Association Between Osteoprotegerin G1181C and T245G Polymorphisms and Diabetic Charcot Neuroarthropathy

A case-control study

DARIO PITOCCO, MD[1]
GIOVANNI ZELANO, MD[2]
GIUSEPPINA GIOFFRÈ, MD[3]
ENRICO DI STASIO, MD[4]
FRANCESCO ZACCARDI, MD[5]
FRANCESCA MARTINI, MD[5]
TITTANIA MUSELLA, MD[5]
GIUSEPPE SCAVONE, MD[5]
MARCO GALLI, MD[6]
SALVATORE CAPUTO, MD[1]
LORENA MANCINI, MD[3]
GIOVANNI GHIRLANDA, MD[1]

OBJECTIVE — Charcot neuroarthropathy is a disabling complication of diabetes. Although its pathogenesis remains unknown, we suppose that genetics may play a relevant role.

RESEARCH DESIGN AND METHODS — We performed a case-control study with 59 subjects with diabetic Charcot neuroarthropathy (Ch group), 41 with diabetic neuropathy without Charcot neuroarthropathy (ND group), and 103 healthy control subjects (H group) to evaluate the impact of two single nucleotide polymorphisms (SNPs) of the osteoprotegerin gene (G1181C and T245G) on the risk of Charcot neuroarthropathy.

RESULTS — Regarding the SNPs of G1181C, we found a significant linkage between the G allele and Charcot neuroarthropathy (Ch vs. ND, odds ratio [OR] 2.32 [95% CI 1.3–4.1], $P = 0.006$; Ch vs. H, 2.10 [1.3–3.3], $P = 0.002$; and ND vs. H, 0.90 [0.7–1.9], $P = 0.452$); similarly, we found a linkage with the G allele of T245G (Ch vs. ND, 6.25 [2.2–19.7], $P < 0.001$; Ch vs. H, 3.56 [1.9–6.7], $P = 0.001$; and ND vs. H, 0.54 [0.6–5.7], $P = 0.304$), supporting a protective role for the allele C and T, respectively. For this reason we investigated the frequency of the protective double homozygosis CC + TT (7% in Ch) that was significantly lower in Ch compared with H (0.18 [0.06–0.5], $P = 0.002$) and with ND (0.17 [0.05–0.58], $P = 0.006$), whereas there was no difference between H and ND (1.05 [0.43–2.0], $P = 0.468$). In a multivariate logistic backward regression model, only weight and the lack of CC and TT genotypes were independently associated with the presence of Charcot neuroarthropathy.

CONCLUSIONS — This is the first study that shows an association between genetic regulation of bone remodeling and Charcot neuroarthropathy.

Diabetes Care **32:1694–1697, 2009**

Charcot neuroarthropathy is a chronic and progressive disease of bone and joints, defined by painful or relatively painless bone and joint destruction, in limbs that have lost sensory innervation; it is characterized by pathological fractures, joint dislocation, and deformity (1). With the decline in numbers of cases of tertiary syphilis, the primary etiology today is diabetes. The incidence is ~0.1–5% in diabetic patients with peripheral neuropathy, but it is likely that many cases are undiagnosed (2). The majority of patients with Charcot neuroarthropathy are from 50 to 60 years old, and most will have had diabetes for at least 10 years (3,4).

The pathogenesis of Charcot neuroarthropathy is still unknown, but it is undoubtedly multifactorial (1,5); probably this is one of the reasons that there is no pharmacological treatment available to stop the progress of the disease. The difference between the higher prevalence of diabetic neuropathy and the lower prevalence of Charcot neuroarthropathy (neuropathy seems to be necessary but not sufficient for its presence) and the different clinical features of the two conditions support the hypothesis of the probable involvement of other factors in its pathogenesis.

