Polymorphisms in the Mannose-Binding Lectin Gene are Associated with Defective Mannose-Binding Lectin Functional Activity in Crohn’s Disease Patients

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Mannose-binding lectin, together with mannose-associated serine proteases, activates the lectin pathway of the complement system and subsequent inflammatory mechanisms. An association between mannose-binding lectin deficiency and anti-\textit{Saccharomyces cerevisiae} antibody levels is observed in Crohn’s disease and this deficiency is frequently associated with a severe Crohn’s disease phenotype. In the present study, we assessed the relationship between serum concentrations of mannose-binding lectin, mannose-binding lectin functional activity, \textit{MBL2} and \textit{NOD2} polymorphisms, anti-\textit{S. cerevisiae} antibody levels and clinical Crohn’s disease phenotype in 69 Crohn’s disease patients and 30 age- and sex-matched healthy controls. The results show that the \textit{MBL2} variant rs5030737 at codon 52 was associated with a low level of mannose-binding lectin and impaired mannose-binding lectin–mannose-associated serine protease (MBL-MASP) functional activity in Crohn’s disease patients. This \textit{MBL2} variant was also associated with a higher level of anti-\textit{S. cerevisiae} antibodies. In addition, the \textit{NOD2} variant rs2066844, which is associated with susceptibility to Crohn’s disease, was significantly correlated with an impairment in MBL-MASP functional activity. These results provide evidence that Crohn’s disease patients have an impairment in MBL-MASP functional activity and that this defect is associated with \textit{MBL2} and \textit{NOD2} variants.

Inflammatory bowel disease is a chronic inflammatory disease of the gastrointestinal tract which includes Crohn’s disease and ulcerative colitis\textsuperscript{1}. Although the aetiology of irritable bowel disease is unclear several studies have showed that genetic susceptibility, the microbiota, environment and immune system are all involved in its pathogenesis\textsuperscript{2–4}. Studies in twins have provided the best evidence for genetic predisposition to irritable bowel disease\textsuperscript{5}. Relatives of patients with Crohn’s disease have a higher risk of developing irritable bowel disease than those of patients with ulcerative colitis\textsuperscript{5}. In addition to the clinical characteristics of irritable bowel disease such as patient age at diagnosis, disease location and disease behaviour, serological markers, in particular anti-\textit{Saccharomyces cerevisiae} antibodies, can improve the accuracy of diagnosis of irritable bowel disease. Anti-\textit{S. cerevisiae} antibodies have the highest sensitivity as serological markers of Crohn’s disease\textsuperscript{6}. Plevy \textit{et al.} showed that incorporating a combination of serological, genetic and inflammatory markers into a diagnostic algorithm improved the accuracy of diagnosis of irritable bowel disease\textsuperscript{7}. The genetic association of \textit{NOD2/CARD15} with Crohn’s disease has established a critical link between innate immune cells, the intestinal epithelium and development of the disease\textsuperscript{8}. In addition, mannose-binding lectin is another critical protein, which has a major role in the innate

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immune defence against pathogens and promotes intestinal homeostasis\(^9,10\). This lectin circulates in the blood as a complex with mannose-associated serine proteases. The mannose-binding lectin–mannose-associated serine protease (MBL-MASP) complex activates the lectin complement pathway after recognition of microorganisms through the carbohydrate recognition domain\(^11,12\). The carbohydrate recognition domain of mannose-binding lectin senses polysaccharide patterns such as D-mannose, L-fucose and N-acetylglucosamine on several clinically relevant pathogens\(^13\). This results in activation of MASP-1 and MASP-2 leading to the complement cascade and to pathogen neutralisation. It has been shown that MASP-1 is the main activator of MASP-2\(^14,15\). Both MASP-1 and MASP-2 can form co-complexes with mannose-binding lectin and are crucial to lectin pathway activation. They are also able to cleave prothrombin, promoting clot formation\(^16-18\).

It has been reported that single nucleotide polymorphisms located within the promoter region and exon 1 of the MBL2 gene are correlated with mannose-binding lectin serum levels and, consequently, are associated with a higher risk of developing infectious disease. Several studies have shown an association between mutations in the MBL2 gene and Crohn's disease\(^9,13\). Seibold et al. suggested that an impaired innate immune system defined by mannose-binding lectin deficiency may lead to increased reactivity to mannan antigens in the microbial cell wall and that this enhanced mannan exposure contributes to the generation of anti-\(S.\ cerevisiae\) antibodies in patients with Crohn's disease\(^20,21\). Uemura et al. showed that mannose-binding lectin is expressed in epithelial cells in the murine small intestine\(^8\). Recently, we showed that mannose-binding lectin is produced locally by human colon epithelial cells in response to intestinal inflammation where it plays a crucial role in the mucosal antifungal defence and intestinal homeostasis\(^10\). Although the association between mannose-binding lectin serum concentrations and MBL2 gene mutations in Crohn's disease patients has been studied previously, the functional activity of the MBL–MASP complex has not yet been investigated in any clinical cohort of Crohn's disease patients.

The present study aimed to investigate the relationship between mannose-binding lectin serum concentrations, mannose-binding lectin functional activity, MBL2, MASP1, MASP2 and NOD2 variants, anti-\(S. cerevisiae\) antibody levels and clinical Crohn's disease phenotype in a cohort of Crohn's disease patients in comparison with healthy subjects.

