Maxillary Sinus Floor Augmentation with Two Different Inorganic Bovine Bone Grafts: an Experimental Study in Rabbits

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Objective: To compare the sequential healing of maxillary sinuses grafted with two different xenogeneic bone substitutes processed at either a low (300°C) or high (1200°C) temperature.

Methods: A sinus augmentation procedure was performed bilaterally in 20 rabbits and two different xenogeneic bone grafts were randomly used to fill the elevated spaces. Healing was studied after 2 and 10 weeks, in 10 rabbits during each period.

Results: After 2 weeks of healing, very small amounts of new bone were observed in both groups, and were mainly confined to close to the sinus bone walls and osteotomy edges. After 10 weeks of healing, new bone was found in all regions, with higher percentages in those close to the bone walls and to the osteotomy. In this period of healing, the proportion of new bone in the 300°C group was 20.0% ± 4.3%, and in the 1200°C group it was 17.2% ± 4.3% (P = 0.162). In the 1200°C group, translucent, dark fog-like shadows in regions of the grafts were hiding portions of new bone (interpenetrating bone network).

Conclusion: Both biomaterials provided conditions that allowed bone growth within the elevated space, confirming that both biomaterials are suitable to be used as a graft for sinus floor augmentation.

Key words: animal study, bone healing, histology, sinus floor augmentation, sinus membrane


Maxillary sinus augmentation is a widely used procedure to restore the bone volume lost in the posterior maxilla in patients who require implant-supported rehabilitation. Several studies have shown that the sinus mucosa tends to return to its original position if implants or biomaterials are not inserted into the space created by its elevation. To counteract the volume shrinkage, the use of biomaterials, implants, or devices has been suggested. Autogenous bone is still considered the filler of choice for sinus floor augmentation; however, high rates of volumetric resorption of autogenous bone have been reported, in addition to the surgical morbidity associated with the donor bed. Several grafting materials have been used to fill the augmented maxillary sinus, among which xenogeneic bone granules, derived from different animal species, have been studied in the literature. Among the xenogeneic graft materials, a deproteinised bovine bone mineral (DBBM) processed at a low temperature (300°C) has been used in several clinical and animal studies. This xenogeneic bone presents slow resorption and excellent osteoconductive properties. Another deproteinised bovine bone produced only from the pure mineral bovine bone phase and sintered at a high temperature...
(> 1200°C) has been used for sinus floor augmentation\textsuperscript{30-35}. This xenogeneic graft has also been employed in animal experiments, using different models and sites such as chambers prepared in skinfolds on the back of hamsters\textsuperscript{36}, critical defects in the rabbit ulna\textsuperscript{37}, circumferential defects around dental implants in minipigs\textsuperscript{38} or critical-size calvarium defects in albino rats\textsuperscript{39}. An animal model using rabbits for maxillary sinus augmentation surgery has been shown to be the most appropriate for experiments, mainly due to its similarity to the human anatomy; both have wide and accessible cavity, well-defined ostium and a mucociliary system with the same characteristics\textsuperscript{40,41}.

A comparative clinical study of Bio-Oss (Geistlich, Wolhusen, Switzerland) and Cerabone (Botiss Biomaterials, Zossen, Germany) used in maxillary sinus augmentation surgery found that Cerabone had larger particles (1:2.7) and a less intense gradual release of calcium ions\textsuperscript{30}. Moreover, in a radiographic evaluation conducted after 4 years, a more pronounced volumetric loss was observed for Bio-Oss compared to Cerabone\textsuperscript{30}. An in vitro study reported a higher level of hydrophilicity for Cerabone compared to Bio-Oss\textsuperscript{42}.

In histological analyses, maxillary sinuses of rabbits filled with DBBM presented a good amount of newly formed bone, providing maintenance of the space created within the elevated area\textsuperscript{2,3,24}. There are no reports in the literature involving maxillary sinus augmentation in animals that compared the results of healing using Bio-Oss and Cerabone as fillers. Thus, the aim of the present study was to compare the sequential healing of maxillary sinuses grafted with two different xenogeneic bone substitutes processed at either a low or high temperature.

