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Original Article

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Isotype-specific Antibody Responses to *Mycobacterium avium paratuberculosis* Antigens Are Associated With the Use of Biologic Therapy in Inflammatory Bowel Disease

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Abstract

Background: The role of *Mycobacterium avium paratuberculosis* [MAP] in inflammatory bowel disease [IBD], especially Crohn's disease [CD] is controversial due conflicting results and lack of reproducibility and standardised tests. The current study focuses on the role of MAP in disease progression and genetic susceptibility, as MAP is likely one of many factors involved in the complex pathogenesis of IBD, potentially affecting a subgroup depending on genetic susceptibility.
Methods: Serum from 812 patients was evaluated with seven immunoglobulin [Ig] isotype-specific serology tests assessing humoral response to three different MAP antigens. For each of these in total 21 tests, the intra-assay and inter-assay coefficients were used to evaluate test accuracy. Reliable assays were subsequently analysed in relation to disease characteristics and need for biologic therapy/surgery. Genome-wide genotyping was available for all participants. Genetic determinants of humoral response to MAP antigens were evaluated using genome-wide association analysis and polygenic risk scores [PRS].
Results: High IgA or IgM response to MAP2609 was associated with increased use of biologic therapy in CD and ulcerative colitis [UC] [odds ratios 2.69; 95% confidence interval 1.44–5.01; and 2.60, 1.46–4.64, respectively]. No associations were seen for risk of surgery [*p*-values > 0.29]. We

could not identify genetic determinants nor polygenic risk scores for MAP response with genome-wide significance.
Conclusions: Extensive assays for serological response to MAP were evaluated using stringent criteria for reliability. Increased IgA and IgM response to MAP antigens was seen in patients exposed to biologic therapy, but no genetic determinants underlying this humoral response were found.

Key Words: *Mycobacterium avium paratuberculosis*; nflammatory bowel disease; disease progression; isotype-specific testing; Crohn's disease; genetics

1. Introduction

Inflammatory bowel disease [IBD], consisting of ulcerative colitis [UC] and Crohn's disease [CD], is a chronic relapsing immune-mediated disease characterised by inflammation and ulceration of the gut mucosa.[1,2] Within its complex aetiology, consisting of an interplay between genetic susceptibility, gut microbiota and environmental exposures, a causative role as environmental trigger has been hypothesised for the *Mycobacterium aviumparatuberculosis* [MAP] for CD repeatedly in past decades.[3–5]

MAP is an intracellular parasitic mycobacterium causing Johne's disease [JD], a disease characterised by chronic granulomatous inflammation primarily of the ileum in several mammalian species, in particular ruminant species [eg, cattle, sheep, goats].[6] Previous studies supportive of a role of MAP in CD have shown the similarities between JD and CD, including clinical manifestations of general symptoms such as diarrhoea and weight loss. Also comparable epidemiological findings, such as the rising disease incidence and symptoms often occurring after a long incubation pattern within a pattern of familial occurrence, were shown. Finally, histological findings overlap, including transmural, diffuse granulomatous inflammation of the ileocaecal region as also shown in CD.[7,8]

However, although these similarities might suggest a causative role of MAP in CD, it is also possible these similarities are coincidental, a position that is supported by the fact that previous studies include only small numbers of patients without blinding of samples in laboratories and with technical difficulties of MAP detection.[9,10] As culturing of MAP was proven largely unsuccessful despite numerous attempts, focus shifted to polymerase chain reactions [PCR] and enzyme-linked immune-sorbent assay [ELISA] techniques.[8] A meta-analysis revealed that patients with CD more often had PCR-detectable MAP DNA in intestinal biopsies compared with patients with UC or healthy controls. In addition, patients with CD more often had a detectable humoral response to MAP in serum using ELISA techniques.[11]However, reliability and reproducibility of the used tests were often not shown.[12] Applied methodologies differ between studies, and primarily promising results are often not validated in follow-up studies.[12] Also, when PCR and ELISA were applied to test for MAP in the same individuals, concordance in MAP detection between both techniques was rather low.[8] PCR, focused on detection of the MAP-specific IS900 insertion element in tissue samples of intestinal mucosa, has shown inconsistency when multiple samples per patients were tested, possibly due to non-homogeneous distributions of [microscopic] lesions in the tissue increasing chances of detection when multiple samples are tested.[9] For serology testing, most studies aim to detect humoral response using protein G conjugates, which allows for a general identification of immunoglobulin [Ig] G, and only two studies investigated antigen-specific Ig-isotypes [A, M, and G] in detail. Using these Ig-isotypes to detect MAP in small sample sizes, no differences were seen between cases or controls, nor in disease progression.[13–15]

Finally, in line with observation that only 10–15% of infected cattle eventually develop JD, genetic factors have been shown to be involved in JD susceptibility.[16] Therefore, it was previously hypothesised that MAP might only be associated with CD in those genetically susceptible, forming one of many hits involved in IBD pathogenesis. Previous studies showed no association of MAP detection with *NOD2* mutations, known for their role in immunological response to mycobacteria as well as CD susceptibility, but no further studies focusing on the genetic factors predisposing to MAP detection in humans have been executed.[17–19]

In this study, we use extensive assays for serological response to MAP using stringent validation standards for test accuracy, and we evaluate the role of MAP status in the course of IBD. Furthermore, we aim to identify both single genetic determinants or combined polygenic risk scores for MAP detection in patients with IBD.

