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Replication of genetic variation in the *MYO9B* gene in Crohn's disease

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ABSTRACT

Various genes that may influence the intestinal barrier have been identified, including *MAGI2, PARD3*, and *MYO9B*. These genes are associated with inflammatory bowel disease (IBD) in several European studies. A total of 2,049 individuals (656 Crohn's disease [CD], 544 ulcerative colitis [UC], and 849 controls) were genotyped and association studies were performed for 1 single nucleotide polymorphism (SNP) in *MAGI2*, 1 SNP in *PARD3*, and 6 SNPs in *MYO9B*. We reported an association between 3 SNPs in *MYO9B* and ileal involvement with rs1457092 as the most significant SNP ($p = 0.0073$, odds ratio [OR] 0.69 [95% confidence interval (95% CI) 0.52–0.90]). The nonsynonymous SNP rs1545620 exhibited a p value of 0.014, OR 0.72 (95% CI 0.55–0.93). *MYO9B* was not associated with UC. *MAGI2* or *PARD3* was not associated with IBD. A 6-SNP haplotype block in *MYO9B* demonstrated association with CD and ileal CD ($p = 0.0030$ and 0.0065, respectively). These data demonstrate an association of *MYO9B* with ileal CD; however, there was no association of MAGI2 and *PARD3* with IBD. Because the direction of association of *MYO9B* in this Canadian study was not consistent with European studies, further studies are needed to elucidate the role of *MYO9B* in IBD.

1. Introduction

Independent studies have suggested that alterations in intestinal permeability are involved in the pathogenesis of inflammatory bowel disease (IBD) [1–3] as well as in other autoimmune diseases [4]. Recent animal studies indicate that in diabetes type 1 (DM type 1), celiac disease, and IBD, increased intestinal permeability was present before the onset of disease [5,6]. In individuals who are at increased risk for the development of IBD, an increased intestinal permeability also occurs in the absence of disease, suggesting that a barrier defect may lead to disease development [1,2,7,8]. Several studies support this hypothesis and point to a genetic predisposition that leads to a mucosal immune regulation defect, barrier leakage, and susceptibility to environmental triggers, including luminal bacteria and specific antigens [9]. Therefore, genes encoding tight junction (TJ) proteins could be highly relevant candidates for autoimmune diseases such as celiac disease, DM type 1, and IBD.

Interestingly, several research groups have reported associations between genetic variations in candidate TJ genes and autoimmune diseases. *MYO9B* is one of the most interesting candidate genes and is reported to be associated with celiac disease [10], DM type 1 [11,12], and IBD [13–16]. *MYO9B* at chromosome 19 encodes a single motor protein [17], which is involved in remodeling of the cytoskeleton and influences TJ assembly [18,19]. Interestingly, increased intestinal permeability *in vivo* was correlated with increased *MYO9B* gene expression in the intestinal tissue of DM type 1 patients, suggesting a link between *MYO9B* expression and intestinal permeability changes [20]. Human myosin IXB is expressed in intestinal epithelial cells [21] and animal studies revealed that overexpression of rat myosin IXB leads to actin filament–related morphologic changes in epithelial cells [22].

Three other interesting TJ candidate genes associated with IBD are *MAGI2, PARD3*, and *CDH1*, at chromosomes 7, 10, and 16, respectively [23,24]. *MAGI2* on chromosome 7 encodes the protein MAGI-2 that localizes to the TJ, where it acts as a scaffold and interacts with proteins such as the lipid phosphatase tumor suppressor phosphatase and tensin homolog [25]. Similarly, *PARD3* on chromosome 10 encodes the protein PAR-3 that regulates epithelial cell polarity and facilitates TJ formation [26].