Results

Association between mannose-binding lectin serum concentrations and clinical phenotype of Crohn's disease. Serum concentrations of mannose-binding lectin were not statistically different between Crohn's disease patients and healthy controls although a slightly elevated mannose-binding lectin level was observed in Crohn's disease patients (\(P = 0.8\)). The concentration of mannose-binding lectin was not associated to the clinical phenotype of the disease (B1, B2, or B3) (Fig. 1A,B).

Association between functional activity of the MBL-MASP complex and clinical phenotype of Crohn's disease. To assess whether mannose-binding lectin is able to bind to mannose-associated serine proteases and then to activate the complement system in the serum of Crohn's disease patients we explored the functional activity of the MBL-MASP complex. The functional activity assay was based on the ability of mannose-binding lectin to bind to \(S. cerevisiae\) mannan and the ability of mannose-associated serine proteases to cleave the fluorogenic substrate of thrombin.

The functional activity of the MBL-MASP complex was measured in 69 Crohn's disease and 30 healthy control sera. No functional activity of the MBL-MASP complex was detected in either Crohn's disease patients or healthy control subjects when the mannose-binding lectin level was <500 ng/mL (Fig. 1C). Furthermore, no significant difference in functional activity of the MBL-MASP complex was observed between healthy controls and Crohn's disease patients. Increased functional activity of the MBL-MASP complex was correlated with the mannose-binding lectin serum level in both healthy controls (\(P < 0.0001, r = 0.8\), Fig. 1D) and Crohn's disease patients (\(P < 0.0001, r = 0.75\), Fig. 1E), particularly when the mannose-binding lectin serum concentrations were >500 ng/mL. This suggests that the serum concentration of mannose-binding lectin has an important impact on mannose-binding lectin functional activity.

Association between high anti-\(S. cerevisiae\) antibody levels and the B2 phenotype. Anti-\(S. cerevisiae\) antibody levels were significantly higher in Crohn's disease patients compared to healthy controls (\(P < 0.0001\)) (Fig. 2A). Furthermore, anti-\(S. cerevisiae\) antibody levels were significantly elevated in Crohn's disease patients with the B2 phenotype compared to patients with the B1 phenotype (\(P < 0.01\)) and there was also a tendency towards elevated anti-\(S. cerevisiae\) antibody levels in Crohn's disease patients with the B3 phenotype (\(P = 0.0516\)) (Fig. 2B). Mannose-binding lectin levels were inversely correlated with anti-\(S. cerevisiae\) antibody levels in Crohn's disease patients with severe clinical phenotypes (\(P < 0.015, r = −0.72\)) (Fig. 2C).

Relationship between the MBL2 mutation at codon 52, mannose-binding lectin serum concentrations, MBL-MASP functional activity and anti-\(S. cerevisiae\) antibody levels in Crohn's disease patients. To explore whether MBL2 polymorphisms are associated with susceptibility to Crohn's disease and to its clinical and serological manifestations, the MBL2 gene and its promoter were genotyped in 69 Crohn's disease patients and 30 healthy controls. Two polymorphisms of the MBL2 gene were identified: rs930508 and rs1800450, which were associated with significant mannose-binding lectin deficiency (\(P < 0.01\) and \(P < 0.0001\), respectively; Fig. 3A,B). The polymorphism rs5030737 (codon 52) was associated with a decrease in mannose-binding lectin serum levels (\(P < 0.0001\)) and a low level of MBL-MASP functional activity (\(P < 0.05\)) in Crohn's disease patients (Fig. 3C,D). In addition, the polymorphism rs5030737 was associated with significantly increased levels of anti-\(S. cerevisiae\) antibodies (\(P < 0.01\)) in Crohn's disease patients (Fig. 3E). In terms of the association between mannose-binding lectin polymorphisms and clinical phenotype of Crohn's disease, the polymorphism rs5030737 was more common in Crohn's disease patients with B2 and B3 phenotypes than in those
with B1 (Table 1). 13% of Crohn's disease patients had heterozygous mutations for the rs5030737 variant, which represent 77.7% for B2 and B3 clinical phenotypes vs. 22.3% for B1.

**Association between the NOD2 polymorphism and functional activity of the MBL-MASP complex.**

To investigate an additional genetic marker for Crohn's disease, the NOD2 gene was genotyped in 69 Crohn's disease patients and 30 healthy controls. A significant association was found between the rs2066847 polymorphism and Crohn's disease \( (P = 0.0177) \) and there was a tendency for the rs2066844 polymorphism to be associated with the disease \( (P = 0.0518) \). In terms of the association between clinical phenotype and the NOD2 polymorphism, the rs2066847 variant was found in 15 Crohn's disease patients with clinical phenotypes B1 (33.3%), B2 (40.1%) and B3 (26.6%), respectively, carrying the heterozygous mutation C_C (Table 2) and in two Crohn’s disease patients.
patients carrying the homozygous mutation (both B3). The NOD2 variant rs2066844 was found in 19 Crohn's disease patients (heterozygous mutations C_T) with B1 (73.3%), B2 (15.8%) and B3 (10.5%), respectively (Table 2). While the NOD2 variant rs2066844 was not associated with mannose-binding lectin serum levels, the polymorphism was associated with functional activity of the MBL-MASP complex (Fig. 3F). Significantly lower functional activity of the MBL-MASP complex was observed in Crohn's disease patients carrying the NOD2 variant rs2066844 (R702W) compared to those with the NOD2 wild-type (P < 0.05) (Fig. 3F). In addition to genotyping the MBL2 and NOD2 genes, the MASP1 gene was also genotyped in this study. No association was found between MASP1 and Crohn's disease (Table 3).

