Original Research
Stomatology

Comparative study of oral and salivary parameters in patients with and without loss of bone mass

Igor Figueiredo PEREIRA[a]
Cláudia Borges BRASILEIRO[a]
Núbia Priscilla KLEPERON[b]
Mauro Henrique Nogueira Guimarães de ABREU[a]
Tarcília Aparecida da SILVA[a]
Ricardo Alves MESQUITA[a]
Tania Mara Pimenta AMARAL[a]

[a]Universidade Federal de Minas Gerais – UFMG, School of Dentistry, Department of Clinical, Pathology and Surgery, Belo Horizonte, MG, Brazil.

[b]Universidade Federal de Minas Gerais – UFMG, School of Dentistry, Department of Pediatric Dentistry and Orthodontics, Belo Horizonte, MG, Brazil.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Corresponding Author:
Igor Figueiredo Pereira
E-mail: figueiredo.ctbmf@gmail.com

https://doi.org/10.1590/1807-3107bor-2018.vol32.0054

Submitted: October 10, 2017
Accepted for publication: April 10, 2018
Last revision: May 04, 2017

Abstract: Osteoporosis is an insidious and increasingly prevalent disease that can cause fractures and affect patients' quality of life. The current study comparatively evaluates patients with and without loss of bone mass in terms of salivary calcium, viscosity, and pH. A controlled cross-sectional study was conducted in two groups of 32 postmenopausal women subjected to a bone densitometry scan and later referred for dental management at the Federal University of Minas Gerais, Brazil. The patients were assigned to two groups: Group 1 - patients with low bone mineral density (BMD) and Group 2 - patients without bone mineral changes. The following salivary parameters were evaluated: calcium concentration, flow rate, viscosity, pH, and average total protein. An oral examination was performed for assessment of DMFT variables and tongue coating. Data were analyzed using descriptive and inferential statistics, adopting a p-value < 0.05. The patients' mean age was 60 years (± 7.35). Salivary flow, pH, and viscosity were similar among the groups. Average total protein was 14.8 mg/mL and 19.0 mg/mL in Groups 1 and 2, respectively. Tongue coating and salivary calcium levels were significantly higher in Group 1 (p < 0.001). Salivary calcium is an important screening tool and may eventually be used for the diagnosis of bone mineral changes.

Keywords: Bone Density; Calcium; Osteoporosis; Saliva.

Introduction

Aging is a heterogeneous process, consisting of several reactions, and is characterized by changes that lead to a reduction in the adaptability of human beings to their environment.[1] Scientific advances, medical interventions, and sanitation have contributed to increasing the longevity of the population[2] and the incidence of degenerative diseases.

Osteoporosis is a common systemic skeletal disease in the elderly, characterized by low bone mineral density (BMD), leading to bone fragility and increased risk of fracture. The disease is asymptomatic unless a fracture occurs.[3,4] Osteoporosis affects mainly menopausal women and increases the risk of certain oral changes, such as xerostomia, which is a subjective sensation of dry mouth directly linked to decreased salivary flow.[5] The prevalence of oral symptoms has been significantly greater (43%) in menopausal women. The method of choice for early detection

of osteoporosis and risk of fracture is dual energy X-ray absorptiometry (DEXA), which is expensive and whose results are difficult to interpret.[6]

This research contributes to the identification of new tools for the diagnosis of low BMD by examining the saliva, which is a constituent of organic and inorganic components and which may vary both qualitatively and quantitatively.[7] Saliva is also important for maintaining the health of oral tissues and has the advantage of being easily obtained non-invasively and without discomfort to the patient. Saliva is increasingly being used as a research material in diagnostic assays.

Saliva is used to assess the level of hormones, drugs, and inflammatory factors. Salivary biomarkers have been used to assess the risk of dental caries, periodontal diseases, and Sjögren's syndrome.[8] Therefore, the overall objective of this study is to comparatively evaluate the oral and salivary parameters of patients with and without loss of bone mass.

Methodology

A controlled cross-sectional study was conducted with postmenopausal women referred for dental treatment at the Dental School of the Federal University of Minas Gerais (UFMG), Brazil. Sample size calculation was based on the findings of Taguchi et al.,[9] who compared mandibular cortical bone thickness in patients with and without bone mineral changes using a 95% confidence interval and an 80% test power. Lumbar spine (LS) and femoral neck (FN) BMD was measured by DEXA using a Lexxos densitometer. The patients were assigned to two groups according to the WHO[10] densitometric diagnostic criteria: Group 1 – women with low BMD and Group 2 – women without bone mineral changes.

