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# Migration of titanium dioxide microparticles and nanoparticles through the body and deposition in the gingiva: an experimental study in rats

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The aim of this experimental work was to evaluate deposition of titanium dioxide ($TiO_2$) microparticles and nanoparticles, which could originate from titanium bioimplants, in the gingiva. Wistar rats were injected intraperitoneally (i.p.) with a suspension of $TiO_2$ particles of different sizes (150, 10, or 5 nm). The rats were killed 12 months post-injection, and the buccal and lingual gingivae were resected and evaluated using light and scanning electron microscopy. Energy-dispersive X-ray spectroscopy (EDS) was used to confirm the presence of titanium in deposits of microparticles and nanoparticles, and the concentration of titanium in tissues was measured using inductively coupled plasma–mass spectrometry (ICP-MS). Histological examination showed that all experimental groups exhibited agglomerates, in the gingiva, of titanium particles of micrometer size range, with no associated inflammatory response. Higher concentrations of titanium traces were shown, by ICP-MS, in both buccal and lingual tissues of all experimental groups compared with their matched controls. Titanium concentrations were significantly higher in the buccal gingiva than in the lingual gingiva, and after injection with 5-nm particles than with 10-nm particles in both localizations. Titanium microparticles and nanoparticles deposit in the gingiva, and mostly on the buccal side. Gingival deposition of titanium could be considered a tissue indicator of tribocorrosion processes of titanium bioimplants.

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Owing to the widespread use of metallic biomaterials in orthopedics and dentistry, the surface of biomedical devices could be a potential source of systemic contamination caused by the release of ions/particles (1–3). Titanium is the most commonly used metallic biomaterial in the manufacture of biomedical implants. It is widely used in implants in oral and maxillofacial surgery in the form of grids, plates, screws, and distracters, among others (4). It is a highly reactive metal, and on exposure to air or fluids it rapidly develops a passivating layer of titanium dioxide ($TiO_2$). This layer is responsible for biocompatibility and forms the interface between the biological milieu and the implant, decreasing material reactivity and partially preventing corrosion (1, 5, 6). However, no metal or metal alloy is completely inert in vivo. Because the metal implant is in contact with body tissues and fluids, ions/particles could be released into the biological milieu. It must be pointed out that ions/particles may be released from metal implants as a result of electrochemical corrosion processes, frictional wear, or a synergistic combination of both (1, 7). Mechanical movement of the components of implants against each other results in friction and wear, the study of which is called tribology. The combined effect of mechanical, biochemical, and electrochemical factors is known as tribocorrosion (8).

In this regard, our research group has reported the presence of titanium particles in peri-implant tissue around failed human dental implants (9), in oral mucosa in contact with implant cover screws (10), in cells exfoliated from peri-implant oral mucosa around titanium dental implants (11), and in reactive lesions of peri-implant mucosa (12). The surface of a metallic medical implant may be a potential source of release of both microparticles (MPs) (>100 nm) and nanoparticles (NPs) (1–100 nm) into the biological milieu. As NPs have a greater surface-to-volume ratio compared with MPs, they are biologically more reactive and potentially more harmful to body tissues. In addition, although MPs and NPs can be chemically similar, their

specific physical–chemical properties may result in different biological responses (3).

Ions/particles released from the surface of titanium metallic implants could migrate systemically and deposit in gingival tissue, as found for other metals. Metallic particles that reach the chorion systemically have typically been found to deposit at the level of the buccal gingiva (13). To our knowledge there are no studies in the literature reporting titanium MPs or NPs migrating systemically and depositing in the gingiva. Therefore, the aim of the present experimental work was to evaluate deposition of $TiO_2$ MPs and NPs, which could originate from titanium bioimplants, in the buccal and lingual gingivae.