Discussion
The MBL-MASP complex is an activator of the lectin pathway of the complement system and subsequent inflammatory mechanisms. In the present study, mannose-binding lectin serum levels did not vary significantly between Crohn's disease patients and healthy controls. In addition, mannose-binding lectin serum levels were not associated with the clinical phenotype of Crohn's disease. These data are consistent with previous clinical studies that show the absence of mannose-binding lectin level changes in Crohn's disease patients. Experimental studies showed that MASP-1 and MASP-2 are involved in blood coagulation. Hajela et al. showed that soluble native human MASP-1 has a thrombin-like substrate specificity, cleaving and activating the coagulation proteins Factor XIII and fibrinogen. In addition, MASP-2 is capable of generating thrombin via prothrombin. In the present study, we explored the functional activity of the MBL-MASP complex using a coagulation protease substrate. The test was based on the ability of mannose-binding lectin to bind to S. cerevisiae mannan through its carbohydrate recognition domain and the ability of the mannose-associated serine protease-associated collagen-like domain of mannose-binding lectin to cleave the fluorogenic protease substrate. We observed a significant correlation between mannose-binding lectin serum concentrations and functional activity of the MBL-MASP complex in both Crohn's disease patients and healthy controls. However, a mannose-binding lectin concentration of < 500 ng/mL was associated with an impairment in MBL-MASP functional activity and the absence of enzymatic activity in serum samples from Crohn's disease patients. To corroborate the functional

Figure 2. Anti-S. cerevisiae antibody levels in healthy control subjects and Crohn's disease patients. (A) Anti-S. cerevisiae antibody level was increased in Crohn's disease patients when compared to healthy controls (P < 0.0001). Scatter plots of these data with the median line are shown. (B) Crohn's disease patients with the clinical phenotype B2 had higher anti-S. cerevisiae antibody levels than those with B1 (P < 0.01) and there was a tendency for Crohn's disease patients with B3 to have higher levels than patients with B1 (P = 0.0516). (C) Correlation between anti-S. cerevisiae antibody levels and mannose-binding lectin concentrations in Crohn's disease patients with B3 (P < 0.015, r = −0.72). The results are expressed in arbitrary units (AU).
activity observed in the fluorogenic thrombin assay, two other assays were performed. The first was based on cleavage of complement C4 protein to C4b fragments and the second on the activation of platelets that had been exposed to the MBL-MASP complex trapped on mannan-coated plates. In these assays, we found a significant correlation between mannose-binding lectin concentrations and functional activity of the MBL-MASP complex in both Crohn’s disease patients and healthy controls. These results are consistent with those from the fluorogenic thrombin assay (Supplementary data).

Anti-\textit{S. cerevisiae} antibodies are important serological markers that can help to differentiate Crohn’s disease from ulcerative colitis\textsuperscript{6}. In the present study, anti-\textit{S. cerevisiae} antibody levels were significantly elevated in Crohn’s disease patients (A–C). Mannose-binding lectin concentration was significantly associated with rs930508 (wild-type C/C, heterozygous C/G or homozygous G/G; \(P < 0.01\)), rs1800450 (wild-type C/C, heterozygote C/T; \(P < 0.001\)) and rs5030737 (wild-type G/G, heterozygote G/A; \(P < 0.0001\)) of \textit{MBL2} polymorphisms in Crohn’s disease patients. (D) Functional activity of the MBL-MASP complex was significantly associated with the rs5030737 \textit{MBL2} polymorphism in Crohn’s disease patients (wild-type G/G, heterozygote G/A; \(P < 0.05\)). (E) Relationship between the rs5030737 \textit{MBL2} polymorphism and anti-\textit{S. cerevisiae} antibody levels in Crohn’s disease patients. Anti-\textit{S. cerevisiae} antibody level was significantly higher in heterozygous Crohn’s disease patients (G/A; \(n = 9\)) than in wild-type patients (G/G; \(n = 60\)) for the rs5030737 \textit{MBL2} variant (\(P < 0.01\)). (F) Association between functional activity of the MBL-MASP complex and the \textit{NOD2} polymorphism in Crohn’s disease patients. Functional activity of the MBL-MASP1 complex was significantly higher in heterozygous Crohn’s disease patients (C/T; \(n = 20\)) when compared to wild-type patients (C/C; \(n = 50\)) for the rs2066844 \textit{NOD2} variant (\(P < 0.05\)).

