**GABRIEL MULINARI DOS SANTOS**

# REPARO ÓSSEO PERI-IMPLANTAR EM TÍBIAS DE RATOS ESPONTANEAMENTE HIPERTENSOS (SHR) TRATADOS COM LOSARTAN: ANÁLISE BIOMECÂNICA, MOLECULAR, MICROTOMOGRÁFICA, DINÂMICA ÓSSEA POR FLUOROCROMOS, IMUNOISTOQUÍMICA E HISTOLÓGICA

**Araçatuba – São Paulo**

**2018**

**GABRIEL MULINARI DOS SANTOS**

# REPARO ÓSSEO PERI-IMPLANTAR EM TÍBIAS DE RATOS ESPONTANEAMENTE HIPERTENSOS (SHR) TRATADOS COM LOSARTAN: ANÁLISE BIOMECÂNICA, MOLECULAR, MICROTOMOGRÁFICA, DINÂMICA ÓSSEA POR FLUOROCROMOS, IMUNOISTOQUÍMICA E HISTOLÓGICA

Dissertação apresentada à Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, para obtenção do Título de MESTRE EM ODONTOLOGIA (Área de concentração em Cirurgia e Traumatologia Bucomaxilofacial).

**Orientadora:** Prof[a]. Adj. Roberta Okamoto

**Coorientador:** Prof. Dr. Leonardo Perez Faverani

**Araçatuba – São Paulo**

**2018**

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Santos, Gabriel Mulinari dos.
S237r Reparo ósseo peri-implantar em tíbias de ratos espontaneamente hipertensos (SHR) tratados com losartan: análise biomecânica, molecular, microtomográfica, dinâmica óssea por fluorocromos, imunoistoquímica e histológica / Gabriel Mulinari dos Santos. – Araçatuba, 2018
46 f. : il.

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientadora: Profa. Roberta Okamoto
Coorientador: Prof. Leonardo Perez Faverani

1. Osso e ossos 2. Hipertensão 3. Ratos endogâmicos SHR 4. Anti-hipertensivos 5. Implantes dentários 6. Losartan I. T.

Black D7
CDD 617.6

Claudio Hideo Matsumoto CRB-8/5550

# ERRATA

SANTOS, Gabriel Mulinari dos. **Reparo ósseo peri-implantar em tíbias de ratos espontaneamente hipertensos (SHR) tratados com losartan: análise biomecânica, molecular, microtomográfica, dinâmica óssea por fluorocromos, imunoistoquímica e histológica.** 2018. 47 f. Dissertação (Mestrado) – Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2018.

| Folha | Linha | Onde se lê | Leia-se |
|---|---|---|---|
| 23 | 22 | | Agradecemos ao laboratório de multiusuários da Faculdade de Odontologia de Araçatuba/UNESP pela utilização do microtomógrafo computadorizado **(processo 01.12.0530.00 FINEP/Proinfra 01/2011)".** |
| | | | |
| | | | |
| | | | |

# DEDICATÓRIA

# Dedicatória

A **Deus**, por guiar e iluminar meus passos, transformando os obstáculos em grandes oportunidades e por sempre me cercar de pessoas boas.

Também dedico esse momento as pessoas mais importantes da minha vida, os quais foram a base de tudo que me formei, aqueles que fundamentam a minha história: **a minha família**.

A meu pai, **Jairo dos Santos**, e minha mãe, **Arleide Mulinari dos Santos**, por me amarem e me criarem buscando oferecer sempre o melhor. Se hoje cheguei até aqui, devo a eles, pois se privaram de muitas coisas para me dar o melhor para o meu futuro. Sem esse amor e cuidado, este trabalho não seria possível. Amo muito vocês!

# AGRADECIMENTOS

# Agradecimentos

A **Deus**, por sempre cuidar de cada detalhe do meu caminho e me cercar de pessoas boas.

À minha orientadora, **Professora Dra. Roberta Okamoto**, pela paciência, orientação, humanidade e profundo conhecimento compartilhado. Obrigado pelo estímulo ao meu crescimento na vida acadêmica e por não poupar esforços em me ajudar. Também por proporcionar a maior experiência de minha vida em Viena! Agradeço por tudo, professora!

Ao **Professor Dr. Carlos Ferreira dos Santos**, por sempre ter acreditado em mim e me incentivado nos horizontes acadêmicos, por todas as orientações e ensinamentos. Acima de tudo, obrigado por ser exemplo a ser seguido, não só como profissional, mas também como pessoa. Espelho-me no senhor e tenho o senhor como referência! Eternamente serei grato, professor!

Ao meu coorientador, **Professor Dr. Leonardo Perez Faverani**, que além da ciência me ensinou também a perseverança, dedicação, respeito e humildade.

Ao **Professor Dr. Francisley Ávila Souza** pela confiança, amizade e oportunidade de aprendizado durante os plantões hospitalares. O senhor é um exemplo de pessoa e profissional! Agradeço ao senhor por todas as conversas e ensinamentos!

À **Professora Dra. Mariza Akemi Matsumoto**, pela amizade e sabedoria compartilhada.

Aos **professores da Cirurgia**: **Prof. Dr. Idelmo Rangel Garcia, Prof. Dr. Francisley Ávila Souza, Prof. Dr. Osvaldo Magro Filho, Profa. Dra. Alessandra Marcondes Aranega, Profa. Dra. Daniela Ponzoni, Profa. Dra. Ana Paula Farnezi Bassi** e **Prof. Dr. André Fabris** pelos ensinamentos e amizade.

Ao **Professsor Dr. Reinhard Gruber**, sua esposa **Professora Dra. Ulrike Kuchler** e ao **Professor Stefan Tangl** pela maior experiência de minha vida em Viena. Serei eternamente grato por todo aprendizado! Em especial ao **Prof. Gruber**, pela orientação e ensinamento, exemplo de professor e ser humano!

Às **alunas de iniciação científica da nossa equipe**: Naara Monteiro, Isabela Gandolfo, Jaqueline Hassumi, Jaqueline Silva, Leticia Pitol, Fernanda Yogui, Ana Claudia Ervolino, Elisa Furquim e Paula Frigério, pela amizade, auxilio e boa vontade, meu muito obrigado!

À **Universidade Estadual Paulista "Júlio de Mesquita Filho" – Faculdade de Odontologia de Araçatuba,** na pessoa do diretor **Professor Dr. Wilson Roberto Poi** pela oportunidade de realização da minha pós-graduação em minha cidade natal.

Ao **CAOE - Centro de Assistência Odontológica à Pessoa com Deficiência**, seus funcionários e pacientes, que me ensinaram o valor da vida e possibilitaram minha formação cirúrgica.

À **Medical University of Vienna**, por ter me permitido desenvolver meu projeto de pesquisa, por ser tão bem recebido e pela grande oportunidade de aprendizado que tive em Viena.

Meus sinceros agradecimentos aos amigos de pós-graduação, em especial ao **Fábio Souza Batista** e ao **Pedro Ferreira,** pela amizade, ajuda desde minha chegada em Araçatuba e execução deste trabalho. Também ao **André Oliva, Ciro Duailibe, Gustavo Momesso, Valthierre Nunes, João Paulo Bonardi, Leonardo Freitas, Erick Neiva, Jadson Conforte, William Ricardo, Mônica Munhõz, Tarik Polo, Jonathan Ribeiro, Sormani Queiroz, Juliana Zorzi, Ricardo Jacob, Luara Colombo, Lara Cervantes, Thiago Machado, Raquel Parra** e **Cássio Figueiredo** pela amizade e aprendizado na pós-graduação.

Aos meus primos, **Neyliowan, Ralderkelys**, **Naysilyer** e **Maisa** por serem também meus amigos e irmãos. A minha tia **Arlete Tomazeli**, por ser minha segunda mãe. Aos meus avós **Valdeci, Neiva** e **Maria** que muito me ensinaram.

Ao meu amigo, **Rafael Brandão**, por ter compartilhado grande parte de minha vida e oferecido uma amizade verdadeira. Agradeço-te, meu irmão!

Aos meus pais, **Jairo dos Santos** e **Arleide Mulinari dos Santos**, por sempre me incentivarem e não medirem esforços para que me meu crescimento, por me darem amor e cuidado, e por sempre acreditarem em mim.

# Agradecimentos Institucionais

Ao **Professor Dr. Sandro Roberto Valentini**, digníssimo reitor da Universidade Estadual Paulista "Júlio de Mesquita Filho";

Ao **Professor Dr. Wilson Roberto Poi**, digníssimo Diretor da Faculdade de Odontologia de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho";

Ao **Prof. Dr. João Eduardo Gomes Filho,** digníssimo Vice-diretor da da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho";

Ao **Prof. Dr. André Luiz Fraga Briso**, digníssimo Coordenador da Pós-Graduação em Odontologia da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho";

A todos os **demais professores e funcionários da FOA-UNESP**, por estarem sempre dispostos a ajudar. Em especial, as secretárias da pós-graduação: **Valéria, Cris e Lilian** e aos técnicos do Departamento de Cirurgia e Clínica Integrada: **Marcos e Renato**;

À **Fundação de Amparo à Pesquisa do Estado de São Paulo** pelo financiamento do meu projeto de mestrado (Processo nº 2016/03245-2) e do meu projeto de pesquisa no exterior – BEPE (Processo nº 2017/08081-0).

Gratidão!

