High-resolution three-dimensional micro-computed tomography assessment of micro-architectural patterns in non-adults with cribra orbitalia: correlation between macro- and micro-scale bone features

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June 06, 2024

Abstract

Skeletal porous lesions such as cribra orbitalia (CO) have long been of interest to bioanthropologists worldwide, mainly due to their high prevalence in osteological material. Previous studies considered CO as an external morphological manifestation, and therefore, research has mainly focused on visible (macroscopic) CO patterns. However, the understanding of CO-induced micro-scale bone changes is still scarce. Therefore, here we performed high-resolution micro-computed tomography imaging to investigate three-dimensional CO-induced micro-architectural patterns in non-adults, with a particular focus on the correlation between macroscopic and micro-architectural orbital features. Cortical and trabecular micro-architectural changes in the orbital roof were analyzed in non-adults up to 15 years of age, using orbital roof samples with and without macroscopic traces of CO (n=28). A widely accepted five-grade macroscopic CO scoring system was applied to analyze CO severity. Areas affected with CO (area 1) and areas without macroscopic CO traces (area 2) were analyzed separately. The conducted high-resolution analysis showed that cortical and trabecular micro-architecture varied with CO presence, lesion severity (CO grade), and the analyzed area. Inter-grade comparisons suggested that most of the analyzed micro-architectural parameters were not significantly different between adjacent CO grades. Based on the micro-architectural evaluation of areas 1 and 2, the porous lesions were much more extensive than revealed by gross examination. Our analysis also revealed that micro-architectural differences were particularly pronounced in younger non-adults. Taken together, the macroscopic examination of CO appears to reflect only the tip of the iceberg, as the micro-architectural changes are much larger than macroscopically identified.

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Short Title:

Cribra orbitalia -induced bone micro-architectural alterations in orbital roof

Research Highlights:

Cribra orbitalia (CO) represents orbital porous lesions. A high-resolution microscopic assessment of CO-induced changes in non-adults was done by micro-computed tomography. The microarchitecture was affected by CO presence, CO grade, area, and age.

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ABSTRACT

Skeletal porous lesions such as *cribra orbitalia* (CO) have long been of interest to bioanthropologists worldwide, mainly due to their high prevalence in osteological material. Previous studies considered CO as an external morphological manifestation, and therefore, research has mainly focused on visible (macroscopic) CO patterns. However, the understanding of CO-induced micro-scale bone changes is still scarce. Therefore, here we performed high-resolution micro-computed tomography imaging to investigate three-dimensional CO-induced micro-architectural patterns in non-adults, with a particular focus on the correlation between macroscopic and micro-architectural orbital features. Cortical and trabecular micro-architectural changes in the orbital roof were analyzed in non-adults up to 15 years of age, using orbital roof samples with and without macroscopic traces of CO (n=28). A widely accepted five-grade macroscopic CO scoring system was applied to analyze CO severity. Areas affected with CO (area 1) and areas without macroscopic CO traces (area 2) were analyzed separately. The conducted high-resolution analysis showed that cortical and trabecular micro-architecture varied with CO presence, lesion severity (CO grade), and the analyzed area. Inter-grade comparisons suggested that most of the analyzed micro-architectural parameters were not significantly different between adjacent CO grades. Based on the micro-architectural evaluation of areas 1 and 2, the porous lesions were much more extensive than revealed by gross examination. Our analysis also revealed that micro-architectural differences were particularly pronounced in younger non-adults. Taken together, the macroscopic examination of CO appears to reflect only the tip of the iceberg, as the micro-architectural changes are much larger than macroscopically identified.

Keywords: *cribra orbitalia*, micro-computed tomography, orbital roof, micro-architecture, non-adults

GRAPHICAL ABSTRACT
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INTRODUCTION

Cribra orbitalia (CO) is an osteological condition characterized by porosity and thinning of the outer surface of the orbital roof, along with expansion of the diploe in the upper orbit (Nathan & Haas, 1966). The term CO was introduced by Toldt (1886) and Welcker (1888), who described its basic osteological appearance. In CO, orbital roofs show a significantly reduced cortical bone density (Naveed, Abed, Davagnanam, Uddin, & Add, 2012), thinning of the cortical lamina that forms porosity in the affected area, enlarged inter-trabecular spaces, and position of trabeculae that are normal to the bone surface (Schultz, 2001; Naveed et al, 2012; Brickley, 2018; Brickley, Ives, Mays, 2020; O’Donnell, Hill, Anderson, Edgar, 2020). These changes likely occur as a result of bone marrow hyperplasia, which can be associated with various conditions, such as acquired or genetic anemia, bone marrow malignancy, infections, and metabolic disorders (Blom et al, 2005; Djuric et al, 2008; Naveed et al, 2012; Brickley, 2018; Brickley et al., 2020; Wang, Dittmar, Inskip, Cessford, Mitchell, 2024).