Figure 3. Association between \textit{MBL2} and \textit{NOD2} polymorphisms, mannose-binding lectin concentration, functional activity of the MBL-MASP complex and anti-\textit{S. cerevisiae} antibody levels in Crohn’s disease patients (A–C). Mannose-binding lectin concentration was significantly associated with rs930508 (wild-type C/C, heterozygous C/G or homozygous G/G; \(P < 0.01\)), rs1800450 (wild-type C/C, heterozygote C/T; \(P < 0.001\)) and rs5030737 (wild-type G/G, heterozygote G/A; \(P < 0.0001\)) of \textit{MBL2} polymorphisms in Crohn’s disease patients. (D) Functional activity of the MBL-MASP complex was significantly associated with the rs5030737 \textit{MBL2} polymorphism in Crohn’s disease patients (wild-type G/G, heterozygote G/A; \(P < 0.05\)). (E) Relationship between the rs5030737 \textit{MBL2} polymorphism and anti-\textit{S. cerevisiae} antibody levels in Crohn’s disease patients. Anti-\textit{S. cerevisiae} antibody level was significantly higher in heterozygous Crohn’s disease patients (G/A; \(n = 9\)) than in wild-type patients (G/G; \(n = 60\)) for the rs5030737 \textit{MBL2} variant (\(P < 0.01\)). (F) Association between functional activity of the MBL-MASP complex and the \textit{NOD2} polymorphism in Crohn’s disease patients. Functional activity of the MBL-MASP1 complex was significantly higher in heterozygous Crohn’s disease patients (C/T; \(n = 20\)) when compared to wild-type patients (C/C; \(n = 50\)) for the rs2066844 \textit{NOD2} variant (\(P < 0.05\)).
Crohn's disease patients with stricture formation and penetrating disease complications indicating that higher anti-\textit{S. cerevisiae} antibody levels could be a predictive marker of Crohn's disease severity. This observation is consistent with clinical studies showing that high levels of anti-\textit{S. cerevisiae} antibodies are associated with a complicated clinical phenotype of Crohn's disease and the need for surgery\textsuperscript{28,29}.

In the present study, the analysis of \textit{MBL2} polymorphisms revealed an association between three variants, rs930508, rs1800450 and rs5030737, and a reduction in mannose-binding lectin serum levels in Crohn's disease patients. In addition, both homozygous and heterozygous \textit{MBL2} mutations were associated with a decrease in mannose-binding lectin concentrations. Swale \textit{et al.} showed that the presence of these variants (rs1800450 and rs5030737) was the major contributing factor for lower mannose-binding lectin concentrations\textsuperscript{30}. However, we found that although the \textit{MBL2} variant rs5030737 was associated with a low level of MBL-MASP functional activity, this variant was related to high anti-\textit{S. cerevisiae} antibody levels in Crohn's disease patients. These data corroborate previous observations, which showed that patients with low serum mannose-binding lectin or mannose-binding lectin deficiency were more often anti-\textit{S. cerevisiae} antibody-positive than patients with normal levels of mannose-binding lectin\textsuperscript{20,31}. The variant rs5030737 was found to be related to Crohn's disease in a paediatric cohort\textsuperscript{19}. In addition, Schoepfer \textit{et al.} showed that a low mannose-binding lectin serum level was highly associated with complicated Crohn's disease\textsuperscript{41}. Altogether, our data emphasise the role of the rs5030737 variant in MBL-MASP functional activity and suggest that this variant affects the binding of mannose-associated serine proteases to the collagen-like domain of mannose-binding lectin. This may alter the innate immune response and increase the risk of developing a complicated Crohn's disease phenotype.

The \textit{NOD2} gene is involved in the innate immune response and is highly associated with Crohn's disease\textsuperscript{32}. Crohn's disease-associated \textit{NOD2} polymorphisms exhibit a reduced capacity to activate NF-\kappaB following muramyl dipeptide stimulation, suggesting that the loss of \textit{NOD2} activation promotes Crohn's disease\textsuperscript{33}. \textit{NOD2} polymorphisms, rs2066844 (R702W) and rs2066845 (G908R and rs2066847 (I1007fs), are the most common genetic variants associated with an increased risk of Crohn's disease\textsuperscript{34}. In addition, these variants alter the structure of either the leucine-rich repeat domain of the protein or the adjacent region\textsuperscript{1}. In the present study, we observed that Crohn's disease patients carrying the \textit{NOD2} rs2066844 variant had low functional activity of the MBL-MASP complex, suggesting that both the \textit{NOD2} rs2066844 variant and impairment of the functional

| \textit{MBL2} rs5030737 | Wild-type (G/G) | Heterozygote (G/A) | Mann-Whitney P (\(\alpha = 0.05\)) |
|------------------------|----------------|------------------|-----------------|
| Number (%)             |                |                  |                 |
| 60 (87%)               | 9 (13%)        |                  |                 |
| Behaviour, n (%)        |                |                  |                 |
| B1                     | 35 (58.3%)     | 2 (22%)          |                |
| B2                     | 12 (20%)       | 4 (44.4%)        |                |
| B3                     | 11 (18.3%)     | 3 (33.3%)        |                |
| IBDU                   | 2 (3.3%)       | –                |                |
| ASCA level (AU)         | 56.55          | 110.4            | <0.05          |
| MBL level (ng/mL)       | 3211.9         | 366.4            | <0.0001        |
| MBL-MASP activity (%EF/ng of MBL) | 0.35 | 0.13 | <0.05 |

Table 1. Evaluation of mannose-binding lectin phenotype and genotype in relation to the Crohn's disease phenotype. IBDU: inflammatory bowel disease not identified; AU: arbitrary unit; ASCA: anti-\textit{Saccharomyces cerevisiae} antibodies; MBL: mannose-binding lectin; MASP: mannose-associated serine protease; EF: emitted fluorescence.