# Epígrafe

*“Bem-aventurado o homem que acha sabedoria, e o homem que adquire conhecimento”.*

***Provérbios 3: 13***

# LISTS AND TABLE OF CONTENTS

# *List of Figures*

# *Lists of Abbreviations*

| | |
|---|---|
| **SHR** | Spontaneous hypertensive rats |
| **µCT** | Micro computed tomography |
| **BV/TV** | Bone volume per tissue volume |
| **BIC** | Bone to implant-contact |
| **nBIC** | New bone to implant-contact |
| **oBIC** | Old bone to implant-contact |
| **Tb.Th** | Trabecular Thickness |
| **Tb.N** | Trabecular Number |
| **Tb.Sp** | Trabecular Separation |
| **Po-tot** | Total Porosity |
| **ct.Th** | Cortical thickness from the periosteal to the endosteal margin |
| **g** | Gram |
| **Rpm** | Rotations per minute |
| **kg/day** | Kilogram per day |
| **N** | Newton |
| **ml** | Milliliter |
| **$mm^3$** | Cubic millimeter |
| **vs** | Versus |
| **µm** | Micrometer |
| **mg** | Milligram |

# *Table of Contents*

**1.1 Title page**

**Title**: Losartan reverses impaired osseointegration in spontaneously hypertensive rats

**Running title**: Osseointegration in hypertensive rats

**Key words**: losartan; osseointegration; spontaneously hypertensive rats; bone; renin–angiotensin system

***Esse trabalho foi formatado de acordo com as normas do periódico Clinical Oral Implants Research***

**1.2 Abstract**

Background: Hypertension is associated with cardiovascular diseases but also with alterations in bone quality. Hypertension therefore might be a risk factor for osseointegration. Preclinical studies suggest that losartan, an angiotensin II receptor blocker widely used to treat hypertension, has a beneficial effect in graft consolidation. However, the effect of hypertension and losartan on osseointegration remains unknown.

Methods: Here we used spontaneously hypertensive rats (SHR) and normotensive Wistar albinus rats receiving losartan (30 mg/kg, p.o.) or left untreated. After one week, titanium miniscrews were inserted into the tibia. Sixty days after implantation, implant stability was evaluated by removal torque measurement considered the primary endpoint. Micro computed tomography and histomorphometric analysis were secondary endpoints.

Results: Losartan increased the removal torque in the hypertensive SHR group to levels of the Wistar controls. While the cortical parameters of osseointegration remained unchanged, losartan increased medullary bone formation. Micro computed tomography revealed a higher bone volume per tissue volume and trabecular thickness in the SHR rats treated with losartan. Histomorphometric analysis further showed that losartan significantly increased the thickness of newly formed bone in medullary area in hypertensive SHR rats. Losartan did not significantly alter the parameters of osseointegration in normotensive rats.

Conclusions: The data presented suggest that the angiotensin II receptor antagonist losartan increases the medullary parameters of osseointegration in a tibia model of spontaneously hypertensive rats.

## 1.3 Introduction

Hypertension is a major risk factor for premature death worldwide, mainly for causing cardiovascular complications (Tibazarwa & Damasceno 2014). Every fifth person worldwide today suffers from hypertension and these numbers increase further (Kearney et al. 2005). In addition to cardiovascular complications, hypertension is associated with impaired calcium metabolism (McCarron et al. 1981, Wright & Rankin 1982), decreased bone mineral density (Javed et al. 2012, Manrique et al. 2012), osteoporosis (Cappuccio et al. 2000), and consequently bone fractures (Vestergaard et al. 2009, Yamamoto et al. 2015). Hypertension also negatively affects bone regeneration (Gealh et al. 2014, Manrique et al. 2015) and alveolar bone quality (Bastos et al. 2010), both crucial elements for the osseointegration of dental implants (Isidor 2006, Schenk & Buser 1998). Therefore, uncontrolled hypertension is supposed to be a risk factor in implant dentistry.

Losartan, the angiotensin II receptor blocker (Al-Majed et al. 2015), is prescribed particular in the population over 60 years with more than half having hypertension (Ong et al. 2007). The possible benefit exceeds the reduction of cardiovascular complications because antihypertensive drugs were associated with increased bone mass (Chen et al. 2015, Izu et al. 2009, Ma et al. 2010, Shimizu et al. 2008, Zhou et al. 2017). Losartan consequently improves the physicochemical properties of bone (Donmez et al. 2016) and reduces the fracture risk (Yamamoto et al. 2015). Losartan further supports fracture healing (Rajkumar et al. 2013) and graft consolidation (Gealh et al. 2014). These findings are consistent with the effects of antihypertensive drugs to increase survival rates of dental implants (Lee et al. 2010, Wu et al. 2016), also after sinus augmentation (Garcia-Denche et al. 2013). However, the possible impact of hypertension and the treatment of losartan on osseointegration remain unclear.

Spontaneously hypertensive rats (SHR) (Okamoto & Aoki 1963, Pinto et al. 1998) are widely used to study the role of losartan and others angiotensin II receptor antagonist in vivo (Gealh et al. 2014, Soltis 1993, You et al. 2008, Zhang et al. 2013). SHR rats develop hypertension around 5–6 weeks of age (Okamoto & Aoki 1963). Losartan at least partially reduced periodontitis (Santos et al. 2015) and orthodontic tooth movement in SHR rats (Moura et al. 2016). When untreated, SHR present delayed alveolar bone healing suggesting that also osseointegration could be negatively affected (Manrique et al. 2015). To test this hypothesis, osseointegration was evaluated in SHR rats treated with losartan. The clinical relevance of this approach with respect to human biology is to define if treatment with losartan under hypertension condition support or even increase the parameters of osseointegration.

**1.4 Material and methods**

*Study design and ethics*

The Ethics Committee in the Use of Animals of Araçatuba Dental School (CEUA-2016-404) approved this study. The study was performed in 2016 at the Department of Oral Surgery and Integrated Clinic of the Araçatuba Dental School in accordance with the ARRIVE guidelines. A total of 32 male rats were used, 16 adult male Wistar rats (*Rattus norvegicus*, albinus) and 16 SHRs (body weight, 275 to 350 g). The animals were kept in cages in an environment with stable temperature (22°C ± 2°C), controlled light cycle (12 hours light, 12 hours dark), balanced feed (Ração Mogiana Alimentos SA, Campinas, Brazil) and controlled amounts of water. The animals were divided into four groups: Wistar, Wistar losartan, SHR, and SHR losartan. Randomization was performed by a computer-generated list. The sample was kept as small as possible, taking into account the statistical planning. All evaluations were performed under calibration and blinding examination.

*Losartan treatment*

Losartan (Biosintetica, São Paulo, Brazil) was applied daily at 30 mg in drinking water per kg body weight, seven days prior to implant placement until euthanasia (Gealh et al. 2014). Systolic blood pressure was checked preoperatively and every day prior to euthanasia by tail-cuff indirect plethysmography using a Physiograph®, MK-III-S (Narco Bio-systems, Houston, Texas), adapted for measurements in rats, according to previous studies (Gealh et al. 2014, Manrique et al. 2015). Excluded were animals that did not consume adequate losartan and animals that failed to lower blood pressure.

*Implant placement*

GMS and FRSB performed the surgeries. As recently reported (Faverani et al. 2017, Ramalho-Ferreira et al. 2015), animals received 50 mg/kg of ketamine intramuscularly (Coopers Brazil Ltda, Cotia, SP, Brazil) and 5 mg/kg xylazine (Coopers Brazil Ltda, Cotia, SP, Brazil) and local anesthesia (Mepivacaina; 0.3 ml/kg 2%, adrenaline 1:100,000, Septodont, Saint-Maur-des Fossés, France). Bone was exposed by an incision of the proximal metaphysis. Ten mm below the knee joint, a hole with a 1.4 mm diameter spiral bur mounted on an electric motor (BLM 600®; Driller, São Paulo, SP, Brazil) at a speed of 1000 rpm under irrigation with 0.9% sodium chloride (Fisiológico®, Laboratórios Biosintética Ltda®, Ribeirão Preto, SP, Brazil). Grade 4 titanium screws with 1.5 mm diameter and 3.5 mm length with an acid-etched surface (Emfills, Itu, São Paulo, Brazil) were implanted bilaterally in each tibia. Wounds were closed with resorbable sutures (Poliglactina 910, Vicryl, Ethicon, Johnson & Johnson Prod, São José dos Campos, Brazil). Animals received a single injection of intramuscular pentabiotic (0.1 ml/kg; Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil) and sodic dipyrone (1 mg/kg; Ariston, Indústrias Químicas e Farmacêuticas Ltda, São Paulo, Brazil). Sixty days after implant placement animals received a lethal dose of thiopental (150 mg/kg body weight; Cristália, Ltda., Itapira, SP, Brazil).

*Biomechanical test*

Removal torque was determined on the left tibias of eight rats per group (Ramalho-Ferreira et al. 2015). Exposed implants were mount in an adapted implant hexagon and the digital torque wrench (Conexão, São Paulo, Brazil) was measured. An anti-clockwise movement was applied by increasing the removal torque until the implant rotates inside the bone tissue at the maximum torque peak in Newton centimeter (Ncm).