2. Methods

2.1. Study population

We performed a cross-sectional study within the longitudinal 1000IBD cohort of the University Medical Center Groningen [UMCG], a tertiary referral centre in The Netherlands.[20] After enrolment, patients in the 1000IBD cohort are prospectively followed, and detailed information is collected such as clinical characteristics and molecular data, as described in detail elsewhere.[20] For a subset of patients, data concerning anti-*Saccharomyces cerevisiae* antibody [ASCA] and anti-neutrophil cytoplasmic antibody [ANCA] were available as part of this cohort. Also, serum samples of 50 healthy controls were included within the process of test development, to determine a baseline response to MAP antigens in non-IBD serum, as well as to measure a-specific background. The primary outcome measures in this study were determined to be the ever need for IBD-related surgical intervention and the ever need for biologic therapy [infliximab, adalimumab, golimumab, vedolizumab, and/or ustekinumab].

2.2. MAP detection

All patients within the 1000IBD cohort were screened for availability of serum samples and intestinal mucosal biopsies, all stored at -80° Celsius in our biobank (PSI-UMCG [IRB no 08/279]). Samples were selected cross-sectional, without previous knowledge of the primary outcome measures of this study. Two different laboratory methodologies for MAP antibody detection were used by laboratory staff blinded to patient characteristics: 1] PCR of biopsies, performed at the UMCG; and 2] ELISA of serum using immunoglobulin isotype-specific tests, performed at the Department of Bacteriology

and Epidemiology, Wageningen Bioveterinary Research, Lelystad, The Netherlands.

2.2.1. ELISA using immunoglobulin isotype specific tests

In total, 812 patients had serum samples available. Antigen-specific antibody responses to three MAP antigens in serum were determined using an indirect ELISA format for seven immunoglobulin [Ig] isotypes; IgA, IgE, IgM, IgG1-4 [Supplementary Methods, available as Supplementary data at *ECCO-JCC* online]. The selection and production of the *E. coli* recombinant MAP antigens [MAP0210c, MAP2942c, and MAP2609] have been described previously in detail.[21,22] All 21 different ELISA tests were performed in duplicate for each patient. Results were expressed as sample/positive ratios [S/P], calculated as the blank corrected optical density [OD] value of the sample divided by the blank corrected OD value of the antigen- and isotype-specific positive controls per plate.

2.2.2. PCR

In addition, 18 patients with paired biopsies from inflamed and non-inflamed ileal or colonic regions were randomly selected. Biopsies were taken at random during disease course. At time of biopsies, all patients were previously exposed to steroids, while 8 patients were previously exposed to biological therapy. PCR for the MAP-unique IS900 insertion fragment was performed as previously described [Supplementary Methods].[23]

2.3. Genetic data

All patients were genotyped using the Global Screening Array [Infinium Global Screening Array, Illumina, San Diego, CA, USA; Supplementary Methods]. In short, the Global Screening Array is a genotyping platform including over 700 000 genetic variants and comprises a multi-ethnic genome-wide backbone combined with content derived from exome-sequencing studies and meta-analyses of several phenotype-specific consortia. Extensive pre-imputation quality control was performed on the genotype data [SupplementaryMethods] and, after pre-phasing with the Eagle2 algorithm, genetic data were imputed to the Haplotype Reference Consortium reference panel using the Michigan Imputation server.[24] After post-imputation quality control measurements were performed, 5 390 911 genetic variants with a minor allele frequency greater than 5% remained. To limit bias from population stratification, only patients with genetic data clustering with individuals from European ancestry were included.

2.4. Statistical analysis

2.4.1. ELISA test accuracy

First, we calculated the intra-assay coefficient of variation and the inter-assay coefficient of variation to evaluate test accuracy.[25] For the intra-assay coefficient of variation, a within-test variation of < 10% was accepted to be accurate. For the inter-assay coefficient of variation, a between-test variation of < 20% was accepted to be accurate. Tests that did not meet these criteria were excluded from further analyses. Next, the percent differences in the S/P between duplicate tests of each isotype-antigen combination were evaluated for each patient. If the difference between duplicates exceeded 10%, patients were excluded from further analyses.

2.4.2. Baseline comparison of clinical data

S/P values of antigen-specific Ig isotype responses were analysed as continuous variables as well as in quartiles, for which cut-offs were based on patients without ever need of biologic therapy or IBD-related surgery. By using the continuous S/P values instead of cut-offs based on controls, potential significant differences highlight relative changes in response to MAP antigens by specific subgroups within this IBD cohort. If needed, logarithmic transformation was used to obtain normal distributed variables. We compared baseline characteristics across MAP antigen-specific isotype responses using univariate testing. For categorical variables, chi square tests were used and for continuous variables, one-way analysis of variance [ANOVA] tests were used. To assess the relation between MAP and the ever need for biologic therapy or IBD-related surgery, we performed logistic regression modelling, adjusting for the potential confounding effect of sex, age [in years], smoking status [current, former, never], and disease duration [in years]. Model assumptions were met. All p-values were two-sided and p-values < 0.05 were considered significant. Statistical analyses were performed using SPSS statistical software package version 23 [SPSS Inc., Chicago, IL, USA].

2.4.3. Genome-wide association analyses

Genome-wide association studies [GWAS] for presence of MAP [continuous data of all isotypes analysed separately] were performed in PLINK [v.1.9], using linear regression models with age and sex as covariates. Associations between genetic variants and MAP with p-values < 5.0×10^{-8} were considered significant.