| \textit{NOD2} rs2066844 | Wild-type (C/C) | Heterozygote (C/T) | Mann-Whitney P (\(\alpha = 0.05\)) |
|------------------------|----------------|------------------|-----------------|
| Number (%)             |                |                  |                 |
| 50 (72.5%)             | 19 (27.5%)     |                  |                 |
| Behaviour              |                |                  |                 |
| B1                     | 23 (46%)       | 14 (73.3%)       |                |
| B2                     | 13 (26%)       | 3 (15.8%)        |                |
| B3                     | 12 (24%)       | 2 (10.5%)        |                |
| IBDU                   | 2 (4%)         | –                |                |
| ASCA level (AU)         | 62.8           | 66.7             | ns              |
| MBL level (ng/mL)       | 3046.5         | 2299.46          | ns              |
| MBL-MASP activity (%EF/ng of MBL) | 0.38 | 0.16 | p < 0.05 |

Table 2. Relationship between the rs2066844 \textit{NOD2} polymorphism and clinical phenotype of Crohn's disease. IBDU: inflammatory bowel disease not identified; AU: arbitrary unit; ASCA: anti-\textit{Saccharomyces cerevisiae} antibodies; MBL: mannose-binding lectin; MASP: mannose-associated serine protease; EF: emitted fluorescence.
activity of the MBL-MASP complex could affect the innate immune response against pathogens, leading to an increased risk of Crohn's disease\textsuperscript{35,36}.

In conclusion, although mannose-binding lectin serum levels did not vary significantly between Crohn's disease patients and healthy controls, we selected 69 Crohn's disease patients for a preliminary polymorphism analysis who reflected the characteristics of the original cohort in terms of the diversity of clinical phenotypes, age and sex. This made it possible to explore multiple genetic targets with a more focused approach (Supplementary data). The number of patients with the B3 phenotype was very low in our initial cohort ($n = 40$). We intend to increase our statistical power by including new Crohn's disease patients with the B3 phenotype. Overall, our findings provide evidence that Crohn's disease patients with severe clinical phenotypes have an impairment of MBL-MASP functional activity and that this defect is associated with $\textit{MBL2}$ and $\textit{NOD2}$ variants. This study will enable us to determine the relationship between MBL2 and NOD2 in Crohn's disease and the way in which each affect the other by studying the signalling pathways.

**Methods**

**Study population.** The Crohn's disease patients included in this study had previously been included in the MINOTOR cohort study at Lille University Hospital. At inclusion, the patients underwent a thorough clinical and laboratory examination. The diagnosis of Crohn's disease was based on standard endoscopic, histological and radiographic findings\textsuperscript{37}. The clinical phenotype of Crohn's disease was determined according to the Montreal classification on the basis of age at onset (A), disease location (L) and behaviour (B)\textsuperscript{38}. B1 corresponds to non-stenosing, non-penetrating disease, B2 to stenosing behaviour and B3 to penetrating behaviour. Sixty-nine Crohn's

| SNP       | Crohn's disease patients vs. healthy controls | European allele frequency | Crohn's disease association $P$ | MBL-MASP activity $P$ |
|-----------|----------------------------------------------|---------------------------|---------------------------------|----------------------|
| MASP1     | Wild-type                                   | Heterozygous              | Homozygous                      | Ref      | Muted     | Ref      | Muted     |
| rs850312  | C/C: 56.5 vs. 54                            | G/T: 27.5 vs. 38          | T/T: 16 vs. 8                   | C: 66     | T: 34     |
| rs72549154| C/C: 94 vs. 88                              | C/A: 6 vs. 12             |                                 | C: 97     | A: 3      |
| rs3774268 | A/A: 64 vs. 78                              | G/A: 32 vs. 19            | G/G: 4 vs. 4                    | A: 15     | G: 85     |
| rs43090319| G/G: 48 vs. 50                              | G/GG: 46 vs. 42           | G/GG: 6 vs. 8                   |          |
| rs78008995| G/G: 87 vs. 88                              | G/T: 13 vs. 8             | T/T: 0 vs. 4                    |          |
| rs72549251| T/T: 97 vs. 92                              | T/C: 3 vs. 8              |                                 |          |
| rs16861895| C/C: 69 vs. 35                              | C/G: 28 vs. 61            | G/G: 3 vs. 4                    |          |
| rs16861896| G/G: 69 vs. 35                              | G/A: 28 vs. 61            | A/A: 3 vs. 4                    |          |
| rs72549254| G/G: 69 vs. 35                              | G/A: 28 vs. 61            | A/A: 3 vs. 4                    |          |
| NOD2      | rs2066844 (R702W)                           | C/C: 72 vs. 92            |                                 | C: 95     | T: 5      | 0.0518   | <0.05    |
| rs2066845 (G908R) | G/G: 88 vs. 88    | G/C: 12 vs. 12            | G: 99                            | C: 1      |          |
| rs2066847 (I1007fs) | C/C: 75 vs. 96    | C/CC: 22 vs. 4           | CC/CC: 3 vs. 0                   | 0.0177    |          |
| rs2076753 | G/G: 35 vs. 50                              | G/T: 36 vs. 46            | T/T: 29 vs. 4                    | 0.0119    |          |
| rs2067085 | C/C: 55 vs. 35                              | C/G: 32 vs. 54            | G/G: 13 vs. 11                   | C: 57     | G: 43     |
| rs2066842 | C/C: 33 vs. 58                              | C/T: 38 vs. 38            | T/T: 29 vs. 4                    | C: 76     | T: 24     | 0.002    |
| rs2066843 | C/C: 33 vs. 58                              | C/T: 38 vs. 38            | T/T: 29 vs. 4                    | C: 76     | T: 24     | 0.002    |
| rs1861759 | T/T: 56 vs. 31                              | T/G: 30 vs. 54            | G/G: 13 vs. 15                   | T: 58     | G: 42     |
| rs5743291 | G/G: 91 vs. 85                              | G/A: 7 vs. 15             | A/A: 1 vs. 0                     | G: 90     | A: 10     |
| rs1077861 | A/A: 55 vs. 4                               | T/A: 33 vs. 69            | T/T: 12 vs. 27                   |          |