These TJs seal the route between the intestinal epithelial cells and therefore play a role in regulating intestinal permeability. Although the exact mechanism by which the different gene variants

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Table 1
Demographic and clinical characteristics of 2281 Caucasian subjects; 754 Crohn patients (CD), 603 ulcerative colitis (UC) patients and 924 controls

Characteristics	CD (n = 754)	UC (n = 603)	Controls (n = 924)
Gender (%)			
Male	395 (52.4)	279 (46.3)	332 (35.9)
Age at diagnosis (yrs) (median, range)	16 (2–62)	23 (1–73)	NA
Young Patients (<19)	441 (58.5)	207 (34.3)	
Jewish heritage (%)			
No	590 (78.2)	498 (82.6)	856 (92.6)
Yes	156 (20.7)	104 (17.2)	68 (7.4)
Family history of IBD in 1st or 2nd degree relative (%)	175 (23.2)	105 (17.4)	3 (0.003)
Smoking history (%)			
No smoking	444 (58.9)	359 (59.5)	149 (16.1)
Current smoker	129 (17.1)	72 (11.9)	3 (0.003)
Previous smoker	40 (5.3)	113 (18.7)	0
Location (%)	57 (7.6)	NA	NA
Unknown			
L1 ileal	181 (24.0)		
L2 colonic	191 (25.3)		
L3 ileocolonic	321 (42.6)		
Disease Extent (%)			
E1 proctitis	NA	16 (2.7)	NA
E2 left sided		121 (20.1)	
E3 extensive		454 (75.3)	
Behavior (%)	366 (48.5)	NA	NA
B1 non-stricturing, non-penetrating	186 (24.7) 195 (25.9)		
B2 stricturing			
B3 penetrating			
Perianal disease			
No	527 (69.9)		
Yes	227 (30.1)		

lead to altered gut barrier is unknown, there are several lines of evidence to support that a defect in the mucosal intestinal barrier might play a pivotal role in the development of autoimmunity.

These 3 genes (*MAGI2, PARD3,* and *MYO9B*) were associated with autoimmune diseases in independent European studies [10,12–16,24,27], and we undertook a replication study in a Canadian IBD cohort.

2. Material and methods

2.1. Methods

The study cohort included 2,281 Caucasian subjects (754 Crohn's disease [CD], 603 ulcerative colitis [UC], and 924 healthy, unrelated controls). Subjects were recruited from either the Hospital for Sick Children (22%) or Mount Sinai Hospital (78%), Toronto, Canada (see Table 1 for characteristics of the cohort). All subjects had a confirmed diagnosis of IBD and fulfilled standard diagnostic criteria [28,29]. Phenotypic characterization of CD patients was based on the Montreal classification [30]. Definitions of L1 and L3 included disease within the small bowel proximal to the terminal ileum and distal to the ligament of Treitz. Study subject phenotypic information and DNA samples were obtained with institutional review board approval for IBD genetic studies at the Hospital for Sick Children and Mount Sinai Hospital in Toronto. Written informed consent was obtained from all participants.

2.2. Single nucleotide polymorphism (SNP) analysis

We performed an independent replication study of the association of *MAGI2, PARD3*, and *MYO9B* with IBD. Only SNPs associated with IBD in earlier reports were included in this replication study [13–16,24]. Because *CDH1* was already analyzed in this population, we did not investigate *CDH1* polymorphisms [23]. All IBD patients were genotyped for 1 SNP in *MAGI2* (rs6962966) [24], 1 SNP in *PARD3* (rs4379776) [24], and 6 SNPs in *MYO9B* (rs1545620, rs2305767, rs1457092, rs962917, rs2305764, and rs2279002) [13–16]. Genotyping of samples was performed using the Illumina Goldengate custom chip genotyping system (Illumina Goldengate, San Diego, CA, USA) and TaqMan (Taqman Applied Biosystems, Foster City, CA, USA) at the Centre for Applied Genomics, Hospital for Sick Children, Toronto.

2.3. Quality control and population stratification

We performed systematic quality control filtering on the raw genotyping data of the 2,281 individuals. To reduce the possibility of population stratification, we limited the analysis to Caucasian subjects. After quality control filtering, a total of 2,049 subjects (656 CD, 544 UC, and 849 controls) were used in the final analysis. The sample call rate for *MAGI2*, *PARD3,* and *MYO9B* was more than 98.51, 97.83, and 97.03%, respectively, of all cases and controls after quality control filtering. None of the SNPs exhibited departure from Hardy–Weinberg equilibrium [31]. One SNP in *MAGI2*, 1 SNP in *PARD3*, and 6 SNPs in *MYO9B* were analyzed.