2.4.4. Polygenetic risk scores

Summary statistics for both CD and UC GWAS were obtained from publicly available repositories and were used as 'base' data to calculate polygenetic risk scores [PRS].[26] Using PRSice2 software, PRS were calculated for both CD and UC, separately [Supplementary Methods].[27] PRS-phenotype associations with an empirical p-value < 0.05 were considered significant.

2.4.5. Ethical considerations

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the UMCG human research committee. Ethical approval for this study was granted in 2017 [no. 2017/473], as well as for the PSI UMCG biobank in 2008 [no. 2008/279]. Written informed consent was obtained for all participants in the Biobank.

3. Results

In Table 1, baseline characteristics of the study cohort are shown. There were no significant differences between patients and controls concerning age or sex.

3.1. ELISA test reliability

ELISA isotype specific tests were performed for 812 patients with IBD: 430 [50.8%] patients with CD, 372 [43.9%] with UC, and 45 [5.3%] IBD-unclassified. As shown in Supplementary Table 1, available as Supplementary data at *ECCO-JCC* online, only four tests showed interpretable serological responses and met set standards for test reliability: IgA-MAP0210c, IgA-MAP2609, IgM-MAP0210c, and IgM-MAP2609. The remaining test results indicated no isotype-specific serological responses to the MAP antigens in patients and controls. Next, in tests which met the overall reliability criteria, patients with a difference between duplicates > 10% were excluded, leaving a sample size of 440 [54.2%] for IgA-MAP0210c, 348 [42.9%] for MAPIgA-2609, 306 [37.7%] for IgM-MAP0210c, and 365 [45.0%] for IgM-MAP2609.

Table 1. Baseline characteristics of study cohort.

	IBD		Controls		
	N = 812		*N* = 50		*p*-value:
Female sex, *n* [%]	460	[56.7]	26	[52.0]	0.52
Age, mean [SD]	48.2	[15.2]	54.0	[9.9]	0.38
IBD subtype					
Crohn's disease, *n* [%]	411	[50.6]			
Ulcerative colitis, *n* [%]	358	[44.1]			
IBD-undetermined, *n* [%]	43	[5.3]			

	Crohn's disease					Ulcerative colitis				
	No TNFα		TNFα use			TNFα use		No TNFα		
	N = 188		*N* = 218		*p*-value	*N* = 296		*N*= 62		*p*-value
Female sex, n [%]	116	[44.6]	114	[55.4]	0.362	147	[82.6]	31	[17.4]	0.823
Age, mean [SD]	49.5	[15.8]	43.8	[14.2]	1.58×10^{-4}	50.6	[14.8]	47.3	[14.7]	0.112
Age at diagnosis, *n* [%]										
≤16 years old	24	[36.9]	41	[63.1]	0.051	33	[86.8]	5	[13.2]	0.773
17–40 years old	120	[45.8]	142	[54.2]		184	[82.1]	40	[17.9]	
>40 years old	44	[57.1]	33	[42.9]		79	[82.3]	17	[17.7]	
Smoking status; ever, *n* [%]	120	[46.5]	138	[53.5]	0.723					
Disease duration, mean [SD]	19.0	[11.8]	16.8	[9.2]	0.034	18.1	[10.7]	15.4	[9.9]	0.066

IBD, inflammatory bowel disease; SD, standard deviation; TNF, tumour necrosis factor.

3.2. ELISA and clinical characteristics

When compared with 50 healthy controls, patients showed a significantly increased humoral response to all four tests [all p-values < 0.032, Supplementary Figure 1, available as Supplementary data at *ECCO-JCC* online]. While patients with CD had a small but significant higher response to log-transformed IgA-MAP0210c (mean -0.22 [standard deviation 0.45] versus 0.27 [0.41], p-value 0.016) and IgM-MAP0210c (-0.09 [0.28] versus -0.14 [0.31], p-value 0.023) compared with patients with UC, no differences were seen between subtypes of IBD when measurements were compared based on quartiles [p-values > 0.06], as shown in Table 2. Next, clinical outcomes were compared across quartiles of each isotype-specific test. IgA-MAP2609 was associated with a higher age [p-value 0.006] and disease extent in UC [p-value 0.003]. Also, patients in higher quartiles of IgA-MP2609 were more often exposed to biologic therapy [p-value 0.013] although no association with the ever need for surgical intervention was shown [p-value 0.287]. No differences were seen between IgA-MAP0210c quartiles [p-values > 0.05] [Table 3]. IgM-MAP0210c was associated with smoking status, showing more ever smokers in Q1 than other quartiles [p-value 0.016]. As with IgA-MAP2609, a higher rate of the ever need for biologic therapy was shown for IgM-MAP0210c [p-value 0.041] as well as for IgM-MAP2609 [p-value 2.64×10^{-4}] [Table 4].