## Material and methods

### Animal treatment protocol

Male Wistar rats ($n = 40$), weighing ~100 g, were injected intraperitoneally (i.p.) with a suspension of $TiO_2$ particles in 5 ml of 0.9% sodium chloride (NaCl) at a dose of 1.6 g of TiO2 particles/1,000 g body weight, following our experimental model (14). The experimental treatments were as follows: $TiO_2$-MPs150 ($n = 10$): i.p. injection with $TiO_2$ MPs (anatase), of average particle size (APS) 150 nm (Sigma Chemical Company, St Louis, MO, USA); $TiO_2$-NPs10 ($n = 10$): i.p injection with $TiO_2$ NPs (anatase), of APS 10 nm (Nanostructured and Amorphous Materials, Los Alamos, NM, USA); and $TiO_2$-NPs5 ($n = 10$): i.p injection with $TiO_2$ NPs (anatase), APS 5 nm (Nanostructured and Amorphous Materials). Control rats ($n = 10$) were injected with an equivalent volume of vehicle (NaCl). All rats were killed 12 months post-injection.

Adequate measures were taken to minimize any pain and discomfort experienced by the rats. All procedures were performed in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (15) and the guidelines of the School of Dentistry of the University of Buenos Aires (Res. (CD) 352/02 and Res. (CD) 694/02).

The protocol was approved by the Institutional Experimentation Committee (School of Dentistry of the University of Buenos Aires, Resolution Number 28/11/2012-37).

### Characterization of particles

Before injection, APS and morphology were confirmed by scanning electron microscopy, using a scanning electron microscope (Carl Zeiss SUPRA 40; Carl Zeiss, Oberkochen, Germany) equipped with a field emission filament. For this purpose, the particles were placed on a conductive carbon tape and were analyzed without being coated. Images were obtained using an in-lens detector and 4-kV acceleration voltage. The particles were chemically identified using energy-dispersive X-ray spectroscopy (EDS) (Oxford Instruments, Bucks, UK).

### Histology

All rats were killed 12 months post-injection by i.p. overdose of ketamine chlorhydrate (Holliday-Scott, Buenos Aires, Argentina). The buccal and lingual, unattached and attached gingivae of the first and second lower molars were resected. The samples were examined macroscopically using a stereo magnifier (Zeiss Stemi 2000-C; Carl Zeiss, Jena, Germany) and half of the samples were used for histologic evaluation. They were fixed in 10% buffered formalin and embedded in paraffin to obtain histological sections, which were stained with hematoxylin and eosin (H&E) or Grenacher's carmine and examined using conventional and polarized light microscopy. The remaining samples were processed for determination of titanium concentration.

### Tissue titanium concentration

The samples used to determine titanium concentration were weighed, dissolved in 65% nitric acid (Merck, Darmstadt, Germany), and then analyzed using inductively coupled plasma–mass spectrometry (ICP-MS) (Elan DRC II; PerkinElmer, Shelton, CT, USA). The titanium concentration was quantified in parts per billion.

### Characterization of particles in tissues

The histologic sections showing particle deposits were evaluated using scanning electron microscopy (Carl Zeiss Supra 40 microscope; Carl Zeiss, Oberkochen, Germany), and the chemical composition of the particles was determined using EDS (Oxford Instruments).

### Immunohistochemistry

An immunohistochemical technique was performed to identify Langerhans cells (using the Langerhans cell marker, CD1a) (Cell Marque, Rocklin, CA, USA). Formalin-fixed tissue sections were deparaffinized, rehydrated in distilled water, and then incubated with monoclonal mouse anti-human CD1a at 20°C for 1 h. Then, the sections were incubated with polymer (alkaline phosphatase)-labeled anti-mouse IgG (Cell Marque) and revealed with a red chromogen (Fast Red; Merck, Darmstadt, Germany) to visualize the reaction. After incubation, the sections were washed in distilled water and counterstained with hematoxylin.

### Statistical analysis

The results were compared employing one-way ANOVA or the Student's *t*-test. Values are expressed as mean and SD. Statistical significance was set at $P < 0.05$.

## Results

None of the experimental or the control rats showed alterations in body weight, behavior, or general health (data not shown).

### Characterization of particles before injection

Morphologic characterization by scanning electron microscopy confirmed that the APS of the particles employed in the experiment was 150, 10, and 5 nm (Fig. 1A–C). The 150-nm particles were mostly spherical, whereas the smaller particles were lentil-shaped and formed agglomerates. Chemical analysis using EDS confirmed the presence of titanium in all the particles (Fig. 1D).