In cases of increased demand for erythropoiesis, such as anemia, malignant tumors of the bone marrow, and various forms of metabolic and infectious diseases, there is expansion of the red bone marrow, and cell proliferation that may cause cortical and trabecular atrophy (Schultz, 2001; Brickley, 2018; Brickley et al., 2020). In this way, thinning of the cortical bone occurs, followed by the appearance of porotic lesions on the outer cortical surface, as well as trabecular proliferation and expansion of the inter-trabecular spaces (Stuart-Macadam, 1985; Schultz, 2001; Blom et al, 2005; Walker, Bathurst, Richman, Gjerdrum, & Andrushko, 2009; Brickley, 2018; Buikstra, 2019). This process is especially emphasized in regions with red (active) bone marrow, predominantly in non-adults, but it is important to note that physiological pattern of successive bone marrow conversion could contribute to this condition during growth (Kricun, 1985; Stuart-Macadam, 1985; Schultz, 2001; Blom et al, 2005; Djuric et al., 2008; Walker et al, 2009; Malkiewicz, Dziedzic, 2012; Watts, 2013; Mays, 2018; Brickley, 2018; Brickley et al., 2020; Buikstra, 2019; McFadden & Oxenham, 2020).

According to previous research, CO is largely considered as an external morphological manifestation (Wapler, Crubezy & Schultz,2004; Naveed et al., 2012), thereby emphasizing ectocranial surface porosity (Orter, 2003; Wapler et al., 2004; Brickey et al., 2020; O’Donnell, et al., 2020; Anderson et al., 2021). Therefore, numerous studies have focused on the macroscopic observation of the presence/absence of CO (Mays, 2018; Rinaldo, Zedda, Bramanti, Rosa, Gualdi-Russo, 2019; Wapler et al., 2004; Djuric et al., 2008), determining the
severity of lesions (Welcker, 1888; Nathan & Haas, 1966; Hengen, 1971; Stuart – Macadam, 1985; Buikstra & Ubelaker, 1994; Jacobi, Danforth, 2002), as well as lesion’s degree of activity or healing (Mensforth, Lovejoy, Lallo, Armelagos, 1978; Mittler & Van Gerven, 1994; Rinaldo et al, 2019; O’Donnell, 2019; O’Donnell et al., 2020). However, with the emphasis on the importance of differential diagnosis in CO research, primarily by Stuart-Macadam (1985), new methods and techniques have been introduced into the analyses of these skeletal manifestations. Thus, in addition to histological analyses (Schultz, 1996, 2001; Wapler et al., 2004; Rühli, Kuhn, Evison, Müller, and Schultz, 2007), other, more extensive morphological studies have been performed, such as various imaging techniques, which redirect the focus to the inner layers of the bone in the orbital roof (Stuart-Macadam, 1985, 1987a,b, 1989, 1992; Exner, Bogusch, and Sokiranski, 2004; Wapler et al., 2004; Kuhn,Schultz, Müller, Rühli, 2007; Rühli et al., 2007; Galea, 2013; Morgan, 2014; Robertson, 2017; Anderson, 2022). Few studies used various types of computed tomography to examine CO-induced bone alterations in adults only (Exner, et al., 2004; Galea, 2013; Anderson et al., 2021; Anderson, 2022), or only in non-adults (O’Donnell et al., 2020; O’Donnell, Buikstra, Hill, Anderson, & O’Donnell, 2023). However, previous micro-computed tomography (micro-CT) studies of CO (Rühli et al., 2007; Galea, 2013; Morgan, 2014; Robertson, 2017) and study design, primarily due to inadequate sample selection and comparison of micro-scale bone changes simultaneously in non-adults and adults, without separating the age groups (Morgan, 2014; Robertson, 2017). Additionally, numerous previous studies have been only concerned with determining the precise etiology of CO lesions (Nice, Daves, & Wood, 1964; Angel, 1966; Stuart-Macadam, 1985; Walker et al., 2009; Rivera, and Lahr, 2017; Brickley, 2018; Hens, Godde, &Macak, 2019; Brickley et al., 2020; O’Donnell et al., 2020, 2023; Rothschild, Zdilla, Jellema, & Lambert, 2021; Zdilla, Nestor, Rothschild, & Lambert, 2022; Wang et al., 2024), but a consensus regarding these mechanisms has not yet been reached. Therefore, many questions dealing with CO-induced alterations in the orbital roof micro-scale bone features remain insufficiently elucidated.