3.3. MAP and use of biologic therapy

To evaluate the association of MAP-specific serological responses with use of biologic therapy more specifically, we used logistic regression modelling. As shown in Table 5, a significant, risk-increasing association was seen for three out of the four isotype-specific tests for IBD. When compared with the lowest quartile, patients in the highest quartile of IgA-MAP2609 had a 2.69-fold [95% confidence interval 1.44–5.01] higher chance of ever using biologic therapy, comparable to the 2.60-fold [95%CI 1.46–4.64] risk increase shown for IgM-MAP2609. Findings were similar when continuous variables were analysed [Table 5]. Analyses evaluating CD and UC separately were limited by sample size, whereas similar trends were seen as for IBD in general [Supplementary Table 2a and b, available as Supplementary data at *ECCO-JCC* online]. For UC, only high IgA response to MAP2609 was associated with use of biologic therapy [5.61;1.14–27.75]. High response to MAP in three out of the four tests were associated with use of biologic therapy in CD, most clear for IgM response to MAP2609 [2.92;1.31–6.51]. For a small subset of 13 patients [CD n = 9, UC n = 3; IBD-U n = 1], serum samples tested were obtained before start of anti-tumour necrosis factor [TNF] treatment. When timing of biologic use was taken into account, separating anti-TNF treatment started after sampling [n = 13] from use before sampling [n = 285], differences with never users were only seen in the latter group [Supplementary Figure 2, available as Supplementary data at *ECCO-JCC* online]. Finally, different types of biologic treatment were evaluated. Of all patients using biologic treatment in this cohort, infliximab [n = 242, 83.4%] and adalimumab [n = 133, 45.9%] were mostly prescribed, with only a limited number of patients using vedolizumab [n = 26, 9.0%] or ustekinumab [n = 22, 7.6%]. When MAP serologic response was analysed for subtypes of biologic treatment, an increased response was seen for IgA-MAP2609, IgM-MAP2609, and IgM-MAP0210c [p-values < 0.003], comparable to associations seen when all biologic are combined [Supplementary Table 3, available as Supplementary data at *ECCO-JCC* online].

Table 2. Isotype-specific MAP serology tests comparing CD and UC.

		IgA—MAP0210c[a]					IgA—MAP2609[a]				
		CD		UC		p-value	CD		UC		p-value
Continuous	mean, SD	-0.16	0.41	-0.24		**0.050**	-0.16	0.33	-0.16	0.32	0.898
Quartiles											
Q1	n, %	27	15.4	46	25.3	0.145	5622.4		36	18.3	0.310
Q2	n, %	46	26.3	41	22.5		4819.2		49	24.9	
Q3	n, %	51	29.1	49	26.9		6024.0		53	26.9	
Q4	n, %	51	29.1	46	25.3		8634.4		59	29.9	
		IgM—MAP0210c[a]					IgM—MAP2609				
		CD		UC		p-value	CD		UC		p-value
Continuous	mean, SD	-0.08	0.28	-0.11	0.32	0.178	1.19	0.56	1.10	0.53	0.095
Quartiles											
Q1	n, %	58	22.1	60	26.9	0.569	4720.7		50	25.4	0.069
Q2	n, %	59	22.4	51	22.9		5323.3		46	23.4	
Q3	n, %	68	25.9	49	22.0		4318.9		50	25.4	
Q4	n, %	78	29.7	63	28.3		8437.0		51	25.9	

Ig,s immunoglobulin; CD,s Crohn's disease; UC, ulcerative colitis; SD, standard deviation.[a]Variables log-transformed for obtaining a normal distribution.

Table 3. Characteristics of participants according to immunoglobulin A response to MAP.

	IgA—MAP0210c				
	Q1:	Q2:	Q3:	Q4:	*p*-value
	n = 77	*n* = 89	*n* = 105	*n* = 101	
Female sex, *n* [%]	44 [57.1]	58 [65.2]	62 [59.0]	61 [60.4]	0.737
Age, mean [SD]	45.1 [16.1]	47.2 [14.8]	46.6 [15.6]	48.9 [14.9]	0.082
Age at diagnosis, *n* [%]					
≤16 years old	14 [18.2]	12 [13.5]	15 [14.3]	9 [8.9]	0.255
17–40 years old	53 [68.8]	58 [65.2]	62 [59.0]	69 [68.3]	
>40 years old	10 [13.0]	19 [21.3]	28 [26.7]	23 [22.8]	
Smoking status; ever, *n* [%]	34 [45.9]	52 [63.4]	65 [64.4]	71 [73.2]	0.004
Disease duration, mean [SD]	17.8 [10.9]	17.0 [10.1]	16.3 [10.7]	17.2 [10.6]	0.366
Disease location [CD], *n* [%]					
Ileum	6 [23.1]	9 [19.6]	15 [30.6]	16 [32.7]	0.465
Colon	5 [19.2]	11 [23.9]	13 [26.5]	15 [30.6]	
Ileum and colon	15 [57.7]	26 [56.5]	21 [42.9]	18 [36.7]	
Disease extent [UC], *n* [%]					
Proctitis	3 [6.8]	6 [15.8]	4 [8.9]	5 [11.4]	0.889
Left-sided colitis	15 [34.1]	10 [26.3]	13 [28.9]	14 [31.8]	
Extensive colitis	26 [59.1]	22 [57.9]	28 [62.2]	25 [56.8]	
Ever need for:					
Biologic treatment, *n* [%]	26 [33.8]	31 [34.8]	47 [45.6]	39 [39.0]	0.329
Surgical intervention, *n* [%]	20 [26.0]	35 [39.3]	34 [32.4]	31 [30.7]	0.316
	IgA—MAP2609				
	Q1:	Q2:	Q3:	Q4:	*p*-value
	n = 94	*n* = 102	*n* = 119	*n* = 149	
Female sex, *n* [%]	54 [57.4]	60 [58.0]	68 [57.1]	77 [51.7]	0.661
Age, mean [SD]	45.8 [16.9]	46.1 [13.7]	48.0 [15.2]	51.4 [14.6]	0.006
Age at diagnosis, *n* [%]					
≤16 years old	13 [13.8]	12 [11.8]	17 [14.3]	11 [7.4]	0.408
17–40 years old	61 [64.9]	72 [70.6]	74 [62.2]	98 [66.2]	
>40 years old	20 [21.3]	18 [17.6]	28 [23.5]	39 [26.4]	
Smoking status; ever, *n* [%]	46 [55.4]	57 [58.8]	71 [62.3]	98 [67.1]	0.311
Disease duration, mean [SD]	16.1 [9.9]	17.1 [10.5]	16.5 [10.1]	18.6 [10.7]	0.408
Disease location [CD], *n* [%]					
Ileum	19 [35.2]	19 [39.6]	13 [22.0]	24 [28.2]	0.333
Colon	11 [20.4]	7 [14.6]	14 [23.7]	24 [28.2]	
Ileum and colon	24 [44.4]	22 [45.8]	32 [54.2]	37 [43.5]	
Disease extent [UC], *n* [%]					
Proctitis	0 [0.0]	4 [9.3]	11 [22.4]	4 [6.8]	0.003
Left-sided colitis	17 [48.6]	9 [20.9]	18 [36.7]	23 [39.0]	
Extensive colitis	18 [51.4]	30 [69.8]	20 [40.8]	32 [54.2]	
Ever need for:					
Biologic treatment, *n* [%]	24 [26.1]	34 [33.3]	51 [42.9]	67 [45.0]	0.013
Surgical intervention, *n* [%]	33 [35.1]	30 [29.4]	40 [33.6]	61 [40.9]	0.287

