Leptin acts on neoplastic behavior and expression levels of genes related to hypoxia, angiogenesis, and invasiveness in oral squamous cell carcinoma

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Abstract
Leptin, one of the main hormones controlling energy homeostasis, has been associated with different cancer types. In oral cancer, its effect is not well understood. We investigated, through in vitro and in vivo assays, whether leptin can affect the neoplastic behavior of oral squamous cell carcinoma. Expression of genes possibly linked to the leptin pathway was assessed in leptin-treated oral squamous cell carcinoma cells and also in tissue samples of oral squamous cell carcinoma and oral mucosa, including leptin, leptin receptor, hypoxia-inducible factor 1-alpha, E-cadherin, matrix metalloproteinase-2, matrix metalloproteinase-9, Col1A1, Ki67, and mir-210. Leptin treatment favored higher rates of cell proliferation and migration, and reduced apoptosis. Accordingly, leptin-treated oral squamous cell carcinoma cells show decreased messenger RNA caspase-3 expression, and increased levels of E-cadherin, Col1A1, matrix metalloproteinase-2, matrix metalloproteinase-9, and mir-210. Leptin treatment favored higher rates of cell proliferation and migration, and reduced apoptosis. Accordingly, leptin-treated oral squamous cell carcinoma cells show decreased messenger RNA caspase-3 expression, and increased levels of E-cadherin, Col1A1, matrix metalloproteinase-2, matrix metalloproteinase-9, and mir-210. In tissue samples, hypoxia-inducible factor 1-alpha messenger RNA and protein expression of leptin and leptin receptor were high in oral squamous cell carcinoma cases. Serum leptin levels were increased in first clinical stages of the disease. In animal model, oral squamous cell carcinoma–induced mice show higher leptin receptor expression, and serum leptin level was increased in dysplasia group. Our findings suggest that leptin seems to exert an effect on oral squamous cell carcinoma cells behavior and also on molecular markers related to cell proliferation, migration, and tumor angiogenesis.

Keywords
Oral squamous cell carcinoma, leptin, cell proliferation, migration, apoptosis, E-cadherin, Col1A1, matrix metalloproteinase

Date received: 15 September 2016; accepted: 24 December 2016

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**Introduction**

Leptin (Lep) is a hormone controlling feeding behavior and energy homeostasis. Besides, it is a pleiotropic cytokine involved in a great variety of physiological and pathological processes in several organs. Functional activities of Lep are mediated through the transmembrane leptin receptor (LepR) by the activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways. Lep has been pointed as a pro-angiogenic factor, and hypoxia is a key signal for induction of angiogenesis. Under these conditions, hypoxia-inducible factor 1-alpha (HIF-1α) expression can be associated with increased Lep expression and also higher mir-210 levels. Indeed, leptin levels are increased under hypoxia in trophoblasts and breast cancer through HIF-1α. However, data on the interrelationship of Lep, HIF-1α, mir-210, and angiogenesis in oral squamous cell carcinoma (OSCC) are scarce and inconclusive.

Lep, due to its mitogenic, antiapoptotic, pro-inflammatory, and angiogenic properties, can favor development and progression of different cancer types. However, there is no consensus as to the effect of Lep on carcinogenesis pathways. Human cancer cells exhibit differential responses to the treatment with leptin, depending upon the organ of derivation. Lep can promote proliferative effect in some cancer cell types, as in esophageal, breast, and prostate cancers. However, treatment with different concentrations of leptin was not found to interfere significantly in prostate cancer cell proliferation.

In OSCC, it is not well understood whether Lep plays a relevant role in disease pathogenesis. Studies have focused on the investigation of leptin plasma levels and gene polymorphisms of Lep or its receptor, LepR, associating the findings with development and progression risk of OSCC. In vitro hypoxia condition, by adding 100 µM Cobalt(II) chloride (CoCl2), to verify whether this condition interferes in cell OSCC proliferation under leptin treatment. Hypoxia condition was not relevant in cell proliferation (Supplementary Figure 1(b)). So, hypoxia mimicking was not considered in vitro phenotype assays.

