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Mycobacterium avium subsp. *paratuberculosis*, Genetic Susceptibility to Crohn's Disease, and Sardinians: the Way Ahead

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The present study was performed to determine what proportion of people in Sardinia with or without Crohn's disease were infected with *Mycobacterium avium* subspecies *paratuberculosis* and had a preponderance of allelic variants of Nod2, an intracellular protein involved in Crohn's disease susceptibility. Genetic analysis of the alleles of the NOD2/CARD15 gene (*insC3020*, G908R, and R702W alleles), linked to susceptibility or genetic predisposition to Crohn's disease in humans, was carried out on specimens from 37 Crohn's disease patients and 34 patients without Crohn's disease. Our results show that more than 70 percent of people in Sardinia with Crohn's disease carry at least one of the susceptibility-associated NOD2/CARD15 alleles and were infected with *Mycobacterium avium* subspecies *paratuberculosis*.

Recent genetic and epidemiological studies provide suggestive evidence for the presence of genetic determinants of susceptibility to Crohn's disease (CD) and its clinical progression. In a recent report, Behr and colleagues documented the coexistence of *Mycobacterium avium* subsp. *paratuberculosis* infection and a permissive NOD2/CARD15 mutant in one individual with CD (1). CD is a disease of unknown etiology linked to immune dysregulation (10). An increasing amount of evidence supports the idea that CD is caused by *Mycobacterium avium* subsp. *paratuberculosis* (8, 11, 12). In 2001, three independent mutations within the NOD2/CARD15 gene were discovered to be strongly linked to CD in Europeans (3, 4, 9). According to the observation of Behr et al., the presence of a gene that is associated with an increased susceptibility to develop CD does not preclude the possibility that the disease may be infectious in etiology (1). It is possible that genetically identifiable subpopulations may have different tendencies to develop CD when exposed to the same infectious agent (3, 6). The following case study suggests an exploitation of these considerations in a group of Mediterranean patients with CD.

Endoscopic mucosal biopsy specimens were obtained from 37 patients with CD and 34 individuals without it who were attending the Clinic of Surgery, University of Sassari, for ileocolonoscopy. Biopsy specimens were taken both from the visibly inflamed regions in the patients with inflammatory bowel disease and from several regions throughout the intestines of control subjects. Informed consent for sampling and publication of the results was obtained. Biopsy specimens were examined for the presence of *Mycobacterium avium* subsp. *paratuberculosis* and the carriage of NOD2/CARD15 mutations. All the individuals examined were living in Sardinia and were of Sardinian origin. Tissue samples were placed into liquid 7H9 medium, transported directly to the laboratory, and processed within 1 h. One biopsy specimen was used to make a smear and examined by Ziehl-Neelsen and rhodamine-auramine staining as previously described (12).

Samples for PCR analysis for bacterial presence were processed as previously described (7). Primers p89 and p92 described in our previous work (11, 12) were used to amplify a 284-bp fragment specific for *Mycobacterium avium* subsp. *paratuberculosis*. The reaction mixture (final volume of 50 μl) comprised primers at a concentration of 0.5 μM, Expand High Fidelity reaction buffer (1×), 200 μM (each) dNTPs, and 3 U of Expand High-Fidelity *Taq* polymerase (Expand High-fidelity PCR system; Roche, Lewes, United Kingdom). Cycling conditions were 1 cycle of 94°C for 3 min and 36 cycles of 94°C for 40 s, 62°C for 40 s, and 72°C for 40 s, followed by a final step of 72°C for 5 min. Amplified fragments were visualized with ethidium bromide on 2.5% agarose-1000 gel (Life Technologies, Grand Island, NY) and purified with a QIAquick gel extraction kit (QIAGEN, Crawley, United Kingdom). Each amplicon was then sequenced in both directions by using p89 and p92 primers, and the sequences obtained had confirmed 100% homology to the IS*900* locus (GenBank accession no. AF416985).

Mycobacterium avium subsp. *paratuberculosis* DNA was detected in 25 CD patients (67.6%) and in 7 controls (20.6%)

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TABLE 1. Patient details[a]