Ig, immunoglobulin; SD, standard deviation; CD, Crohn's disease; UC, ulcerative colitis.

3.4. MAP and response to ASCA/ANCAs

To evaluate the hypothesis that the presence of an autoimmune disease such as IBD causes a falsely increased serological response to MAP, potential associations between this serological response and ASCA and ANCA positivity were explored. ANCA and ANCA status was available in a subset of 367 patients [42.6%]. Although no association was seen for ANCA and MAP serology, patients with a positive ASCA test were shown to have a higher IgA response to MAP0210c and MAP2609 [*p*-values < 0.02] as shown in Supplementary Table 4, available as Supplementary data at *ECCO-JCC* online. Furthermore, multivariate logistic regression showed that ASCA was associated with an increased risk of the use of biologics [1.63; 1.02–2.60] and need for surgery [2.23; 1.38–3.61] in patients with IBD [Supplementary Table 5, available as Supplementary data at *ECCO-JCC* online]. When ASCA was added to the multivariate regression model associating MAP with the use of biologic treatment however, the risk-increasing association of IgA-MAP2609 [3.22; 1.38–7.52] and IgM-MAP [2.30; 1.06–4.97] remained significant. Similar findings were seen when the multivariate adjusted model was corrected for ASCA as well as ANCA [Table 5].

3.5. Genetic determinants of humoral response to MAP

Finally, we interrogated genetic determinants of the humoral response to MAP infection in patients with IBD. GWAS for the four different ELISA tests did not yield associations exceeding

Table 4. Characteristics of participants according to immunoglobulin M response to MAP.