Table 3. Frequency of MASP1 and NOD2 polymorphisms in 69 Crohn's disease patients and 30 healthy controls. SNP: single nucleotide polymorphism; MBL: mannose-binding lectin; MASP: mannose-associated serine protease.
disease patients (42 females/27 males; age at diagnosis: 10–50 years; B1 = 37, B2 = 16, B3 = 14, unclassified = 6) were included in the cohort. Thirty healthy control subjects (14 females/16 males; age 19–40 years) were also included. All healthy controls were free of symptoms and had a normal clinical examination. Details of the clinical phenotype of the Crohn’s disease patients including: age at diagnosis (A1: <16 years, A2: 17–40 years, A3: >40 years), disease location (L1: ileal, L2: colonic, L3: ileocolonic) and behaviour (B1: non-stricturing, non-penetrating, B2: structuring, B3: penetrating) are shown in Table 4.

All subjects were informed about the study and gave their written consent to participate. The study protocol was reviewed and approved by the Ethics Committees of Lille University Hospital (CP 05/86). The study was conducted according to the principles expressed in the Declaration of Helsinki.

### Measurement of mannose-binding lectin and anti-\textit{S. cerevisiae} antibody concentrations

Mannose-binding lectin concentrations were measured by enzyme-linked immunosorbent assay according to the manufacturer’s instructions (BioPorto, Denmark) and are expressed as ng/mL. A concentration of <500 ng/mL was considered to represent mannose-binding lectin deficiency, low levels were 500–1000 ng/mL, normal levels were 1000–4000 ng/mL and a high mannose-binding lectin level was >4000 ng/mL.

Anti-\textit{S. cerevisiae} antibodies were also detected by enzyme-linked immunosorbent assay (IBDX gASCA; Glycominds, Israel)\textsuperscript{39}. Briefly, 50 μL of 1:100 diluted serum was added to the coated wells. Absorbance was read at 450 nm (reference filter, 620 nm) in a microplate reader (Bio-Rad) after addition of tetramethylbenzidine\textsuperscript{40}. Results are expressed as arbitrary units (AU). Crohn’s disease patients were declared anti-\textit{S. cerevisiae} antibody-positive when serum levels were >50 AU.

### Assessment of the functional activity of the MBL-MASP complex

Activity of the MBL-MASP complex in serum was determined using a modified version of a method described previously\textsuperscript{27}. This assay is based on the thrombin-like activity of mannose-associated serine protease to cleave thrombin substrate. Briefly, 96-well plates (Nunc-ImmuNo, Maxisorp, Germany) were coated with 50 μL of 1:100 diluted serum. Absorbance was read at 450 nm (reference filter, 620 nm) in a microplate reader (Bio-Rad) after addition of tetramethylbenzidine\textsuperscript{40}. Results are expressed as arbitrary units (AU). Crohn’s disease patients were declared anti-\textit{S. cerevisiae} antibody-positive when serum levels were >50 AU.

### Sequencing analysis

DNA extractions were performed as described previously\textsuperscript{10}. DNA was extracted using a commercial kit according to the manufacturer’s instructions (Kit Nucleon BACC3; GE Healthcare). AmpliSeq libraries were prepared using an ion AmpliSeq library kit 2.0 and ion AmpliSeq custom panel (Life Technologies). AmpliSeq technologies were used to design a custom NGS library including 110 amplicons in two pools, covering all targets of interest (19.87 kb covered at 99.42%). The targets of interest were the \textit{MBL2}, \textit{NOD2} and \textit{MASP1} genes. The design is available in the supplementary data. 10 ng of each DNA sample was used as a template to prepare the library. Quality control of all libraries was performed with an Agilent bioanalyser using high sensitivity.

