original article

Genetic polymorphism and failure of dental implants: a pilot study

Abstract / Objective: *To investigate the relationship between genetic polymorphisms related to cytokines in bone remodeling osteoprotegerin (OPG) and receptor activator of nuclear factor kappa ß ligand (RANKL). It also aims to relate the presence of OPG and RANKL polymorphisms with clinical failure of dental implants over time and to determine which combinations of OPG and RANKL polymorphisms are associated with failure of dental implants.* **Methods:** *Twenty patients of both sexes, over eighteen years, rehabilitated with thirty-four dental implants were evaluated during 24 months after implant-supported crown placement. After clinical and radiographic examination, patients were included in a failure group if one or more of the following criteria were identified: mobility, persistent subjective complaints, recurrent peri-implantitis with suppuration, continuous radiolucency around the implant, probing depth ≥ 5 mm and bleeding on probing. Peripheral blood was collected for analysis of cytokine OPG and RANKL polymorphisms by polymerase chain reaction (PCR).* **Results:** *There were no statistically significant differences between the failure group in relation to genotypes OPG and RANKL.* **Conclusion:** *OPG and RANKL polymorphisms did not influence dental implants failure in the investigated sample.*

Keywords: *Dental implants. Genetic polymorphism. Osteoprotegerin. RANK-Ligand.*

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How to cite this article: Teixeira CEC, Matheus TCU, Souza PRE, Donos N, Cimões R. Genetic polymorphism and failure of dental implants: a pilot study. Dental Press Implantol. 2014 Oct-Dec;8(4):68-78. doi: http://dx.doi.org/10.14436/2237-650X.8.4.068-078.oar

Submitted: September 10, 2014 - **Revised and accepted:** October 05, 2014

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» The authors report no commercial, proprietary or financial interest in the products or companies described in this article.

INTRODUCTION

Clinical evidence on osseointegration has changed the field of Implantodontics and, as a result, has rendered osseointegrated implants the best esthetic and functional alternative for edentulism.[1] Osseointegration, as a means of direct anchorage of implant placement around the receiving bone site, appears to be the most important feature of long-term implant placement clinical success.[2,3] Osseointegration success relies on biological (absence of infection, peri-implantitis, bone loss) and technical factors (absence of overload and fracture).[4,5]

Although osseointegration yields predictable, reproducible and stable long-term outcomes with high success rates ranging from 85 and 94% 10 years after dental implant placement,[6] implant failure still occurs. Furthermore, the growing demand for this type of procedure in the last few years has increased the number of cases involving failure and related complications.[7] The success rate goes down to 61% when well-established criteria are used to determine whether an implant was successful or not.[8]

Integration and maintenance of dental implants into the bone are associated with ongoing bone tissue renewal which, in turn, results from the process of resorption and tissue neoformation. Bone remodeling is driven, whether positively or negatively, by a number of factors, namely: diseases, medication, systemic hormones (parathormone and calcitonin), local cytokines (interleukins: IL-1 and IL), growth factors (TNF), bone metabolism mediators (receptor activator of nuclear factor kappa ß-RANK), its RANKL ligand and osteoprotegerin (OPG).[9,10]

RANKL is a cytokine belonging to the TNF family and is essential for osteoclastogenesis.[9] Osteoprotegerin (OPG) is a protein that prevents osteoclasts activation and production by competing with RANKL through RANK receptors on the surface of pre-osteoclasts and/or osteoblasts.[11]

Genetic polymorphism is a variation in the sequence of nucleotides, and is located in a specific portion of a DNA (deoxyribonucleic acid) molecule with at least two structural variations (alleles). It is found in the overall population with a frequency slightly greater than 1%.[12] Single nucleotide polymorphisms (SNPs) are the main DNA variation in the human genome, and polymorphic alleles have been reported a major cause of human complex disease susceptibility.[13]

Presently, little is known about genetic susceptibility and polymorphisms associated with peri-implant complications.[14] Studies assessing a potential relationship between bone metabolism markers and peri-implant mucositis or peri-implantitis are rare.[15] Thus, a few studies have made an attempt to establish a relationship between cytokines concentration in peri-implant crevicular fluid and peri-implant diseases[16,17] so as to identify specific genotypes and alleles as markers of implant loss.

The present pilot study aimed at investigating the relationship established between bone remodeling polymorphisms and dental implant failure.