A common feature of Charcot neuroarthropathy is bone reabsorption, and the association between diabetes and osteoporosis could contribute to the presence of Charcot neuroarthropathy (6–8). Indeed, the study of bone turnover markers in acute Charcot neuroarthropathy shows that there is an increase in osteoclast activity compared with osteoblast activity (9); this can lead to osteopenia, which could predispose to fracture, even as a consequence of minimal trauma.

New insights into the regulation of osteoclastogenesis have resulted from the discovery of three members of the tumor necrosis factor (TNF) and TNF receptor superfamily; one of these receptors, osteoprotegerin (OPG), is an important regulator of bone remodeling (10). OPG gene single nucleotide polymorphisms (SNPs) have been associated with osteoporosis (11,12) and are considered early predictors of cardiovascular disease (13). Two of the most studied polymorphisms are G1181C (located in exon I) and T245G (located in the promoter region); the latter is in complete linkage with A163G and G209A polymorphisms (14). Because of their regulatory function in bone remodeling and for their involvement in the pathogenesis of osteoporosis, we focused our investigation on these two

From the [1]Institute of Internal Medicine, Policlinico "A. Gemelli," Rome, Italy; the [2]Institute of Human Anatomy and Cell Biology, Policlinico "A. Gemelli," Rome, Italy, and the Rehabilitation Research Center "Armonia," Latina, Italy; the [3]Foot Care Unit, Policlinico "A. Gemelli," Rome, Italy; the [4]Institute of Biochemistry, Policlinico "A. Gemelli," Rome, Italy; the [5]Diabetes Center, Policlinico "A. Gemelli," Rome, Italy; and the [6]Institute of Orthopedic Surgery, Policlinico "A. Gemelli," Rome, Italy.
Corresponding author: Dario Pitocco, dario.pitocco@rm.unicatt.it.
Received 11 February 2009 and accepted 1 June 2009.
Published ahead of print at http://care.diabetesjournals.org on 5 June 2009. DOI: 10.2337/dc09-0243.
D.P. and G.Z. contributed equally to this study.

OPG SNPs to evaluate their possible association with Charcot neuroarthropathy.

RESEARCH DESIGN AND METHODS

— The study was performed in our department. We included 59 consecutive Caucasian subjects with diabetic Charcot neuroarthropathy (Ch group), 41 consecutive Caucasian subjects with diabetic neuropathy without Charcot neuroarthropathy (ND group), and 103 healthy Caucasians as the control subjects (H group). All diabetic subjects had type 2 diabetes.

An assessment of vibration perception threshold was performed with a biothesiometer, according to Young et al. (15). All diabetic subjects had a definite diagnosis of peripheral neuropathy with a Neuropathy Disability Score >5 (16) and a pathological conduction velocity. Autonomic neuropathy was diagnosed according to the standardized procedure of Ewing and Clarke (17), including four cardiovascular autonomic tests. A radiological evaluation of both feet was performed in all diabetic subjects (Charcot neuroarthropathy and neuropathy only subjects) to confirm the diagnosis or the absence of Charcot neuroarthropathy.

Clinical and radiological findings (bone fracture, fragmentation, or destruction and joint subluxation, dislocation, or destruction, seen on X rays taken in two different projections, according to the protocol described by Cavanagh et al. [18]) were considered as indicative of a diagnosis of Charcot neuroarthropathy. If the diagnosis was doubtful, magnetic resonance imaging of the feet was performed (19).

This study was conducted in agreement with the Declaration of Helsinki and was approved by the ethics committee of our hospital. All subjects provided informed consent.