	IgM—MAP0210c				
	Q1:	Q2:	Q3:	Q4:	*p*-value
	n = 127	*n* = 112	*n* = 123	*n* = 144	
Female sex, *n* [%]	67 [52.8]	62 [55.4]	71 [57.7]	92 [63.9]	0.286
Age, mean [SD]	52.2 [15.6]	45.7 [14.9]	46.3 [14.2]	45.9 [15.3]	0.948
Age at diagnosis, *n* [%]					
≤16 years old	13 [10.2]	19 [17.0]	21 [17.2]	20 [13.9]	0.328
17–40 years old	80 [63.0]	67 [59.8]	82 [67.2]	92 [63.9]	
>40 years old	34 [26.8]	26 [23.2]	19 [15.6]	32 [22.2]	
Smoking status; ever, *n* [%]	86 [70.5]	60 [54.1]	58 [51.8]	80 [57.1]	0.016
Disease duration, mean [SD]	18.9 [12.1]	16.1 [10.4]	17.6 [9.1]	16.2 [9.5]	0.197
Disease location [CD], *n* [%]					
Ileum	15 [26.3]	19 [32.8]	23 [34.3]	21 [26.9]	0.927
Colon	12 [21.1]	12 [20.7]	15 [22.4]	19 [24.4]	
Ileum and colon	30 [52.6]	27 [46.6]	29 [43.3]	38 [48.7]	
Disease extent [UC], n [%]					
Proctitis	3 [5.4]	6 [12.5]	5 [10.6]	9 [15.5]	0.248
Left-sided colitis	26 [46.4]	16 [33.3]	12 [25.5]	17 [29.3]	
Extensive colitis	27 [48.2]	26 [54.2]	30 [63.8]	32 [55.2]	
Ever need for:					
Biologic treatment, *n* [%]	43 [33.9]	43 [38.4]	43 [35.5]	70 [49.3]	**0.041**
Surgical intervention, *n* [%]	52 [41.3]	34 [30.4]	44 [35.8]	49 [34.0]	0.352
	IgM—MAP2609				
	Q1:	Q2:	Q3:	Q4:	*p*-value
	n = 101	*n* = 105	*n* = 97	*n* = 144	
Female sex, *n* [%]	47 [46.5]	67 [63.8]	51 [52.6]	92 [63.9]	0.018
Age, mean [SD]	51.7 [15.0]	47.9 [15.3]	46.0 [14.2]	44.8 [13.7]	0.269
Age at diagnosis, *n* [%]					
≤16 years old	16 [15.8]	13 [12.4]	12 [12.4]	21 [14.6]	0.450
17–40 years old	58 [57.4]	73 [69.5]	69 [71.1]	97 [67.4]	
>40 years old	27 [26.7]	19 [18.1]	16 [16.5]	26 [18.1]	
Smoking status; ever, *n* [%]	53 [58.2]	62 [63.3]	53 [55.8]	81 [58.3]	0.756
Disease duration, mean [SD]	20.4 [11.5]	17.1 [10.1]	17.5 [9.2]	16.4 [9.2]	0.384
Disease location [CD], *n* [%]					
Ileum	12 [26.1]	18 [34.6]	12 [27.9]	18 [21.7]	0.684
Colon	11 [23.9]	13 [25.0]	9 [20.9]	18 [21.7]	
Ileum and colon	23 [50.0]	21 [40.4]	22 [51.2]	47 [56.6]	
Disease extent [UC], *n* [%]					
Proctitis	6 [12.5]	4 [10.0]	5 [10.9]	4 [8.7]	0.777
Left-sided colitis	19 [39.6]	11 [27.5]	12 [26.1]	16 [34.8]	
Extensive colitis	23 [47.9]	25 [62.5]	29 [63.0]	26 [56.5]	
Ever need for:					
Biologic treatment, *n* [%]	28 [28.0]	38 [36.2]	34 [35.1]	77 [53.8]	2.64×10^{-4}
Surgical intervention, *n* [%]	41 [40.6]	37 [35.2]	31 [32.0]	55 [38.2]	0.608

Ig, immunoglobulin, SD indicates standard deviation; CD,s Crohn's disease; UC, ulcerative colitis.

the genome-wide significance threshold [all *p*-values > 5.0×10^{-8}] [Supplementary Tables 2–5]. We constructed a total of eight PRS models [PRS targeted on phenotypes]. Table 6 gives the estimates from all GRS linear regression analyses. As displayed in Table 6, no GRS models remained significantly associated with MAP isotypes after 10 000 permutations [all empirical *p*-values > 0.20].
<<Table 6 near here>>

3.6. PCR

We performed MAP-specific IS900 PCR in a small sample of 18 patients with IBD for whom paired inflamed and non-inflamed mucosal biopsies were available, including six patients with ileal CD, six with colonic CD, and six with UC, as this method was often used in previous studies.[28] Biopsies were analysed for the presence of IS900 [413bp]. We could not detect MAP DNA in any of the samples tested [Supplementary Figure 1].

4. Discussion

In this study, we have performed extensive serological assays for humoral response to MAP in patients with IBD. After evaluation of seven isotype-specific ELISA tests to three different MAP antigens, solid IgA and IgM responses were observed against two antigens. These four assays were deemed reliable, based on stringent criteria. High response for either two of these four tests was associated with previous treatment with biologic therapy in patients with IBD, and

Table 5. Risk of biologic therapy based on MAP serology in patients with inflammatory bowel disease.