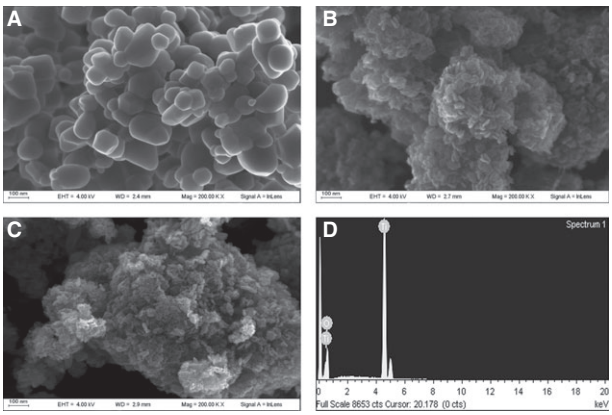


*Fig. 1.* Titanium dioxide ($TiO_2$) observed by scanning electron microscopy (SEM). Note the different shape and average particle size (APS) of the particles: (A) 150 nm APS, (B) 10 nm APS, and (C) 5 nm APS. Original magnification, ×200000. (D) Energy-dispersive X-ray spectroscopy (EDS) analysis of the particles; the spectrum corresponding to titanium is shown.

### Macroscopic and light-microscopic study of tissues

Macroscopic examination using a stereo magnifier revealed no changes in the texture and/or color of the gingivae in any of the study groups.

Light-microscopic examination of the buccal and lingual gingivae of the experimental group showed orthokeratotic epithelium with an evident granular layer, elongation of the epithelial rete ridges, and slight stratification of the basal cells. No vascular alterations or inflammatory infiltrate was observed in the underlying connective tissue. Agglomerates of particles with no giant-cell reaction were observed in both tissues (Fig. 2A–D). Agglomerates of particles contained in cellular elements were observed inside the epithelium (Fig. 2B). Polarized light microscopy showed the deposits to be birefringent (Fig. 3), and allowed identification of small birefringent particles in the tissue that could not be visualized under a light microscope. Gingival samples from the control group showed no morphologic alterations and had no particle deposits.

### Scanning electron microscopy and chemical characterization of particles in gingival tissues

Scanning electron microscopy demonstrated the presence of agglomerates corresponding to the different size and shape of particles in the epithelium and connective tissue (Fig. 2E–H). Elemental analysis by EDS confirmed that the deposits observed were titanium (Fig. 4). Energy-dispersive X-ray spectroscopy mapping (Fig. 2G,H) revealed areas in the epithelial tissue with a higher concentration of titanium (indicated by the higher density of dots); these areas were consistent with the deposits found using light microscopy (Fig. 2C,D).

### Titanium concentration in gingiva

Inductively coupled plasma–mass spectrometry showed higher traces of titanium in both buccal and lingual gingivae in all the experimental groups compared with