Restriction fragment–length polymorphism-PCR analysis

Genomic DNA was isolated from whole blood by standard procedures. Genotyping of the G1181C OPG exon 1 polymorphism was performed using a mismatched oligonucleotide approach. A 570-bp fragment was amplified with primers 5′-TGCGTCCGGATCTTGGCTGGATCGG-3′ (sense) and 5′-GGGCGCGGCGGGCGCGCCCAGGGACTTACCACGAGCGCGCAGCACAGCTA-3′ (antisense), containing a T instead of an A nucleotide two bases before the 3′ end that corresponds to the third base of codon 3 that encodes lysine in exon 1 of the OPG gene, thereby introducing an artificial *Xsp*I restriction site in the presence of the mutant allele. The PCR mixture (30 μl) contained genomic DNA (100 ng), 1× PCR buffer, 0.2 mmol/l each of the four deoxyribonucleotides, 1.0–2.5 mmol/l $MgCl_2$, 0.42 μmol/l each of the two oligonucleotide primers, and 0.6 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial 12 min at 95°C, followed by 35 cycles of 60 s at 94°C, 30 s at 56°C, and 60 s at 72°C, and finally, 7 min at 72°C. After PCR amplification, 5 μl of each PCR product (5 μl) was digested with 3 units *Xsp*I (Cambrex Bio Science, Apen, Germany) for 16 h and subsequently analyzed on a 3% agarose gel and visualized by ethidium bromide staining. In the presence of a C nucleotide at position 1181, the 570-bp PCR product was cleaved into 522- and 48-bp fragments, respectively.

Genotyping of the T245G OPG promoter polymorphism was performed by amplification of a 271-bp fragment with oligonucleotide primers 5′-CGA ACC CTA GAG CAA AGT GC-3′ (sense) and 5′-TGT CTG ATT GGC CCT AAA GC-3′ (antisense). The PCR mixture (30 μl) contained genomic DNA (100 ng), 1× PCR buffer, 0.2 mmol/l each of the four deoxyribonucleotides, 1.0–2.5 mmol/l $MgCl_2$, 0.42 μmol/l each of the two oligonucleotide primers, and 0.6 unit of AmpliTaq Gold polymerase (Applied Biosystems). Cycling conditions consisted of an initial 12 min at 95°C, followed by 35 cycles of 60 s at 94°C, 30 s at 56°C, and 60 s at 72°C, and finally, 7 min at 72°C. After PCR amplification, 5 μl of each PCR product was digested with 3 units *Hinf*I restriction endonuclease (New England Biolabs, Beverly, MA) for 16 h and subsequently analyzed on a 3% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Statistical analysis was performed with SPSS (release 15.0; SPSS, Chicago, IL). Continuous variables are expressed as means ± SD, categorical variables are displayed as frequencies, and the appropriate parametric or nonparametric test (*t* test and ANOVA for continuous normally distributed variables, Mann-Whitney *U* test for nonnormally distributed variables, or corrected χ^2 or Fisher's exact test for categorical data) was used to assess the significance of the differences between subgroups. Analysis of genotype data was performed using PowerMarker software (version 3.25) (20). Hardy-Weinberg equilibrium was assessed by a χ^2 test or Fisher's exact test as appropriate. D' was calculated to evaluate linkage disequilibrium for all pairwise SNP combinations. Multivariate binary logistic analysis was performed to evaluate the relationship between the presence of Charcot disease and genotypes and clinical/laboratory findings. The coefficients obtained from the logistic regression were expressed in terms of odds ratios (ORs) with 95% CI. The statistical significance was set at $P < 0.05$.