*Micro computerized tomography (µCT)*

The right tibias of eight rats per group were fixed in 10% buffered formalin (Reagentes Analíticos®, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 48 hours, washed in running water for 24 hours, and stored in 70% alcohol. Tibiae were scanned in the longitudinal plane with a SkyScan 1172 (Bruker microCT, Aartselaar, Belgium) at 70 kV/114 mA with an integration time of 1 x 380 ms in a standard configuration (tube current 165uA, image pixel size 9.92um, filter for beam hardening aluminum-copper 0.5 mm, frame averaging 4, rotation step 0.6 degree). Region of interest (ROI) was a 0.5 mm high and 0.8 mm wide rectangular area in the most central slice of the first two medullary implant threats with 50 slices in the proximal and distal direction, as previous described (Faverani et al. 2017). Images were converted to gray scale values between 70 and 100 representing trabecular bone but not titanium. Morphological parameters were calculated with a software (SkyScan, Leuven, Belgium) according to the American Society of Bone and Mineral Research (Dempster et al. 2013); bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.S), and trabecular number (Tb.N).

*Sample processing and histomorphometric analysis*

Subsequently to µCT, these samples were dehydrated in ascending grades of alcohol and embedded in light-curing resin (Technovit 7200 VLC þ BPO, Kulzer & Co., Hanau, Germany). Undecalcified thin ground sections were prepared along the longitudinal axis of the implant and the shaft of the tibia according to Donath (Donath 1988). Following a Levai-Laczko stain, the specimens were digitized with the Olympus dotSlide 2.4 (Olympus, Tokyo, Japan) at a resolution of 0.312 µm/pixel. A 200 µm ROI parallel to the contour of the implant was defined as described elsewhere (Kuchler et al. 2011). Using the Definiens Developer XD2® software (Version 2.0.0; Munich, Germany), bone and soft tissue were classified from digital images. The classified areas were manually corrected using Adobe Photoshop® software (Adobe, San Jose, CA). For histomorphometric analysis the cortical thickness from the periosteal to the endosteal margin (ct.Th), the percentage of newly formed bone per tissue volume (nBV/TV), the percentage of new bone to implant-contact (nBIC), the percentage of old bone to implant-contact (oBIC), were evaluated in the cortical compartment. In the medullary compartment the thickness of the newly formed layers of bone on the implant surface (nB.Th), nBIC and nBV/TV and were calculated.

*Statistics*

The statistical tests were performed in the GraphPad Prism 7 program (GraphPad Software; La Jolla; USA). For the quantitative parameters obtained from biomechanics (removal torque) and µCT (BV/TV; Tb.Th; Tb.N; Tb.S), a normality and homoscedasticity test was applied to verify the distribution of the data in the normality curve. From this, ANOVA and with post-hoc Tukey test were chosen. Means of the histomorphometric parameters were compared in the medullary and cortical compartments by ANOVA and with post-hoc Kruskal-Wallis test. In case of a significant interaction effect, post hoc multiple-tests were conducted to test the losartan effect both in the control and the hypertensive group. P-values of <0.05 were considered significant for all analyses. The Benjamin–Hochberg procedure was applied to correct for multiple testing, and significance was assigned at the 5% level.

**1.5 Results**

*Biomechanics*

We first determined whether hypertension in the SHR negatively affects the resistance against removal by the torque wrench. In support of this hypothesis, the removal torque was significantly lower in the SHR group compared to the Wistar group (6.0 ± 2.1 Ncm versus 13.0 ± 2.5 Ncm; p=0.006), respectively (Figure 1). Importantly, losartan reversed the negative impact of hypertension on the removal torque to levels of Wistar animals (12.0 ± 2.3 Ncm versus 13.0 ± 2.1 Ncm; p=0.48). Surprisingly in the Wistar group, there was a trend towards a reduction of removal torque by losartan that, however, not reached the level of significance (p=0.51). Thus, biomechanical testing exposed the beneficial effects of losartan on implant stability in hypertensive rats (Figure 1).

*µCT*

Consistent with the biomechanical findings, losartan increased the mean BV/TV in the hypertensive SHR group compared to the untreated SHR rats (52.5 ± 0.6% versus 45.6 ± 0.6%; p=0.02) but not in the normotensive Wistar control group (p=0.98; Figure 2A). The anabolic effect of losartan on BV/TV was caused by increasing the thickness of the bone trabeculae in the losartan treated SHR animals compared to the respective SHR controls (0.13 ± 0.129 mm versus 0.096 ± 0.003 mm; p=0.01; Figure 2B). The number of bone trabeculae remained unchanged in this setting (Figure 2C). The anabolic changes by losartan only caused a moderate decrease in trabecular separation (Figure 2D). Taken together, µCT structural analysis suggests that losartan exerts anabolic effects on periimplant bone in a hypertensive rat model.

*Histology*

Figure 3 provides an overview of the bicortical implant integration, with a small seam of bone formation occurring in the medullary compartment. Considerable bone formation was observed on the periosteal

and the endosteal surface of the cortical bone. No obvious differences were visible when comparing the four groups. At a higher magnification, plexiform bone characterized the periosteal region of the cortical compartment (Figure 4), while a thin layer of woven bone was found in the medullary compartment (Figure 5). Periimplant bone had undergone bone remodeling. Erythrocytes indicate the presence of the blood vessels. No signs of inflammations were observed. Again, these histological findings applied to all four groups.

*Histomorphometry of the medullary compartment*

Losartan significantly increased the average thickness of the newly formed layers of bone on the implant surface in the medullary compartment (nB.Th) of the hypertensive SHR group and, surprisingly, also compared to the two groups of normotensive animals (p=0.0008; Kruskal Wallis; Figure 6A). There was also a trend of a lower medullary nBIC in hypertensive SHR compared to normotensive Wistar rats, with no considerable changes caused by losartan (p=0.599; Figure 6B). The medullary nBV/TV was similar among all four groups (p=0.592, Figure 6C). Thus, losartan increased the thickness of the periimplant bone, but has no impact on the coverage of the implant surface.

*Histomorphometry of the cortical compartment*

As expected, the thickness of the cortical bone was lower in the hypertensive SHR compared to normotensive Wistar rats (p=0.0152, Figure 7A). However, losartan failed to return the cortical thickness to levels of normotensive rats (Figure 7A). Hypertension had no significant effects on the other parameters of osseointegration, e.g. BV/TV (p=0.8112, Figure 7B) and BIC (p=0.7892, Figure 7C, D), thus no effects of losartan were noticed.

**1.6 Discussion**

Hypertension delays bone regeneration in extraction sockets (Manrique et al. 2015) and antihypertensive drugs improve fracture healing (Rajkumar et al. 2013). Considering that osseointegration follows the principles of bone regeneration and remodeling we have raised the hypothesis that antihypertensive drugs reverse impaired osseointegration under hypertensive conditions. Support for this hypothesis comes from epidemiological studies where antihypertensive drugs improve implant survival (Garcia-Denche et al. 2013, Wu et al. 2016). The underlying mechanism of antihypertensive drugs has not been reported so far and may be related to the stimulation of bone regeneration and remodeling during osseointegration. In support of this hypothesis, we report here that losartan increased implant stability in hypertensive rats as determined by biomechanical testing. Histomorphometric and µCT analysis at least help to explain the biomechanical data on the structural level.

The cortical compartment mainly accounts responsible for the biomechanical stability of implants (Miyamoto et al. 2005). Our data showing that losartan failed to return the cortical thickness to levels of normotensive rates and had no significant effects on the other cortical parameters of osseointegration were unexpected. Explanations for the changes in biomechanical stability can thus not be solely based on the amount of bone that was newly formed; it is the biomechanical properties of the cortical that may provide some explanations. At day 60, which is the selected time point of our study, bone defects in a rat model are already undergoing remodeling (Puricelli et al. 2010). Thus, the mechanical properties of bone are increasing even when the net amount of bone remains constant. Our data showing that losartan failed to return the cortical thickness to levels of normotensive rates and had no significant effects on the other cortical parameters of osseointegration support this hypothesis. We can thus speculate that losartan caused a positive shift in bone remodeling leading to higher implant stability; while the amount of cortical bone remains rather constant.

The medullary compartment also contributes to the biomechanical stability of the implants as is reflected by µCT and histomorphometric analysis. Even though the µCT analysis is underpowered, there is a clear trend toward a beneficial effect of losartan to increase medullary bone thickness being associated with the BV/TV in hypertensive SHR rats. Considering also the lower trabecular number, the data suggest that losartan causes a positive shift in bone remodeling under hypertensive conditions. Losartan not necessarily increases the formation of new bone on the implant surface. In support of this concept are our findings from histomorphometry where losartan increased medullary bone thickness in hypertensive SHR rats, even above the levels of normotensive controls. Again, losartan had no impact on the parameters that reflect the formation of new bone on the implant surface. Taken together, these data led us to suggest that losartan increases the amount of existing bone in the medullary compartment while not being responsible for the initiation of bone formation.

The clinical relevance of our findings that losartan supports osseointegration in a hypertensive rat model greatly corroborate with clinical observations namely that antihypertensive drugs support implant survival (Garcia-Denche et al. 2013, Wu et al. 2016). Clinical observations were from implants loaded at least 1 year, not including implants that are lost during the early period prior to functional loading (Garcia-Denche et al. 2013, Wu et al. 2016). Our model at least partially reflects this clinical scenario ,because the 60 days observation period integrates bone regeneration but also the continuous process of bone remodeling (Puricelli et al. 2010). Support for this hypothesis comes from studies showing that hypertension is linked to osteoporosis, and antihypertensive drugs reduce the fracture risk, respectively (Cappuccio et al. 2000, Vestergaard et al. 2009). The clinical relevance of the present study is maybe twofold: first, losartan prevents systemic bone loss in hypertensive patients, thereby also supporting the quality of the alveolar bone before implants are placed (Bastos et al. 2010). Secondly, losartan supports bone remodeling after implants were placed causing a better biomechanical stability in the long term.