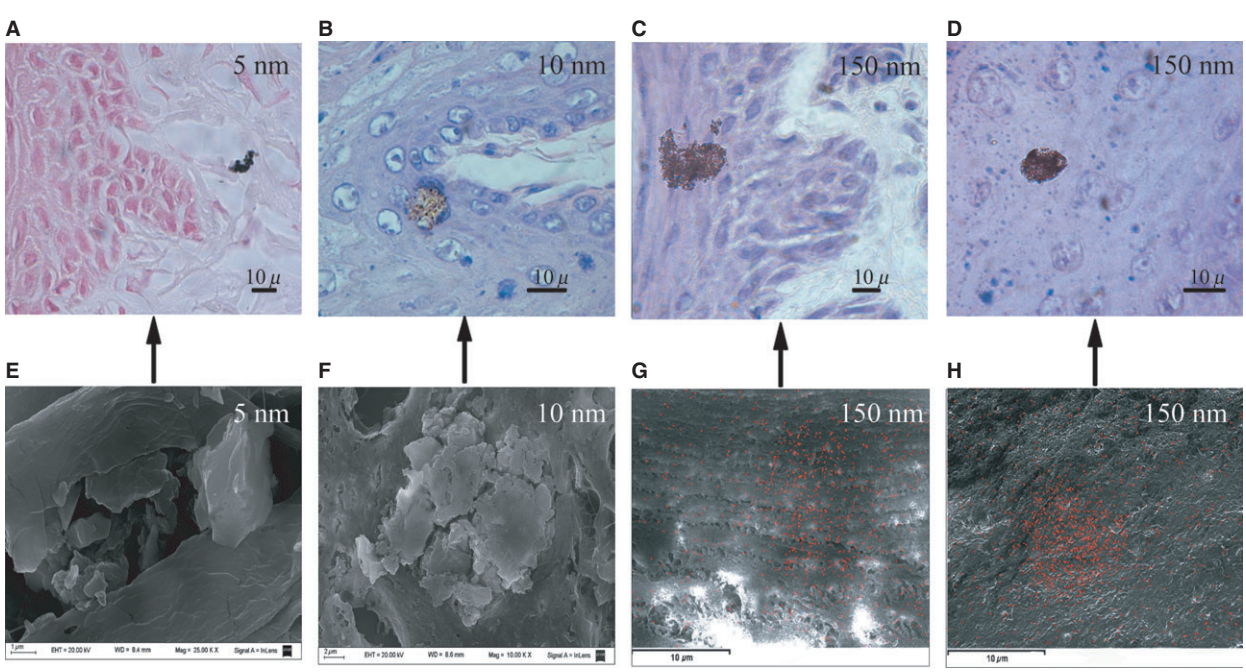


*Fig. 2.* Histological analysis of the gingiva. (A–D) Light microscopy. Titanium deposits can be seen in the connective (A) and epithelial (B–D) tissues. (A) Grenacher's carmine. Original magnification ×1000. (B–D) Hematoxylin and eosin. Original magnification ×1000. (E–H) Scanning electron microscopy examination of the microparticle and nanoparticle deposits. The images correspond to the deposits shown in the upper panel in the microphotographs. (G,H) Energy-dispersive X-ray spectroscopy (EDS) mapping. 5, 10, and 150 nm correspond to average particle size (APS).

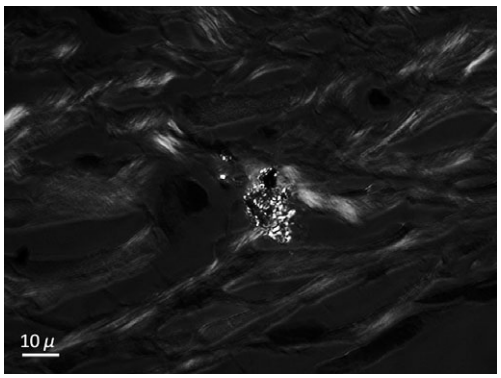


*Fig. 3*. Particle deposits, as observed by polarized light microscopy. Note the birefringence of the particles inside a phagocytic cell. Original magnification ×1000.

controls (Fig. 5A). Titanium traces were significantly higher in both buccal and lingual gingivae in the 5-nm APS group compared with the 10-nm APS group (buccal gingiva: 4.12 ± 0.77 mg $kg^{-1}$ vs. 2.00 ± 0.80 mg $kg^{-1}$; lingual gingiva: 2.21 ± 0.60 mg $kg^{-1}$ vs. 1.22 ± 0.17 mg $kg^{-1}$; $P < 0.05$) (Fig. 5A). The titanium concentration was significantly higher in the buccal gingiva compared with the lingual gingiva (3.07 ± 1.46 mg $kg^{-1}$ vs. 1.61 ± 0.68 mg $kg^{-1}$, respectively; $P < 0.05$) (Fig. 5B).

### Immunohistochemistry

Immunohistochemical staining for CD1a was negative, and no Langerhans cells were detected in any of the samples.