RESULTS

— Table 1 shows the clinical and laboratory characteristics of the Ch, H, and ND groups. Comparison of OPG polymorphisms showed significant differences in the frequencies of alleles between Ch versus ND and Ch versus H, whereas ND and H were not different (Table 2). We found a positive association with the G allele of G1181C in Ch compared with ND and H (OR 2.32 [95% CI 1.3–4.1], $P = 0.006$ and 2.10 [1.3–3.3], $P = 0.002$, respectively), whereas H and ND were overlapped (0.90 [0.7–1.9], $P = 0.452$); regarding T245G, we showed a strong positive association with the G allele in Ch compared with ND and H (6.25 [2.2–19.7], $P < 0.001$ and 3.56 [1.9–6.7], $P = 0.001$, respectively), whereas there were no differences between H and ND (0.54 [0.6–5.7], $P = 0.304$). Because the frequencies of C (G1181C) and T (T245G) alleles were lower in Ch, we analyzed the distribution of the protective double homozygosis CC + TT, which was significantly lower in Ch (frequency 7%) compared with that in H (0.18 [0.06–0.5], $P = 0.002$) and ND (0.17 [0.05–0.58], $P = 0.006$), whereas there was no difference between H and ND (1.05 [0.43–2.0], $P = 0.468$). Thus, the risk to have Charcot neuroarthropathy in diabetic and neuropathic subjects with CC/TT homozygosis is approximately sixfold lower (1/OR TT + CC [0.17]). In a multivariate logistic backward regression model built using Charcot disease as a dependent variable and SNPs and clinical/laboratory values as independent variables (Table 1), only weight and the lack of CC and TT genotypes were independently associated with the presence of Charcot neuroarthropathy (1.07 [1.03–1.12], $P = 0.001$; 0.17 [0.04–0.71], $P = 0.015$; and 0.06 [0.01–0.36], $P = 0.002$, respectively). For example, in our population, subjects without TT polymor-

Table 1—*Clinical and laboratory characteristics of Charcot, neuropathic + diabetic, and healthy subjects*

	Ch group	ND group	H group	*P**
A1C (%)	8.2 ± 2.4[1]	8.0 ± 1.8[2]	5.1 ± 0.4[1,2]	<0.001
Disease duration (years)	20 ± 11	21 ± 10		
Weight (kg)	97 ± 16[1,2]	84 ± 17[1,3]	74 ± 4[2,3]	<0.001
Waist circumference (cm)	115 ± 14[1,2]	106 ± 12[1,3]	97 ± 5[2,3]	<0.001
Age (years)	59 ± 9[1]	64 ± 10[1]	62 ± 6	0.013
Sex (male/female)	39/19	26/13	63/40	NS
Total cholesterol (mg/dl)	178 ± 47	182 ± 38	182 ± 38	NS
LDL cholesterol (mg/dl)	105 ± 35	103 ± 35	103 ± 35	NS
HDL cholesterol (mg/dl)	43 ± 9	45 ± 12	45 ± 12	NS
Triglycerides (mg/dl)	161 ± 98[1]	144 ± 67[2]	144 ± 67[1,2]	<0.001
Conduction velocity (m/s)	32.2 ± 4.5	33.2 ± 4.2		NS
Conduction amplitude (μV)	1.2 ± 0.4	1.3 ± 0.4		NS
NDS: 6-7-8-9-10†	10-13-17-13-6	7-8-12-11-3		NS
ANS: 3-4-5-6-7-8†	8-16-14-14-5-2	6-12-10-9-3-1		NS

Data are means ± SD. *Significance of difference for the correspondent row. If the *P* value is significant (<0.05), apex numbers locate the difference between corresponding row subgroups. †Data regarding Neuropathy Disability Score (NDS) (16) and Autonomic Neuropathy Score (ANS) (17) are expressed as frequencies of patients belonging to each score (e.g., Charcot: NDS 7, 13 patients; ND: ANS 4, 12 patients).

phisms have a 16-fold higher risk of Charcot neuroarthropathy (1/OR TT [0.06]), indicating the protective role played by the alleles C and T, respectively.

Conduction velocity and amplitude, Neuropathy Disability Score, and Autonomic Neuropathy Score were similar between Ch and ND (Table 1); moreover, no significant difference was found in a comparison of these four variables in relation to OPG SNPs (data not shown).

Genetic distribution of both SNPs were in Hardy-Weinberg equilibrium. There was a weak linkage disequilibrium between the two SNPs ($D' = 0.330$) analyzed.