The WHO10 classification of osteoporosis evaluates the prevalence of the disease according to the following criteria, based on bone densitometry findings:

Normal bone density: T-score greater than or equal to -1.0; Osteopenia (low bone mass): T-score between -1.0 and -2.5; and osteoporosis: T-score less than -2.5. Patients with osteopenia or osteoporosis were allocated to Group 1 and those without any bone mineral changes (normal) were allocated to Group 2.

Exclusion criteria

Patients younger than 45 years, who were smokers, who had diabetes, who were not in menopause, who were taking continuous medication other than that for the treatment of osteoporosis, and who refused to sign the informed consent form were excluded from the study.[11]

Saliva collection

Unstimulated and stimulated saliva samples were collected. Patients spent 5 minutes without making chewing movements, swallowing, or speaking, in a sitting position, facing forward. Unstimulated saliva was collected for 5 minutes in a 50-mL sterile tube (model K19-0050 KASVI – Centrifuge Tubes, China). For stimulated saliva sampling, patients chewed hyperboloids (silicone chewing instruments that are nontoxic, tasteless, and odorless) for 5 minutes before collection.[12] Salivary flow rate was recorded as mL/min, used for the physical evaluation of all collected samples.

Salivary viscosity and pH

Salivary viscosity was measured using an Ostwald (D-445/D-446/U.E) viscometer, immersed (Annex A1) in water at 36°C, whose value was obtained in seconds. After 5 minutes in a thermostatic bath, the saliva was evaluated for viscosity and the average value was determined after performing three evaluations for each sample. Viscosity was assessed at a constant temperature of 36ºC, using thermostatic bath for all evaluations. The final viscosity value was calculated according to the time recorded on Ostwald viscometer and to saliva density.[13] Salivary pH was measured with a digital pH meter, using at least 2 mL of each sample.[14] The final value corresponded to the average of three measurements.

Oral health status

The oral health status was represented by two parameters: DMFT (decayed, missing, filled teeth), widely used in studies for oral health assessment, and tongue coating. The assessment was performed by a single examiner, calibrated by direct visual observation of the oral cavity, using gloves, a mirror, and a clinical probe.[15] Tongue coating was assessed

according to its presence or absence, as verified by clinical examination by the calibrated examiner.[16] DMFT was calculated by means of an index that numerically indicates the prevalence of tooth decay individually.

Salivary calcium concentration

The collected saliva was centrifuged (3,000 g) (Microcentrifuge Eppendorf 5415D) and the supernatants were separated. Simultaneously, the saliva was frozen until analysis. Calcium concentrations were measured by atomic absorption spectrometry (for color analysis). The results were expressed in mg/dL.7 Note that all tubes used for collection were washed with deionized water to avoid interference with the calcium assay, which was performed at the Laboratory of Experimental Pathology II of the Faculty of Dentistry of the UFMG. Calcium concentration was determined by colorimetry using Bioclin® commercial kit (basic cresolphthalein complexone method, Quibasa Chemical Ltd., Belo Horizonte, Brazil), which measures the intensity of the color produced by the calcium compound at an alkaline pH.

Salivary protein concentration

Total concentration of salivary proteins was determined by the Bradford method, which consisted in using the "Coomassie brilliant blue" (BG-250) dye (a registered trademark of Imperial Chemical Industries, London, England). This method is based on the interaction between the BG-250 dye and protein macromolecules containing basic amino acids or aromatic side chains. This assay was performed in a sterile tube (model K19-0050 KASVI - Centrifuge Tubes, Beijing, China). The interaction consisted of 0.1 mL of the saliva sample and 3 mL of BG-250.

Statistical analysis

Data were analyzed descriptively and inferentially using the Statistical Package for Social Sciences (SPSS) software, version 23.0, with a 95% confidence interval. Quantitative variables were tested for normality by graphical analysis (histogram, Q-Q plot) and by the Kolmogorov-Smirnov test ($p < 0.05$). Association tests (Student's t or Mann-Whitney tests) were performed to compare the quantitative variables in both groups. Pearson's chi-square test was used to compare the presence of tongue coating in both groups. Spearman's correlation coefficients were employed to compare salivary parameters and bone density in the group with osteoporosis.