## Discussion

The present work sought to evaluate titanium MP and NP deposits in the gingiva, resulting from systemic transport from the injection site. Previous experimental work conducted at our laboratory showed that titanium particles are transported in the bloodstream by plasma proteins or mononuclear phagocytic cells, and are deposited in organs with macrophagic activity, such as the liver, spleen, and lungs (16). As a result of tribocorrosion processes, titanium ions/particles released from metallic implants (such as coxofemoral prostheses, dental implants, fracture plates and screws, and metal plates used to reconstruct bone defects, among others) could migrate systemically and deposit in body tissues, including the gingiva. Studies in the field of orthopedics have shown that titanium ions enter the neighboring tissues, reach the internal environment, and are excreted in the urine (17, 18).

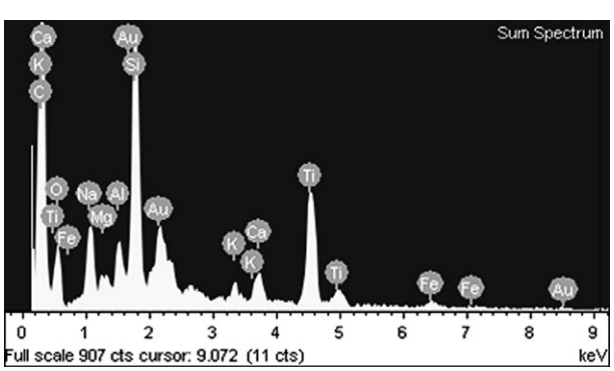


*Fig. 4*. Energy-dispersive X-ray spectroscopy (EDS) of the microparticle and nanoparticle deposits. The spectrum corresponding to titanium is shown. Al, aluminium; Au, gold; C, carbon; Ca, calcium; Fe, iron; K, potassium; Mg, magnesium; Na, sodium; O, oxygen; Si, silicon; Ti, titanium.

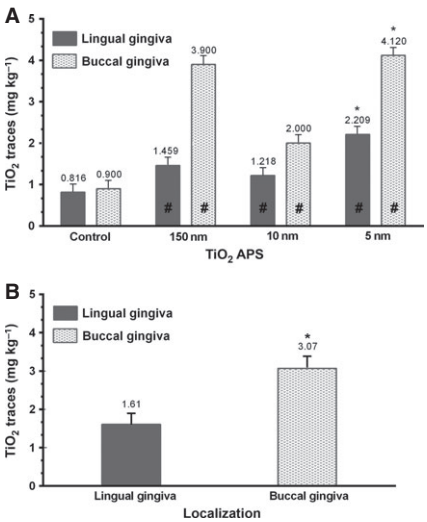


*Fig. 5*. Concentration of titanium in lingual and buccal gingivae, determined using inductively coupled plasma–mass spectrometry (ICP-MS). (A) Significantly higher concentrations of titanium traces were found in both lingual and buccal gingivae in all experimental groups compared with the controls (#$P < 0.05$). The titanium concentration was significantly higher in the group injected intraperitoneally (i.p.) with titanium dioxide ($TiO_2$) nanoparticles (anatase) of 5-nm average particle size (APS) than in the group injected with $TiO_2$ nanoparticles (anatase) of 10-nm APS in both lingual and buccal gingivae (A; *$P < 0.05$). (B) The titanium concentration was significantly higher in the buccal gingiva than in the lingual gingiva (*$P < 0.05$).

Previous work in dental research carried out by our team demonstrated the presence of particles originating from a titanium dental implant in cells exfoliated from the peri-implant sulcus (11). Hence, the deposition of titanium in the gingiva may involve yet another mechanism, which would seemingly be associated with the migration of particles through the epithelium. In agreement with other reports in the literature, the particles might have reached the connective tissue by entering between or through epithelial cells (inter- and intracellular routes) (3, 19, 20). Another possible source of particles deposited in the gingiva could be implant cover screws, as we previously showed in human oral mucosa adjacent to these prosthetic structures (10). In addition, the involvement of exogenous sources (such as food products, toothpastes,

prophylaxis pastes, and abrading and polishing agents, as reported in oral biopsies) in the deposition of $TiO_2$ particles in the gingiva, should not be disregarded (21). In the present study, no pigmentation was observed macroscopically in either the buccal or the lingual gingiva tissue samples. However, the presence of particles in the epithelium and connective tissue was shown histologically; the particles were confirmed to be titanium by EDS and ICP-MS. Hence, the absence of pigmentation on clinical examination would not be indicative of the absence of deposits.