Materials and methods

Ethical statements

The experimental protocol was approved by the Ethical Committee of the Faculty of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil on 8 April 2019 (protocol #2019.1.113.58.1). The article was written according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. The Brazilian rules for animal care were followed strictly.

Animal sample

A total of 20 adult male New Zealand white rabbits weighing approximately 3.5 to 4.0 kg and aged 5 to 6 months were used in the present study following a previously published methodology\textsuperscript{43}.

Study design and sample calculation

A randomised split-mouth design was used to eliminate interferences between subjects in the same group. Sinus augmentation was performed bilaterally and two different bovine xenogeneic bone materials, sintered at 300°C (Bio-Oss group) or 1200°C (Cerabone group), were used to fill the two augmented spaces. Two healing periods were analysed, namely 2 and 10 weeks, with 10 animals each period.

The sample size was determined considering data from a previous study available at the time of sample size calculation\textsuperscript{28}, applying alpha = 0.05, power = 0.8 and a correlation between measures of 0.5. Thus, for this configuration, the sample size was 10 animals per group (two groups, n = 10) to enable the authors to find statistical significance between the experimental groups.

Biomaterials

Bio-Oss is a DBBM with sinterisation at 300°, porosity of 75% to 80%, pores of 20 to 200 µm and a mean particle size of 0.5 to 1.0 mm\textsuperscript{44}. Cerabone, meanwhile, is formed completely by hydroxyapatite from bovine cancellous bone, with sinterisation at 1200°C, porosity of 65% to 80%, pores of 600 to 900 µm and a mean particle size of 0.5 to 1.0 mm\textsuperscript{44}.

Randomisation and allocation concealment

Randomisation between groups and periods of healing was performed electronically (randomization.com) by an author who was not involved in the selection and handling of animals and/or surgical procedures (SPX) on 5 May 2019. The treatment allocations were secured in opaque sealed envelopes and revealed to the surgeon (VFB) immediately after completion of the osteotomy and sinus mucosa elevation.

Surgical procedures

The surgical procedures were performed by one expert surgeon (VFB), preceded by injection of acepromazine maleate (1.0 mg/kg; Acepran, Vetnil, Louveira, SP, Brazil), xylazine (3.0 mg/kg; Dopaser, Hertape Calier, Juatuba, MG, Brazil) and ketamine hydrochloride (50 mg/kg: Ketamin Agener, União Química Farmacêutica Nacional, Embu-Guaxu, São Paulo, Brazil). Through an incision in the nasal dorsum, the nasal bone was exposed, and osteotomies were created bilaterally to the naso-incisal suture (Fig 1a). The sinus mucosa was elevated and the subantral spaces were randomly filled.
with similar amounts of the two biomaterials (Fig 1b). The osteotomies were subsequently covered using a collagen membrane (Bio-Gide, Geistlich; Fig 1c), and the wounds were closed with sutures.

**Maintenance care**

The animals were housed in individual cages placed in climatised rooms with access to food and water ad libitum. The biological functions and the wounds were monitored by veterinarians over the whole period of the experiment.

**Euthanasia**

The rabbits were first anaesthetised following similar procedure exposed above and then euthanised in a closed transparent acrylic box containing gas carbon dioxide (CO2). The region of interest was harvested and fixed in 10% formaldehyde.

**Microcomputed tomography (microCT) evaluations**

A 1172 microcomputed tomography (microCT) system (Bruker, Kontich, Belgium) was used to take microCT scans of the specimens. The parameters used were 9.92 μm isotropic pixel, 60 KV/165, 134 μA, filter Al 0.5 mm, exposure time 596 ms, rotation step 0.4 degrees, frame average 4, and random movement 10. The analyses were performed using DataViewer (Bruker).

**Histological preparation**

Precision cutting/grinding equipment (Exakt, Apparatebau, Norderstedt, Germany) was used to prepare two histological slides from each biopsy specimen after dehydration, inclusion in resin (LR White Hard Grid, London Resin, Berkshire, UK) and polymerisation. The small screw placed in the naso-incisal suture was used as a reference. The two slides were stained with either toluidine blue or Stevenel’s blue and alizarin red.

