

# Analysis of the association of polymorphism in the osteoprotegerin gene with susceptibility to chronic kidney disease and periodontitis

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**Background and Objective:** Chronic kidney disease (CKD) is a complex disorder, which results in several complications involving disturbance of mineral metabolism. Periodontal disease is an infectious disease that appears to be an important cause of systemic inflammation in CKD patients. Periodontal disease is characterized by clinical attachment loss (CAL) caused by alveolar bone resorption around teeth, which may lead to tooth loss. Osteoprotegerin (OPG) is a key regulator of osteoclastogenesis. Polymorphisms are the main source of genetic variation, and single nucleotide polymorphisms (SNPs) have been reported as major modulators of disease susceptibility. The aim of this study was to investigate the association of a polymorphism located at position –223 in the untranslated region of the *OPG* gene, previously known as –950, with susceptibility to CKD and periodontal disease.

**Material and Methods:** A sample of 224 subjects without and with CKD (in hemodialysis) was divided into groups with and without periodontal disease. The *OPG* polymorphism was analyzed by polymerase chain reaction and restriction fragment length polymorphism.

**Results:** No association was found between the studied *OPG* polymorphism and susceptibility to CKD or periodontal disease.

**Conclusion:** It was concluded that polymorphism *OPG*–223 (C/T) was not associated with CKD and periodontal disease in a Brazilian population. Studies on other polymorphisms in this and other genes of the host response could help to clarify the involvement of bone metabolism mediators in the susceptibility to CKD and periodontal disease.

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Chronic kidney disease (CKD) is a complex disorder that combines environmental and genetic effects (1). It represents a progressive and irreversible deterioration of the kidney's functional units, nephrons. It is characterized by reduction of renal mass, leading to structural hypertrophy of the remaining nephrons. It results from a wide spectrum of diseases, such as glomerulonephritis, diabetes, hypertension and autoimmune disorders (2,3), but its clinical manifestations are largely independent of the initial insult that damaged the kidneys. Loss of renal function arises with accumulation of metabolic waste products, which in turn change the normal homeostatic mechanisms that control electrolytic balance (4). Dialysis or renal transplant is required to remove toxic products of metabolism from the blood, the latter being the ideal form of treatment (5). In 2005, the prevalence of patients with CKD was 19.2 million in the USA (6) and two million in Brazil (7).

Renal patients are prone to infectious complications (8). In fact, chronic infections appear to be important causes of persistent systemic inflammation in CKD patients, which in turn have been considered a major risk factor for CKD patients' morbidity and mortality (9,10). Regarding complications of an infectious nature, periodontal disease has been referred to as a major infectious focus that could enhance levels of systemic inflammation, increasing patients' morbidity (11).

Periodontal disease or periodontitis is an infectious disorder, in which putative periodontopathogens trigger chronic inflammatory and immune responses that are thought to determine the clinical outcome of the disease (12). It is characterized by irreversible loss of tissue support around the teeth, which often leads to tooth loss. The main clinical sign that characterizes periodontitis is clinical attachment loss (CAL) caused by alveolar bone resorption (13).

Periodontitis has environment and genetics as determinant factors that contribute to the individual variation (14). Heritable risk factors may be

related to inflammatory or immune mechanisms that, if rendered ineffective or hyperactive, could enhance the pathogenic potential of bacterial plaque in susceptible individuals (15). According to the American Academy of Periodontology (16), 5–15% of people suffer from severe periodontal disease and 50% of adults have at least a moderate type of periodontitis. In Brazil, 50% of the population between 35 and 44 years old present some form of periodontal disease, according to the Brazil Oral Health Project (7). Periodontitis has been considered a CKD complication (17,18), and its prevalence and severity are suggested to be increased in CKD patients (19).

With the increasing number of patients in hemodialysis, studies have been focusing on CKD complications, mainly related to disturbance of mineral bone metabolism, such as secondary hyperparathyroidism (20), extra-osseous calcification (21) and bone diseases (22). The basic molecular mechanisms underlying bone metabolism in CKD and periodontal disease must be balanced to avoid bone damage. In this context, studies focusing on mediators of bone metabolism could contribute to the understanding of mechanisms involved in the outcome of CKD (23) and periodontal disease (13).

Bone is a dynamic tissue, which is continuously renovating in a process called 'remodelling' (24). The process of co-ordinated formation and resorption of bone may be up- or downregulated by a wide spectrum of factors, such as diseases, drug usage, systemic hormones [parathormone (PTH), calcitriol], local cytokines [interleukin (IL)-1, IL-6] and growth factors [such as tumour necrosis factor (TNF)], bone metabolism mediators [receptor activator of nuclear factor  $\kappa$ B (RANK) and RANK ligand (RANKL)] and genetic polymorphisms (25).