| Table 4. Clinical characteristics of the Crohn’s disease patients. Values shown are n, or n (%). A: age; L: disease location; B: behaviour. |
| Mean age of onset (years) | Crohn’s disease patients (n = 69) |
|----------------------------|-----------------------------------|
| Female/male                | 42/27                             |
| Montreal classification    |                                   |
| 16–years (A1)              | 17 (24.6%)                        |
| 16–40 years (A2)           | 48 (69.6%)                        |
| >40 years (A3)             | 3 (4.3%)                          |
| Terminal ileum (L1)        | 15 (21.7%)                        |
| Colon (L2)                 | 14 (20.3%)                        |
| Ileocolon (L3)             | 34 (49.3%)                        |
| Non-stricturing/non-penetrating (B1) | 37 (53.6%) |
| Stricturing (B2)           | 16 (23.2%)                        |
| Penetrating (B3)           | 14 (20.3%)                        |
| Perianal lesions           | 23 (33.3%)                        |
| Surgery                    | 42 (60.9%)                        |
| Predisposed subjects       | 14 (40.6%)                        |
S. cerevisiae lectin levels were determined with Haploview software41. Technologies). Allelic frequencies and their association with anti-S. cerevisiae antibody and mannose-binding genome and base calling were performed using Torrent Suite software. Identification of variants was performed following the manufacturer's protocol using ion 318 v2 chips (Life Technologies). Thirty-two barcoded samples were loaded per chip to ensure an average depth of 1500. For data analysis, alignment of the sequences to the human genome build 19 reference genome and base calling were performed using Torrent Suite software. Identification of variants was performed with an ion torrent variant caller and coverage analysis was generated using coverage analysis plugins (Life Technologies). Allelic frequencies and their association with Crohn's disease or anti-S. cerevisiae antibody levels were determined with Haplovip software41.

**Statistical analysis.** Statistical analysis was performed using Prism 4.0 from GraphPad and XLSTAT. Data were analysed using either the Kruskal-Wallis or Mann-Whitney U test to compare pairs of groups and the Chi²-test for comparison of two independent groups with categorical data. Differences were considered significant when the P value was as follows: P < 0.05; P < 0.01; P < 0.001. Fitting the Hardy-Weinberg equilibrium, allelic frequencies and their association with Crohn's disease or anti-S. cerevisiae antibody levels were determined with Haplovip software.

**References**

1. Baumgart, D. C. & Sandborn, W. J. Inflammatory bowel disease: clinical aspects and established and evolving therapies. _Lancet_ 369, 1641–1657 (2007).

2. Darfeuille-Michaud, A. et al. Presence of adherent _Escherichia coli_ strains in ileal mucosa of patients with Crohn's disease. _Gastroenterology_ 115, 1405–1413 (1998).

3. Hugot, J. P. et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. _Nature_ 411, 599–603 (2001).

4. Hart, A. L. et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. _Gastroenterology_ 129, 50–65 (2005).

5. Halne, L. et al. Family and twin studies in inflammatory bowel disease. _World J. Gastroenterol._ 12, 3668–3672 (2006).

6. Sendid, B. et al. Specific antibody response to oligomannosidic epitopes in Crohn's disease. _Clin. Diag. Lab. Immunol._ 3, 219–226 (1996).

7. Plevy, S. et al. Combined serological, genetic, and inflammatory markers differentiate non-IBD, Crohn's disease, and ulcerative colitis patients. _Inflamm. Bowel Dis._ 19, 1139–1148 (2013).

8. Lesage, S. et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. _AJHG._ 70, 845–857 (2002).

9. Sebold, F. et al. Genetic variants of the mannan-binding lectin are associated with immune reactivity to mannans in Crohn's disease. _Gastroenterology_ 127, 1076–1084 (2004).

10. Choteau, L. et al. Role of mannose-binding lectin in intestinal homeostasis and fungal elimination. _Mucosal Immunol._ doi: 10.1038/mi.2015.100 (2015).

11. Matsumita, M. & Fujita, T. Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. _J. Exp. Med._ 176, 1497–1502 (1992).

12. Hajela, K. et al. The biological functions of MBL-associated serine proteases (MASPs). _Immunobiology_ 205, 467–475 (2002).

13. Wei, W. L., Dickamer, K. & Hendrickson, W. A. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. _Nature_ 360, 127–134 (1992).

14. Heja, D. et al. Revised mechanism of complement lectin-pathway activation revealing the role of serine protease MASP-1 as the exclusive activator of MASP-2. _Proc. Natl. Acad. Sci._ USA 109, 10498–10503 (2012).

15. Megyeri, M. et al. Quantitative characterization of the activation steps of mannann-binding lectin (MBL)-associated serine proteases (MASPs) points to the central role of MASP-1 in the initiation of the complement lectin pathway. _J. Biol. Chem._ 288, 8922–8934 (2013).

16. Jenny, L., Dobo, J., Gal, P. & Schroeder, V. MASP-1 of the complement system promotes clotting via prothrombin activation. _Molec. Immunol._ 65, 398–405 (2015).

17. Krarup, A., Wallis, R., Presanis, J. S., Gal, P. & Sim, R. B. Simultaneous activation of complement and coagulation by MBL-associated serine protease 2. _PloS One_ 2, e623 (2007).

18. Takahashi, K. et al. Mannose-binding lectin and its associated proteases (MASPs) mediate coagulation and its deficiency is a risk factor in developing complications from infection, including disseminated intravascular coagulation. _Immunobiology_ 216, 96–102 (2011).

19. Bak-Romaniszyn, L. et al. Mannan-binding lectin deficiency in pediatric patients with inflammatory bowel disease. _Scand. J. Gastroenterol._ 46, 1275–1278 (2011).