**Histometric evaluations**

Photomicrographs were taken under the microscope (Leica DMLB, Wetzlar, Germany) using a digital camera (Digital Sight DS-2Mv, Nikon, Tokyo, Japan).

The following regions were analysed within the elevated space (Fig 2): medial bone wall (M), lateral bone wall (L), sub-sinus region (S), central area (C) and osteotomy region (A).

A grid of 80 squares was superimposed on the images of the histological slides using the software Image J 1.50i (National Institutes of Health, Bethesda, MD, USA) and a point-counting procedure at 100× magnification was adopted for morphometric measurements. New bone (not covered by graft shadows), interpenetrating bone network (IBN; see-through bone graft in the Cerabone group) and residual xenogeneic bone were assessed. Total bone was calculated as the sum of new bone and IBN. Percentages were calculated with respect to the total area of the region evaluated and a mean value was obtained for the five regions. Before taking the measurements, calibration with another expert examiner (SPX) was performed until an inter-class correlation coefficient \( k > 0.9 \) was achieved for tissue recognition.

**Data analysis**

The primary variable was the mean total bone percentage in the full elevated space. The data from the various
regions were used to establish a more detailed description of bone formation.

Prism 9.1.1 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. The normal distribution of the variables was assessed with a Shapiro-Wilk test for both paired and unpaired variables. Either a paired t test or a Wilcoxon test was used to evaluate the differences between the Bio-Oss and Cerabone groups. Differences between the two healing periods were evaluated using either an unpaired t test or a Mann-Whitney test.

A Spearman two-tailed correlation coefficient was applied to measure the strength of the correlation between the outcomes of the histological and microCT analyses. GraphPad Prism 9.1.1 was used. The correlation coefficient and P values were reported. P < 0.05 was considered statistically significant.

The tables listed mean values, standard deviations, P values, medians and 25% and 75% percentiles, whereas in the text only mean values were reported.

Results

Descriptive histological evaluation

In the histological analysis, the surface of the biomaterial was well defined for the Bio-Oss granules whereas for Cerabone, translucent dark fog-like shadows gave the granules an undefined periphery. In some instances, especially at the 10-week period, these shadows hid the presence of new bone that was unveiled when light intensity was increased when the photomicrographs were taken under the microscope (IBN; Figs 3a to d). After 2 weeks of healing, very small amounts of new bone were observed in both groups, mainly confined to close to the sinus bone walls and osteotomy edges, whereas the other regions were practically devoid of new bone (Figs 4a and b). The graft material occupied almost half of the elevated space.

Ridges of new bone sprouting from the sinus bone walls towards the centre of the elevated space incorporated the nearest granules in both the Cerabone (Fig 5a) and Bio-Oss (Fig 5b) groups. The granules furthest from the bone walls were instead surrounded by soft tissue, presenting a dense layer of fibroblast-like cells disposed along the graft surface. Some osteoclast-like cells were also seen on the surface of both biomaterials (Figs 5a and b). The osteotomy was still covered by the collagen membrane (Figs 4a and b) involved in degradation processes. New bone was forming from the edges of the osteotomies, aiming to close the defect (Figs 5c and d). A few granules were found in some specimens beyond the osteotomy in both groups.

After 10 weeks of healing, new bone was found in all regions, with higher percentages recorded in regions close to the bone walls and the osteotomy (Figs 6a and b). The granules presented a higher grade
of incorporation into newly formed bone compared to the previous healing period (Figs 7a and d). The regions subjacent to the sinus mucosa presented the lowest amounts of new bone. Most of the osteotomies were closed by newly formed bone with signs of remodelling. The degradation of the collagen membrane was not completed yet (Figs 6a and b). Some granules were found beyond the osteotomy and several perforations of the sinus mucosa were observed for both biomaterials.

Histometric evaluation

After 2 weeks of healing (Table 1), IBN was almost absent such that both new bone and total bone fractions were 0.7% and 0.3% for the Bio-Oss and Cerabone groups, respectively ($P = 0.098$). The residual graft fraction was 43.4% for the Bio-Oss group and 48.8% for the Cerabone group ($P = 0.037$).