According to the literature, deposition of metal in gingival tissues has typically been reported to occur on the buccal side (13). In agreement with such reports, our results show that the concentration of titanium in tissues, as determined by ICP-MS, was significantly higher on the buccal side than on the lingual side. This finding could be related to the higher density of blood and/or lymph vessels in the buccal gingiva, in addition to it having a greater tissue volume.

The observed variability in the concentration of deposits found in this study could be associated with the physical–chemical properties of the particles. Hence, variables such as particle size and distribution, aggregation state, shape, crystal structure, chemical compositions, surface area and load, and porosity (22, 23) could explain the differences observed. Regarding size, the tissue concentrations of 150- and 5-nm APS NPs were similar, probably because of the capacity of the smaller NPs (5 nm) to aggregate and form larger structures that behave like MPs. The lower concentration of 10-nm APS NPs could be attributed to a greater clearance capacity for NPs of this size. The ability of NPs to form larger superstructures of aggregates/agglomerates has been extensively noted in the literature (3, 24). The aggregation state and the surface area of some nanoparticulate materials can change once they are placed in the biological milieu. The levels of particle aggregation should be taken into account when considering size- and dose-dependent toxicity (25). The shape of the particles may have effects on the kinetics of deposition and absorption in the body (22). The shapes of the particles used in the present study [i.e. the round spherical (150 nm) particles and the lentil-shaped (10 and 5 nm) particles] may also have had an effect on deposit concentration.

Our results showed that the particles deposited formed agglomerates in the MP size range, which caused no inflammatory reaction. Although it is well documented that particle agglomeration in the tissues triggers giant cell recruitment (3), giant cells were not observed in this study. Such an inflammatory state may also be associated with the physical-chemical properties of the particles, as well as with the individual response of the host (21, 26). The agglomerated NPs seemed to act as MPs with different roughness on their surfaces. This finding seems to be very important because the observed lack of inflammatory response may be caused by the body recognizing the agglomerated particles as MPs instead of NPs.

In several cases, the agglomerates of particles in the epithelial tissue were surrounded by cellular elements, which seemed compatible with Langerhans cells. Chan *et al.* (27) showed that dendritic cells are able to take up titanium(IV) ions, which bind specifically to phosphorous-containing molecules. However, in our study, immunolabeling with anti Cd1a, used to analyze and determine the possible role of these cells in the transport mechanism through the epithelium, was negative and ruled out their involvement.

Given that NPs have a larger surface to volume ratio, they are biologically more reactive and potentially more harmful to human health than MPs. Although MPs and NPs can be chemically similar, their specific physical-chemical properties may stimulate different biological responses (3). The ions/particles resulting from corrosion/tribocorrosion processes of titanium could trigger different biologic effects. Research has shown that metal corrosion/tribocorrosion can affect the close contact between the implant and the bone tissue (1, 18, 28, 29). In addition, other studies have reported hypersensitivity reactions to titanium, probably as a result of exposure to ions/particles originating from an implant (30–34). In previous work, we confirmed the presence of macrophages and T-lymphocytes associated with metallic particles originating from implant cover screws, suggesting the occurrence of a cell-mediated immune response (10). It is thus possible that the presence of MPs or NPs in the peri-implant bed could trigger an immune response and the subsequent release of inflammatory mediators, which would result in progressive bone loss. Foreign-body reactions could be caused, for example, by corrosive by-products or excess cement in soft tissues (35) and these may contribute to crestal or marginal bone loss.

Regarding carcinogenic potential, there are scant reports on the potential development of malignant tumors associated with prosthetic structures in humans (36). Features such as ionic valence, particle concentration and size, and hypersensitivity, have been proposed to explain the potential association between malignant transformation and a metallic implant (36). Regarding titanium specifically, there are reports of neoplasia, such as squamous cell carcinoma (37), osteosarcoma (38), and plasmacytoma of the mandible (39), in association with dental implants. With the currently available data, the contention that MPs or NPs alone might be a contributory factor for cancer formation does not hold (40). Interestingly, however, the International Agency for Research on Cancer has classified $TiO_2$ as a potential occupational carcinogen (41).