MATERIAL AND METHODS

Study design and patients

A therapeutic prognosis cohort study was conducted from November 2012 to November 2013 at the Federal University of Pernambuco (UFPE) postgraduate program clinic. A total of 20 male and female patients who underwent rehabilitation treatment with 34 single dental implants took part in this research. In selecting the sample, the following inclusion criteria were applied: patients older than 18 years of age who

underwent rehabilitation treatment with single dental implants (Straumann, Basel, Switzerland) and were in good periodontal health conditions. Patients were initially assessed one year after prostheses placement, and subsequently every 6 months, thereby totalizing a 24-month follow-up. The following exclusion criteria were applied: patients presenting systemic conditions, such as diabetes mellitus, and periodontal disease; patients who missed three assessment sessions or were attending other dental premises but the Federal University of Pernambuco (UFPE) postgraduate program clinic. All research subjects signed TCLE consent form soon after the study was approved (CEP-UFPE) under protocol CAAE 03534012.0.0000.5208.

Clinical and radiographic assessment

All patients were assessed by a previously calibrated researcher using a North Carolina periodontal probe (Trinity, São Paulo, Brazil). Probing depth (PD) and clinical attachment loss (CAL) were measured and recorded on four different implant surfaces (mesial, distal, lingual, buccal). Presence and absence of mobility were recorded and periapical intraoral radiographic examination was carried out using the parallel technique and a positioner. Patients were advised on oral hygiene and prophylaxis.

After clinical and radiographic examination, patients were divided into two groups (Table 1). The implant success group (control) was set up once the criteria established by Ong et al[18] were applied, whereas the implant failure group (study) was set up when one or more criteria pertaining to the failure group was identified.

Sampling and DNA isolation

For the first assessment session, 8 ml of peripheral blood were obtained and transferred to cartridges filled with EDTA. Blood samples were stored at -20 ºC for subsequent DNA isolation and analysis.

Table 1. Dental implants success and failure criteria according to Ong et al.[18]

Success group	Failure group
» Absence of mobility;[19] » Absence of subjective complaints (pain, foreign body sensation, dysesthesia);[19] » Absence of peri-implant infection associated with suppuration;[19] » Absence of radiolucency around the implant;[19] » Probing depth not greater or equal to 5 mm;[20,21] » Absence of bleeding on probing.[20]	» Presence of bleeding on probing; » Probing depth greater or equal to 5 mm; » Presence of radiolucency around the implant;[19] » Presence of subjective complaints (pain, foreign body sensation, dysesthesia);[19] » Presence of mobility. <u>Peri-implantitis</u> » Probing depth greater than or equal to 5 mm, bleeding on probing, purulent discharge and radiographic bone loss.[22]

DNA was isolated from patient's blood samples using QIAamp DNA mini kit (Qiagen, Hilden, Germany) in compliance with the manufacturer's instructions. After the isolation procedure, DNA remained stored at – 20 °C until PCR processing.

PCR processing for OPG

The promoter region (-950 element) of the OPG23 gene was amplified using the following pair of primers: forward 5'-CCC AGG GGA CAG ACA CCA C-3' and reverse 5'-GCG CGC AGC ACA GCA ACT T-3'.[10]

PCR reactions were prepared using GoTaq Green Master Mix solution (Promega, Madison, USA), as follows: 10.0 µL of water; 1.0 µL of forward primer; 1.0 µL of reverse primer; 10.0 µL of Master Mix; and 3.0 µL of DNA; totaling 25.0 µL total volume. All reactions included an amplification reaction without DNA sample used as negative control, so as to examine the potential for contamination.

The thermocycler (Biocycler) settings were as follows: Initialization step, hot start (95 °C for 5 minutes); second step divided into three steps for 35 cycles: [1] target DNA denaturation by heating (95 °C for 1 minute), [2] annealing of primer (57 °C for 1 minute), [3] extension (72 °C for 1 minute); and third step, final elongation (72 °C for 7 minutes).

Polymorphism analysis was performed by means of the restriction fragment length polymorphism (RFLP) technique. To this end, 5.0 µl of the product were subjected to digestion by 4 U of HincII restriction enzyme (Promega) at 37 °C overnight, totaling 10.0 µl total volume.

Subsequently, 10.0 µl of PCR product were added to 8.0 µl of GelRed^{M} fluorescent stain (Biotium, California, USA) and subjected to electrophoresis in 2.5% agarose gel. Electrophoresis runs were detected under ultraviolet light and photographed for future analysis. Molecular mass marker (100 bp ladder, LGC Biotecnologia) was added to the electrophoresis run.