	IgA—MAP0210c						
	Q1:	Q2:	Q3:	Q4:	p_{trend}	Continuous	*p*-value
	n = 77	*n* = 89	*n* = 105	*n* = 101		*n* = 464	
Median	-0.670	-0.405	-0.151	0.221		-0.293	
Cases [*n*]	26	31	47	39		143	
Unadj. OR [95% CI]	1.0 ref.	1.05 [0.55–1.99]	1.65 [0.89–3.03]	1.25 [0.68–2.33]	0.265	1.58 [0.94–2.65]	0.083
MV-adj. model 1 OR [95% CI]	1.0 ref.	1.03 [0.52–2.03]	1.61 [0.85–3.06]	1.19 [0.62–2.30]	0.368	1.59 [0.92–2.75]	0.098
MV-adj. model 2 OR [95% CI]	1.0 ref.	1.20 [0.44–3.26]	**3.10 [1.20–8.06]**	1.13 [0.44–2.93]	0.462	1.40 [0.63–3.09]	0.408
	IgA—MAP2609						
	Q1:	Q2:	Q3:	Q4:	p_{trend}	Continuous	*p*-value
	n = 94	*n* = 102	*n* = 119	*n* =149		*n* = 462	
Median	-0.556	-0.328	-0.140	0.157		-0.179	
Cases [*n*]	24	34	51	67		176	
Unadj. OR [95% CI]	1.0 ref.	1.42 [0.76–2.64]	2.13 [1.18–3.83]	2.32 [1.31–4.08]	**0.002**	2.84 [1.56–5.17]	0.001
MV- =adj. model 1 OR [95% CI]	1.0 ref.	1.53 [0.79–2.97]	2.43 [1.29–4.60]	2.69 [1.44–5.01]	0.001	3.29 [1.71–6.31]	3.49×10^{-4}
MV-adj. model 2 OR [95% CI]	1.0 ref.	1.18 [0.46–3.02]	3.23 [1.33–7.87]	3.23 [1.38–7.56]	0.002	3.78 [1.49–9.57]	0.005
	IgM—MAP0210c						
	Q1:	Q2:	Q3:	Q4:	p_{trend}	Continuous	*p*-value
	n = 127	*n* = 112	*n* = 123	*n* = 144		*n* = 506	
Median	-0.453	-0.193	-0.004	0.222		-0.081	
Cases [*n*]	43	43	43	70		199	
Unadj. OR [95% CI]	1.0 ref.	1.22 [0.72–2.07]	1.08 [0.64–1.82]	1.90 [1.16–3.11]	0.018	2.44 [1.33–4.49]	0.004
MV-adj. model 1 OR [95% CI]	1.0 ref.	0.99 [0.57–2.07]	0.91 [0.52–1.60]	1.56 [0.93–2.62]	0.095	2.09 [1.10–3.95]	0.024
MV-adj. model 2 OR [95% CI]	1.0 ref.	0.99 [0.45–2.17]	0.86 [0.40–1.83]	1.13 [0.55–2.30]	0.815	1.52 [0.67–3.42]	0.314
	IgM—MAP2609						
	Q1:	Q2:	Q3:	Q4:	p_{trend}	Continuous	*p*-value
	n = 101	*n* = 105	*n* = 97	*n* = 144		*n* = 447	
Median	0.482	0.838	1.229	1.737		1.082	
Cases [*n*]	28	38	34	77		177	
Unadj. OR [95% CI]	1.0 ref.	1.46 [0.81–2.63]	1.39 [0.76–2.54]	3.00 [1.74–8.18]	7.80×10^{-5}	1.99 [1.39–2.83]	1.52 × 10-4
MV-adj. model 1 OR [95% CI]	1.0 ref.	1.29 [0.69–2.41]	1.25 [0.66–2.34]	2.60 [1.46–4.64]	0.001	1.80 [1.24–2.62]	0.002
MV-adj. model 2 OR [95% CI]	1.0 ref.	1.20 [0.52–2.78]	1.05 [0.43–2.58]	2.26 [1.04–4.91]	0.040	1.64 [0.97–2.79]	0.066

MV-model 1: adjusted for sex, age [in years], history of smoking [current, former, never] and disease duration [in years]. MV-model 2: adjustments from model 1, ASCA status and ANCA status.
Ig, immunoglobulin; OR, odds ratio; CI, confidence interval; MV, multivariable; ANCA, antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; ref. reference value.

Table 6. Overview of GRS-MAP associations.

GRS	MAP test	No. SNPs in optimal GRS	R^2	Coefficient; SE	Empirical *p*-value[a]
CD	IgA—MAP0210c	23 117	0.79%	-144; 84	0.52
	IgA—MAP2609	69 507	1.10%	-258; 112	0.20
	IgM—MAP0210c	343 804	0.46%	-427; 276	0.66
	IgM—MAP2609	317	0.60%	-10; 6	0.56
UC	IgA—MAP0210c	34 068	1.09%	248; 122	0.38
	IgA—MAP2609	4 551	0.46%	42; 28	0.80
	IgM—MAP0210c	3 638	1.14%	-57; 23	0.23
	IgM—MAP2609	2 788	0.52%	-51; 33	0.68

SNP, single nucleotide polymorphism; GRS, genetic risk score; SE, standard error; CD, Crohn's disease; UC, ulcerative colitis.
[a]Empirical *p*-value refers to *p*-value after 10 000 rounds of permutation.

also after correction for ASCA/ANCA antibodies. We could not identify genetic determinants underlying this altered humoral response.

Although multiple studies evaluated the role of MAP in the aetiology of CD, the role of MAP in disease course of IBD was studied scarcely in the past. In contrast to a previous meta-analysis, this study did not show a great difference of humoral response to MAP in CD compared with UC.[11] In contrast to previous studies using Protein G to measure a general IgG response, we observed no test reaction when IgG subtypes were evaluated separately.[13] Therefore, these tests were excluded from further analyses in the current study. It is possible that these tests show no results when no specific IgG is present in the studied population, for example when no structural isotype switch occurs, or when IgM represents recent or repeated short-lived immune responses leading to little formation of memory B cells. This discrepancy with previous studies could be the result of the decreased precision when Protein G is used, combined with the small study populations, increasing risk of random findings. In line with previous studies however, no associations for humoral response to MAP antigens measured by isotype-specific ELISA and disease location or need for surgical resection were observed.[15] Unfortunately, this previous study was performed before the introduction of biologic therapy in treatment of IBD and was limited by a small sample size. To our knowledge, ours is the first study to show an association between the use of biologic therapy, mostly anti-tumour necrosis factor α [anti-TNFα], and humoral response to MAP. In the past, TNFα was proven to be of importance in the immunological response to tuberculosis [TB], leading to the consensus on screening for TB before the start of anti-TNFα therapy.[29,30] The role of Pregnane X receptor [PXR] activation might also explain these findings, as previous studies have showed that, in patients with severe IBD, compensatory PXR activation counteracts inflammation, while also being shown to augment mycobacterium survival, potentially explaining the high serologic response shown in this subset of patients.[31,32]

However, the current study includes only a small number of patients who started biologic treatment after sampling for the current study. Therefore, it remains unclear whether the increased humoral response as shown is present before the start of biological treatment. Longitudinal measurements including sampling before and after start of biologic treatment might shed light on whether the altered humoral response increases risk of starting biologic treatment. Also, these measurements would allow the evaluation of whether the use of biologic treatment either alters the measured humoral response, or increases risk of infection with MAP, as well as the possible differing effect of different types of biologic treatments used.