New bone was mostly located close to the sinus bone walls (Table 2). A slight tendency towards higher bone
formation was seen in the Bio-Oss group compared to the Cerabone group in all regions. The submucosa region was virtually devoid of new bone in both groups. None of the differences between groups were statistically significant considering the various regions.

After 10 weeks of healing (Table 1), small fractions of IBN were found in the Cerabone group (1.3%). Total bone was found in proportions of 20.0% in the Bio-Oss group, and 17.2% in the Cerabone group ($P = 0.162$).

The residual graft decreased with respect to the previous healing period to 36.4% and 35.3% ($P = 0.846$) in the Bio-Oss and Cerabone groups, respectively (Fig 8). In both groups, the differences between periods in total bone and residual graft percentages were statistically significant ($P < 0.05$).

In both groups, the regions presenting the greatest amount of new bone were those close to the window, followed by those close to the sinus bone walls.
In these regions, the total bone percentage was higher in the Bio-Oss group; however, only the difference at the next-to-window wall region was statistically significant ($P = 0.018$). The lowest percentage of new bone was found in the submucosa regions, followed by the central region.

**microCT evaluation**

The evaluation was performed at two different thresholds of grey levels to identify bone tissue from xenogeneic bone graft, namely 60 to 80 and 70 to 100. After 2 weeks of healing (Figs 9a to c), the tissue vol-

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**Table 1** Percentage of new bone and graft remnants within the full elevated area.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Grafting material</th>
<th>New bone, %</th>
<th>IBN, %</th>
<th>Total bone, %</th>
<th>Residual graft, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median (25%; 75%)</td>
<td>$P$ value</td>
<td>Mean ± SD</td>
<td>Median (25%; 75%)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>Bio-Oss</td>
<td>0.7 ± 0.8</td>
<td>0.4 (0.2; 0.6)</td>
<td>0.098</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Cerabone</td>
<td>0.3 ± 0.3</td>
<td>0.3 (0.1; 0.5)</td>
<td>0.0 ± 0.1</td>
<td>0.0 (0.0; 0.0)</td>
</tr>
<tr>
<td>10 weeks</td>
<td>Bio-Oss</td>
<td>20.0 ± 4.3</td>
<td>21.4 (18.1; 22.1)</td>
<td>0.162</td>
<td>36.4 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Cerabone</td>
<td>15.8 ± 4.0</td>
<td>14.5 (13.8; 19.6)</td>
<td>1.3 ± 0.6</td>
<td>1.5 (1.2; 1.5)</td>
</tr>
</tbody>
</table>

**Table 2** Total bone percentages after 2 weeks of healing. The various regions of the augmented area were evaluated.

<table>
<thead>
<tr>
<th>Grafting material</th>
<th>Full augmented area</th>
<th>Next-to-window</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median (25%; 75%)</td>
<td>$P$ value</td>
</tr>
<tr>
<td>Bio-Oss</td>
<td>0.7 ± 0.8</td>
<td>0.4 (0.2; 0.6)</td>
<td>0.159</td>
</tr>
<tr>
<td>Cerabone</td>
<td>0.3 ± 0.3</td>
<td>0.3 (0.1; 0.5)</td>
<td>0.0 ± 0.1</td>
</tr>
</tbody>
</table>

**Table 3** Percentage of new bone after 10 weeks of healing. The various regions of the augmented area were assessed.

<table>
<thead>
<tr>
<th>Grafting material</th>
<th>Full elevated area</th>
<th>Next-to-window</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median (25%; 75%)</td>
<td>$P$ value</td>
</tr>
<tr>
<td>Bio-Oss</td>
<td>20.0 ± 4.3</td>
<td>21.4 (18.1; 22.1)</td>
<td>0.162</td>
</tr>
<tr>
<td>Cerabone</td>
<td>17.2 ± 4.3</td>
<td>15.8 (14.1; 21.1)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

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**Fig 8** New bone formation and xenogeneic bone graft resorption between 2 and 10 weeks of healing.
Correlation between 10-week histological and microCT analyses

Applying a grey level of 60 to 80 for bone tissue evaluation, a weak positive correlation for the Bio-Oss group (0.091; $P = 0.811$) and a weak negative correlation for the Cerabone group ($-0.333; P = 0.349$) were found. Using a grey level of 70 to 100, a strong positive correlation for the Bio-Oss group (0.709; $P = 0.027$) and a weak positive correlation for the Cerabone group (0.212; $P = 0.560$) were found.