Our primary aim was to explore the differences in cortical and trabecular micro-architecture between non-adults with and without macroscopic signs of CO. This study also aimed to determine whether there is a correlation between CO severity (macroscopic grades) and micro-architectural orbital bone features. Furthermore, we examined the differences between various parts of the orbital roof, that is, whether the macroscopically visible CO lesions correspond to micro-scale internal bone changes. Our additional aim was to determine whether age affects micro-architectural changes in non-adults with and without CO.

MATERIAL AND METHODS

In this study, samples of orbital roofs were collected from the Osteological Collection of the Center of Bone Biology, Faculty of Medicine, University of Belgrade. Considering the high prevalence of non-adults with CO traces in archaeological populations (Djuric et al., 2008; Nikolić, Đukić, Penezić, 2021; Mikašinović, Đukić, Penezić, 2023), only orbital roof samples from non-adults under 15 years of age were selected for this study. Bone samples with the presence of taphonomic changes and macroscopic signs of confounding pathological conditions were excluded from the study.

Based on well-recognized bioanthropological standards for age determination in non-adults, specifically dental developmental status (Ubelaker, 1984), epiphyseal union time (Brothwell, 1981), and diaphyseal length (Maresh, 1955, 1970; Schaffer, Black, Scheuer, 2009), all available preserved orbital roof samples from non-adults under 15 years of age from the Osteological Collection were selected for this study. Considering the state of preservation of the skeletal material, our study encompassed a total of 28 orbital roof samples (Figure 1A).

In accordance with our aims, this study was designed in four main phases. The first phase refers to the examination of differences in cortical and trabecular micro-architectural features between samples with macroscopically visible traces of CO (CO group, n=23) and those without signs of CO (control group, n=5) (Figure 1).

The second phase aimed to explore relations between the severity of macroscopically visible CO lesions and internal bone micro-architecture. For this purpose, we applied a widely used five-grade scoring sys-
tem for the macroscopic evaluation of CO proposed by Stuart-Macadam (1985). The macroscopic grades were evaluated using stereo magnification Leica MS5 x 4 equipped with HDMI digital camera (HY – 2307, Shenzhen Hayer Electronics Co). All observations were done under the neon light. During macroscopic CO analyses, we followed most recent recommendations of the International Meeting on Porous Skeletal Lesions (pslmeet2023).

Our samples were divided into the following groups (Figure 1B):

Grade A (slight/light) — slight porosity with barely visible sporadic pores, in the central part of the orbital roof, often scattered across the orbital roof (n=6);

Grade B (moderate/medium) — intermediate degree of porosity, manifested as small and large isolated pores, in some cases slightly linked to the trabecular structure (n=6);

Grade C (severe) — porotic lesions damaging the cortical surface and grouped pores with clearly visible unconnected trabeculae; the trabecular region is small, as is the area with thinned cortical bone (n=5);

Grade D — the presence of macro-morphological features such as expansion of trabecular bone and new bone formation on almost the entire surface of the orbital roof; the entire area is affected by porous lesions caused by so-called “trabecularization” of the cortical bone (n=6).

Grade 0 (control group) — no visible macroscopic traces of CO (n=5).

The third phase referred to the determination of the micro-scale bone features in two separate areas on the same orbit (area 1 and 2). Area 1 was defined as the area with the most dominant changes at the macroscopic level, whereas area 2 was selected at the periphery of the affected orbital roof (lateral part of the orbit), where no changes were visible at the macroscopic level (Figure 1C).

Finally, the fourth phase of our research focused on examination of cortical and trabecular micro-architectural differences between two age groups of non-adults, namely infans I (0–7 years of age, n=14) and infans II (8–15 years of age, n=14).