New insight on the role of losartan in implant dentistry also leads to new questions. As already stated, what remains to be determined is if the positive effect of losartan on biomechanical implant stability is a consequence of a positive balance of bone remodeling. Moreover, we can not explain if the beneficial effects of losartan on osseointegration are indirect via the control of blood pressure or if losartan also exerts direct effects on cells involved in bone regeneration and remodeling. For example, losartan decreases the suppressive effects of angiotensin II on osteogenic differentiation markers in vitro (Nakai et al. 2015). Our data are in favor of the indirect effect as losartan fails to push osseointegration in normotensive rats. Moreover, it remains unclear if antihypertensive drugs other than losartan cause similar changes in a SHR model, and if the effects observed can be reproduced in other models of hypertension (Zhou et al. 2017). Finally, the rat model only partially reflects the clinical situation. Also the long bones of rats may not respond similarly as the alveolar bone to losartan and that one time-point of observation provides limited insight into the sequential process of osseointegration. Clearly further research is necessary to further reveal this positive effect of losartan on osseointegration.

**1.7 Conclusion**

In conclusion, evidence presented herein suggests that losartan can reverse impaired osseointegration under hypertensive condition. The present preclinical data add to the accumulating knowledge that hypertension is a risk factor in dental implantology and that losartan, being an antihypertensive drug, can help to overcome these limitations.

**1.8 Acknowledgements**

The project was financially supported by the “São Paulo Research Foundation” (process number 2016/03245-2) and (process number 2017/08081-0). The authors are grateful for the dental implants provided by the “Emfils Indústria e Comércio”.

**1.9 References**


Al-Majed, A. R., Assiri, E., Khalil, N. Y. & Abdel-Aziz, H. A. (2015) Losartan: Comprehensive profile. *Profiles Drug Subst Excip Relat Methodol* **40**: 159-194.

Bastos, M. F., Brilhante, F. V., Goncalves, T. E., Pires, A. G., Napimoga, M. H., Marques, M. R. & Duarte, P. M. (2010) Hypertension may affect tooth-supporting alveolar bone quality: A study in rats. *J Periodontol* **81**: 1075-1083.

Cappuccio, F. P., Kalaitzidis, R., Duneclift, S. & Eastwood, J. B. (2000) Unravelling the links between calcium excretion, salt intake, hypertension, kidney stones and bone metabolism. *J Nephrol* **13**: 169-177.

Chen, S., Grover, M., Sibai, T., Black, J., Rianon, N., Rajagopal, A., Munivez, E., Bertin, T., Dawson, B., Chen, Y., Jiang, M. M., Lee, B., Yang, T. & Bae, Y. (2015) Losartan increases bone mass and accelerates chondrocyte hypertrophy in developing skeleton. *Mol Genet Metab* **115**: 53-60.

Dempster, D. W., Compston, J. E., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., Ott, S. M., Recker, R. R. & Parfitt, A. M. (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: A 2012 update of the report of the asbmr histomorphometry nomenclature committee. *J Bone Miner Res* **28**: 2-17.

Donath, K. (1988) Die trenn-dünnschliff-technik zur herstellung histologischer präparate von nicht schneidbaren geweben und materialien. Der präparator.

Donmez, B. O., Unal, M., Ozdemir, S., Ozturk, N., Oguz, N. & Akkus, O. (2016) Effects of losartan treatment on the physicochemical properties of diabetic rat bone. *J Bone Miner Metab*.

Faverani, L. P., Polo, T. O., Ramalho-Ferreira, G., Momesso, G. A., Hassumi, J. S., Rossi, A. C., Freire, A. R., Prado, F. B., Luvizuto, E. R., Gruber, R. & Okamoto, R. (2017) Raloxifene but not alendronate can compensate the impaired osseointegration in osteoporotic rats. *Clin Oral Investig*.

Garcia-Denche, J. T., Wu, X., Martinez, P. P., Eimar, H., Ikbal, D. J., Hernandez, G., Lopez-Cabarcos, E., Fernandez-Tresguerres, I. & Tamimi, F. (2013) Membranes over the lateral window in sinus augmentation procedures: A two-arm and split-mouth randomized clinical trials. *J Clin Periodontol* **40**: 1043-1051.

Gealh, W. C., Pereira, C. C., Luvizuto, E. R., Garcia-Junior, I. R., Antoniali, C. & Okamoto, R. (2014) Healing process of autogenous bone graft in spontaneously hypertensive rats treated with losartan: An immunohistochemical and histomorphometric study. *J Oral Maxillofac Surg* **72**: 2569-2581.

Isidor, F. (2006) Influence of forces on peri-implant bone. *Clin Oral Implants Res* **17 Suppl 2**: 8-18.

Izu, Y., Mizoguchi, F., Kawamata, A., Hayata, T., Nakamoto, T., Nakashima, K., Inagami, T., Ezura, Y. & Noda, M. (2009) Angiotensin ii type 2 receptor blockade increases bone mass. *J Biol Chem* **284**: 4857-4864.

Javed, F., Khan, S. A., Ayers, E. W., Aziz, E. F., Akram, M. S., Nadkarni, G. N., Sabharwal, M. S., Ahmad, Z., Benjo, A. M. & Herzog, E. (2012) Association of hypertension and bone mineral density in an elderly african american female population. *J Natl Med Assoc* **104**: 172-178.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K. & He, J. (2005) Global burden of hypertension: Analysis of worldwide data. *Lancet* **365**: 217-223.

Kuchler, U., Spilka, T., Baron, K., Tangl, S., Watzek, G. & Gruber, R. (2011) Intermittent parathyroid hormone fails to stimulate osseointegration in diabetic rats. *Clin Oral Implants Res* **22**: 518-523.

Lee, H. J., Kim, Y. K., Park, J. Y., Kim, S. G., Kim, M. J. & Yun, P. Y. (2010) Short-term clinical retrospective study of implants in geriatric patients older than 70 years. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **110**: 442-446.

Ma, L., Ji, J. L., Ji, H., Yu, X., Ding, L. J., Liu, K. & Li, Y. Q. (2010) Telmisartan alleviates rosiglitazone-induced bone loss in ovariectomized spontaneous hypertensive rats. *Bone* **47**: 5-11.

Manrique, N., Pereira, C. C., Garcia, L. M., Micaroni, S., Carvalho, A. A., Perri, S. H., Okamoto, R., Sumida, D. H. & Antoniali, C. (2012) Alveolar bone healing process in spontaneously hypertensive rats (shr). A radiographic densitometry study. *J Appl Oral Sci* **20**: 222-227.

Manrique, N., Pereira, C. C., Luvizuto, E. R., Sanchez Mdel, P., Okamoto, T., Okamoto, R., Sumida, D. H. & Antoniali, C. (2015) Hypertension modifies opg, rank, and rankl expression during the dental socket bone healing process in spontaneously hypertensive rats. *Clin Oral Investig* **19**: 1319-1327.

McCarron, D. A., Yung, N. N., Ugoretz, B. A. & Krutzik, S. (1981) Disturbances of calcium metabolism in the spontaneously hypertensive rat. *Hypertension* **3**: I162-167.