Discussion

The present study aimed to compare the sequential healing of maxillary sinuses grafted with two different xenogeneic bone substitutes sintered at either a low or high temperature. After 10 weeks of healing, the histological
evaluation showed that a tendency towards higher bone formation was found in the Bio-Oss group compared to the Cerabone group; however, the difference was not statistically significant. In both groups, the highest percentages of new bone were observed in the regions close to the bone walls and the osteotomy.

In the Cerabone group, several graft particles presented dark regions with fog-like shadows that hid the hard and soft tissues. These tissues were shown more clearly under the microscope when overexposing the image to the light whereas, when adjusting the focus, no improvement was made in the identification. This method made it possible to identify new bone hidden by the shadows and was illustrated in other articles in which biphasic beta tricalcium phosphate/hydroxyapatite ($\beta$-TCP/HA) were used for sinus floor augmentation in sheep\textsuperscript{46} and rabbits\textsuperscript{47} and recently in human biopsy specimens to unveil bone covered by shadows around Cerabone\textsuperscript{48}. The total amount of bone reported represented the sum of new bone outside the dark shadows and that covered by those shadows (IBN). The structure of the IBN recalled that of an “interpenetrating polymer network”\textsuperscript{49} and consequently, a similar name was adopted.

After 2 weeks of healing, the percentage of new bone in the full elevated area was $< 1\%$ in both groups. The inclusion of an early period of healing in the analysis of the present study enabled the differences in the regions close to the source of new bone to be evaluated and, at the same time, provided the opportunity to assess dimensional variations over time. After 2 weeks of healing, the postsurgical oedema that occurs after sinus floor augmentation\textsuperscript{50-53} appeared to be resolved. The resorptive processes were still in an early phase, resulting in little effect on dimensional changes. During this healing period in both groups, bone was formed from the lateral sinus walls while, in the submucosa area, no new bone was found. This is in agreement with several other reports that showed the absence of participation of the sinus mucosa in bone formation during this early period of healing\textsuperscript{8,9,54-55}.

After 10 weeks, new bone proportions increased in both groups in all regions included in the analyses. In this healing period, the proportion of new bone was 20.0\% in the Bio-Oss group and 17.2\% in the Cerabone group. A comparison of the results of healing between Bio-Oss and Cerabone after sinus floor augmentation was also performed in a clinical study\textsuperscript{31}. Biopsy specimens were harvested after 6 months from the augmented sinus, and the proportion of new bone was 41.4\% in the Bio-Oss group and 39.2\% in the Cerabone group; however, the difference was not statistically significant\textsuperscript{31}. These proportions of new bone were approximately double for both groups compared to the present study. The difference in percentage might be related to the different model used, but also to the fact that the biopsy specimens in the animal study comprised all regions of the sinus, including those with very little bone such as that subjacent to the submucosa. In the previous study, only the region close to the base of the sinus was included in the analysis\textsuperscript{31}, i.e., closer to an important source of new bone\textsuperscript{50,51}.

In another clinical study, the analysis performed on biopsy specimens harvested after 8 months from sinus augmentation revealed higher proportions of new bone in the Cerabone group (29.1\%) compared to the Bio-Oss group (24.6\%)\textsuperscript{56}. Again, the difference was not statistically significant.