RFLP is formed by a single base (T/C) of the OPG gene that creates a restriction site for Hinc II. Alleles resulting from cleavage by Hinc II are termed "C" (presence of Hinc II site, presence of two fragments 248 pb and 83 pb) or "T" (absence of Hinc II site, with one fragment 331 pb).[10]

PCR processing for RANKL156 and RANKL438

The promoter region of RANKL156 (rs9533156) and RANKL438 (rs2277438) genes was amplified using the ARMS-PCR technique, as described by Kadkhodazadeh et al.[11] RANKL156 gene is located in the promoter region,[24] whereas RANKL438 is at 5' untranslated region (5' -UTR).[25]

The following primers were used:

» rs2277438, allele primer: X GTTGGGGACATAAAGACTCTTGCA, allele primer: Y GGGGACATAAAGACTCTTGCG, common primer: CTGCTATTTAATACAGTGTGACTTAAGAA;

» rs9533156, allele primer: X CCCTTTACCCTTTTCTCTGCACC, allele primer: Y CCCTTTACCCTTTTCTCTGCACT, common primer: GCCTATAGACACCAACTCTGACTTTATAA.

ARMS-PCR reactions were prepared using GoTaq Green Master Mix solution (Promega, Madison, USA), as follows: 5.0 µL of water; 0.3 µL of primer X; 0.3 µL of common primer; 4.4 µL of Master Mix; and 2.0 µL of DNA; totaling 12.0 µL total volume. The same protocol was also applied to primer Y. All reactions included an amplification reaction without DNA sample used as negative control, so as to examine the potential for contamination.

The thermocycler (Biocycler) settings for RANKL156 were as follows: Initialization step,

hot start (95 °C for 5 minutes); second step divided into three steps for 35 cycles: [1] target DNA denaturation by heating (95 °C for 1 minute), [2] annealing of primer (55 °C for 1 minute), [3] extension (72 °C for 1 minute); and third step, final elongation (72 °C for 7 minutes). For RANKL438: Initialization step, hot start (95 °C for 5 minutes); second step divided into three steps for 35 cycles: [1] target DNA denaturation by heating (95 °C for 1 minute), [2] annealing of primer (53 °C for 1 minute), [3] extension (72 °C for 1 minute); and third step, final elongation (72 °C for 7 minutes).

Subsequently, 5.0 µl of PCR product were added to 4.0 µl of GelRedM fluorescent stain (Biotium, California, USA) and subjected to electrophoresis in 2.0% agarose gel. Electrophoresis runs were detected under ultraviolet light and photographed for future analysis. Molecular mass marker (100 bp ladder, LGC Biotecnologia) was added to the electrophoresis run.

Statistical analyses

Absolute distribution, percentages and statistical values of mean, standard deviation and median were obtained for data analysis. McNemar's test was used to compare categorical variables; F (ANOVA) with Bonferroni correction was used to assess repeated measurements; Pearson's chi-squared or Fisher's exact test were also used. The conditions for the chi-squared test were not examined. Margin of error was set at 5%. Data were processed in Excel spreadsheet. SPSS software (Statistical Package for the Social Sciences) version 21.0 was used for statistical analyses.

RESULTS

Patient's individual assessment

Patients were aged between 25 and 69 years old, with a mean age of 41.90 ± 11.05 and median of 40.50. A total of 55% of patients were females.

Table 2 shows that, for OPG, most patients (70%) had genotype TT. As for RANKL156, most patients (80%) were CT; and for RANKL438, the majority was AG (75%).

Table 2 also shows that, for OPG, most patients had T allele (80%); whereas for RANKL156, more than half (55%) had C allele and for RANKL438 more than half (57.5%) had G allele.

Results according to implant

Based on assessment of 34 implants in place, Table 3 presents the results for mobility, subjective complaint, infection associated with suppuration, radiolucency, probing ≥ 5 mm, bleeding on probing (BP), annual vertical bone loss and implant outcomes (failure or success). Most implants presented bleeding on probing; however, this percentage decreased at each new assessment as follows: 94.1%(assessment 1), 79.4% (assessment 2), 58.8% (assessment 3); with $P < 0.05$ between the first and third assessments and between the second and third assessments. Most implants were classified as cases of failure; however, this percentage decreased at each new assessment as follows: 94.1%(assessment 1), 79.4% (assessment 2) and 61.8% (assessment 3); with $P < 0.05$ between the first and third assessments.

Table 4 presents mean and standard deviation values, as well as probing depth (PD) and clinical attachment loss (CAL) median values for each assessment phase. Importantly, the mean values of both measurements reduced at each assessment phase, with significant differences revealed by multiple comparison tests (between pairs), and difference between the first and third phases for each measurement individually. From the first to the third assessment phase, PD reduced from

2.76 to 2.38 mm; whereas CAL reduced from 2.90 to 2.53 mm.