**Material and methods**

**Cell culture**

Two human OSCC cell lines, SCC-9 and SCC-4 (CRL-1629 and CRL1624, American Type Culture Collection (ATCC) cell bank, USA), were used in this study. HaCaT cell line of human immortalized keratinocytes (CCL-185, ATCC cell bank) was the comparison group. Cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 or DMEM medium (Gibco, USA) and supplemented with 10% fetal bovine serum and 0.4 µg/mL hydrocortisone (Gibco, South America), according to ATCC protocol. All experiments were performed in triplicate and ran independently at least three times.

**Cell proliferation assay**

A density of 2 × 105 OSCC cells was plated on a dish and incubated at 37°C, 5% CO2 for approximately 24h to establish adherent monolayers. OSCC cells were treated with 100 ng/mL human recombinant leptin (Invitrogen, USA), for 72 h. Previously, we performed a dose–response curve to establish leptin concentration and time for treatment (Supplementary Figure 1(a)). Trypan-blue exclusion test determined cell proliferation. In addition, we mimicked in vitro hypoxia condition, by adding 100 µM Cobalt(II) chloride (CoCl2), to verify whether this condition interferes in cell OSCC proliferation under leptin treatment. Hypoxia condition was not relevant in cell proliferation (Supplementary Figure 1(b)). So, hypoxia mimicking was not considered in vitro phenotype assays.

**Migration assay**

Cell migration was assayed by wound healing method, as previously described. Briefly, at the full confluence, the cells were scraped away horizontally using a 200 µL tip. Culture medium was then replaced by serum-free medium, adding 100 ng/mL leptin.

In order to measure the wound covered area by migrating cells, images of the wounded cell monolayers were taken using an Olympus IX81 Inverted Microscope (Olympus, Center Valley, PA, USA) coupled with camera SC30 (Olympus) at 0 and 72 h after wounding.

**Invasion assay**

A two-level 24-well cell invasion assay chamber containing precoated membrane with Matrigel (BD Biosciences, USA) was used for these experiments. Then, in the top chamber, 2 × 105 OSCC cells were plated onto each well and treated with 100 ng/mL leptin for 72 h. In lower compartment, 10% fetal bovine serum was added as a chemoattractant factor for cells. The membrane containing invading cells was fixed using formaldehyde and stained with Giemsa. Non-invading cells on the upper side of the membrane were removed with a cotton swab. Images of cell invasion were captured by microscope FSX100 (Olympus) and counted using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA; http://imagej.nih.gov/ij/).

**Cell death/viability assay**

A volume of 25 µL of cell suspension (2.0 × 106 cells/mL) was incubated with 1.0 µL of a solution containing 5 mg/mL of acridine orange (AO, Sigma) and ethidium bromide stock (EB, Sigma). Each sample was mixed just before
microscopic analysis and quantification. Samples were evaluated immediately. A volume of 10µL of cell suspension was placed onto a microscopic slide and covered with a glass coverslip. The slide was observed under a fluorescence microscope FSX100 (Olympus). Intense EB staining (Ex360-370, Em420-460, filter DM400) indicates cell death, while intense AO (Ex460-495, Em510-550, filter DM505) indicates live cells. In addition to staining, nuclear morphology was also discernible.

**Assay in animal model**

In order to better understand the relationship between Lep and its receptor in OSCC, we induced stages of oral carcinogenesis progression, in mice Swiss males, using 4-nitroquinolinone-1-oxide (4NQO; Sigma-Aldrich, USA). This methodology was adopted from Czerninski et al. In each stage, as hyperplasia, dysplasia, and neoplasia, we quantified messenger RNA (mRNA) LepR expression in cryopreserved tissues of animals, and also serum leptin levels. Immunohistochemical expression of Lep and LepR was also investigated. All animal studies were carried out according to the National Council for the Control of Animal Experimentation (CONCEA, Brazil), and the study was approved by Ethics Committee for Experimentation and Animal Welfare of State University of Montes Claros, Brazil (CEEBEA; Protocol No. 087). Further details on in vivo experiments are displayed in Supplementary material.