In previous studies, evaluation of humoral response through ELISA tests was often executed through the use of Protein G conjugates to measure IgG antibody response.[13] In the current study, seven different Ig isotypes are tested. While clear signals are shown for IgA and IgM, no signal was observed for the isotype-specific tests of IgG1-4, indicating no evident added value of Protein G conjugate in this context. Our data rather suggest a difference in local response to repeated MAP exposure in a subgroup of patients, as shown by the associations described for IgA, known for its role in mucosal membranes. Identification of this subgroup might be of interest in the context of using anti-mycobacterial drugs and/or [anti-TNF] biologics. Whereas a previous randomised controlled trial found only a potential short-term but no prolonged beneficial effect of 2 years of anti-mycobacterial treatment, baseline testing for MAP was not performed.[33] It is therefore possible that the evaluated treatment is only efficient in those with an altered humoral response, and is no longer observed when efficacy within the entire IBD population is evaluated.

Also, one might hypothesise that the serological response to MAP in patients with IBD is falsely elevated due to an decreased barrier function and increased exposure and immune-reactivity, especially in IBD patients with more severe disease that needed biologic treatment. This hypothesis is supported by our finding that also ASCA-positive patients have an increased IgA response to MAP antibodies, suggesting that at least part of the increased serological response shown to MAP is due to this increased exposure and stimulation of the immune system. However, when multivariate logistic regression models associating MAP response with biologic use were corrected for the presence of autoantibodies ASCA and ANCA, the risk-increasing results remained similar, indicating that it is possible that, whereas overall immune reactivity is involved in the serological response to MAP, there could still be a MAP-specific association with use of biologic therapy. Future studies comparing these findings in patients with IBD with patients with other autoimmune diseases previously associated with MAP, such as multiple sclerosis and diabetes mellitus type 1, would potentially further our knowledge on whether the found association is IBD-specific.[34,35]

Finally, we evaluated possible genetic determinants underlying this altered humoral response to MAP, using two distinct approaches. Using genome-wide association studies, we could not identify genome-wide significant associations. Given the limited number of samples in our study and the potential small effect sizes of individual genetic variants, we then constructed genetic risk scores of CD and UC. Genetic risk scores aggregate the effects of thousands of trait-associated genetic variants discovered in GWAS. By combining the effects of many genetic variants with small effects sizes, genetic risk scores have the potential to uncover genetic contributions to phenotypes. However, neither the composite genetic risk of CD,

nor that of UC, was significantly associated with immunoglobulin-specific humoral response to MAP.

We acknowledge several limitations to the current study. Although multiple tests aimed at MAP detection were performed for each patient, tests were all performed using one sample per patient, studying cross-sectional associations where no statements can be made concerning causal relationships. Also, we used a novel method of evaluating the humoral response of IBD patients to different MAP antigens through isotype-specific tests. Therefore, future studies in independent laboratories using similar methodologies are highly needed to confirm reliability of the current method and validate our findings.

Finally, as this study was nested within the 1000IBD cohort, including only patients treated at a tertiary referral hospital, it is possible that severe phenotypes of disease are over-represented in the current study. However, the 1000IBD cohort also provides the great advantage of including only patients with a confirmed IBD diagnosis and strict follow-up by IBD specialists in line with current guidelines, hindering misclassification due to the use of ICD codes. Another strength of the current study is the use of different methodological approaches of MAP detection. In previous studies, repeatability of promising findings in independent laboratories often failed.[12] Strikingly, none of these previous studies have shown measures of test accuracy and repeatability, therefore leaving room to doubt reliability of published results.[7,36] By using stringent measures for test accuracy, this is the first study to use a quality check of MAP serology data before evaluating its association with disease.

In this study, we used extensive high-quality methodologies to measure humoral response to MAP, showing an association between an increased humoral immune response use of biologic therapy, whereas no genetic determinants underlying this response were identified. The data underlying this article will be shared on reasonable request to the corresponding author.

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Conflict of Interest

RKW: unrestricted research grants from Takeda, Johnson and Johnson, Tramedico, and Ferring Pharmaceutical; consultant for Takeda Pharmaceuticals; received speaker's fees from MSD, Boston Scientific, Abbvie, and Janssen Pharmaceuticals. GD: unrestricted research grants from Abbvie and Takeda; advisory boards for Mundipharma and Pharmacosmos; received speaker's fees from Takeda and Janssen Pharmaceuticals. The other authors have no disclosures.

Author Contributions

KWJS: study design, data collection, data analysis, writing first draft of manuscript. MDV: study design, data collection, data analysis, writing first draft of manuscript. TB: data collection, critical revision of the manuscript. AD: data collection, critical revision of the manuscript. LR: data collection, critical revision of the manuscript. MCV: data collection, critical revision of the manuscript. HMvD: data collection, critical revision of the manuscript. EAMF: data collection, critical revision of the manuscript. BZA: study design, critical revision of the manuscript. CLB: study design, critical revision of the manuscript. RKW: data collection, critical revision of the manuscript. HvG: data collection, critical revision of the manuscript. APK: study design, data collection, critical revision of the manuscript. GD: study design, data collection, critical revision of the manuscript.

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Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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