2.4. Genetic analysis

Haploview (Haploview, MIT Broad Institute Cambridge, MA, USA) [32] was used to obtain LD patterns, obtaining descriptive statistics and summaries of the SNPs. In Supplementary Table 1 the pairwise linkage disequilibrium between the 6 *MYO9B* SNPs is given by the D' statistics computed with the genotype data of the 924 healthy unrelated control patients. Plink version 1.06 (PLINK MIT, Cambridge, MA, USA) [33] was applied to test for Hardy–Weinberg equilibrium [31] for each marker based on Pearson's χ^2 test. Descriptive statistics of demographic variables were generated using SAS version 9.2 (SAS Institute, Cary, NC). The Wilcoxon rank-sum test and χ^2 test were used to identify differences in demographic variables between subgroups.

2.5. Association analysis

For each SNP, association analyses were applied to detect the phenotype–genotype associations of different outcomes, such as IBD versus healthy controls (HC), CD versus HC, and UC versus HC. Logistic regression models were applied for the additive genetic model, and Pearson χ^2 tests were applied for dominant and recessive genetic models. Although we used an additive genetic model for primary analysis [34], we also explored dominant and recessive genetic models for sensitive analysis (data not shown). Throughout the report the p values are for the additive genetic model. We are aware of the risk of inflated false-positive results caused by multiple comparisons. However, the candidate markers are in high linkage disequilibrium and are not independent, so a simple Bonferroni adjustment is too conservative. Considering that the discovery stage is exploratory and hypothesis generating, all statistical tests will be two-sided with the significance level defined as 0.01. p values between 0.05 and 0.01 are defined as nominal signals. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated for the risk group compared with the referent HC group. Associations of IBD phenotype with SNP genotypes were tested by logistic regression (SAS v9.2).

2.6. Subgroup analysis

In addition to the major outcomes of comparing IBD with HC, CD with HC, and UC with HC, we performed subgroup analysis to evaluate the genetic effect in these populations. The comparisons tested included ileal only (Montreal classification L1) versus HC, any ileal (Montreal classification L1/L3) versus HC, colon only (Montreal classification L2) versus HC, colon any (Montreal classification L2/L3) versus HC, perianal disease versus HC, young (diagnosis age ≤18 years) versus HC, ileal only (Montreal classification L1) versus colon only (Montreal classification L2), young IBD pa-

tients (diagnosis age ≤18 years old) versus old IBD patients (diagnosis age >18 years old), and perianal disease versus no perianal disease [30]. Different genetic models were used to test for single marker associations between each of the subgroup comparisons. In addition, we applied multivariate analysis adjusting for factors such as Jewish heritage.

Haplotype association analysis was applied using Plink [33]. The haplotype analysis was applied separately on IBD, CD, and UC. Both omnibus analysis (overall analysis) and haplotypes-specific analysis were applied.

3. Results

We reported an association between 3 *MYO9B* SNPs and patients with ileal only involvement (Montreal classification L1). SNP rs1457092 demonstrated the strongest association ($p = 0.0073$, OR 0.69 [95% CI 0.52–0.90]). No *MYO9B* SNPs were associated with IBD patients (Table 2A), UC patients (Table 2B), or any of the subgroups of CD patients other than ileal only patients (Table 3). The nonsynonymous SNP rs1545620 demonstrated a nonsignificant p value of 0.014 (OR 0.72 [95% CI 0.55–0.93]) (Table 3A, Supplementary Table 4A).

In the other subgroups an association was observed between *MYO9B* rs2279002 and perianal disease ($p = 0.038$, OR 0.78 [95% CI 0.61–0.99], Table 3C) and between *MYO9B* rs2305767 and young age at diagnosis ($p = 0.039$, OR 1.17 [95% CI 1.01–1.37], Table 3D).

Haplotype analysis of *MYO9B* indicated that the 6-SNP haplotype block was associated with CD ($p = 0.0030$, p omnibus = 0.032; affected, 28%, vs unaffected HC, 34%) and ileal only CD ($p = 0.0065$, p omnibus = 0.13; Supplementary Table 2).

No associations were observed between *MAGI2* or *PARD3* and IBD in any of the IBD subgroups (Tables 2 and 3).

The results using multivariate analysis adjusting for Jewish heritage are consistent with the analysis without adjustment (results not shown).