**Micro-CT scanning, reconstruction, and evaluation procedures**

Orbital roof samples of approximately 2 × 0.5 cm were consistently oriented (lateral part of the orbital roof facing upwards), placed on a standard sample holder, and scanned using a micro-computed tomography system (Skyscan μCT 1172, Bruker, Belgium) at an isotropic resolution of 9.95 μm, following previously established methodological approach (Djukić, Milovanović, Milenković, Djurić, 2020). In short, scan parameters were set at 45 kV and 222 μA, exposure time of 1,200 ms, 0.5 mm Al filter, 2K camera binning, rotation step 0.4 degrees, frame averaging 3, and random motion 5. NRecon software (ver. 1.6.9.8) with the InstaRecon engine (ver. 2.0.2.5) was used to reconstruct the images applying appropriate corrections for thermal drift, misalignment, ring artifacts, and beam hardening.

The cortical and trabecular micro-architecture was analyzed using 64-bit CT-Analyzer software (ver. 1.20.3.0, Bruker, Belgium), developed by Skyscan for the evaluation of three-dimensional (3D) bone morphology. As shown in Figure 1C and 1D, the position and size of volume of interest (VOI) were standardized using manual region of interest (ROI) demarcation. For each sample, trabecular and cortical bone VOIs were manually marked in a uniform manner (700 central slices each). A single researcher conducted all manual VOI demarcation procedures to avoid inter-observer error. To ensure adequate inter-individual comparison, the same threshold value (40/255) was set to differentiate the mineralized tissue and non-mineralized bone marrow spaces. In addition, a despeckle procedure was performed (white speckles less than 14,000 voxels in the 3D space were removed).

Using CT-Analyzer software, we analyzed the micro-architectural parameters of the trabecular and cortical bone for each sample. We analyzed the following trabecular parameters: bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), trabecular number (Tb.N, 1/mm), and connectivity density (Conn.Dn, 1/mm³). For the cortical compartment, we determined the following
parameters: total porosity (Po.tot, %), open porosity (Po.op, %), closed porosity (Po.cl, %), pore diameter (Po.Dm, 1/mm), pore spacing (Po.Sp, mm), cortical thickness (Ct.Th, mm), specific cortical bone surface (Ct.BS/BV, 1/mm), and cortical bone surface density (Ct.BS/TV, 1/mm); we also computed the number of closed pores per bone volume (Po.cl per BV, %).

Statistical analysis
The Kolmogorov–Smirnov test revealed that our data did not follow normal distribution. Therefore, repeated-measures analysis of variance (ANOVA) was carried out using rank-transformed data at the statistical significance level of 0.05. The analysis revealed the effects of CO presence or CO grade, analyzed area, and age group, as well their interactions, on the micro-architectural parameters. Post-hoc tests with Bonferroni correction were conducted for pairwise comparisons between the CO grades. All analyses were performed in SPSS software for Windows operative system (version 21, IBM Corp, Armonk, NY, USA).

RESULTS
3.1. Comparison of 3D bone micro-architecture between the control group (orbits with no macroscopic traces of CO) and the CO group (orbits with macroscopic traces of CO)
Analysis of micro-architecture showed significant micro-architectural alterations in both cortical and trabecular bone compartments in the CO group relative to the control group (Figure 2A). Our data revealed a significantly higher Po.Dm (p=0.004), Po.op (p<0.001), Po.tot (p<0.001), and Conn.Dn (p=0.017), while Ct.Th (p=0.002) and Tb.Th (p=0.019) were significantly lower in the CO group than in the control group (Figure 2B, C).

3.2. Severity of macroscopically visible lesions and internal orbital micro-architectural features
Our results revealed that micro-architectural features of cortical bone were different between various macroscopic CO grades (Figure 3). Significant inter-grade differences were noted in Po.Dm (p<0.001), Ct.Th (p=0.003), Po.op (p<0.001), and Po.tot (p<0.001). Specifically, there were a trend of gradual increase in Po.Dm with CO grade and a trend of decreasing Ct.Th with increasing CO grade (Figure 3A). In the case of Ct.Th, the inter-grade comparison showed a statistically significant difference between grade 0 and grades C and D (p<0.005; Figure 3A). Furthermore, the Po.op and Po.tot values increased with CO grades (p<0.005; Figure 3A). Inter-grade differences in trabecular bone were not as prominent as those in cortical bone (Figure 3B), but they were almost significant for Conn.Dn (p=0.060).