Miyamoto, I., Tsuboi, Y., Wada, E., Suwa, H. & Iizuka, T. (2005) Influence of cortical bone thickness and implant length on implant stability at the time of surgery--clinical, prospective, biomechanical, and imaging study. *Bone* **37**: 776-780.
Moura, A. P., Montalvany-Antonucci, C. C., Rodrigues de Albuquerque Taddei, S., Queiroz-Junior, C. M., Biguetti, C. C., Garlet, G. P., Ferreira, A. J., Teixeira, M. M., Silva, T. A. & Andrade, I., Jr. (2016) Effects of angiotensin ii type i receptor blocker losartan on orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* **149**: 358-365.
Nakai, K., Kawato, T., Morita, T., Yamazaki, Y., Tanaka, H., Tonogi, M., Oki, H. & Maeno, M. (2015) Angiotensin ii suppresses osteoblastic differentiation and mineralized nodule formation via at1 receptor in ros17/2.8 cells. *Arch Med Sci* **11**: 628-637.
Okamoto, K. & Aoki, K. (1963) Development of a strain of spontaneously hypertensive rats. *Jpn Circ J* **27**: 282-293.
Ong, K. L., Cheung, B. M., Man, Y. B., Lau, C. P. & Lam, K. S. (2007) Prevalence, awareness, treatment, and control of hypertension among united states adults 1999-2004. *Hypertension* **49**: 69-75.
Pinto, Y. M., Paul, M. & Ganten, D. (1998) Lessons from rat models of hypertension: From goldblatt to genetic engineering. *Cardiovasc Res* **39**: 77-88.
Puricelli, E., Corsetti, A., Ponzoni, D., Martins, G. L., Leite, M. G. & Santos, L. A. (2010) Characterization of bone repair in rat femur after treatment with calcium phosphate cement and autogenous bone graft. *Head Face Med* **6**: 10.
Rajkumar, D. S., Faitelson, A. V., Gudyrev, O. S., Dubrovin, G. M., Pokrovski, M. V. & Ivanov, A. V. (2013) Comparative evaluation of enalapril and losartan in pharmacological correction of experimental osteoporosis and fractures of its background. *J Osteoporos* **2013**: 325693.
Ramalho-Ferreira, G., Faverani, L. P., Prado, F. B., Garcia, I. R., Jr. & Okamoto, R. (2015) Raloxifene enhances peri-implant bone healing in osteoporotic rats. *Int J Oral Maxillofac Surg* **44**: 798-805.
Santos, C. F., Morandini, A. C., Dionisio, T. J., Faria, F. A., Lima, M. C., Figueiredo, C. M., Colombini-Ishikiriama, B. L., Sipert, C. R., Maciel, R. P., Akashi, A. P., Souza, G. P., Garlet, G. P., Rodini, C. O., Amaral, S. L., Becari, C., Salgado, M. C., Oliveira, E. B., Matus, I., Didier, D. N. & Greene, A. S. (2015) Functional local renin-angiotensin system in human and rat periodontal tissue. *PLoS One* **10**: e0134601.
Schenk, R. K. & Buser, D. (1998) Osseointegration: A reality. *Periodontol 2000* **17**: 22-35.
Shimizu, H., Nakagami, H., Osako, M. K., Hanayama, R., Kunugiza, Y., Kizawa, T., Tomita, T., Yoshikawa, H., Ogihara, T. & Morishita, R. (2008) Angiotensin ii accelerates osteoporosis by activating osteoclasts. *Faseb j* **22**: 2465-2475.
Soltis, E. E. (1993) Alterations in vascular structure and function after short-term losartan treatment in spontaneously hypertensive rats. *J Pharmacol Exp Ther* **266**: 642-646.
Tibazarwa, K. B. & Damasceno, A. A. (2014) Hypertension in developing countries. *Can J Cardiol* **30**: 527-533.
Vestergaard, P., Rejnmark, L. & Mosekilde, L. (2009) Hypertension is a risk factor for fractures. *Calcif Tissue Int* **84**: 103-111.
Wright, G. L. & Rankin, G. O. (1982) Concentrations of ionic and total calcium in plasma of four models of hypertension. *Am J Physiol* **243**: H365-370.
Wu, X., Al-Abedalla, K., Eimar, H., Arekunnath Madathil, S., Abi-Nader, S., Daniel, N. G., Nicolau, B. & Tamimi, F. (2016) Antihypertensive medications and the survival rate of osseointegrated dental implants: A cohort study. *Clin Implant Dent Relat Res* **18**: 1171-1182.
Yamamoto, S., Kido, R., Onishi, Y., Fukuma, S., Akizawa, T., Fukagawa, M., Kazama, J. J., Narita, I. & Fukuhara, S. (2015) Use of renin-angiotensin system inhibitors is associated with reduction of fracture risk in hemodialysis patients. *PLoS One* **10**: e0122691.
You, D., Cochain, C., Loinard, C., Vilar, J., Mees, B., Duriez, M., Levy, B. I. & Silvestre, J. S. (2008) Hypertension impairs postnatal vasculogenesis: Role of antihypertensive agents. *Hypertension* **51**: 1537-1544.
Zhang, Y. F., Qin, L., Kwok, T. C., Yeung, B. H., Li, G. D. & Liu, F. (2013) Effect of angiotensin ii type i receptor blocker losartan on bone deterioration in orchiectomized male hypertensive and normotensive rats. *Chin Med J (Engl)* **126**: 2661-2665.
Zhou, Y., Guan, X., Chen, X., Yu, M., Wang, C., Shi, J., Liu, T. & Wang, H. (2017) Angiotensin ii/angiotensin ii receptor blockade affects osteoporosis via the at1/at2-mediated camp-dependent pka pathway. *Cells Tissues Organs* **204**: 25-37.

**2.1 Figures**

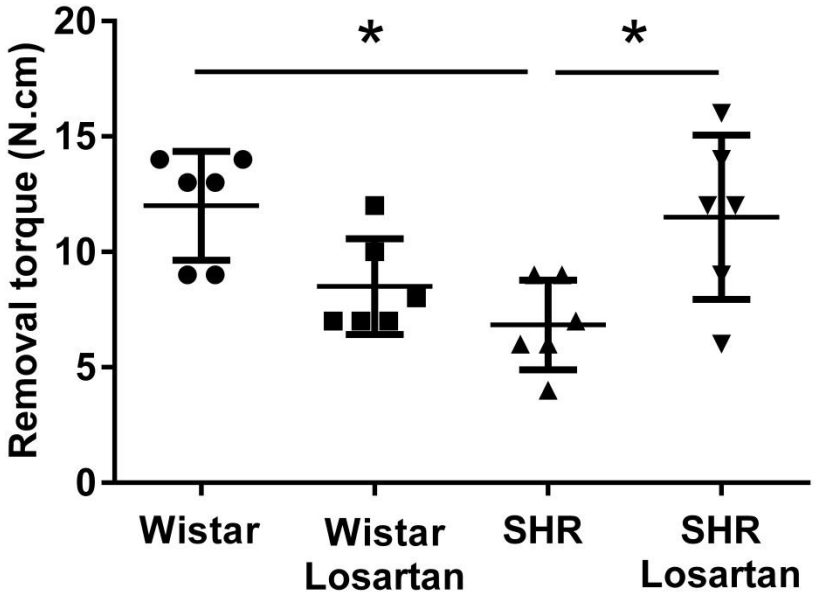


***Figure 1.*** *Biomechanical evaluation of removal torque*

Sixty days after implant placement animals removal torque was determined on the left tibias of eight rats for each of the four experimental groups: Wistar, Wistar Losartan, spontaneously hypertensive rats (SHR), and SHR Losartan. Removal torque was increased until the implant rotated inside the bone and the maximum torque in Newton centimeter (Ncm) is reported.

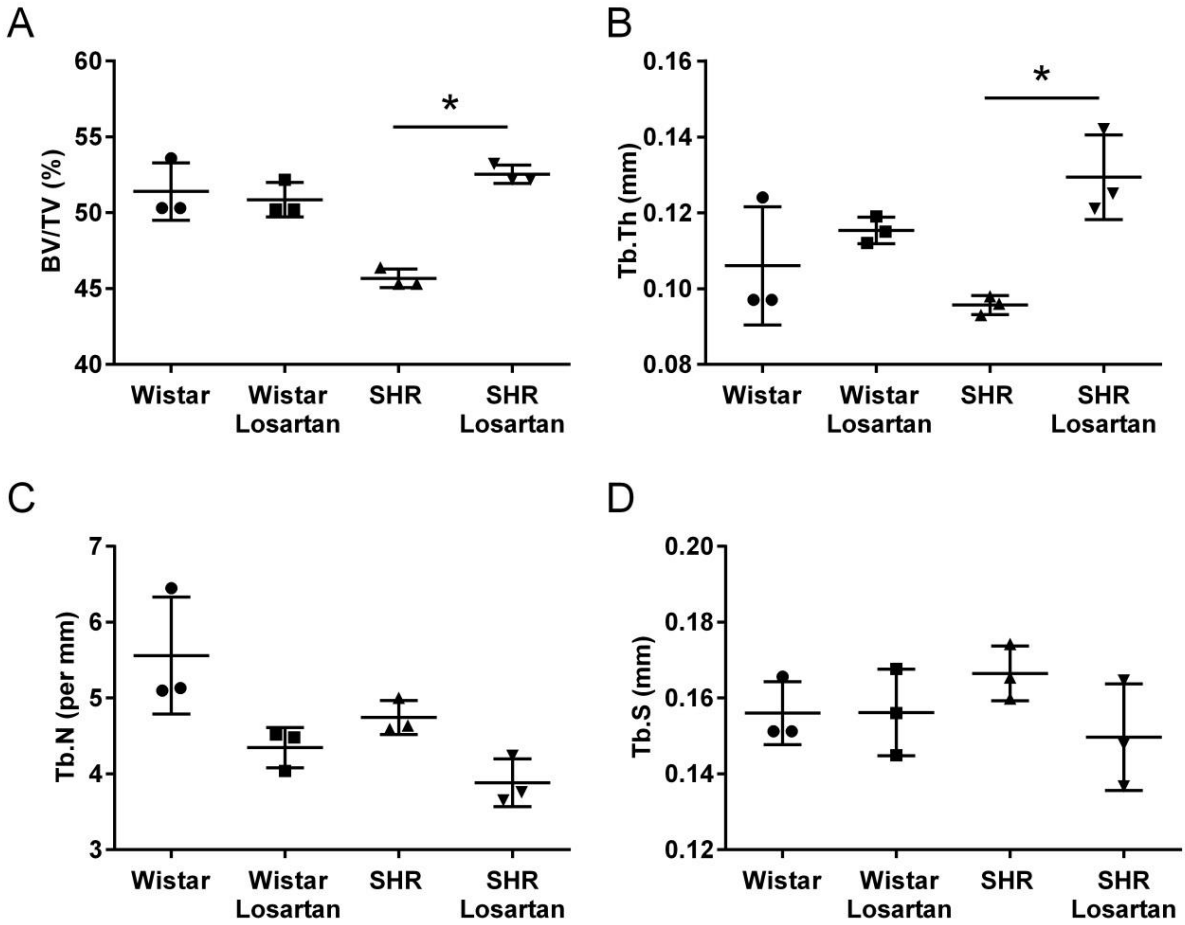


***Figure 2**. Micro computerized tomography of the medullary periimplant bone*

The formalin fixed right tibias of three rats per group were scanned in the longitudinal plane with a region of interest 0.5 mm high and 0.8 mm wide in the most central slice of the first two medullary implant threads with 50 slices in the proximal and distal direction. Morphological parameters were calculated and reported as bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.S), and trabecular number (Tb.N).