DISCUSSION

During the last decades, dental implants have become a treatment alternative commonly applied to cases of complete or partial tooth loss. Implant treatment prognosis is often reported by means of survival rates, i.e., based on the time an implant remains in one's mouth.[26] In order to describe treatment results as accurately as possible, implants clinical conditions must be considered.[27-30]

According to various authors,[19,31,32] no consensus has been reached regarding the exact definition of implant success; nevertheless, a number of criteria has been proposed. These criteria are based on clinical as well as radiographic parameters and are used to determine implant survival rates. Since no exact definition of implant success has been determined, long-term studies are considered of paramount importance.[18,33]

The present study found high failure rates, especially for the first assessment; however, these rates decreased over time,

Table 2. Patients' features in terms of genotype and allele.

Variable	n	%
• **OPG genotype**		
TC	4	20.0
TT	14	70.0
CC	2	10.0
• **RANKL156 genotype**		
CT	16	80.0
TT	1	5.0
CC	3	15.0
• **RANKL438 genotype**		
AG	15	75.0
GG	4	20.0
AA	1	5.0
Total	**20**	**100.0**
• **OPG alleles**		
T	32	80.0
C	8	20.0
• **RANKL156 alleles**		
T	18	45.0
C	22	55.0
• **RANKL438 alleles**		
A	17	42.5
G	23	57.5
Total	**40**	**100.0**

Table 3. Assessment of criteria applied to determine dental implant failure or success.

Variable	Assessment						P value
	1		2		3		
	n	%	n	%	n	%	
TOTAL	**34**	**100.0**	**34**	**100.0**	**34**	**100.0**	
• **Mobility**							
Yes	-	-	2	5.9	-	-	**
No	34	100.0	32	94.1	34	100.0	**
							**
• **Subjective complaint**							
Yes	-	-	-	-	-	-	**
No	34	100.0	34	100.0	34	100.0	**
							**
• **Infection associated with suppuration**							
Yes	-	-	1	2.9	-	-	**
No	34	100.0	33	97.1	34	100.0	**
							**
• **Radiolucency**							
Yes	1	2.9	1	2.9	1	2.9	$p^{(1)} = 1.000$
No	33	97.1	33	97.1	33	97.1	$p^{(2)} = 1.000$
							$p^{(3)} = 1.000$
• **Probing ≥ 5 mm**							
Yes	6	17.6	5	14.7	4	11.8	$p^{(1)} = 1.000$
No	28	82.4	29	85.3	30	88.2	$p^{(2)} = 0.688$
							$p^{(3)} = 1.000$
• **Bleeding on probing**							
Yes	32	94.1	27	79.4	20	58.8	$p^{(1)} = 0.125$
No	2	5.9	7	20.6	14	41.2	$p^{(2)} < 0.001$*
							$p^{(3)} = 0.039$*
• **Annual vertical bone loss**							
Yes	1	2.9	1	2.9	1	2.9	$p^{(1)} = 1.000$
No	33	97.1	33	97.1	33	97.1	$p^{(2)} = 1.000$
							$p^{(3)} = 1.000$
• **Results according to implant**							
Failure rate	32	94.1	27	79.4	21	61.8	$p^{(1)} = 0.125$
Success rate	2	5.9	7	20.6	13	38.2	$p^{(2)} = 0.001$*
							$p^{(3)} = 0.070$

(*): Significant difference at 5%.
(**): Not applicable due to differences among assessment categories.
(1): By means of McNemar test, between the first and second assessment phase.
(2): By means of McNemar test, between the first and third assessment phase.
(3): By means of McNemar test, between the second and third assessment phase.

Table 4. Assessment of criteria applied to determine dental implant failure or success.

Variable	Assessment			P-value
	1	2	3	
	Mean ± SD (Median)	Mean ± SD (Median)	Mean ± SD (Median)	
• Probing depth	2.76 ± 0.65 (2.50) [A]	2.66 ± 0.82 (2.50) [AB]	2.38 ± 0.52 (2.38) [B]	p[(1)] = 0.006*
• Clinical attachment loss	2.90 ± 0.71 (2.69) [A]	2.79 ± 0.85 (2.75) [AB]	2.53 ± 0.55 (2.50) [B]	p[(1)] = 0.005*

(*): Significant difference at 5%.
(1) By means of F test (ANOVA) for repeated measures.
Note: Different letters between brackets reveal evidence of significant difference between assessments compared by Bonferroni correction.

with significant difference between the first and third assessment. Low success rates probably occurred due to the thorough failure criteria employed in this study; including, for instance, bleeding on probing found in several patients during the first assessment and which decreased over time despite being found in the last assessment phase.