CONCLUSIONS

The difference between the high prevalence of diabetic neuropathy compared with the low prevalence of Charcot neuroarthropathy and the different clinical features of the diseases seems to support the hypothesis of a not sufficient role of diabetic neuropathy in the pathogenesis of Charcot neuroarthropathy, with the probable involvement of other factors (i.e., genetics). A peculiar clinical trait of Charcot neuroarthropathy is the low bone mineral density of the foot's bones, probably as a consequence of a continuous bone resorption linked to unbalanced osteoclast/osteoblast activity; in this balance the receptor activator for NF-κB (RANK)-RANK ligand (RANKL) system and its decoy receptor OPG play a pivotal role. Genes of the OPG/RANKL/RANK axis and their polymorphisms, which have already been involved in the pathogenesis of osteoporosis, might be identified as important mediators for paracrine signaling in bone metabolism of Charcot neuroarthropathy. This is the first study that has investigated the possible relationship between genetics and Charcot neuroarthropathy, demonstrating a strong association between OPG G1181C and T245G polymorphisms, located in the exon 1 region and promoter region, respectively, and Charcot neuroarthropathy. The significant association between OPG SNPs and Charcot neuroarthropathy (comparing Ch with ND groups) confirmed that SNPs are an additional risk factor, besides diabetes and neuropathy, for the presence of Charcot disease. Variations in exon 1 of the OPG gene could result in a qualitative alteration of OPG synthesis, thus compromising its function as a decoy receptor, whereas variations in the sequence of the promoter region of the OPG gene could result in altered binding of different transcription factors, thus affecting the expression of OPG (quantitative alteration) and consequently the OPG/RANKL ratio.

Furthermore, we should envision Charcot neuroarthropathy as a microenvironment characterized by a persistent inflammation, linked to a probable neuropathic dysregulation of the inflammation ("inflammatory reflex") (21), with an increased level of cytokines such as TNF-α, interleukin-1β, and interleukin-6 that can promote osteoclastogenesis in synergy with RANKL, exceeding the protective capacity of OPG (22). From this point of view, both genetics and persistent inflammation might compromise the balanced process of bone remodeling and be involved in the pathogenesis of osteolytic bone disorders. Finally, the role of overweight (confirmed by our meta-analysis data) could be double: mechanical (constant weight bearing on the foot) (23) and metabolic ("metabolic inflammation" of obesity) (24).

In summary, our results support the hypothesis that Charcot neuroarthropathy is a disease in which genetics plays an essential role.

Table 2—*Frequencies of G1181C and T245G genotypes*

	ND group	Ch group	H group
G1181C			
CC	15 (36.5)	6 (10.1)	34 (33)
GC	19 (46.3)	34 (57.6)	50 (48.5)
GG	7 (17.2)	19 (32.3)	19 (18.5)
T245G			
GG	0 (0)	7 (11.9)	2 (1.9)
GT	4 (9.75)	16 (27.1)	14 (13.6)
TT	37 (90.25)	36 (61)	87 (84.5)

Data are absolute number (%). The differences between the groups (Ch vs. ND, $P < 0.001$; ND vs. H, NS; and Ch vs. H, $P < 0.001$) were analyzed with the χ^2 test.

Acknowledgments— No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 68th Scientific Session of the American Diabetes Association, San Francisco, California, 6–10 June 2008, and at the Charcot Neuroarthropathy Workshop, Bethesda, Maryland, 17–18 September 2008.

We are grateful to Anna Crescenzi for her technical support and to Fabrizia Toscanella and Cristiana Di Campli of Immacolata Dermatologica Institute for their clinical support.

References

1. Edmonds ME. Progress in care of the diabetic foot. Lancet 1999;354:270–272
2. Sanders LJ, Frykberg RG. Diabetic neuropathic osteoarthropathy: Charcot foot. In *The High Risk Foot in Diabetes Mellitus*. Levin ME, O'Neal LW, Bowker JH, Eds. New York, Churchill Livingstone, 1991, p. 297–338
3. Sinha S, Munichoodappa G, Kozak GP. Neuroarthropathy (Charcot joints) in diabetes mellitus: clinical study of 101 cases. Medicine (Baltimore) 1972;52:191–210
4. Rajbhandari SM, Jenkins RC, Davies C,