3.3. Micro-architectural changes in different areas of the same sample
As noted above, there were significant micro-architectural alterations in the CO group compared with the control group, regardless of the analyzed area (Figure 2B). Regardless of the group, there were micro-architectural differences between the two observed areas of the orbital roof. In the cortical bone, statistically significant differences were recorded for most of the analyzed parameters (p<0.05). Significantly higher Po.Dm (p=0.08), Po.op (p=0.034), and Po.tot (p=0.045), and insignificantly lower Ct.Th (p=0.098) and Po.cl (p=0.060) were noted in area 1 compared with area 2 (Figure 2). In the trabecular bone, despite the absence of statistical significance, greater micro-architectural changes were noted in area 1, with a decreasing trend of Tb.Sp, Conn.Dn, and SMI, and an increasing trend in BV/TV and Tb.N, compared with area 2 (Figure 2).

We also analyzed the interaction of area with other factors (presence of CO or CO grade, age). Specifically, in the cortical bone, the interaction between area and CO presence (Figure 2B) was significant for Po.op (p=0.046), and almost significant for Po.tot (p=0.052). In contrast, the interaction between area and CO grade was not significant for any of the examined micro-architectural parameters of the cortical bone (Figure 3A); however, in the trabecular bone, the interaction was borderline for Tb.Th (p=0.057), with greater trabecular thinning in area 1 in grades 0, A, and D (Figure 3B).

3.4. Micro-architectural differences between two age groups of non-adults
Orbital roof cortical micro-architectural properties were not significantly different between the two non-adult groups (infants I and infants II, Figure 4). However, significantly higher SMI ($p<0.005$) along with a trend toward higher Tb.N ($p=0.066$) was noted in the infants I group compared with the infants II age group.

We also analyzed the interaction of age group with other factors (CO presence, CO grade, and observed area). Our analysis of the interaction of CO presence and the age group showed a trend toward greater CO-induced trabecular thinning in younger than in older non-adults, but without reaching statistical significance ($p=0.074$). Additionally, our results showed an interaction between CO grade and age group in both the cortical and trabecular bone. Specifically, statistical significance was observed in Po.cl ($p=0.031$), Po.cl/BV ($p=0.026$), and Conn.Dn ($p=0.021$), whereby the micro-architectural differences in the cortical bone conditioned by the severity of the lesion were more pronounced in younger than in older non-adults, while in the trabecular bone the pattern was reversed.

Analysis of the interaction between area and age group showed that the micro-architectural changes in the observed areas were conditioned by age. Specifically, Po.Dm increased particularly in area 1 in younger compared with older non-adults ($p=0.008$) (Figure 4).

The multiple interactions between area, CO presence, and age group showed a mutual relationship between these three factors, but only in the case of the cortical Po.cl, where an approximate statistical significance was recorded ($p=0.066$). In area 1, in cases without CO, the percentage of Po.cl was higher in younger non-adults, while in older non-adults, Po.cl values were higher when CO was present. In area 2, the opposite pattern was observed.

Finally, we analyzed the interaction between area, grade, and age. There was a significant interaction but only for individual cortical and trabecular parameters. Specifically, in the cortical bone, the interaction was significant for Ct.Th ($p=0.008$), which in older non-adults increased in area 1 and decreased in area 2, depending on the grade. In the trabecular bone, Tb.Sp ($p=0.047$) changed significantly. This micro-architectural parameter was more pronounced in younger non-adults, regardless of the area, but in relation to the grades.