Compliance with ethical standards

This study met all ethical standards required by Resolution 466/12 of the National Health Council (CNS, Brazil), which deals with ethics in research involving humans. Only patients who properly completed and signed the free and informed consent form were included in this study. This research was approved by the UFMG Platform Brazil / Ethics Committee (process: CAAE – 60082916.0.0000.5149).

Results

The study included 64 patients, 32 with low BMD (case group) and 32 without bone mineral changes (control group). Table 1 shows the descriptive analysis of the variables. Average values were adopted for the variables with normal distribution ($p > 0.05$), along with the minimum and maximum values for the subsequent variables that followed a non-normal distribution ($p < 0.05$).

Table 2 presents the bivariate analysis between the groups and the variables under study ($p < 0.05$). Both average and median values were adopted. Salivary calcium levels and the presence of tongue coating were statistically significant in Group 1 ($p < 0.001$).

Table 1. Descriptive analysis of the variables. Belo Horizonte, 2017.

Variables	Values
Mean age ± SD	60.3 ± 7.3
Unstimulated saliva mL/min	0.6 ± 0.3
Stimulated saliva mL/min	1.8 ± 0.7
DMF-T	25 ± 5
Presence of tongue coating (%)	48.4
pH	7.2 ± 0.4
Viscosity (median, Min - Max)	0.7 (0.4–2.2)
Total proteins mg/mL	18.6 ± 11.0
Calcium mg/dL	10.3 (4.7–177.6)

SD: standard deviation; Min–Max = Minimum–maximum.

Table 2. Characteristics and bivariate association between the variables for both groups. Belo Horizonte, 2017.

Variables	Case (n = 32)	Control(n = 32)	p-value
Mean age ± SD	60.6 ± 6.8	60.2 ± 8.0	0.801*
Unstimulated saliva mL/min	0.6	0.6	0.468**
Stimulated saliva mL/min	1.6	2.0	0.189***
DMF-T	26	23	0.777***
Presence of tongue coating (%)	78.1	18.9	< 0.001**
pH	7.2 ± 0.4	7.1 ± 0.4	0.738**
Viscosity	0.7	0.7	0.619***
Total proteins mg/mL	14.8	19.0	0.095***
Calcium mg/dL	28.5	8.5	< 0.001***

* Student's t test; **Pearson's Chi-square test; ***Mann-Whitney U test,

The Spearman's correlation coefficients show a weak correlation between bone density and unstimulated saliva (rs = -0.287), stimulated saliva (rs = -0.068), pH (rs = -0.190), calcium (rs = 0.097), protein (rs = 0.377), and viscosity (rs = 0.161).

Discussion

Women's health needs are different mainly because fluctuations in hormone levels occur throughout their lives, affecting the physiology of the entire body, including the oral cavity. Thus, menopause may directly affect metabolism, changing salivary parameters and quality of life.[17]

Regarding salivary calcium levels, the present study found a statistically significant greater amount in low BMD patients than in those without bone mineral changes, thus corroborating the findings of other studies that used similar samples, in which salivary calcium levels proved to be significantly higher in patients with bone mineral changes when compared to controls.[18,19,20]

Some studies have tried to establish methods for the diagnosis or prognosis of oral diseases using salivary analysis.[8] Calcium is one of the most important salivary electrolytes because of its effective role in bone structures; it is also the most important ion in the composition of this structure; and it plays a significant role in bone regeneration, which is directly related to osteoporosis, a change that may occur as a consequence of reduced absorption of this electrolyte, which may occur in either the bone or saliva.[21]

Rabiei et al.[7] studied a group of similar patients and assessed salivary calcium, applying a cutoff point of 6.1 mg/dL. Salivary calcium concentration demonstrated that about 67.5% of the patients had osteoporosis, while 60% of women with salivary calcium levels below the cut-off point were free of osteoporosis. They came to the conclusion that salivary calcium can be used to diagnose bone mineral changes, thus obviating the need for bone densitometry. Moghadam et al.[21] did not find any correlation between salivary calcium and low BMD. This difference in results may be due to the different age of patients in each sample and to the study designs as well.