**Patients and tissue samples**

Twenty-six fresh human OSCC specimens and clinical data were obtained from Bank of Human Biological Materials of North of Minas Gerais (Institutional Biobank-State University of Montes Claros, Brazil/National Commission of Ethics in Research, Registration: B-013). Diagnoses were confirmed by histopathological analysis based on the World Health Organization (WHO). Twenty-five fragments of oral mucosa (OM) without clinical evidence of inflammation or epithelial changes were collected during third molar extractions, and it was used as a control group. Clinical data are shown in Supplementary Table 2. Ethical approval for this study was obtained from the relevant Institutional Review Board (Protocol No. CAAE: 35440514.2.0000.5146), and signed informed consent form was obtained from all patients.

**Immunohistochemical staining**

Immunohistochemistry was performed as described before with necessary adaptations. Briefly, 3-µm serial sections were deparaffinized with xylene and rehydrated with alcohol solutions. Antigen retrieval was carried out in a Pascal pot at 121°C for 10 min on Trilogy (Cell Marque Corporation, Sigma-Aldrich). Endogenous peroxidase activity was blocked with two baths of 0.3% hydrogen peroxide for 15 min. The following primary rabbit polyclonal antibodies were used: anti-ob (clone A-20, sc-842; Santa Cruz Biotechnology, USA), obR (clone H-300, sc-8325; Santa Cruz Biotechnology), and CD31 (clone ab-28364; Abcam, USA). All primary antibodies were incubated for 18 h at 4°C. The primary antibodies were detected using the LSAB kit (LSAB-Kit Plus Peroxidase; Dako, USA). Signals were developed with 3,3′-diaminobenzidine tetrahydrochloride hydrate for 3 min and counterstained with Mayer’s hematoxylin for 50 s. For staining quantification, 10 fields of tumor parenchyma and stroma were photographed at 400× magnification in a microscope FSX100 (Olympus). Cell counting was then performed by the ImageJ software (http://rsweb.nih.gov/ij/). Positive and negative cells in the 10 fields were summed, and the proportions of positive cells in neoplastic parenchyma were used to quantify the staining. Breast cancer samples were used as positive controls.

Microvascular density (MVD) was assessed using CD31 labeling, following the method described previously. Images of the selected fields were then captured at 400× magnification, using the same microscope. Vascular structures were counted if they had a brownish-marked lumen and were clearly separated from other adjacent vascular structures.

**Serum leptin dosage**

Serum leptin level was measured in humans by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Leptin Human ELISA-LDN®, Nordhorn, Germany). The dosage in humans was aimed to compare serum leptin levels in 11 OSCC patients and 13 disease-free patients. Additional patient data involved in this analysis are shown in Supplementary Table 3. All patients had body mass index within normal values.

**Analyses of gene expression**

To verify the effect of leptin treatment on gene expression in OSCC cells, quantitative real-time PCR (qRT-PCR) was carried out to analyze the mRNA expression levels of Lep, LepR, HIF-1α, E-cadherin, matrix metalloproteinase-2 (MMP2), MMP9, CollA1, caspase-3, Ki67, and mir-210. All these genes are related to the leptin pathway and/or angiogenesis. Changes in the expression of such genes were also analyzed in tissue samples of OSCC and OM.

Total RNA was isolated with TRIzol reagent according to the manufacturer’s protocol (Invitrogen). Reverse transcription was conducted with 1.5µg of total RNA, using Reverse Transcription Kit (Invitrogen, Life Technology, USA). RT-synthesized complementary DNA (cDNA) was amplified using primers as specified in Supplementary Table 1. qRT-PCR was performed using SYBER Green following the manufacturer’s instructions on a StepOne system (Applied Biosystems, USA). Mir-210 expression was analyzed using Taqman™ MicroRNA Assay (000512;
Tumor Biology

The comparative Ct method was used for analyses.29

Statistical analyses

The analyses were carried out using SPSS 17.0 software and GraphPad Prism software (Version 5.0, GraphPad Software Inc., USA). Shapiro–Wilk test was performed to evaluate data distribution. Statistical tests were selected according to the sample characteristics and distribution of variables; probability values <0.05 were considered to be statistically significant.