Patient	Sex	Disease or group	PCR result (presence of *M. avium* subsp. *paratuberculosis*)	Genetic analysis result for indicated allele		
				insC3020	R702W	G908R
4	F	Crohn's disease	Pos	WT	WT	WT
6	F	Crohn's disease	Pos	HET	WT	WT
9	M	Crohn's disease	Pos	WT	WT	WT
10	M	Crohn's disease	Pos	WT	HET	WT
15	M	Crohn's disease	Pos	HET	WT	HET
16	M	Crohn's disease	Pos	WT	WT	WT
19	F	Crohn's disease	Pos	WT	HET	WT
24	F	Crohn's disease	Pos	WT	WT	WT
25	F	Crohn's disease	Pos	HOM	WT	HOM
28	F	Crohn's disease	Pos	WT	WT	WT
29	M	Crohn's disease	Neg	WT	WT	WT
55	F	Crohn's disease	Pos	WT	HET	HET
62	M	Crohn's disease	Neg	HET	WT	WT
63	M	Crohn's disease	Pos	WT	WT	WT
66	F	Crohn's disease	Pos	WT	WT	WT
67	M	Crohn's disease	Pos	WT	WT	HET
69	F	Crohn's disease	Neg	WT	WT	WT
72	M	Crohn's disease	Neg	WT	WT	WT
74	M	Crohn's disease	Pos	HET	HET	WT
78	F	Crohn's disease	Pos	WT	WT	WT
79	F	Crohn's disease	Neg	WT	WT	HET
81	M	Crohn's disease	Neg	WT	WT	WT
82	F	Crohn's disease	Pos	HET	WT	WT
83	M	Crohn's disease	Pos	WT	WT	WT
91	F	Crohn's disease	Neg	WT	WT	WT
107	M	Crohn's disease	Neg	WT	WT	WT
110	F	Crohn's disease	Neg	WT	WT	HET
111	F	Crohn's disease	Pos	WT	WT	WT
113	F	Crohn's disease	Pos	HET	WT	WT
117	F	Crohn's disease	Pos	WT	WT	WT
120	F	Crohn's disease	Neg	HET	WT	WT
125	M	Crohn's disease	Neg	WT	HET	WT
127	F	Crohn's disease	Neg	WT	WT	WT
130	F	Crohn's disease	Pos	HET	HET	WT
132	F	Crohn's disease	Pos	WT	HET	WT
133	F	Crohn's disease	Pos	HET	WT	WT
135	R	Crohn's disease	Pos	WT	HET	WT
5	M	Control	Pos	WT	HET	WT
26	F	Control	Neg	WT	WT	WT
54	M	Control	Pos	WT	WT	WT
56	M	Control	Pos	WT	WT	WT
57	M	Control	Neg	WT	WT	WT
58	M	Control	Neg	HET	WT	WT
59	M	Control	Neg	WT	WT	WT
60	F	Control	Neg	WT	WT	WT
64	F	Control	Neg	WT	WT	WT
65	F	Control	Neg	WT	WT	WT
68	F	Control	Neg	WT	WT	WT
70	M	Control	Neg	WT	WT	WT
71	M	Control	Neg	WT	WT	WT
73	M	Control	Neg	WT	WT	HET
75	M	Control	Neg	WT	WT	WT
77	F	Control	Neg	WT	WT	WT
84	M	Control	Neg	WT	WT	WT
85	F	Control	Neg	WT	WT	WT
87	F	Control	Neg	WT	WT	WT
88	F	Control	Neg	HET	WT	WT
89	F	Control	Neg	WT	WT	HET
93	F	Control	Neg	WT	WT	WT
103	F	Control	Neg	WT	WT	WT
105	M	Control	Neg	WT	WT	WT
108	M	Control	Neg	WT	WT	WT
114	F	Control	Pos	WT	WT	WT
116	M	Control	Pos	WT	WT	WT
118	M	Control	Pos	WT	WT	WT
128	F	Control	Neg	WT	WT	WT
131	F	Control	Pos	HET	WT	WT
137	M	Control	Neg	WT	WT	WT
138	F	Control	Neg	WT	HET	WT
139	F	Control	Neg	WT	WT	WT
140	F	Control	Neg	WT	WT	WT

[a] Abbreviations: M, male; F, female. Results of genetic analysis of the alleles of the NOD2/CARD15 gene are reported as wild type (WT), heterozygous for the mutation (HET), and homozygous for the mutation (HOM). Results of PCR for *M. avium* subsp. *paratuberculosis* DNA are shown as positive (Pos) or negative (Neg).

(Table 1). The results indicated that the presence of *Mycobacterium avium* subsp. *paratuberculosis* is significantly associated with CD ($P = 0.001$; odds ratio [OR], 4.94 [95% confidence interval $\{CI_{95}\}$, 1.85 to 13.29]).