20. Sebold, F. et al. Association of deficiency for mannann-binding lectin with anti-mannan antibodies in Crohn's disease: a family study. _Inflamm. Bowel Dis._ 13, 1077–1082 (2007).

21. Muller, S. et al. Mannann-binding lectin deficiency results in unusual antibody production and excessive experimental colitis in response to mannann-expressing mild gut pathogens. _Gut_ 59, 1493–1500 (2010).

22. Uemura, K. et al. L-MBP is expressed in epithelial cells of mouse small intestine. _J. Immunol._ 169, 6945–6950 (2002).

23. Petersen, S. V., Thiel, S., Jensen, L., Steffensen, R. & Jensenius, J. C. An assay for the mannann-binding lectin pathway of complement activation. _J. Immunol. Methods_ 257, 107–116 (2001).

24. Nielsen, R. G. et al. Genetic polymorphisms of mannann binding lectin (MBL), serum levels of MBL, the MBL associated serine protease and H-ficolin in patients with Crohn's disease. _Gut_ 56, 311–312 (2007).

25. Hoffmann, C. et al. Is there a role for mannann-binding lectin in the diagnosis of inflammatory bowel disease? _Immunogenetics_ 62, 231–235 (2010).

26. Ambrus, G. et al. Natural substrates and inhibitors of mannann-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. _J. Immunol._ 170, 1374–1382 (2003).

27. Presanis, J. S., Hajela, K., Ambrus, G., Gal, P. & Sim, R. B. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. _Molec. Immunol._ 40, 921–929 (2004).

28. Papp, M. et al. New serological markers for inflammatory bowel disease are associated with earlier age at onset, complicated disease behavior, risk for surgery, and NOD2/CARD15 genotype in a Hungarian IBD cohort. _Am. J. Gastroenterol._ 103, 665–681 (2008).

29. Ferrante, M. et al. New serological markers in inflammatory bowel disease are associated with complicated disease behaviour. _Gut_ 56, 1394–1403 (2007).

30. Swale, A. et al. Serum mannose-binding lectin concentration, but not genotype, is associated with Clostridium difficile infection recurrence: a prospective cohort study. _Clin. Infect. Dis._ 59, 1429–1436 (2014).
31. Schoepfer, A. M. et al. Low Mannan-binding lectin serum levels are associated with complicated Crohn’s disease and reactivity to oligomannan (ASCA). *Am. J. Gastroenterol.* **104**, 2508–2516 (2009).
32. Kullberg, B. J. et al. Crohn’s disease patients homozygous for the 3020insC NOD2 mutation have a defective NOD2/TLR4 cross-tolerance to intestinal stimuli. *Immunology* **123**, 600–605 (2008).
33. Inohara, N. et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn’s disease. *J. Biol. Chem.* **278**, 5509–5512 (2003).
34. Hoefkens, E. et al. Genetic association and functional role of Crohn disease risk alleles involved in microbial sensing, autophagy, and endoplasmic reticulum (ER) stress. *Autophagy* **9**, 2046–2055 (2013).
35. Baptista, M. L. et al. CARD15 and IL23R influences Crohn’s disease susceptibility but not disease phenotype in a Brazilian population. *Inflamm. Bowel Dis.* **14**, 674–679 (2008).
36. Ningappa, M. et al. NOD2 gene polymorphism rs2066844 associates with need for combined liver-intestine transplantation in children with short-gut syndrome. *Am. J. Gastroenterol.* **106**, 157–165 (2011).
37. Nikolaus, S. & Schreiber, S. Diagnostics of inflammatory bowel disease. *Gastroenterology* **133**, 1670–1689 (2007).
38. Silverberg, M. S. et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can. J. Gastroenterol.* **19** Suppl A, 5A–36A (2005).
39. Sendel, B. et al. Antibodies against glucan, chitin, and *Saccharomyces cerevisiae* mannan as new biomarkers of *Candida albicans* infection that complement tests based on *C. albicans* mannan. *Clin. Vacc. Immunol.* **15**, 1868–1877 (2008).
40. Jawhara, S. et al. Colonization of mice by *Candida albicans* is promoted by chemically induced colitis and augments inflammatory responses through galectin-3. *J. Infect. Dis.* **197**, 972–980 (2008).
41. Barrett, J. C., Fry, B., Maller, J. & Daly, M. J. Haplview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).

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Author Contributions
L.C. developed the test, collected and tested the patients’ sera, prepared the diagrams and contributed to the interpretation of the results. F.V. contributed to the interpretation/presentation of the data and to the statistical analysis. F.L. and M.F. contributed to the mannose-binding lectin genotyping and the interpretation of the results. L.D. and D.P. contributed to the interpretation/presentation of the data. C.G.-R. and J.-F.C. were the principal investigators of the Minotor cohort study and J.-F.C. was the reference physician throughout the study. B.S. contributed to the interpretation/presentation of the data and to the statistical analysis. S.J. designed and managed the study, managed the completion of the data and the writing of the manuscript.

Additional Information
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Corrigendum: Polymorphisms in the Mannose-Binding Lectin Gene are Associated with Defective Mannose-Binding Lectin Functional Activity in Crohn’s Disease Patients

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In the Introduction section of this Article, some instances of ‘inflammatory bowel disease’ are incorrectly written as ‘irritable bowel disease’.

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