4. Discussion

A recent UC genome-wide association study (GWAS) meta-analysis [3], genetic studies in CD [23,35,36], and studies of intestinal permeability of patients with CD with NOD2 polymorphisms strongly suggest that the regulation of barrier defense is important in the pathogenesis of IBD [1,37]. Initial genetic and functional studies of *MYO9B* make it an attractive gene in the pathogenesis of a number of diseases [10,18,19,38]. The results of this large cohort study indicate that 3 SNPs in *MYO9B* were associated with ileal involvement. The most significant CD-associated SNP in our cohort was rs1457092. No associations were reported between *MAGI2* or *PARD3* and IBD.

Our most significant ileal only (Montreal classification L1) associated SNP was rs1457092. In 3 other studies [13,15,16] this SNP was also associated with IBD (both UC and CD) but with an opposite direction of association. Similarly, our ileal only CD-associated SNP rs962917 was also observed to be associated in a European IBD study, but once again with an opposite direction [14]. Thus, interestingly, all previous studies (including DM type 1) reported an inverse relationship of association in all *MYO9B* SNPs compared with our study.

Although we reported an association between rs2305764 and the subgroup of ileal CD, no association was demonstrated in CD patients, in concordance with previous studies [13,14]. However, this SNP was strongly associated with celiac disease [10] and with UC in 2 studies [15,16].

There is no straightforward explanation for the different direction of association between the former studies and this study. When replication is expected, it is assumed that the disease-causing gene variant is shared. However, risk allele frequencies may vary between

Table 2
A. MAGI2, PARD3, and MYO9B polymorphisms in patients with inflammatory bowel disease and controls

					Genotype Add Model			
SNP	Gene	Min/maj	MAF affected	MAF control	*p*	OR	L95	U95
rs6962966	*MAGI2*	G/A	0.51	0.48	0.471	1.050	0.919	1.199
rs4379776	*PARD3*	A/G	0.37	0.39	0.172	0.909	0.793	1.042
rs2305767	*MYO9B* (intron 14)	G/A	0.44	0.41	0.273	1.078	0.942	1.234
rs962917	*MYO9B*	A/G	0.36	0.39	0.374	0.940	0.820	1.078
rs1545620	*MYO9B* (exon 20)	C/A	0.40	0.42	0.297	0.931	0.813	1.065
rs1457092	*MYO9B* (intron 20)	A/C	0.36	0.39	0.347	0.937	0.817	1.074
rs2305764	*MYO9B* (intron 28)	A/G	0.41	0.42	0.734	0.977	0.854	1.118
rs2279002	*MYO9B* (intron 32)	G/A	0.31	0.33	0.173	0.906	0.785	1.044
B. MAGI2, PARD3, and MYO9B polymorphisms in patients with Crohn's disease and controls								
rs6962966	*MAGI2*	G/A	0.50	0.48	0.770	1.023	0.877	1.194
rs4379776	*PARD3*	A/G	0.37	0.39	0.271	0.914	0.779	1.073
rs2305767	*MYO9B* (intron 14)	G/A	0.45	0.41	0.108	1.136	0.973	1.328
rs962917	*MYO9B*	A/G	0.35	0.39	0.144	0.887	0.756	1.042
rs1545620	*MYO9B* (exon 20)	C/A	0.38	0.42	0.0988	0.875	0.747	1.025
rs1457092	*MYO9B* (intron 20)	A/C	0.35	0.39	0.138	0.886	0.755	1.040
rs2305764	*MYO9B* (intron 28)	A/G	0.39	0.42	0.279	0.917	0.784	1.073
rs2279002	*MYO9B* (intron 32)	G/A	0.29	0.33	0.061	0.852	0.721	1.007
C. MAGI2, PARD3, and MYO9B polymorphisms in Canadian patients with ulcerative colitis and controls								
rs6962966	*MAGI2*	G/A	0.15	0.48	0.3371	1.083	0.920	1.275
rs4379776	*PARD3*	A/G	0.37	0.39	0.2268	0.901	0.760	1.067
rs2305767	*MYO9B* (intron 14)	G/A	0.42	0.41	0.8971	1.011	0.856	1.194
rs962917	*MYO9B*	A/G	0.38	0.39	0.9543	1.005	0.851	1.187
rs1545620	*MYO9B* (exon 20)	C/A	0.42	0.42	0.9953	1	0.848	1.180
rs1457092	*MYO9B* (intron 20)	A/C	0.38	0.39	0.9933	0.999	0.846	1.180
rs2305764	*MYO9B* (intron 28)	A/G	0.43	0.42	0.5353	1.054	0.893	1.242
rs2279002	*MYO9B* (intron 32)	G/A	0.33	0.33	0.7522	0.972	0.817	1.157