Osteoprotegerin (OPG), also known as factor of osteoclastic inhibition, is a secreted basic glycoprotein with 401 amino acid residues that belongs to the TNF receptor superfamily, and is considered as a bone-regulating protein with the capacity to decrease bone resorption (26). It is expressed by a

variety of organs and tissues, such as heart, lung, kidney, blood vessel wall, intestine, stomach, brain, thyroid gland, spinal marrow and bone (27). This protein has a function to antagonize RANKL, the main regulator of osteoclastogenesis (28). It is a critical cytokine for the differentiation, activation and survival of the osteoclasts and acts as a regulator of osteoblast–osteoclast cross-talk and homeostasis (29).

The OPG gene was cloned and characterized by Morinaga *et al.* (30). The gene, located on chromosome 8q23-24, represents a single copy gene with five exons spanning 29 kb. The translation termination codon is located in exon 5, and a typical poly(A) addition signal resides 173 nucleotides downstream of the translation termination codon. A major transcription initiation site is present 67 nucleotides upstream of the initiation ATG codon (30).

Genetic polymorphisms refer to the existence of two or more alleles at a given locus, with an allele frequency of more than 1% in a population. Single nucleotide polymorphisms (SNPs) represent the most common form of DNA variation in the human genome, and polymorphic alleles have been implicated in the augmentation of susceptibility to complex human diseases (31,32). Polymorphisms in genes of the host bone metabolism have been associated with CKD and periodontitis (33,34). However, to our knowledge, there are no studies investigating the association between polymorphisms in the OPG gene and CKD, and only few studies exploring the relationship between OPG polymorphisms and periodontitis (66,67,68). Thus, the aim of this study was to investigate the association between a polymorphism in the untranslated region (UTR) of the OPG gene and the susceptibility to chronic kidney disease and periodontitis.

## Methods

### Study population

A convenient sample of 224 unrelated subjects of either sex, mean age

Table 1. Baseline characteristics in all groups

	Group 1 (n = 60)	Group 2 (n = 50)	Group 3 (n = 50)	Group 4 (n = 64)
Ethnic group (n; %)				
Caucasoid	47 (78.3)	38 (76)	35 (70)	44 (68.8)
Afro-American	4 (6.7)	11 (22)	13 (26)	5 (7.8)
Mullato	9 (15.0)	1 (2)	2 (4)	15 (23.4)
Age (years; range)	37.8 ± 9.6 (20–70)	40.8 ± 9.4 (20–61)	45.2 ± 12.9 (23–74)	54.5 ± 12.2 (26–77)
Sex (n; %)				
Female	43 (71.7)	33 (66)	16 (34)	23 (35.9)
Male	17 (28.3)	17 (34)	34 (66)	41 (64.1)

Group 1, healthy patients; group 2, without CKD and with periodontal disease; group 3, with CKD and without periodontal disease; and group 4, presenting CKD and periodontal disease. The difference observed among groups in the mean age and sex is due to most CKD patients being older and male.

44.9 years (range 23–77 years), was selected from the Dental Clinics of Pontifical Catholic University of Paraná (PUCPR) and Pro-Renal Foundation, Curitiba, PR, Brazil. The patients were from Southern Brazil (Table 1). Subjects completed personal, medical and dental history questionnaires. The study was approved by the Ethical Committee in Research at PUCPR. Subjects signed a consent form after being advised of the nature of the study (approved under protocol 264/10184).

Kidney disease is a complex disorder and so is periodontitis. When more than one complex disease is investigated, it is important to consider two possible scenarios: (1) the investigated complex diseases present the same genetic predisposing background; or (2) one of the complex diseases predisposes to the other or both predispose to each other. Thus, four groups were selected, roughly equal in size: one control group that we termed 'negative control group' (60 individuals without CKD and without periodontal disease, group 1); groups with only one of each disease (group 2, 50 patients without CKD and with periodontal disease; group 3, 50 patients with CKD, in hemodialysis, and without periodontal disease); and group 4, composed of 64 patients with CKD, in hemodialysis, and with periodontal disease.

Patients without CKD presented glomerular filtration rate > 90 mL/min, estimated according to the

Modification of Diet Renal Disease (MDRD; 35).