***Figure 3.*** *Histology of the bicortical implants*

Histological images at 60 days of the bicortical implant integration for Wistar, Wistar Losartan, SHR and SHR Losartan. Formation of a thin layer of bone occurred in the medullary compartment. Considerable bone formation was observed on the periosteal and the endosteal surface of the cortical bone. All groups showed similar osseointegration. Undecalcified thin-ground sections were prepared along the implant axis and Levai–Laczko stained.

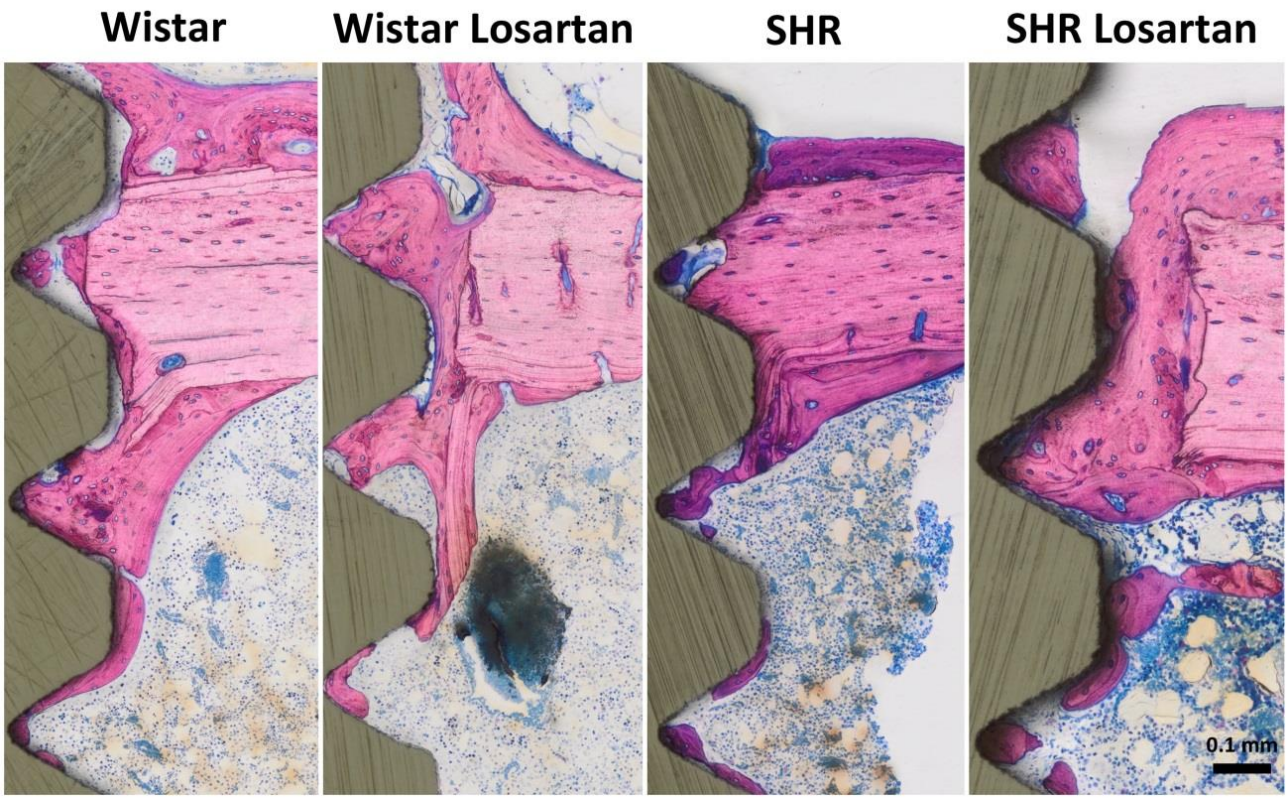


***Figure 4.*** *Histology of the cortical compartment*

Histological pictures of the implant in the tibia, showing the cortical compartment of each group at a higher magnification. It a Plexiform bone is observed in the periosteal region and the the cortical compartment. Undecalcified thin-ground sections were prepared along the implant axis and Levai–Laczko stained.

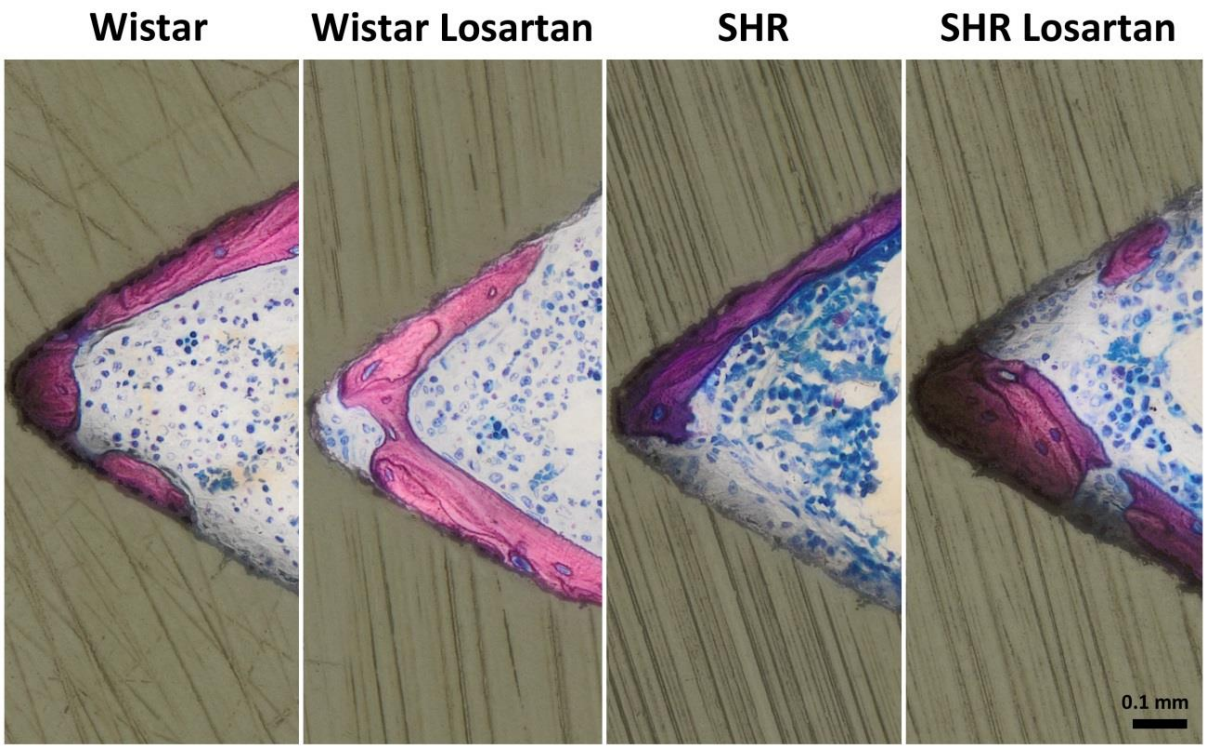


***Figure 5.*** *Histology of the medullary compartment*

Histological pictures of the implant in the tibia, depicting the medullary compartment of each group at a higher magnification. A thin layer of woven bone characterized the bone formation in the medullary compartment. Undecalcified thin-ground sections were prepared along the implant axis and Levai–Laczko stained.

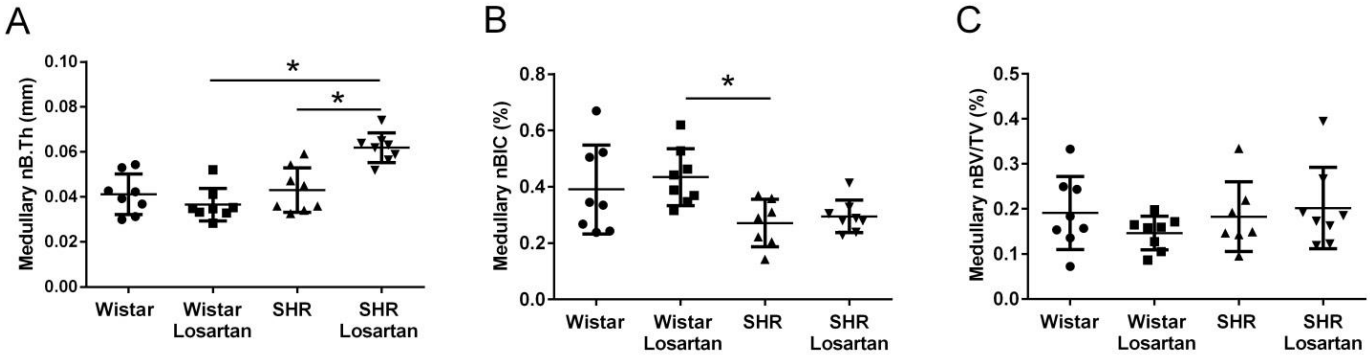


***Figure 6.*** *Histomorphometric results of the medullary compartment*

Scatter-plots summarizing the histomorphometric parameters in the medullary compartment. The data represent 200 µm wide areas immediately adjacent to the implant surfaces. The new bone to implant contact (nBIC), the percentage of new bone volume per tissue volume (nBV/TV) and the thickness of newly formed bone on the implant surface (nB.Th) were calculated.