MAF = minor allele frequency; OR = odds ratio; SNP = single nucleotide polymorphism; L95 = lower limit of 95% confidence interval; U95 = upper limit of 95% confidence interval.

Table 3
A. Disease susceptibility analysis in Crohn's disease (CD) patients with ileal only disease (L1 phenotype according to the Montreal classification of CD [30]) compared with healthy unrelated controls

SNP	Gene	Min/maj	MAF affected	MAF control	Genotype Add Model			
					p	OR	L95	U95
rs6962966	*MAGI2*	G/A	0.55	0.48	0.051	1.286	0.999	1.656
rs4379776	*PARD3*	A/G	0.36	0.39	0.336	0.878	0.673	1.145
rs2305767	*MYO9B* (intron 14)	G/A	0.47	0.41	0.053	1.292	0.996	1.676
rs962917	*MYO9B*	A/G	0.31	0.39	0.008	0.690	0.525	0.908
rs1545620	*MYO9B* (exon 20)	C/AG/T	0.35	0.42	0.014	0.715	0.548	0.934
rs1457092	*MYO9B* (intron 20)	A/C	0.31	0.39	0.007	0.687	0.522	0.904
rs2305764	*MYO9B* (intron 28)	A/G	0.34	0.42	0.009	0.700	0.536	0.913
rs2279002	*MYO9B* (intron 32)	G/A	0.26	0.33	0.014	0.697	0.523	0.929
B. Disease susceptibility analysis in Crohn's disease (CD) patients with ileal any disease (combined L1 and L3 phenotype according to the Montreal classification of CD [30]) compared with healthy unrelated controls								
rs6962966	*MAGI2*	G/A	0.51	0.48	0.231	1.104	0.939	1.299
rs4379776	*PARD3*	A/G	0.37	0.39	0.195	0.893	0.755	1.059
rs2305767	*MYO9B* (intron 14)	G/A	0.45	0.41	0.090	1.155	0.978	1.364
rs962917	*MYO9B*	A/G	0.35	0.39	0.076	0.857	0.723	1.016
rs1545620	*MYO9B* (exon 20)	C/AG/T	0.39	0.42	0.068	0.856	0.725	1.012
rs1457092	*MYO9B* (intron 20)	A/C	0.35	0.39	0.067	0.853	0.720	1.011
rs2305764	*MYO9B* (intron 28)	A/G	0.40	0.42	0.212	0.900	0.763	1.062
rs2279002	*MYO9B* (intron 32)	G/A	0.29	0.33	0.024	0.816	0.684	0.974
C. Disease susceptibility analysis in inflammatory bowel disease patients with young disease (≤18 years of age) compared with healthy unrelated controls								
rs6962966	*MAGI2*	G/A	0.49	0.48	0.725	1.027	0.886	1.191
rs4379776	*PARD3*	A/G	0.38	0.39	0.501	0.948	0.812	1.107
rs2305767	*MYO9B* (intron 14)	G/A	0.45	0.41	0.039	1.173	1.008	1.365
rs962917	*MYO9B*	A/G	0.36	0.39	0.100	0.878	0.753	1.025
rs1545620	*MYO9B* (exon 20)	C/AG/T	0.39	0.42	0.073	0.870	0.747	1.013
rs1457092	*MYO9B* (intron 20)	A/C	0.36	0.39	0.094	0.876	0.751	1.023
rs2305764	*MYO9B* (intron 28)	A/G	0.40	0.42	0.189	0.903	0.775	1.052
rs2279002	*MYO9B* (intron 32)	G/A	0.30	0.33	0.089	0.869	0.739	1.022
D. Disease susceptibility analysis in Crohn's disease (CD) patients with perianal disease (according to the Montreal classification of CD [30]) compared with healthy unrelated controls								
rs6962966	*MAGI2*	G/A	0.48	0.48	0.865	0.982	0.791	1.217
rs4379776	*PARD3*	A/G	0.37	0.39	0.415	0.911	0.727	1.141
rs2305767	*MYO9B* (intron 14)	G/A	0.43	0.41	0.368	1.105	0.889	1.373
rs962917	*MYO9B*	A/G	0.34	0.39	0.081	0.817	0.651	1.025
rs1545620	*MYO9B* (exon 20)	C/AG/T	0.38	0.42	0.079	0.819	0.656	1.024
rs1457092	*MYO9B* (intron 20)	A/C	0.34	0.39	0.074	0.813	0.648	1.020
rs2305764	*MYO9B* (intron 28)	A/G	0.38	0.42	0.153	0.852	0.684	1.061
rs2279002	*MYO9B* (intron 32)	G/A	0.28	0.33	0.038	0.777	0.612	0.986