In the present study, in both groups, the highest amounts of new bone were located close to the bone walls and the osteotomy. Both biomaterials allowed new bone formation and, after 2 weeks, the first signs of incorporation of the neighbouring granules were already noted close to the bone walls. After 10 weeks, incorporation of the granules was observed in all regions. This event has been described in other experimental
In an experiment on sinus augmentation in rabbits, the sequential events of bone-to-graft contact at DBBM granules were analyzed after 7, 14, 20 and 40 days of healing. After 7 days, small amounts of bone were found close to the bone walls, whereas the DBBM granules were surrounded by soft tissue containing fibres and fibroblast-like cells arranged in layers in direct contact with the biomaterial surface, and after 14 days, several DBBM granules were covered by newly formed bone; however, the majority were surrounded by dense tissues, similar to those observed after 7 days of healing, and the regions between granules were occupied by loose tissue, poor in cells but rich in vessels. In the following healing periods, further granules were enclosed by newly formed bone, and the surfaces not covered by bone presented dense tissues. The regions including those between granules, first occupied by loose tissues, underwent a transformation into primitive bone marrow.

In the present study, after 10 weeks of healing, new bone in the whole area of the Bio-Oss group reached a proportion of 20.0%. This result is similar to that reported in another study in which Bio-Oss granules were treated with Argon plasma or left untreated before being used to fill the subantral space in rabbits. The percentages of new bone after 10 weeks were 23.5% in the plasma group and 21.3% in the untreated group. The histological analyses were also performed in the bone wall and central regions of the sinuses. The proportion of new bone in the group not treated with argon plasma was 26.3% in the bone wall region, and 13.2% in the central regions. These data are consistent with those of the present study, with the proportion of new bone being approximately 22.0% to 26.0% in the bone wall region and 13.8% in the central region.

In both groups in the present study, the submucosa region exhibited lower amounts of new bone compared to the other regions examined, namely 6.9% and 9.7% in the Bio-Oss and Cerabone groups, respectively. Other similar experiments on sinus augmentation in rabbits reported data on bone formation in this region. The presence of new bone subjacent to the sinus mucosa cannot exclude its contribution to bone formation; however, if this contribution does exist, it is limited. It is also necessary to consider that several perforations of the sinus mucosa were observed with relation to the biomaterial particles in both groups. It has been shown in a rabbit model that the presence of sinus mucosa perforations compromised new bone formation in the adjacent regions within the sinus.

The difference in percentage between 2 and 10 weeks of healing was 7.0% for Bio-Oss and 13.5% for Cerabone. This means that part of the biomaterial was resorbed or lost through the osteotomy or the sinus mucosa. Indeed, several osteoclastic-like zones were observed around both biomaterials at the 2-week evaluation period whereas after 10 weeks, osteoclastic-like cells were rarely observed. This observation agrees with other reports that showed a progressively decreasing percentage of osteoclasts within the augmented space over time.
Cerabone. The model used represents another limitation of the study considering the dimensions of the sinus and the lower thickness of the sinus mucosa compared to humans. Moreover, healing in rabbits has been shown to be faster compared to humans, thus, any inferences about humans must be taken with caution.

Conclusion

The present study illustrated that both biomaterials provided conditions that allowed bone growth within the elevated space and confirmed that both biomaterials are suitable to be used as graft materials for sinus floor augmentation. The overexposure to the microscope light in the histological preparation might help to identify the tissues veiled by the dark shadows surrounding Cerabone particles.

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Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Vitor FERREIRA BALAN, Daniele BOTTICELLI, David PEÑARROCHA-OLTRA and Samuel Porfirio XAVIER contributed to the conceptualization of the study; Vitor FERREIRA BALAN and Eduardo PIRES GODOY contributed to surgical procedures; Dr Vitor FERREIRA BALAN contributed to the histological and microCT analyses; Drs Vitor FERREIRA BALAN, Daniele BOTTICELLI and Katsuhiko MASUDA contributed to the data analysis; Drs Daniele BOTTICELLI and Samuel Porfirio XAVIER contributed to the supervision of the project; Drs Vitor FERREIRA BALAN and Daniele BOTTICELLI contributed to the manuscript draft; Drs David PEÑARROCHA-OLTRA, Katsuhiko MASUDA, Daniele BOTTICELLI and Samuel Porfirio XAVIER contributed to finalising the article. All authors have read and approved the published version of the manuscript.

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