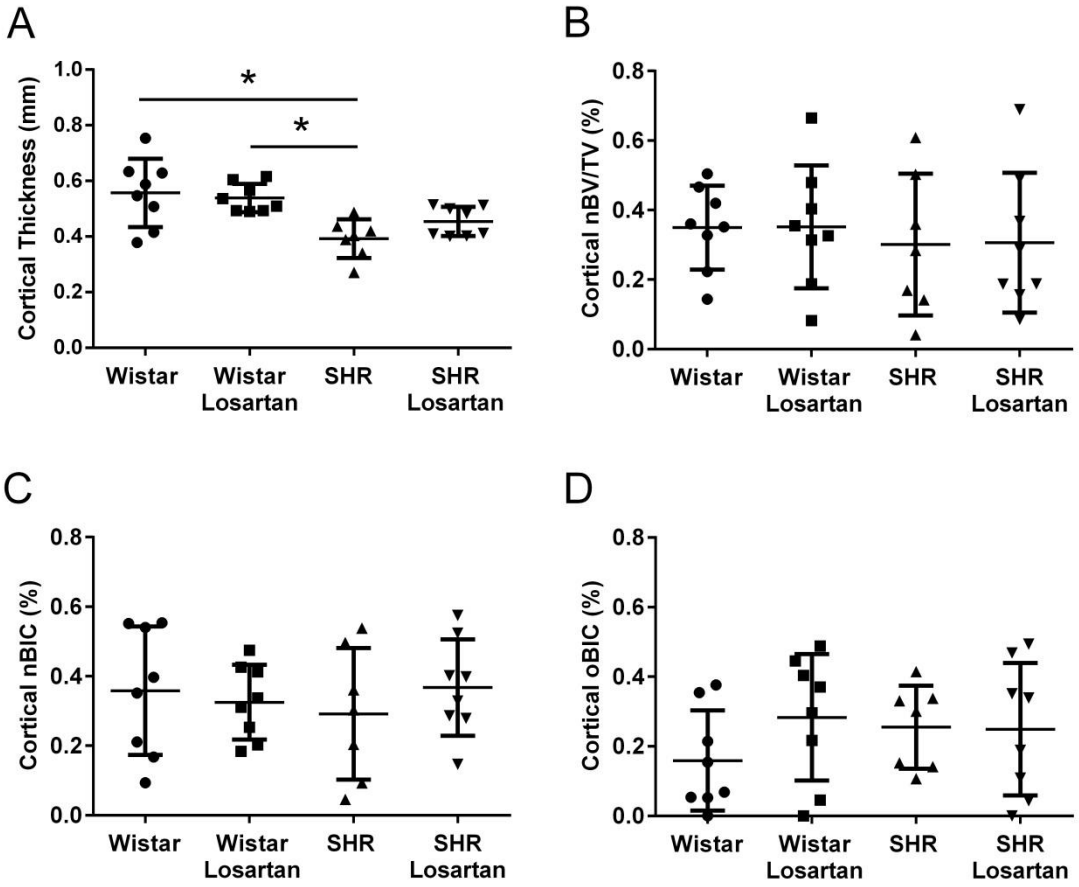


***Figure 7.*** *Histomorphometric results of the cortical compartment*

Scatter-plots summarizing the histomorphometric parameters in the cortical compartment. The data represent 200 µm wide areas immediately adjacent to the implant surfaces. The cortical thickness (Ct.Th), the percentage of newly formed bone per tissue volume (nBV/TV), the percentage of new bone to implant-contact (nBIC), the percentage of old bone to implant-contact (oBIC), were evaluated.

# ANNEXES

## 3.1 Annexe A – Ethical Committee Approval

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

**CEUA - Comissão de Ética no Uso de Animais**
**CEUA - Ethics Committee on the Use of Animals**

**CERTIFICADO**

Certificamos que o Projeto de Pesquisa intitulado **"Reparo ósseo peri-implantar em tibias de ratos espontaneamente hipertensos (SHR) tratados com losartan: Análise biomecânica, molecular, microtomográfica, dinâmica óssea por fluorocromos, imunohistoquimica e histológica"**, Processo FOA nº 00404-2016, sob responsabilidade de Roberta Okamoto apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 14 de Junho de 2017.

**VALIDADE DESTE CERTIFICADO:** 07 de Julho de 2019.
**DATA DA SUBMISSÃO DO RELATÓRIO FINAL:** até 07 de Agosto de 2019.

**CERTIFICATE**

We certify that the study entitled **"Peri-implant bone healing in the of spontaneously rats (SHR) treated with losartan: A biomechanical, molecular, microtomography, bone dynamics by fluorochromes, immunohistochemical and histological analysis"**, Protocol FOA nº 00404-2016, under the supervision of Roberta Okamoto presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on June 14, 2017.

**VALIDITY OF THIS CERTIFICATE:** July 07, 2019.
**DATE OF SUBMISSION OF THE FINAL REPORT:** August 07, 2019.

**Prof. Ass. Dr. Leonardo Perez Faverani**
Coordenador da CEUA
CEUA Coordinator

**CEUA - Comissão de Ética no Uso de Animais**
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

## 3.2 Annexe B – Author Guidelines of the Clinical Oral Implants Research

Author Guidelines

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Submission of Manuscripts, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance.

Useful Websites: Submission Site, Articles published in Clinical Oral Implants Research, Author Services, Wiley-Blackwell's Ethical Guidelines, Guidelines for Figures

The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.

1. GENERAL

Clinical Oral Implants Research conveys scientific progress in the field of implant dentistry and its related areas to clinicians, teachers and researchers concerned with the application of this information for the benefit of patients in need of oral implants. The journal addresses itself to clinicians, general practitioners, periodontists, oral and maxillofacial surgeons and prosthodontists, as well as to teachers, academicians and scholars involved in the education of professionals and in the scientific promotion of the field of implant dentistry.

Clinical Oral Implants Research publishes:

Original research articles of high scientific merit in the field of material sciences, physiology of wound healing, biology of tissue integration of implants, diagnosis and treatment planning, prevention of pathologic processes jeopardizing the longevity of implants, clinical trials on implant systems, stomatognathic physiology related to oral implants, new developments in therapeutic concepts and prosthetic rehabilitation.

Review articles by experts on new developments in basic sciences related to implant dentistry and clinically applied concepts.

Case reports and case series only if they provide or document new fundamental knowledge.

Novel developments if they provide a technical novelty for any implant system.

Short communications of important research findings in a concise format and for rapid publication.

Treatment rational by experts with evidence-based treatment approach.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in Clinical Oral Implants Research. Authors are encouraged to visit Wiley-Blackwell Author Services for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

Clinical Oral Implants Research adheres to the below ethical guidelines for publication and research.

2.1. Authorship and Acknowledgements

Authors submitting a paper do so on the understanding that the manuscript have been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal. ALL named authors must have made an active contribution to the conception and design and/or analysis and interpretation of the data and/or the drafting of the paper and ALL must have critically reviewed its content and have approved the final version submitted for publication. Participation solely in the acquisition of funding or the collection of data does not justify authorship.

Clinical Oral Implants Research adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

Up to 6 authors are accepted without need for justification. In the case of a specific and detailed justification of the role of every author, up to 8 authors may be mentioned. It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited. Acknowledge only persons who have made substantive contributions to the study. Authors are responsible for obtaining written permission from everyone acknowledged by name because readers may infer their endorsement of the data and conclusions.

2.2. Ethical Approvals

Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version, 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editor reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

2.3 Clinical Trials

Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material.

Clinical Oral Implants Research encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials, http://isrctn.org/. The clinical trial registration number and name of the trial register will then be published with the paper.

2.4 Conflict of Interest and Source of Funding

Clinical Oral Implants Research requires that sources of institutional, private and corporate financial support for the work within the manuscript be fully acknowledged, and any potential conflicts of interest noted. Suppliers of materials should be named and their location (town, state/county, country) included. Information concerning conflict of interest and sources of funding should be included under Acknowledgements.

2.5 Appeal of Decision

The decision on a paper is final and cannot be appealed.

2.6 Permissions

If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

2.7 Copyright Assignment

Authors submitting a paper do so on the understanding that the work and its essential substance have not been published before and is not being considered for publication elsewhere.

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions http://authorservices.wiley.com/bauthor/faqs_copyright.asp

For authors choosing OnlineOpen

If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements(OAA):

Creative Commons Attribution Non-Commercial License OAA

Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://authorservices.wiley.com/bauthor/faqs_copyright.asp and visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by The Wellcome Trust and members of the Research Councils UK (RCUK) you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with Wellcome Trust and Research Councils UK requirements. For more information on this policy and the Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

For RCUK and Wellcome Trust authors click on the link below to preview the terms and conditions of this license:

Creative Commons Attribution License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://authorservices.wiley.com/bauthor/faqs_copyright.asp and visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

2.8 OnlineOpen

OnlineOpen is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive. For the full list of terms and conditions, see

http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms

Any authors wishing to send their paper OnlineOpen will be required to complete the payment form available from our website at:

https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

3. SUBMISSION OF MANUSCRIPTS

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/coir. The use of an online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts. Complete instructions for submitting a paper is available online and below. Further assistance can be obtained from the Editorial Assistant Ms. Brigitte Baur. E-mail: coir@zmk.unibe.ch

3.1. Getting Started

Launch your web browser (supported browsers include Internet Explorer 6 or higher, Netscape 7.0, 7.1, or 7.2, Safari 1.2.4, or Firefox 1.0.4) and go to the journal's online Submission Site: http://mc.manuscriptcentral.com/coir

• Log-in or click the 'Create Account' option if you are a first-time user.

