypes of *SFTPA1* and *SFTPA2* are involved in susceptibility to CAP [17]. When we measured LD among *MBL2, SFTPA1, SFTPA2* and *SFTPD* genes, some SNPs and haplotypes of both *SFTPA* genes were found to be in LD with *MBL2* alleles. Interestingly, an additive effect of the *MBL2 XA* variant with variants at *SFTPA1, SFTPA2* and *SFTPD* genes on protection against P-CAP was observed. Whether the observed association is due either to epistatic effects or to the existence of an extended protector haplotype encompassing the four genes, or both, is not known. In any event, our results suggest that the trend towards a protective effect of the *MBL2* genotype *XA/O* on susceptibility to P-CAP might be due to LD with protector haplotypes on the genes of the studied SP. Likewise, the reported associations between the *MBL2 O/O* genotype with

susceptibility to P-CAP might also be spurious, and due to LD with the SP genes. Wide genetic studies of this region are required to characterise the extent of LD among these genes and its relevance for genetic association studies.

In this and previous studies, *XA/O+O/O* genotypes and serum MBL-deficiency were associated with a poor prognosis of CAP [14] or pneumococcal disease [25]. Some objections to these results may be argued. How MBL can influence severity of P-CAP if it does not bind to pneumococcus? The rationale for the involvement of MBL deficiency in the severity of pneumonia could be its role in apoptosis [31], or its capacity to bind peptidoglycan and to inhibit peptidoglycan-induced pro-inflammatory cytokine production by macrophages [32]. However, only a weak statistical significance of MBL genotypes with severity or outcome was observed in our study, which could be even lower if the results were corrected for multiple comparisons. *SFTPA1*, *SFTPA2* and *SFTPD* variants were found to be involved in severity of CAP [17] and, hence, LD of *MBL2* with SP genes could underlie the association of *MBL2* deficiency with higher severity and/or poor outcome in CAP and P-CAP. Vaccination against pneumococcus may obviously affect susceptibility to pneumococcal disease, particularly among high-risk individuals. Unfortunately, no data about the vaccination status of most patients against pneumococcus were available. However, pneumococcal vaccine is not included in the Spanish vaccination schedule and most adults have not been vaccinated against pneumococcus.

Overall, our data and several lines of evidence do not support a role of MBL deficiency on susceptibility to P-CAP or to IPD. By contrast, our results suggest that MBL deficiency may be associated with higher severity of P-CAP. However, studies aimed to analyse the role of genetic variability of the MBL gene in infectious diseases, particularly respiratory, should be aware of the existing LD among *MBL2* and the genes of SP-A1, -A2 and -D. Identification of new pathways and molecules involved in susceptibility to and severity of respiratory infectious diseases could lead to new therapeutic approaches, and the therapeutic use of MBL and SPs has been advocated [15, 16, 33]. Large studies designed to analyse the genetic variability in the region of chromosome 10 containing these genes are desirable in order to unravel their role in susceptibility and severity of pneumococcal infection, particularly P-CAP.

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STATEMENT OF INTEREST

None declared.

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