4. DISCUSSION

The study of porous changes in bone has long been of interest, predominantly because of their high prevalence in archaeological skeletal material from different periods and locations (Djuric et al., 2008; Mikašinović et al., 2023), as well as in contemporary population (Beatrice, Soler, 2016; Steyn, Voeller, Botha, and Ross, 2016; O’Donnell et al., 2020). In contrast to numerous bioanthropological studies, the examination of CO in clinical studies in contemporary population is rare (Steyn et al., 2016; Hens et al., 2019; O’Donnell et al., 2020; Rothschild et al., 2021; Anderson et al., 2021). The exceptions are studies that have used available clinical information to evaluate porotic lesions (Steyn et al., 2016; Anderson et al., 2021), lesion morphology (O’Donnell et al., 2023), or the etiology of these skeletal manifestations (Nice et al., 1964; Hens et al., 2019; O’Donnell et al., 2020, 2023). In addition to these previous evaluations, radiological and histological assessments of CO-induced bone alterations were previously conducted (Stuart-Macadam, 1985, 1987a, b, 1992; Schultz, 2001; Wapler, et al., 2004). Recently, the analysis of CO-induced lesions has been improved by the application of different CT imaging techniques (Exner et al., 2004; Wapler et al., 2004; Kuhn et al., 2007; Rühl et al., 2007; Anderson, 2022; O’Donnell et al., 2023). Additionally, scientists agreed that micro-CT could contribute to better understanding of CO-induced bone lesions (Schultz, 2001; Brickley et al., 2020), considering its high resolution and 3D-approach in histomorphometric bone assessment. However, previous research regarding CO-induced micro-architectural changes has not been published in international scientific journals, but has only been included in master’s or PhD theses (Galea, 2013; Morgan, 2014; Roberston, 2017). These previous studies have focused on parts or even whole skulls of adults or a wide range of non-adult populations (up to 19 years of age) and on evaluating several general features (Robertson, 2017), such as trabecular thickness, size and shape of the diploe, and thickness of the outer table of the orbital roof, in relation to the spatial distribution of CO, grades, or degree of activity (Galea, 2013; Morgan, 2014; Roberston, 2017). However, these studies were mostly qualitative due to the inability to compare data since
non-uniform sampling strategies or low scanning resolution were used, highlighting the need for more detailed analysis.

Our results showed that the presence of CO affected cortical and trabecular micro-architecture, in terms of increased cortical porosity, Po.Dm, and Conn.Dn, and decreased Ct.Th and Tb.Th in the CO group. Such results are expected and may speak in favor of bone marrow hyperplasia in CO, initiating an osteoclastic response in the form of thinning of both cortical and trabecular bone (Stuart-Macadam, 1985; Brickley et al., 2020), which is additionally manifested by greater perforation and resorption of trabecular elements and removing fine trabeculae (Stuart-Macadam, 1985; Resnick, Nivaiama, 1988; Ragsdale, Lehmer, 2012; Naveed et al., 2012; Brickley et al., 2020). Furthermore, our data showed that micro-architectural changes in the orbital roof were conditioned by CO grade, suggesting that the micro-architecture of the orbital roof changes with lesion progression (Figure 5). Such results are supported by other micro-CT studies dealing with the analysis of interrelation of macroscopic CO expression and internal orbital changes (Morgan, 2014; Robertson, 2017). However, except for the noted overall trend in micro-scale changes, no significant micro-architectural differences were revealed between adjacent CO grades (Figure 5). The absence of micro-architectural differences between adjacent CO grades may suggest that the changes are gradual and/or that there is some overlap between CO grades, so that a five-stage scoring system fails to fully, and reliably reflect micro-architectural changes related to CO. Nevertheless, the inter-grade comparison showed that the most pronounced micro-architectural differences were recorded between the grades 0 and D (Figure 5). In particular, the most pronounced micro-architectural differences in grade D compared with the other CO grades may indicate an intense proliferative process at the time of death (O’Donnell et al., 2023), which macroscopically manifested with newly formed bone. According to O’Donnell et al. (2023), such a phenomenon could occur due to prolonged illness or deprivation of a person’s immune response. On the other hand, Galea (2013) suggests that proliferation occurs in earlier stages, while in more progressive forms, increased resorption is recorded. However, macroscopic examination of our samples still supports O’Donnell et al. (2023) and Morgan (2014), who additionally emphasized that in more severe CO forms, there was a common occurrence of resorption and new bone formation, which is characteristic of bone marrow hyperplasia (Stuart-Macadam, 1987b), which is also detected at the micro-scale level. In this way, there may be increased osteoclastic activity, followed by a simultaneous osteoblastic response with the aim of replacing resorbed elements (Ortner, 2003; Buikstra, 2019) or micro-damage with newly formed bone in the same place (Brickley et al., 2020). Practically, the expansion of the subperiosteal bone marrow results in the formation of new bone that covers the cortical porous bone created due to bone marrow hyperplasia (Brickley et al., 2020).