Regarding the clinical parameters evaluated in the present study, the DMFT found in the case group was higher than that of the control group, but without any statistical significance. Conversely, tongue coating was significantly greater in patients with bone mineral changes. Research into tongue coating began in China and is very popular in Asia today.[22] Primary and most recent studies also use this parameter for an early diagnosis of some diseases, including osteoporosis. Association of this parameter with patients with osteoporosis is scarce in the literature, but tongue coating has been associated with patients' predisposition to

diseases, such as cancer[23] and cardiopulmonary disease.[24] In a Japanese study, tongue coating showed was positively correlated with salivary viscosity ($p < 0.001$).[25] It is believed that more viscous saliva favors the accumulation of mainly anaerobic gram-negative bacteria, thus contributing to the formation of tongue coating.[26] Therefore, further studies comparing salivary parameters with tongue coating are warranted, since the first results for salivary viscosity proved to be significant. The surface of the tongue is formed by a bacterial plaque, a structure firmly bonded not only to the tooth surface, but also to other structures, which can be found in the mouth. Salivary calcium plays an important role in the inorganic matrix of the bacterial plaque.[27] According to this study, tongue coating was greater in patients with low BMD and salivary calcium, showing a positive correlation between these variables. Regarding salivary flow, the present study found no statistically significant difference when the groups were compared with and without bone mineral changes. This result is in line with literature findings, which point to no significant differences when postmenopausal women were assessed.[11,17]

The sensation of dry mouth is one of the main complaints of many older people and is strongly associated with menopause. The exact mechanisms that cause this feeling in women have not been firmly established, but there is good reason to believe that this may well be related to the decrease in estrogen levels, which occurs with advancing age.[28] Another study, which analyzed the saliva of women of advanced age, showed dependence on estrogen and found that decreased salivary flow was positively correlated with salivary calcium concentration and inversely correlated with serum estrogen, thus explaining the lower salivary flow in postmenopausal patients. Furthermore, estrogen deficiency accelerates bone turnover, thereby interfering with calcium uptake by the intestines and increasing calcium excretion.[6]

The analysis of other salivary parameters, such as viscosity, pH, and protein concentration, revealed no significant differences between the groups. Viscosity depends on important macromolecules, such as proteins, especially mucins such as MUC5B, which also influence other parameters, such as salivary flow and pH.[29] One explanation for these results is that these parameters proved to be similar between the groups, regardless of the decrease or absence of bone mass.

The average salivary pH of the patients in the present study proved to be quite similar between the groups (7.1 to 7.2), which was within normal salivary values. These values were also similar to those observed by Preoteasa et al.,[14] who evaluated male and female patients aged over 50 years. These authors found normal salivary pH values and determined that factors, such as salivary flow, diet, and oral hygiene, do influence salivary pH.[30]

This research sought to conduct a study using similar groups. An important factor in these types of study is age; in the present study, the average age was 60 in both groups. Rabiei et al.[7] conducted a similar study and found that patients' age and time since menopause may reduce the accuracy of the results, that is, the more disparate the age and time since menopause, the less effective the tools (*e.g.*, salivary calcium) will be in detecting osteoporosis.

Therefore, it is important to seek new tools for the screening and early diagnosis of osteoporosis, thus improving patient quality of life, including calcium and other salivary parameters as important factors in this process, along with the assessment of oral health status.[6] Despite the encouraging results, the present research used a small sample and, therefore, longitudinal studies with a larger sample size are recommended for confirming whether postmenopausal women with salivary changes tend to have osteoporosis and whether these findings change over the years.

Conclusions

Salivary calcium was an important screening tool and could eventually be used to diagnose bone mineral changes, given the statistically significant results obtained for the group with low BMD and the larger and statistically significant presence of tongue coating among patients with decreased BMD.