• If you are creating a new account.

- After clicking on 'Create Account', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.

- Enter your institution and address information as appropriate, and then click 'Next.'

- Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your area of expertise. Click 'Finish'.

• If you have an account, but have forgotten your log in details, go to Password Help on the journals online submission system http://mc.manuscriptcentral.com/coir and enter your e-mail address. The system will send you an automatic user ID and a new temporary password.

• Log-in and select Corresponding Author Center.

3.2. Submitting Your Manuscript

• After you have logged in, click the 'Submit a Manuscript' link in the menu bar.

• Enter data and answer questions as appropriate. You may copy and paste directly from your manuscript and you may upload your pre-prepared covering letter.

• Click the 'Next' button on each screen to save your work and advance to the next screen.

• You are required to upload your files.

- Click on the 'Browse' button and locate the file on your computer.

- Select the designation of each file in the drop-down menu next to the Browse button.

- When you have selected all files you wish to upload, click the 'Upload Files' button.

• Review your submission (in HTML and PDF format) before sending to the Journal. Click the 'Submit' button when you are finished reviewing.

3.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the entire manuscript including title page, abstract, text, references, tables, and figure legends, but no embedded figures. In the text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.4. Blinded Review

All manuscripts submitted to Clinical Oral Implants Research will be reviewed by two experts in the field. Clinical Oral Implants Research uses single blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

3.5. Suggest a Reviewer

Clinical Oral Implants Research attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, please suggest the names and current email addresses of one potential international reviewer whom you consider capable of reviewing your manuscript. In addition to your choice the journal editor will choose one or two reviewers as well.

3.6. Suspension of Submission Mid-way in the Submission Process

You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation email after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our email server (uranus.scholarone.com) to their whitelist.

3.8. Manuscript Status

You can access ScholarOne Manuscripts (formerly known as Manuscript Central) any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

3.9. Submission of Revised Manuscripts

To submit your revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision' . Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original research articles of high scientific merit in the field of material sciences, physiology of wound healing, biology of tissue integration of implants, diagnosis and treatment planning, prevention of pathologic processes jeopardizing the longevity of implants, clinical trials on implant systems, stomatognathic physiology related to oral implants, new developments in therapeutic concepts and prosthetic rehabilitation.

Review articles by experts on new developments in basic sciences related to implant dentistry and clinically applied concepts. Reviews are generally by invitation only and have to be approved by the Editor-in-Chief before submission.

Case reports and case series, but only if they provide or document new fundamental knowledge and if they use language understandable to the clinician.

Novel developments if they provide a technical novelty for any implant system.

Short communications of important research findings in a concise format and for rapid publication.

Treatment rational by experts with evidence-based treatment approach.

Proceedings of international meetings may also be considered for publication at the discretion of the Editor.

## 5. MANUSCRIPT FORMAT AND STRUCTURE

### 5.1. Page Charge

Articles exceeding 10 published pages are subject to a charge of USD 160 per additional page. One published page amounts approximately to 5,500 characters (excluding figures and tables).

### 5.2. Format

Language: The language of publication is English. Authors for whom English is a second language might choose to have their manuscript professionally edited by an English speaking person before submission to make sure the English is of high quality. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication

Abbreviations, Symbols and Nomenclature: The symbol % is to be used for percent, h for hour, min for minute, and s for second. In vitro, in vivo, in situ and other Latin expressions are to be italicised. Use only standard abbreviations. All units will be metric. Use no roman numerals in the text. In decimals, a decimal point and not a comma will be used. Avoid abbreviations in the title. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement. In cases of doubt, the spelling orthodoxy of Webster's third new international dictionary will be adhered to.

Scientific Names: Proper names of bacteria should be binomial and should be singly underlined on the typescript. The full proper name (e.g., Streptococcus sanguis) must be given upon first mention. The generic name may be abbreviated thereafter with the first letter of the genus (e.g., S. sanguis). If abbreviation of the generic name could cause confusion, the full name should be used. If the vernacular form of a genus name (e.g., streptococci) is used, the first letter of the vernacular name is not capitalised and the name is not underlined. Use of two letters of the genus (e.g., Ps. for Peptostreptococcus) is incorrect, even though it might avoid ambiguity. With regard to drugs, generic names should be used instead of proprietary names. If a proprietary name is used, it must be attached when the term is first used.

5.2. Structure

All manuscripts submitted to Clinical Oral Implants Research should include Title Page, Abstract, Main Text and Acknowledgements, Tables, Figures and Figure Legends as appropriate.

Title Page: should contain the title of the article, full name(s) of the authors (no more than 6) and institutional affiliation(s), a running title not exceeding 60 letters and spaces, and the name, telephone and fax numbers, email and complete mailing address of the author responsible for correspondence. The author must list appropriate key words for indexing purposes.

Abstract: should not to exceed 250 words. This should be structured into: objectives, material and methods, results, conclusions, and no other information.

Main Text of Original Research Article should include Introduction, Material and Methods, Results and Discussion.

Introduction: Summarise the rationale and purpose of the study, giving only strictly pertinent references. Do not review existing literature extensively. State clearly the working hypothesis.

Material and Methods: Material and methods should be presented in sufficient detail to allow confirmation of the observations. Published methods should be referenced and discussed only briefly, unless modifications have been made. Indicate the statistical methods used, if applicable.

Results: Present your results in a logical sequence in the text, tables, and illustrations. Do not repeat in the text all data in the tables and illustrations. The important observations should be emphasised.

Discussion: Summarise the findings without repeating in detail the data given in the Results section. Relate your observations to other relevant studies and point out the implications of the findings and their limitations. Cite other relevant studies.

Main Text of Short Communications: Short communications are limited to two printed pages including illustrations and references and need not follow the usual division into material and methods, etc., but should have an abstract.

Acknowledgements: Acknowledge only persons who have made substantive contributions to the study. Authors are responsible for obtaining written permission from everyone acknowledged by name because readers may infer their endorsement of the data and conclusions. Sources of financial support should be acknowledged.

5.3. References

References should quote the last name(s) of the author(s) and the year of publication (Black & Miller 1988). Three or more authors should always be referred to as, for example, (Fox et al. 1977).

A list of references should be given at the end of the paper and should follow the recommendations in Units, symbols and abbreviations: a guide for biological and medical editors and authors (1988), p. 52, London: The Royal Society of Medicine.

a) The arrangement of the references should be alphabetical by author's surname.

b) The order of the items in each reference should be:

(i) for journal references:

name(s) of author(s), year, title of paper, title of journal, volume number, first and last page numbers.

(ii) for book references:

name(s) of author(s), year, title of book, edition, volume, chapter and/ or page number, town of publication, publisher.

c) Author's names should be arranged thus: Daniels, J.A., Kelly, R.A. & Til, T.C.

Note the use of the ampersand and omission of comma before it. Author's names when repeated in the next reference are always spelled out in full.

d) The year of publication should be surrounded by parentheses: (1966).

c) The title of the paper should be included, without quotation marks.

f) The journal title should be written in full, italicised, and followed by volume number in bold type, and page numbers.

Examples:

Tonetti, M. S., Schmid, J., Hämmerle,C. H. & Lang, N. P. (1993) Intraepithelial antigen-presenting cells in the keratinized mucosa around teeth and osseointegrated implants. Clinical Oral Implants Research 4: 177-186.

Poole, B., Ohkuma, S. & Warburton, M. (1978) Some aspects of the intracellular breakdown of erogenous and endogenous proteins. In: Segal, H.S. & Doyle, D.J., eds. Protein turnover and lysosome function, 1st edition, p. 43. New York: Academic Press.

We recommend the use of a tool such as Reference Manager for reference management and formatting. Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp

5.4. Tables, Figures and Figure Legends

Tables: Tables should be numbered consecutively with Arabic numerals. Type each table on a separate sheet, with titles making them self-explanatory. Due regard should be given to the proportions of the printed page.

Figures: All figures should clarify the text and their number should be kept to a minimum. Details must be large enough to retain their clarity after reduction in size. Illustrations should preferably fill a single-column width (81 mm) after reduction, although in exceptional cases 120mm (double-column) and 168 mm (full page) widths will be accepted. Micrographs should be designed to be reproduced without reduction, and they should be dressed directly on the micrograph with a linear size scale, arrows, and other designators as needed. Each figure should have a legend

Preparation of Electronic Figures for Publication: Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: lineart: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi.

Further information can be obtained at Wiley-Blackwell's guidelines for figures: http://authorservices.wiley.com/bauthor/illustration.asp

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp

Permissions: If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

## 6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

### 6.1 Proof Corrections

The corresponding author will receive an email alert containing a link to a web site. A working email address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following Web site: www.adobe.com/products/acrobat/readstep2.html. This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within three days of receipt.

Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained by the publisher. Please note that the author is responsible for all statements made in his work, including changes made by the copy editor.

Articles should not normally exceed 10 printed pages, including illustrations and references. Additional pages will be charged to the author(s) at the rate of USD 160 per page.

### 6.2 Early View (Publication Prior to Print)

Clinical Oral Implants Research is covered by Wiley-Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

6.3 Author Services

Online production tracking is available for your article through Wiley-Blackwell's Author Services. Author Services enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The author will receive an e-mail with a unique link that enables them to register and have their article automatically added to the system. Please ensure that a complete e-mail address is provided when submitting the manuscript. Visit http://authorservices.wiley.com/bauthor/ for more details on online production tracking and for a wealth of resources including.