Our micro-CT analysis revealed the presence of micro-architectural changes in both of the observed areas in individuals with CO relative to those without CO. These changes were certainly conditioned by the presence of CO, but they were surprisingly recorded even in the area without macroscopically visible traces of CO (area 2), especially in the cortical bone. These data suggest that macroscopic examination of CO appears to reflect only the tip of the iceberg, since micro-architectural alterations are much more extensive than macroscopically identified (Figure 5). It is possible that orbital morphology and variation in cortical thickness could be the structural basis for this finding (Schats, 2021).

Trabecular and cortical micro-architectural variations were predominantly pronounced in younger non-adults (Figure 5). The reason for the more intense changes in the younger population could be related to morphological and physiological bone and bone marrow requests during growth (Dunnill, Anderson, Whitehead, 1967; Cristy, 1981; Kricun, 1985; Stuart-Macadam 1985; Okada et al., 1989; Simonson & Kao, 1992; Goldman, McFarlin, Cooper, Thomas, Clement, 2009). Primarily, the observed micro-architectural changes may arise from the normal physiological development of bone and bone marrow (Goldman et al., 2009; Brickley et al., 2020). More precisely, the reason for greater micro-architectural variations in both bone compartments may be related to the fact that in younger non-adults, the bone marrow is more reactive to changes (Kricun, 1985; Stuart-Macadam, 1985; Schultz, 2001; Blom et al., 2005; Djuric et al., 2008; Walker et al, 2009; Malkiewicz, Dziedzic, 2012; Watts, 2013; Mays, 2018; Brickley, 2018; Brickley et al., 2020; Buikstra, 2019; McFadden & Oxenham, 2020), and with growth it becomes more resistant to mechanical stress (Djuric et al., 2008; Malkiewicz, Dziedzic, 2012; Brickley, 2018; Buikstra, 2019; McFadden & Oxenham, 2020). In addition, the
observed bone changes can be associated with the greater reactivity of trabecular bone to metabolic disorders that lead to bone marrow hyperplasia, and at the same time with the fact that cortical bone becomes more rigid and resistant to mechanical loads with aging (Boskey, Coleman, 2010).

Despite the disadvantages of our research, the main advantage of this study is the highest resolution of micro-CT scanning of CO-induced bone applied thus far. Additionally, given that the areas on the orbital roof affected and non-affected by CO were analyzed exclusively in non-adult population, we ensure consistent sample. However, in addition to the some strengths of our study, it is important to note its limitations. The cross-sectional study design is a clear limitation of our study, considering that we were not able to follow individual-specific disease progression over time. Moreover, considering the chosen study design, we were unable to assess the covariant effect of various diseases (especially those that do not leave traces on bone). A larger sample size could contribute to a better understanding of the specificity of micro-architectural differences between grades in non-adults with CO, as well as further elucidating the impact of CO on bone micro-architecture. Finally, our study is of limited informative value regarding etiopathogenetic mechanisms of CO-induced bone alterations in non-adults, highlighting the need for further investigation.

5. CONCLUSION

According to our results, the presence of CO in non-adults significantly affects cortical and trabecular micro-architecture, which may be consistent with initiating bone marrow hyperplasia, followed by greater porosity and resorption of trabecular elements. Analyzing how micro-architectural differences are affected by the severity of CO lesions, we concluded that they are conditioned by the CO grade, especially in the cortical bone. Inter-grade comparisons revealed a trend of micro-architectural variation from the lightest to the most progressive form of CO. However, most of the analyzed micro-architectural variables did not show significant variation between grades in non-adults with CO, as well as further elucidating the impact of CO on bone micro-architecture. Finally, our study is of limited informative value regarding etiopathogenetic mechanisms of CO-induced bone alterations in non-adults, highlighting the need for further investigation.

FUNDING: Authors received financial support from the Science Fund of the Republic of Serbia (GRANT No.7394, FirPanGen [PRISMA program]; GRANT No. 7749444, BoFraM [IDEAS program]), and the Ministry of Science of the Republic of Serbia (GRANT No. 451-03-66/2024-03/200110, institutional funding to Faculty of Medicine University of Belgrade; and support to the Center of Bone Biology as the center of excellence).

COMPETING INTEREST: Authors have no conflicts of interest to declare.

DATA AVAILABILITY: All data that support findings generated during this study are available from the corresponding author upon request.

CODE AVAILABILITY: Not applicable.