Results

Leptin increases cell migration and proliferation of OSCC cell lines but not cell invasion

Cell proliferation of SCC-9 and SCC-4 was significantly increased following the treatment with 100 ng/mL leptin, for 72h compared with untreated control (Figure 1(a)). HaCaT cells show similar results. Supporting the proliferation data, we identified a lower number of dead cells in the leptin-treated group (Figure 1(b)). Accordingly, mRNA caspase-3 was significantly decreased in the leptin-treated group (Figure 1(c)).

Cell migration was significantly relevant in leptin-treated OSCC cells for 72h, in wound healing assay (Figure 1(d)). These results were similar for 96h of treatment, but no difference was found between groups during 48h of treatment (Supplementary Figure 1(c)). Interestingly, OSCC cells treated with leptin were not more invasive than the control group (Figure 1(e)).

Leptin-treated OSCC cells show high mRNA expression of genes related to the proliferation, migration, and angiogenesis

We assessed changes in the expression of several genes to understand the underlying mechanisms responsible for
growth and migration of OSCC cells. Figure 2 shows expression levels of genes involved in different processes, such as cell proliferation, angiogenesis, cell invasion, and metastasis. In both SCC-4 and SCC-9, increased mRNA levels of mir-210, E-cadherin, Col1A1, MMP2, and MMP9 were observed in the leptin-treated group. LepR expression was similar between the groups. Interestingly, no difference regarding Ki67 expression was found between the treated and non-treated groups.

Supplementary Figure 1(d) and (e) shows increased expression of HIF-1α and LepR in leptin-treated cells, under hypoxia conditions.
Serum leptin levels are higher in early events of oral carcinogenesis and LepR in the late stages in an animal model

The 4NQO-induced oral carcinogenesis resulted in the progressive appearance of tumoral lesions in the tongue and OM, which was preceded by dysplastic lesions. Histological analysis revealed epithelial dysplasia and OSCC (Figure 3(a) and Supplementary Figure 2).

We then investigated serum leptin levels and tissue LepR mRNA expression in the dysplastic and neoplastic lesions to verify whether these genes were differentially
expressed in carcinogenesis. OSCC-induced mice show higher mRNA LepR expression than dysplasia and normal OM groups. Serum leptin levels in epithelial dysplasia were also greater than normal OM. The body weight was reduced in oral carcinogenesis model (Figure 3(b)). Immunohistochemical analysis revealed expression of Lep and LepR proteins in healthy tissues, dysplastic, and neoplastic samples. Figure 3(c) illustrates the representative immunohistochemical images of distinct expression profiles of Lep and LepR in different stages of 4NQO-induced oral carcinogenesis.

**Immunohistochemical expression of Lep and LepR is higher in OSCC than in OM**

Lep and LepR proteins were evident in OSCC and OM, being both proteins highly expressed in OSCC parenchyma (Figure 4(a)). Conversely, in the stroma, Lep and LepR immunoreactivity was not different between OSCC and OM (Supplementary Figure 3(a)).

**Expression of mRNA LepR in OSCC and OM is related to the angiogenesis markers**

Interestingly, mRNA expression of LepR and HIF-1α was significantly increased in OSCC compared to the OM. There was no difference in the mir-210 expression between OSCC and OM (Figure 4(b)). Low or no mRNA Lep expression was observed in OM and OSCC (data not shown).

A scatter dot graphic fitted to OSCC and OM was generated to show correlation analysis between the studied genes. Results showed a statistically significant positive correlation between LepR and angiogenic mir-210, and

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**Figure 4.** Lep and LepR expression in biological samples of oral squamous cell carcinoma (OSCC) and oral mucosa. (a) Immunohistochemical expression of Lep and LepR. (b) mRNA expression of LepR, HIF-1α, and mir-210. (c) Expression of Lep and LepR proteins in endothelial cells. (d) mRNA expression of LepR, HIF-1α, and mir-210 in different TNM stages. (e) Serum leptin dosage in OSCC patients and disease-free patients. (f) Serum leptin dosage in different TNM stages. Kruskal–Wallis or Mann–Whitney test (symbol — p < 0.05). Immunohistochemistry expression of Lep and LepR was higher in OSCC than in oral mucosa. mRNA expression of LepR and HIF-1α was significantly increased in OSCC compared to the OM. Serum leptin level was not statistically different in all OSCC patients and control patients, but it was importantly increased in the early stages of cancer.
also HIF-1α (p<0.05), as well as between HIF-1α and mir-210 (p<0.05; Supplementary Figure 3(b)).