References

1. Schmidt MI, Duncan BB, Silva GA, Menezes AM, Monteiro CA, Barreto SM et al. Chronic non-communicable diseases in Brazil: burden and current challenges. Lancet. 2011 Jun;377(9781):1949-61. https://doi.org/10.1016/S0140-6736(11)60135-9
2. Baccaro LF, Conde DM, Costa-Paiva L, Pinto-Neto AM. The epidemiology and management of postmenopausal osteoporosis: a viewpoint from Brazil. Clin Interv Aging. 2015 Mar;10(10):583-91. https://doi.org/10.2147/CIA.S54614
3. Hajian-Tilaki K, Heidari B, Firouzjahi A, Bagherzadeh M, Hajian-Tilaki A, Halalkhor S. Prevalence of metabolic syndrome and the association with socio-demographic characteristics and physical activity in urban population of Iranian adults: a population-based study. Diabetes Metab Syndr. 2014 Jul-Sep;8(3):170-6. https://doi.org/10.1016/j.dsx.2014.04.012
4. Govindraju P, Chandra P. Radiomorphometric indices of the mandible: an indicator of osteoporosis. J Clin Diagn Res. 2014 Mar;8(3):195-8. https://doi.org/10.7860/JCDR/2014/6844.4160
5. Friedlander AH. The physiology, medical management and oral implications of menopause. J Am Dent Assoc. 2002 Jan;133(1):73-81. https://doi.org/10.14219/jada.archive.2002.0025
6. Singh B, Pallagatti S, Narang RS, Kaur K, Sheikh S, Manchanda A et al. Evaluation of serum calcium and serum parathyroid levels in post-menopausal women with and without oral dryness [d]. Gerodontology. 2016 Jun;33(2):240-6. https://doi.org/10.1111/ger.12148
7. Rabiei M, Masooleh IS, Leyli EK, Nikoukar LR. Salivary calcium concentration as a screening tool for postmenopausal osteoporosis. Int J Rheum Dis. 2013 Apr;16(2):198-202. https://doi.org/10.1111/1756-185X.12003
8. Navazesh M, Kumar SK. Measuring salivary flow: challenges and opportunities. J Am Dent Assoc. 2008 May;139 Suppl:35S-40S. https://doi.org/10.14219/jada.archive.2008.0353
9. Taguchi A, Suei Y, Sanada M, Ohtsuka M, Nakamoto T, Sumida H et al. Validation of dental panoramic radiography measures for identifying postmenopausal women with spinal osteoporosis. AJR Am J Roentgenol. 2004 Dec;183(6):1755-60. https://doi.org/10.2214/ajr.183.6.01831755
10. World Health Organization - WHO. The world health report 2004: changing history. Geneva: World Health Organization; 2004.
11. Agha-Hosseini F, Mirzaii-Dizgah I, Moghaddam PP, Akrad ZT. Stimulated whole salivary flow rate and composition in menopausal women with oral dryness feeling. Oral Dis. 2007 May;13(3):320-3. https://doi.org/10.1111/j.1601-0825.2006.01288.x
12. Amaral TMP, Campos CC, Santos TPM, Leles CR, Teixeira AL, Teixeira MM et al. Effect of salivary stimulation therapies on salivary flow and chemotherapy-induced mucositis: a preliminary study. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012 May;113(5):628-37. https://doi.org/10.1016/j.oooo.2011.10.012
13. Animireddy D, Reddy Bekkem VT, Vallala P, Kotha SB, Ankireddy S, Mohammad N. Evaluation of pH, buffering capacity, viscosity and flow rate levels of saliva in caries-free, minimal caries and nursing caries children: an in vivo study. Contemp Clin Dent. 2014 Jul;5(3):324-8. https://doi.org/10.4103/0976-237X.137931
14. Preoteasa E, Tâncu AM, Iosif L, Melescanu Imre M, Murariu-Măgureanu C, Preoteasa CT. Salivary changes related to systemic diseases in the edentulous patients. J Med Life. 2014 Oct-Dec;7(4):577-80.
15. Lambert MJ, Vanobbergen JS, Martens LC, De Visschere LM. Socioeconomic inequalities in caries experience, care level and dental attendance in primary school children in Belgium: a cross-sectional survey. BMJ Open. 2017 Jul;7(7):e015042. https://doi.org/10.1136/bmjopen-2016-015042
16. Miyazaki H, Sakao S, Katoh Y, Takehara T. Correlation between volatile sulphur compounds and certain oral health measurements in the general population. J Periodontol. 1995 Aug;66(8):679-84. https://doi.org/10.1902/jop.1995.66.8.679
17. Saluja P, Shetty V, Dave A, Arora M, Hans V, Madan A. Comparative evaluation of the effect of menstruation, pregnancy and menopause on salivary flow fate, pH and gustatory function. J Clin Diagn Res. 2014 Oct;8(10):ZC81-5. https://doi.org/10.7860/JCDR/2014/9935.5071
18. Sewón L, Laine M, Karjalainen S, Doroguinskaia A, Lehtonen-Veromaa M. Salivary calcium reflects skeletal bone density of heavy smokers. Arch Oral Biol. 2004 May;49(5):355-8. https://doi.org/10.1016/j.archoralbio.2003.11.004
19. Saha MK, Agrawal P, Saha SG, Vishwanathan V, Pathak V, Saiprasad SV et al. Evaluation of Correlation between salivary calcium, alkaline phosphatase and osteoporosis: a prospective, comparative and observational study. J Clin Diagn Res. 2017 Mar;11(3):ZC63-6.
20. Agha-Hosseini F, Mirzaii-Dizgah I, Moosavi MS. Relationship of serum and saliva calcium, phosphorus and alkaline phosphatase with dry mouth feeling in menopause. Gerodontology. 2012 Jun;29(2):e1092-7. https://doi.org/10.1111/j.1741-2358.2012.00619.x
21. Moghadam SA, Zakeri Z, Fakour SR, Moghaddam AA. Does salivary calcium and phosphate concentrations adequately reflect bone mineral density in patients with chronic periodontitis? Glob J Health Sci. 2016 Oct;8(10):56723. https://doi.org/10.5539/gjhs.v8n10p282
22. Kishimoto N, Stegaroiu R, Shibata S, Ito K, Inoue M, Ohuchi A. Changes in the oral moisture and the amount of microorganisms in saliva and tongue coating after oral ingestion resumption: a pilot study. Open Dent J. 2016 Mar;10(10):79-88. https://doi.org/10.2174/1874210601610010079