AUTHOR CONTRIBUTION: Conceptualization: Ksenija Djukic and Marija Djuric; Methodology: Jelena Jadzic and Petar Milovanovic; Investigation: Veda Mikasinovic, Petar Milenkovic; Formal analysis: Veda Mikasinovic and Petar Milovanovic; Data interpretation: All authors; Visualization: Veda Mikasinovic and Jelena Jadzic; Writing-original draft: Veda Mikasinovic; Writing – Review and Editing: Ksenija Djukic, Jelena Jadzic, Petar Milovanovic, Petar Milenkovic, and Marija Djuric; Supervision: Marija Djuric; Project administration and Funding acquisition: Ksenija Djukic and Marija Djuric; Final approval of the submitted manuscript: all authors.

ETHICS APPROVAL: Sample collection and other procedures performed during this study were in accordance with the ethical standards of the Ethics Committee of the Faculty of Medicine, University of Belgrade, Serbia, and under the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.
The Institutional Ethics Committee waived the requirement of informed consent for the collection of the samples included in the study.

ANIMAL RESEARCH: Not applicable.

CONSENT TO PARTICIPATE: Not applicable.

CONSENT FOR PUBLICATION: Not applicable.

REFERENCES


**Figure legends:**
Figure 1. Representation of the methodology used in the present study: bone sampling (A), macroscopic CO scoring system (B), differentiation between area affected with CO and that without CO (C), and trabecular and cortical ROI demarcation procedure (D)

For interpretation of the colors in the figure legend, the reader is referred to the web version of the article.

Abbreviations: CO – cribra orbitalia, the ROI – region of interest

Figure 2. Comparison of representative micro-architectural findings of the orbital roof samples between the CO group and the control group (A): cortical (B) and trabecular (C) micro-architectural properties

Analysis of variance (ANOVA) on ranks was done to assess the inter-group difference in rank-transformed data (*** indicates significant difference between the CO group and the control group). For interpretation of the colors in the figure legend, the reader is referred to the web version of the article.

Abbreviations: Ct.Po.tot – cortical total porosity; Ct.Po.op – cortical open porosity; Ct.Po.cl – cortical close porosity; Ct.Po.cl/BV – cortical close porosity per bone volume; Po.Dm – pore diameter; Ct.Th – cortical thickness; BV/TV - bone volume fraction; Tb.N – trabecular number; Tb.Th – trabecular thickness; Tb.Sp – trabecular separation; Conn.Dn - connectivity density; SMI – structure model index

Figure 3. Comparison of cortical (A) and trabecular (B) micro-architectural properties between individuals with various macroscopic CO grades

Analysis of variance (ANOVA) on ranks was done to assess the inter-grade difference in rank-transformed data (ˆˆˆ indicates significant difference between grade B and other CO grades, ### indicates significant difference between grade C and other CO grades, *** indicates significant difference between grade D and other CO grades). For interpretation of the colors in the figure legend, the reader is referred to the web version of the article.

Abbreviations: Ct.Po.tot – cortical total porosity; Ct.Po.op – cortical open porosity; Po.Dm – pore diameter; Ct.Th – cortical thickness; BV/TV – bone volume fraction; Tb.N – trabecular number; Conn.Dn – connectivity density

Figure 4. Comparison of cortical (A) and trabecular (B) orbital micro-architecture between two non-adults’ age groups

Analysis of variance (ANOVA) on ranks was done to assess the inter-group difference in rank-transformed data (** indicates significant difference between CO group and control group). For interpretation of the colors in the figure legend, the reader is referred to the web version of the article.

Abbreviations: Ct.Po.tot – cortical total porosity; Ct.Po.op – cortical open porosity; Ct.Po.cl – cortical close porosity; Ct.Po.cl/BV – cortical close porosity per bone volume; Po.Dm – pore diameter; Ct.Th – cortical thickness; BV/TV – bone volume fraction; Tb.N – trabecular number; Tb.Sp – trabecular separation; Conn.Dn - connectivity density; SMI – structure model index

Figure 5. Schematic representation of CO-induced micro-architectural alterations in orbital roof collected from non-adult individuals

The presence of CO in non-adults significantly affected cortical and trabecular micro-architecture. The most prominent micro-architectural changes were noted in younger non-adults, especially in the cortical bone, in both of the observed areas. Inter-grade comparison of CO showed no statistically significant differences between adjacent CO grades, but there was a trend of micro-architectural decline in advanced CO grades. The most prominent micro-architectural changes were noted in the cortical bone of individuals with grade D.

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