Specimens of OM and OSCC exhibited positive labeling for CD31; no statistical difference was identified between groups. Likewise, microvasculature showed positive staining for Lep and LepR proteins in healthy and neoplastic tissues (Figure 4(c)).

**OSCC is associated with serum leptin and tissue LepR mRNA**

Analysis regarding tumor–node–metastasis (TNM) stages and LepR mRNA revealed higher expression of this transcript in stage III/IV. A similar result was found in HIF-1α expression. No difference regarding mir-210 expression was observed between the different TNM stage groups (Figure 4(d)).

Serum leptin level was not statistically different in all OSCC patients and control patients (Figure 4(e)), but it was significantly increased in the early stages of cancer (Figure 4(f)). In plasma analysis of Lep, all patients had normal body mass index.

**Discussion**

Various studies have suggested that leptin can be involved in the development of several human cancer types. In vitro assays performed in this study revealed that leptin-treated OSCC cells showed increased proliferation and migration, but it did not affect Ki67 expression in these cells. Some previous studies reported leptin-induced proliferation of various other cells, such as skin keratinocytes, lung epithelial cells, hemopoietic cells, pancreatic beta cells, monocytes, and endothelial cells. Our study also revealed that leptin reduced OSCC cell death, decreased caspase-3 mRNA expression, and EB/OA staining. This phenotypic effect was similar to what was previously shown. So, we suggested that increased proliferation of leptin-treated OSCC cells seemed to occur due to the downregulation of events in cell cycle arrest or apoptosis rate and not necessarily due to the increase in Ki67-associated proliferation.

Hypoxia in microtumoral ambient plays a major role in cell survival and angiogenesis, being HIF-1α a signaling molecule involved in the initiation of angiogenesis. High HIF-1α expression in OSCC can be regarded as an independent prognostic biomarker. In this study, HIF-1α mRNA expression was higher in OSCC than in OM.

A previous study emphasized that leptin is a major regulator of the tumor angiogenesis in breast cancer cells. Under hypoxic conditions, we identified higher HIF-1α expression in the leptin-treated group than control group. It was shown that leptin signaling activates HIF-1α, increasing its expression. Furthermore, LepR expression was increased in conditions of normoxia and hypoxia, being activated directly by HIF-1α. We identified that there is a significant positive correlation between HIF-1α and LepR. Although this relationship does not necessarily suggest a causal or functional relationship, it may point to a possible action of leptin in OSCC angiogenesis through HIF-1α. In pancreatic cancer, LepR levels were significantly correlated with HIF-1α levels, further supporting the HIF-1α role in the regulation of LepR. In our study, we observed that LepR and HIF-1α were more expressed in the advanced stages of tumor development. These results corroborate the literature data for other cancer types, which shows that LepR overexpression was significantly correlated with the higher tumor sizes and late TNM stages.

Although hypoxia induces tumor angiogenesis, we did not identify differences in CD31 staining between OM and OSCC. CD31 is a characteristic marker of all endothelial cells instead of only neovessels. However, our findings revealed endothelial cells expressing Lep and LepR proteins. It can be indicative of the involvement of these proteins in the angiogenic phenomenon. Also, our results suggest that leptin in OSCC cells was associated with high hypoxamir-210 expression. Previous results showed that HL-1 cells transduced with miR-210 could release several angiogenic factors compared to control cells, including leptin. The positive correlation between LepR and hypoxamir-210, and also HIF-1α, as well as between HIF-1α and mir-210, leads us to hypothesize that leptin pathway acts in the angiogenic process via HIF-1α. Mir-210 is regulated by HIF-1α and could thus be regarded as a marker for the activation of HIF-1α, and potentially tissue hypoxia.