Subjects could not have any of the following exclusion criteria: chronic usage of anti-inflammatory drugs; HIV infection; immunosuppressive chemotherapy; history of any diseases known to severely compromise immune function (for groups 1 and 2); active infection; current pregnancy or lactation; diseases of the oral hard or soft tissues, except caries (and periodontal disease for groups 1 and 3); use of orthodontic appliances; or present

necrotizing ulcerative gingivitis and periodontitis.

General clinical aspects of CKD patients are shown in Table 2.

### Clinical parameters of periodontitis

Diagnosis of periodontal disease was made on the basis of clinical parameters, such as probing pocket depth (PPD) and assessment of clinical attachment loss (CAL). Measurements of PPD and CAL were recorded at four points around each tooth. Subjects with CAL ≥ 5 mm, in at least three teeth, in at least two quadrants, were considered affected (36). The following parameters were recorded: the gingival index (37); the plaque index (38); the calculus index (39); and mobility (present or absent). The periodontal status of all subjects is shown in Table 3.

### Collection and purification of DNA

Cells were obtained using a mouthwash with 3% glucose solution and scraping of the oral mucosa with a sterile spatula (40). The DNA was extracted from epithelial buccal cells with 10 M ammonium acetate and 1 mM EDTA (41).

Table 2. Baseline clinical parameters of the chronic kidney disease patients

	Without periodontal disease (n = 50)	With periodontal disease (n = 64)
Main cause of CKD (n; %)		
Chronic glomerulonephritis	19 (38)	21 (32.8)
Hypertensive nephropathy	14 (28)	10 (15.9)
Diabetic nephropathy	7 (14)	14 (22.2)
Other/unknown	10 (20)	19 (30.2)
Duration of hemodialysis treatment (months) <sup>a</sup>	47.8 ± 48.0	47.2 ± 43.3
Systemic condition (n; %)		
Diabetes	7 (14)	17 (26.9)
Hepatitis	11 (22)	17 (26.9)
Cardiovascular disease	10 (20)	17 (26.5)
Hypertension	33 (66)	54 (85.7)
Current medication (n; %)		
Antihypertensives	35 (70)	50 (78.1)
Diuretics	10 (2)	23 (36.5)
Calcium carbonate	34 (68)	47 (73.4)
Vitamin D (calcitriol)	9 (18)	7 (11.1)
Antiplatelet agents	3 (6)	5 (7.9)
Others	41 (82)	51 (80.9)
Habits (n; %)		
Smoking	11 (22)	16 (25.3)

<sup>a</sup>Mean ± SD.

Table 3. Periodontal status of the study population

	Group 1 (n = 60)	Group 2 (n = 50)	Group 3 (n = 50)	Group 4 (n = 64)	p value
Gingival index	0.2 ± 0.4	1.5 ± 0.9	0.5 ± 0.6	1.7 ± 0.7	0.0001
Plaque index	0.3 ± 0.4	1.3 ± 1.0	0.5 ± 0.8	1.0 ± 0.9	0.0001
Calculus index	0.2 ± 0.2	1.0 ± 0.9	0.3 ± 0.5	0.7 ± 0.9	0.0001
PPD (mm)	1.5 ± 2.4	4.6 ± 1.0	2.0 ± 2.0	3.6 ± 1.1	0.0001
CAL (mm)	2.2 ± 2.6	6.1 ± 0.9	2.6 ± 2.7	5.3 ± 1.3	0.0001
Mobility (yes/no)	0/60	21/29	0/50	32/32	0.0001

Values are presented as means ± SD, with *p* values derived from the Kruskal–Wallis test.

### Analysis of OPG polymorphism

A 331 bp fragment (GenBank accession number AB008821) was amplified by polymerase chain reaction (PCR) using the following primer pair: (forward 5'-CCC AGG GGA CAG ACA CCA C-3' and reverse 5'-GCG CGC AGC ACA GCA ACT T-3'). Reaction conditions and cycling parameters were as follows. One microlitre of the genomic DNA was used for PCR amplification in a reaction mixture containing 22.5 µL PCR Supermix (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.3 µL of each primer. The reactions were performed in a Techne T-512 thermal cycler and consisted of denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 95°C for 1 min, annealing at 57°C for 1 min and elongation at 72°C for 1 min, with a final extension at 72°C for 7 min. Restriction fragment length polymorphism (RFLP) technique was performed in a final reaction volume of 20 µL, using 1 U of *HincII* (5'-GTPyT↓PuAC-3'; Invitrogen Life Technologies), and 10 µL aliquot of PCR products, digested at 37°C overnight. The digested products were separated by 1.7% agarose gel electrophoresis and visualized by ethidium bromide–UVB illumination. The genotypes were determined by comparing the restriction length polymorphism band patterns with a 1 kb plus DNA ladder (Invitrogen Life Technologies). The RFLP is formed by a single base transition (T/C) of the OPG gene that creates a *HincII* restriction site. The alleles which result from the cleavage of *HincII* are designated 'C' (*HincII* site present, with two fragments of 248 and 83 bp) or 'T' (*HincII* site absent, with one fragment of 331 bp).