Tesfaye S. Charcot neuroarthropathy in diabetes mellitus. Diabetologia 2002;45:1085–1096
5. Frykberg RG, Kozak GP. The diabetic Charcot foot. In *Management of Diabetic Foot Problems*. 2nd ed. Kozak GP, Campbell DR, Fryberg RG, Haberwshaw GM, Eds. Philadelphia, WB Saunders, 1995, p. 88–97
6. Childs M, Armstrong DG, Edelson GW. Is Charcot arthropathy a late sequela of osteoporosis in patients with diabetes mellitus? J Foot Ankle Surg 1998;37:437–439
7. Saller A, Maggi S, Romanato G, Tonin P, Crepaldi G. Diabetes and osteoporosis. Aging Clin Exp Res 2008;20:280–289
8. Räkel A, Sheehy O, Rahme E, LeLorier J. Osteoporosis among patients with type 1 and type 2 diabetes. Diabetes Metab 2008;34:193–205
9. Piaggesi A, Rizzo L, Golia F, Costi D, Baccetti F, Ciaccio S, De Gregorio S, Vignali E, Trippi D, Zampa V, Marcocci C, Del Prato S. Biochemical and ultrasound tests for early diagnosis of active neuro-osteoarthropathy (NOA) of the diabetic foot. Diabetes Res Clin Pract 2002;58:1–9
10. Khosla S. Minireview: The OPG/RANKL/RANK system. Endocrinology 2001;142:5050–5055
11. Arko B, Prezelj J, Komel R, Kocijancic A, Hudler P, Marc J. Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis. J Clin Endocrinol Metab 2002;87:4080–4084
12. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Center JR, Nguyen TV, Bagger Y, Gulcher JR, Eisman JA, Christiansen C, Sigurdsson G, Kong A, Thorsteinsdottir U, Stefansson K. Multiple genetic loci for bone mineral density and fractures. N Engl J Med 2008;358:2355–65
13. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. Circ Res 2004;95:1046–1057
14. Kim JG, Kim JH, Kim JY, Ku SY, Jee BC, Suh CS, Kim SH, Choi YM. Association between osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK), and RANK ligand (RANKL) gene polymorphisms and circulating OPG, soluble RANKL levels, and bone mineral density in Korean postmenopausal women. Menopause 2007;14:913–918
15. MJ Young, Breddy JL, Veves A, Boulton AJM. The prediction of diabetic neuropathy foot using vibration perception threshold: a prospective study. Diabetes Care 1994;17:557–560
16. Young MJ, Boulton AJM, Macleod AF, Williamd DRR, Sonksen PK. A multicentre study of the prevalence of diabetic peripheral neuropathy in the UK hospital clinic population. Diabetologia 1993;36:150–154
17. Ewing DJ, Clarke BF. Diagnosis and management of diabetic autonomic neuropathy. Br Med J 1982;285:916–918
18. Cavanagh PR, Young MJ, Adams JE, Vickers KL, Boulton AJM. Radiographic abnormalities in the feet of patients with diabetic neuropathy. Diabetes Care 1994;17:201–209
19. Tomas MB, Patel M, Marvin SE, Palestro CJ. The diabetic foot. Br J Radiol 2000;73:443–450
20. Liu K, Muse SV. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 2005;21:2128–2129
21. Tracey KJ. The inflammatory reflex. Nature 2002;420:853–859
22. Mabilleau G, Petrova NL, Edmonds ME, Sabokbar A. Increased osteoclastic activity in acute Charcot's osteoarthropathy: the role of receptor activator of nuclear factor-κB ligand. Diabetologia 2008;51:1035–1040
23. Stuck RM, Sohn MW, Budiman-Mak E, Lee TA, Weiss KB. Charcot arthropathy risk elevation in the obese diabetic population. Am J Med 2008;121:1008–1014
24. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology 2007;132:2169–2180