We found increased expression of LepR mRNA in OSCC compared to the OM, which may suggest that leptin plays an important role as a local growth factor in neoplastic tissues. However, low or no Lep mRNA expression was identified in both OSCC and OM. Interestingly, increased immunohistochemical expression of Lep and LepR was observed in OSCC compared to OM. Despite low levels of Lep transcript, its protein product was significantly expressed in the neoplastic lesions. These findings lead us to think about the existence of post-translational modifications associated with leptin expression. Post-translational phenomena involving leptin is not clarified in the literature. However, a study suggested that leptin is subjected to suffer conformational changes by phosphorylation followed by the increased ubiquitination; however, such molecular change needs to be better understood.

Serum leptin can exercise its biological effects on responsive cells of a classical endocrine mode. In this study, leptin contributed more efficiently to the initiation of oral cancer and first events of disease (stages I/II). In our animal study of oral-induced carcinogenesis, serum leptin levels and tissue protein expression were increased in epithelial dysplasia. LepR mRNA expression was gradually increased from hyperplasia to epithelial dysplasia and OSCC lesions in Swiss mice. Increased plasma levels...
of leptin were identified in patients with oral premalignant lesions but not in head and neck squamous cell carcinoma (HNSCC) patients. Significant reduction in leptin levels in OSCC patients was previously observed, and it was correlated with weight loss and cachexia status, but there are controversies about this fact in the literature.21

Our results suggested a possible involvement of serum leptin as well tissue protein expression and LepR mRNA in OSCC. We believe that leptin can act not only by endocrine actions but also by an autocrine pathway. Besides serum leptin, we identified a significant protein expression of Lep and LepR in OSCC samples.

The literature has addressed that leptin is capable of stimulating cell migration of human oral mucosal epithelial cells. Our molecular analysis was performed in an attempt to explain the migration process triggered by leptin in OSCC. Interestingly, under leptin treatment, a significant increase in E-cadherin expression was observed in the migrating OSCC cells. E-cadherin is an intercellular adhesion molecule frequently lost in human epithelial cancers. However, it has well been shown in epithelial ovarian tumors that E-cadherin expression is much more elevated than normal ovaries.56 Therefore, it is suggested that E-cadherin may also trigger intracellular activation of proliferation and survival signals.57 Leptin increases E-cadherin expression in breast cancer through MAPK activation, cyclic adenosine monophosphate–response element binding protein (CREB), and Sp1 transcriptional factors. In our study, increased E-cadherin expression was associated with increased cell proliferative and migratory phenotypes.

MMPs are critical enzymes involved in tumor invasion, and studies have shown that leptin may enhance tumor invasion by upregulating MMP-2. In this study, leptin promoted a significant increase in the expression of MMPs. The effect of leptin in the expression of MMP2, MMP9, and Coll1A1 suggests that Lep not only can favor the formation of a matrix to serve as a base for cell growth, but also can stimulate matrix degradation pathway through MMPs, allowing cell migration.

Taken together, our findings point Lep/LepR pathway as an important target involved in neoplastic behavior and progression of OSCC. Leptin was identified to be probably associated with the initial stages of OSCC. We highlight the need for more studies targeting leptin signaling to better understand its real importance in OSCC pathogenesis. Besides, genes involved in this molecular signaling can be suggested as interesting molecular markers for future investigations of new therapeutic targets in OSCC.

Acknowledgements
A.L.S.G. and L.C.F. have contributed equally to this work

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval
All applicable international, national, and institutional guidelines for the care and use of animals were followed. The animal study was approved by Ethics Committee for Animal Experimentation of Universidade Estadual de Montes Claros, Brazil (Protocol No. 087). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Furthermore, this study was approved by the local ethics committee of Universidade Estadual de Montes Claros, Brazil (Protocol No. CAAE: 35440514.2.0000.5146).

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. A.L.S.G, R.S.G., and A.M.B.D.P are research fellows of the CNPq.

Informed consent
Informed consent was obtained from all individual participants included in the study.

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