Corrosion/tribocorrosion is not only a local problem because the particles released during the process can migrate to distant sites (17, 18). In this regard, previous work conducted at our laboratory (23), using rats injected i.p. with 150, 10, or 5 nm APS $TiO_2$ particles showed the presence of foci of necrosis in liver sections at 3 and 12 months only in rats injected with 10 and 5 nm APS particles. Interestingly, a hemorrhagic exudate with infiltration of mononuclear cells and polymorphous nuclear neutrophils was observed in the rats

injected with particles of 5-nm APS. The results are in line with studies in the literature reporting similar histological changes (42). There are reports in the literature showing alterations in the lungs. In keeping with such findings, Chen *et al*. (43) reported that $TiO_2$ NP deposits caused thickening of the alveolar septa and neutrophil infiltrate. Other studies in the literature have shown a relationship between the size of the surface area of the particle and the severity of its effects on health (44). Despite the kidney being exposed to the effect of NPs during ultrafiltration, the impact of NPs on the kidneys remains to be clarified (3). In previous work we also encountered deposits of NPs in the kidney, although they did not cause histological alterations (23). Nevertheless, some studies found NPs to cause glomerular edema and accumulation of protein in renal tubules, as a consequence of retention of $TiO_2$ particles (43).

All in all, the potential risk of corrosion/tribocorrosion, and the possible detrimental consequences of by-products in tissues, are issues of clinical importance (3).

As recently stated by Summer *et al*. (45), biomarkers can serve as informative predictors of disease onset in asymptomatic individuals (predictive biomarkers), indicators of disease incidence and progression (diagnostic/progression biomarkers), and measures of response to treatment or surgical intervention (response biomarkers). It must be pointed out that identification of predictive/diagnostic biomarkers associated with the deposition of particles resulting from implant wear and/or corrosion would contribute to early detection of biological sequelae (pathology). For example, determination of bone turnover and/or inflammatory biomarkers would allow identification of osteolysis, and would permit disease progression to be monitored. Similarly, blood cells loaded with titanium might serve as early bioindicators of corrosion/tribocorrosion (16).

The metals that deposit in the oral mucosa, especially heavy metals, have been attributed little importance per se. Nevertheless, the significance of oral mucosal pigmentation associated with heavy metals lies primarily in the recognition and treatment of the underlying cause to avoid severe systemic toxic effects (13, 46).

In the specific case of titanium, the presence of deposits in the gingiva could be a tissue indicator of tribocorrosion processes of biomedical devices and possible systemic contamination. Further research must be conducted to analyze the potential long-term biologic effects of such deposits.

In conclusion, our study showed that $TiO_2$ NPs deposit in the gingiva, forming agglomerates in the micro size range. The titanium concentration in the gingiva was higher in rats exposed to the smaller NPs (5 nm), and was similar to that observed with MPs of 150-nm APS.

The agglomerated NPs seem to act as MPs with different roughness on their surfaces. This finding seems to be very important because the observed lack of inflammatory response may be caused by the body recognizing the agglomerated particles as MPs instead of NPs.

Further studies quantifying titanium in saliva or blood samples collected near the study sites, at different experimental time points, should be conducted to clarify whether the concentration of titanium differs with time. Moreover, taking into account that there are morphological differences in gingiva from anterior to posterior areas, and that the rate of gingival pigmentation caused by dental implants is higher in the esthetic zone, it would be of particular interest to study this region of the oral mucosa. In addition, further studies should be conducted to evaluate the bone tissue response to particle deposition and its relationship with the data presented here, given the close association between tribocorrosion debris and peri-implantitis. Gingival deposition of titanium could be considered a tissue indicator of tribocorrosion of titanium bioimplants.

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*Conflicts of interest* – The authors report no conflicts of interest related to this study.

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