### Statistical analysis

The differences in observed frequencies of polymorphism among the groups were assessed by standard chi-squared test ( $\chi^2$ ) and considered significant when the *p*-value was <0.05. Continuous variables were expressed as means and standard deviations. Comparisons of continuous variables were performed using one-way analysis of variance (ANOVA). Kruskal–Wallis test was used for non-parametric multiple comparisons for independent variables. Statistical analysis was performed using statistical software BioEstat 2.0 for Windows, SPSS (Statistical Package for the Social Sciences) 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

### Results

The study polymorphism was observed to be located at position –223 in the UTR region of OPG gene (Fig. 1). This polymorphism was referred to as a polymorphism in the OPG gene

promoter (position –950) by Brändström *et al.* (42) and other authors (43).

No statistically significant association was found between the polymorphism in the OPG gene and CKD or periodontal disease. Moreover, there no association of the polymorphism was found with clinical parameters of periodontal disease. The allele frequencies and genotype distributions of the OPG polymorphism for all groups are shown in Table 4.

### Discussion

The identification of the OPG–RANKL–RANK system as the dominant, final mediator of osteoclastogenesis represents a major advance in bone biology. The initial cloning and characterization of OPG as a soluble, decoy receptor belonging to the TNF receptor superfamily was the first step that eventually led to an unraveling of this system. Soon thereafter, the molecule blocked by OPG, called RANKL, was identified as the key

cctcagagccccgcggagacagcagccgctgttctcagccccggtggcttttttccccctgctctcccaggg  
gacagacaccaccgccccaccctcagccccacctccctggggatCCTTTCCGCCCCAGCC  
CTGAAAGCgtaaT/CCCTGGAGCTTTCTGCACACCCCCGACCGCTCCCGC  
CCAAGCTTCCTAAAAAGAAAGGTGCAAAGTTTGGTCCAGGATAGAAAAAT  
GACTGATCAAAGGCAGGCGATACTTCCTGTTGCCGGGACGCTATATATAA  
CGTGATGAGCGCACGGGCTGCGGAGACGCACCGGAGCGCTCGCCCAGC  
CGCCGCCTCCAAGCCCCTGAGGTTTCCGGGGACCACA\*atgaacaagttgctgtgc  
tgccgctcgtgtaagtccctgggccagccgacgggtgccggcgctggggaggctgctgccacctggtc  
tcccaacctcccagcggaccggcggggagaaggctccactcgctccctccaggagaggctggggtagg  
ctggagcaggaaaccgcttcaagttatgccatgctcccctagggt

Fig. 1. Nucleotide sequence of the human OPG gene (AB008821). The underlined bases represent the primers for the OPG polymorphism. Capital letters indicate 5' UTR. The asterisk represents the beginning of the first exon. Italicized nucleotides show the restriction site for *HincII*. Boldface bases represent the polymorphism (T/C).

Table 4. Allele frequency and genotype distribution of the OPG SNP

SNP (n; %)	Group 1 (n = 60)	Group 2 (n = 50)	Group 3 (n = 50)	Group 4 (n = 64)	chi-squared
<b>Genotypes</b>					
T T	47 (78.3)	41 (82.0)	43 (86.0)	52 (81.3)	$\chi^2 = 3.86$
T C	5 (8.3)	6 (12.0)	3 (6.0)	4 (6.2)	$p = 0.69$
C C	8 (13.4)	3 (6.0)	4 (8)	8 (12.5)	
<b>Alleles</b>					
T	99 (82.5)	88 (88.0)	89 (89.0)	108 (84.4)	$\chi^2 = 2.50$
C	21 (17.5)	12 (12.0)	11 (11.0)	20 (15.6)	$p = 0.47$

mediator of osteoclastogenesis in both a membrane-bound form expressed on preosteoblastic/stromal cells and a soluble form. In turn, RANKL was shown to bind its receptor, RANK, on osteoclast lineage cells. The important role played by these factors in regulating bone metabolism was demonstrated by the findings of extremes of skeletal phenotypes (osteoporosis and osteopetrosis) in mice with altered expression of these molecules (44).