Because of the recently published data showing genetic susceptibility to CD, we also performed genetic testing, aimed at susceptibility-linked alleles of the NOD2/CARD15 gene (*insC3020*, G908R, and R702W alleles), as previously described (2). Our results indicated that 19 (51.35%) of the CD patients were carriers of at least one of the susceptibility-associated NOD2/CARD15 alleles (10 carried the *insC3020* mutation, 8 the R702W mutation, and 6 the G908R mutation), whereas only 7 (20.6%) of the healthy controls carried at least one of the mutated NOD2/CARD15 alleles. A significant association between the carriage of at least one NOD2/CARD15 mutation and development of CD was observed ($P = 0.007$; OR = 4.07 [CI_{95}, 1.42 to 11.66]). The results confirm for the first time the associations of NOD2/CARD15 mutations and CD in the Sardinian population.

Among the CD patients who carried at least one of the susceptibility-associated NOD2/CARD15 alleles, 14 (73.7%) had evidence for the presence of *Mycobacterium avium* subsp. *paratuberculosis* DNA. This association was marginally significant ($P < 0.04$; OR = 1.7 [CI_{95}, 0.92 to 3.2]) in our study group. Since several findings indicate that the presence of certain bacteria in the face of permissive NOD2/CARD15 mutations is necessary for development of CD (5, 7), it is possible that in individuals with NOD2/CARD15 mutations, the innate response to mycobacterial exposure would be inadequate, permitting establishment of a persistent mycobacterial infection. Chronic infection could then activate the inflammatory response which is characteristic of CD..

Collectively, our findings support and substantiate the suggestions of Behr et al. that these cases may illustrate a potential conceptual approach to CD etiology, mostly based on tandem searches for bacterial trigger and host susceptibility (1). However, further studies involving a larger number of cases and controls on a global scale are clearly needed to elucidate the mechanism behind the etiology of CD.

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REFERENCES

1. **Behr, M. A., M. Semret, A. Poon, and E. Schurr.** 2004. Crohn's disease, mycobacteria, and NOD2. Lancet Infect. Dis. **4:**136–137.
2. **Gazouli, M., P. Zacharatos, G. J. Mantzaris, C. Barbatis, I. Ikonomopoulos, A. J. Archimandritis, J. C. Lukas, E. Papalambros, and V. Gorgoulis.** 2004. Association of NOD2/CARD15 variants with Crohn's disease in a Greek population. Eur. J. Gastroenterol. Hepatol. **16:**1177–1182.
3. **Hampe, J., A. Cuthbert, P. J. Croucher, M. M. Mirza, S. Mascheretti, S.**

Fisher, H. Frenzel, K. King, A. Hasselmeyer, A. J. MacPherson, S. Bridger, S. van Deventer, A. Forbes, S. Nikolaus, J. E. Lennard-Jones, U. R. Foelsch, M. Krawczak, C. Lewis, S. Schreiber, and C. G. Mathew. 2001. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet **16:**1925–1928.

4. **Hugot, J. P., M. Chamaillard, H. Zouali, S. Lesage, J. P. Cezard, J. Belaiche, S. Almer, C. Tysk, C. A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J. F. Colombel, M. Sahbatou, and G. Thomas.** 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature **411:**599–603.
5. **Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S. J. Foster, A. P Moran, J. L. Fernandez-Luna, and G. Nunez.** 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J. Biol. Chem. **278:**5509–5512.
6. **Inoue, N., K. Tamura, Y. Kinouchi, Y. Fukuda, S. Takahashi, Y. Ogura, N. Inohara, G. Nunez, Y. Kishi, Y. Koike, T. Shimosegawa, T. Shimoyama, and T. Hibi.** 2002. Lack of common NOD2 variants in Japanese patients with Crohn's disease. Gastroenterology **123:**86–91.
7. **Maeda, S., L. C. Hsu, H. Liu, L. A. Bankston, M. Iimura, M. F. Kagnoff, L. Eckmann, and M. Karin.** 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. Science **307:**734–738.
8. **Naser, S. A., G. Ghobrial, C. Romero, and J. F. Valentine.** 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet **364:**1039–1044.
9. **Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerr, J. P. Achkar, S. R. Brant, T. M. Bayless, B. S. Kirschner, S. B. Hanauer, G. Nunez, and J. H. Cho.** 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature **411:**603–606.
10. **Podolsky, D. K.** 2002. Inflammatory bowel disease. N. Engl. J. Med. **347:** 417–429.
11. **Sechi, L. A., M. Mura, F. Tanda, A. Lissia, A. Solinas, G. Fadda, and S. Zanetti.** 2001. Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. J. Clin. Microbiol. **39:**4514–4517.
12. **Sechi, L. A., A. M. Scanu, P. Molicotti, S. Cannas, M. Mura, G. Dettori, G. Fadda, and S. Zanetti.** 2005. Detection and isolation of *Mycobacterium avium* subspecies *paratuberculosis* from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia. Am. J. Gastroenterol. **100:**1529–1536.