Implant failure tends to increase over time; however, the definition of failure varies widely among studies, with many of them considering the presence of peri-implantitis as the only determining factor. Thus, assessment considering bleeding on probing alone, which is not necessarily associated with peri-implantitis, increased the failure rates found in the present study. Decreased bleeding on probing over time and consequent increase in success rates might have occurred due to improvements in patient's oral hygiene. Once bleeding is acknowledged during the first assessment session, patients tend to improve biofilm control, especially after being advised by the dentist at each session. This is in accordance with other findings[34] reporting the significant effects produced by satisfactory oral hygiene on implant success and survival.

A prospective study conducted with patients diagnosed with severe periodontitis assessed[35] mucositis, peri-implantitis, implant success and survival rates. The criteria established for implant success were similar to those used in the present study. The group comprising patients without periodontal disease had a failure rate of 50%, similar to the results of the present study (61.8%) by end of the third assessment phase.

In the present study, PD ≥ 5 mm was rarely found at the third assessment phase; with a statistically non-significant decrease from one phase to the other. In agreement with another study,[32] PD was associated with other criteria, such as bone loss, subjective complaint, infection associated with suppuration, and bleeding; since PD alone has little diagnostic value and does not necessarily suggest intraosseous implant disease.

Similarly to other variables, both PD and CAL values decreased from one assessment phase to the other. A previous study[36] found a mean PD value of 5.55 mm and clinical attachment loss value of 4.85 mm in the

peri-implantitis group. Another study[11] found a mean PD value of 6.81 mm and CAL value of 4.44 mm in the peri-implantitis group. The aforementioned results differ from the present study probably due to the latter presenting a lower number of patients with PD ≥ 5 mm.

Some studies[37-43] establish a correlation between genetic alterations and peri-implant bone loss; however, they assessed different chemokines and polymorphic regions, populations of varied ethnicity and a limited number of patients. In addition, these studies did not establish patterns for clinical characterization, did not differentiate peri-implant mucositis from peri-implantitis, and did not conduct long-term follow-ups to assess implant survival rates after initial assessment. The present study aimed at establishing a correlation between implant failure and OPG and RANKL cytokines. Identifying the OPG-RANKL-RANK system as the final mediator of osteoclastogenesis is a major advance in bone biology.

This study did not find statistically significant differences when OPG, RANKL156 and RANKL438 polymorphisms were associated with dental implant failure within 24 months. Considering the criteria for implant failure used in the present study, it is reasonable to assert that the literature does not comprise other studies employing the same criteria and making an attempt to establish an association between the aforementioned cytokines polymorphisms.

In this research, the genotype most frequently found for OPG was TT, while the T allele was most frequently found and associated with implant failure. No statistically significant relationship was established between TT genotype – T allele and implant failure or SS and PS ≥ 5 mm clinical parameters, although this genotype was the most frequently found in implant failure cases in the three assessment phases. A study[10] established a relationship between periodontal disease (PD) and OPG polymorphism and found high percentages of TT genotype in the PD group. In the healthy group, the T allele was predominant, despite non-significant statistically relevance.

A study associating peri-implantitis with RANKL156 and RANKL438 polymorphism[11] found significant association between RANKL156 CT genotype and peri-implantitis; however, such relationship was not established between RANKL438 and peri-implantitis. This is in agreement with the present study, since, despite not being statistically significant, the CT RANKL156 genotype was the genotype most frequently found in implant failure cases in the three assessment phases. On the other hand, in the present study, RANKL156 T allele was most frequently found in the peri-implantitis group.[11] The C allele was the genotype most frequently associated with implant failure; however, with no statistically significant relevance in allele frequency in either one of the studies.

The present study might not have found significant relationship between polymorphism and implant failure due to the limited number of patients and implants investigated. Large study populations are paramount to avoid potential bias resulting from samples of heterogeneous nature.[44]

CONCLUSION

The present findings did not establish a significant association between OPG – RANKL polymorphism and dental implant failure. Thus, further studies with a longer follow-up and a higher number of patients are essential to yield clearer results.

ACKNOWLEDGEMENTS

This study was carried out with the Coordination for Improvement of Higher Education Personnel (CAPES) Institutional Postdoc 2011 funds.

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