The RANK–RANKL–OPG regulatory axis is also involved in inflammatory bone destruction induced by pro-inflammatory cytokines, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (45). In addition, a number of other mediators of bone metabolism, such as TGF- $\beta$  (46), PTH (47), 1,25-dihydroxyvitamin D<sub>3</sub> (48), glucocorticoids (49) and estrogen (50), exert their effects on osteoclastogenesis by regulating osteoblastic/stromal cell production of OPG and RANKL. However, not all regulation of osteoclasts is exclusively via the osteoblast, since calcitonin acts directly on osteoclastic cells (51) and estrogen has been shown to induce apoptosis of osteoclasts (52).

Osteoprotegerin might protect bone against intensive bone loss resulting from the imbalance of bone kinetics in CKD hemodialysis patients (4). Higher serum OPG and lower serum RANKL were found in CKD patients in hemodialysis. Increased serum OPG levels in hemodialysis patients are believed to partly reflect a compensatory response to increased bone loss (53). The determination of serum OPG levels in association with PTH levels could be useful in the diagnosis of bone turnover in renal patients (24). Besides, it could contribute to prevent patients

from developing vascular calcification, a major risk factor for cardiovascular diseases, which in turn is an important mortality indicator in CKD patients (54).

The OPG expression from gingival tissue was higher in chronic periodontitis than in healthy patients (12). Human periodontal ligament cells stimulated with lipopolysaccharide could inhibit osteoclastogenesis by producing higher levels of OPG than RANKL via the induction of IL-1 $\beta$  and TNF- $\alpha$  (55). In contrast, an increased concentration of RANKL and a decreased concentration of OPG were detected in gingival crevicular fluid from patients with periodontitis (56). Also, osteoblasts in culture exposed to a stimulus of periodontopathogens showed increased expression of RANKL and decreased expression of OPG (57). However, levels of OPG in saliva did not show a relationship with periodontal disease and were not correlated with periodontal indices (58). *Porphyromonas gingivalis* upregulated the expression of OPG in human microvascular endothelial cells via a nuclear factor  $\kappa$ B-dependent pathway; thus, these endothelial cells may act as a source of OPG and thereby may play a role in regulating bone metabolism in periodontitis (59). Thus, changes in the levels of this regulator of osteoclast differentiation may play a major role in the bone loss observed in periodontitis (60).

A number of polymorphisms in the OPG gene have been described in previous investigations and associated with bone mineral density (61), vertebral fractures (62), coronary artery disease (43), Paget's disease (63), osteoarthritis (64) and osteoporosis

(65) in different populations. To our knowledge, this is the first study investigating the association between polymorphisms in the OPG gene and CKD. We found no association between the study OPG polymorphism and CKD. We have recently identified an association of a polymorphism in the vitamin D receptor (VDR) gene with CKD (34). However, other polymorphisms in the OPG gene and in other genes of the host bone metabolism response may also be involved in the determination of susceptibility to and/or progression of CKD.

With regard to periodontitis, there are a few association studies investigating polymorphisms in the OPG gene. No association was found between aggressive (66) or chronic periodontitis (67,68) and OPG polymorphisms. Lack of association between an OPG polymorphism and chronic periodontitis was also observed in our study. It is worth mentioning that the investigated polymorphisms in our study and in the two other studies reporting periodontitis are limited to the upstream region of the OPG gene. A physical study considering linkage disequilibrium blocks with a number of polymorphisms representing the whole gene could facilitate understanding of the real involvement of this gene in the determination of susceptibility to periodontal diseases. Besides, other polymorphisms in genes of the immune-inflammatory and bone metabolism host response may be involved in the modulation of periodontal diseases.

In relation to functionality of this polymorphism, although the study polymorphism is located 129 bp upstream from the TATA box, 13 bp downstream from the activating protein 2-binding site and 32 bp upstream from a specific protein 1-binding site, it does not seem to interfere with transcription activity of this gene (43).

Complex diseases, such as CKD (69) and periodontal disease (70), have a genetic basis that combines effects of the interaction of sequence variation of multiple genes with the environment (71). Even though no association of the study OPG gene polymorphism was

found with either chronic kidney disease or periodontitis, osteoprotegerin may have an impact in basic molecular mechanisms underlying bone metabolism in both CKD and periodontal disease. Additional studies investigating other polymorphisms in this and other genes of the host response could help to clarify the involvement of bone metabolism mediators in the determination of susceptibility to CKD and periodontal disease.

It was concluded that polymorphism OPG-223 (C/T) was not associated with CKD and periodontal disease in a Brazilian population.

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