MAF = minor allele frequency; OR = odds ratio; SNP = single nucleotide polymorphism; L95 = lower limit of 95% confidence interval; U95 = upper limit of 95% confidence interval.

different populations, as consistently reported in this study. All former studies were performed in European populations with frequencies of risk alleles that varied significantly compared with the Canadian population (with differences in minor allele frequencies up to 6% in controls). Even within populations, these founder effects may underlie differences in allele frequencies [39]. Another explanation is that our study and most other studies [13–16] are relatively small and might be underpowered to detect small effects of genes. However, none of the variants was associated in GWAS [3,40–44], indicating that if the association with *MYO9B* is not a false positive, it must be associated with a subphenotype not studied in these GWAS.

We report an association in the opposite direction compared with 4 European IBD studies, but all associations were significant and we believe that *MYO9B* has an important function in the pathogenesis of IBD. In support of this are functional studies in patients with type 1 diabetes, indicating a correlation between *MYO9B* expression and intestinal permeability. Increased zonulin levels were reported in diabetic patients. Zonulin is a protein that modulates intestinal permeability by disassembling the intercellular TJ [45,46]. Zonulin correlated with increased intestinal permeability *in vivo* and with an increased *MYO9B* gene expression in intestinal tissue, suggesting a link between *MYO9B* expression and intestinal permeability changes [20]. Furthermore, a trend toward abnormal intestinal permeability in patients with CD carrying the rs1545620 risk allele further supports this hypothesis [14]. Although the exact mechanism by which *MYO9B* influences the intestinal barrier is unknown, the combination of functional data and the results of different independent association studies in various autoimmune diseases point to a role for *MYO9B*.

Rs1545620 is one of our CD-associated SNPs (although not significant after correction); it is a nonsynonymous SNP and therefore induces a coding variant (Alanine1011Serine) in the third IQ domain of *MYO9B*, which is involved in the binding of calmodulin [17,47]. Calmodulin regulates the motor activity of MYO9B on actin filaments and might therefore influence the velocity of the MYO9B protein. Genetic variants in *MYO9B* might be involved in actin remodeling in epithelial enterocytes [17,21,22] and might be linked to a defect in barrier function that appears to be required before the development of disease. This coding variant of rs1545620 might be the causative genetic variant. However, it also is possible that this genetic variant might be in linkage disequilibrium with another disease-causing mutation.

The most significant associated *MYO9B* SNP rs1457092 in our study is located in an intron and not in a conserved coding se-

quence. According to the splicing prediction program (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html), it does not create any alternative splicing sites. It is therefore unlikely that this SNP is the causal mutation, but rather that it is a marker of disease in linkage disequilibrium with a causative variant.

Our study has some limitations. First, although 2,281 individuals were included, the relatively small number of patients in the subgroups makes it difficult to detect genes with small effects. Second, we may not have investigated all relevant genetic variations because many more genes might be involved in intestinal permeability and innate immunity.

In conclusion, our data point to a role of the *MYO9B* gene in the development of ileal CD, although the direction of association is different from that reported in European studies [13–16]. Future joint analysis of GWAS of different cohorts will increase the power to detect small effects of genes and therefore will likely reveal whether *MYO9B* is indeed a risk variant.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.humimm.2011.03.025.

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