23. Han S, Yang X, Qi Q, Pan Y, Chen Y, Shen J et al. Potential screening and early diagnosis method for cancer: tongue diagnosis. Int J Oncol. 2016 Jun;48(6):2257-64. https://doi.org/10.3892/ijo.2016.3466
24. Liang W, Li X, Li Y, Li C, Gao B, Gan H et al. Tongue coating microbiome regulates the changes in tongue texture and coating in patients with post-menopausal osteoporosis of Gan-shen deficiency syndrome type. Int J Mol Med. 2013 Nov;32(5):1069-76. https://doi.org/10.3892/ijmm.2013.1485
25. Ueno M, Takeuchi S, Takehara S, Kawaguchi Y. Saliva viscosity as a potential risk factor for oral malodor. Acta Odontol Scand. 2014 Nov;72(8):1005-9. https://doi.org/10.3109/00016357.2014.938115
26. Park MS, Chung JW, Kim YK, Chung SC, Kho HS. Viscosity and wettability of animal mucin solutions and human saliva. Oral Dis. 2007 Mar;13(2):181-6. https://doi.org/10.1111/j.1601-0825.2006.01263.x
27. Sewón LA, Karjalainen SM, Söderling E, Lapinleimu H, Simell O. Associations between salivary calcium and oral health. J Clin Periodontol. 1998;25(11):915-9. https://doi.org/10.1111/j.1600-051X.1998.tb02390.x
28. Singh R, Pallagatti S, Sheikh S, Singh B, Arora G, Aggarwal A. Correlation of serum oestrogen with salivary calcium in post-menopausal women with and without oral dryness feeling. Gerodontology. 2012 Jun;29(2):125-9. https://doi.org/10.1111/j.1741-2358.2011.00580.x
29. Koshimune S, Awano S, Gohara K, Kurihara E, Ansai T, Takehara T. Low salivary flow and volatile sulfur compounds in mouth air. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003 Jul;96(1):38-41. https://doi.org/10.1016/S1079-2104(03)00162-8
30. Hohensinn B, Haselgrübler R, Müller U, Stadlbauer V, Lanzerstorfer P, Lirk G et al. Sustaining elevated levels of nitrite in the oral cavity through consumption of nitrate-rich beetroot juice in young healthy adults reduces salivary pH. Nitric Oxide. 2016 Nov;60:10-5. https://doi.org/10.1016/j.niox.2016.08.006