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Research Article

Leishmania amazonensis infection impairs VLA-4 clustering and adhesion complex assembly at the adhesion site of J774 cells

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Abstract

Cutaneous leishmaniasis is an infectious disease that may lead to a single or multiple disseminated cutaneous lesions. The mechanisms involved in *Leishmania* dissemination to different areas of the skin and the internal organs remain poorly understood. Evidence shows that Very Late Antigen-4 (VLA-4)-dependent phagocyte adhesion is impaired by *Leishmania* infection, which may be related to the mechanisms of parasite dissemination. We investigated factors potentially associated with decreased VLA-4-mediated adhesion in *Leishmania*-infected macrophages, including lipid raft-mediated VLA-4 mobilization along the cellular membrane, integrin cluster formation at the cell base (adhesion site), and focal adhesion complex assembly. Phagocytes treated with Methyl-β-Cyclodextrin (MβCD) demonstrated reduced adhesion, similarly to *Leishmania amazonensis*-infected J774 cells. Infected and MβCD-treated macrophages presented decreased VLA-4 mobilization to the adhesion plane, as well as reduced integrin clustering. *Leishmania amazonensis*-infected cells exhibited talin depletion, as well as a decreased mobilization of adhesion complex proteins, such as talin and viculin, which were associated with lower VLA-4 concentrations at the adhesion site and limited cell-spreading. Our results suggest that *Leishmania* infection may modulate the firm adhesion phase of the cell-spreading process, which could contribute to the bloodstream dissemination of infected cells.

Keywords: leishmaniasis, integrin, leukocyte adhesion

Introduction

Once *Leishmania* is inoculated into the skin by sandflies, neutrophils, monocytes, and macrophages migrate to the site of infection where they phagocytose the parasite. Infected cells may remain at the inoculation site or disseminate through the body, causing lesions in the dermis, mucosae, or internal organs (Scott and Novais 2016, Scorza et al 2017, Burza et al 2018). The capability of infected phagocytes to remain at the infection site or to migrate to other regions of the body is relevant to the clinical presentation of leishmaniasis. For phagocytes to migrate from the infection site, adhesion occurs, followed by detachment from cells, as well as from extracellular components present in inflammatory infiltrate. This sequence of events results in a directional movement of the phagocyte towards lymphatic endothelial cell junctions, transmigration to the lymphatic vessel lumen and transport to draining lymph nodes (Janatpour et al. 2001, Palframan et al. 2001, Randolph et al 2005). In the lymph node, using similar adhesion and detachment mechanisms, infected phagocytes exit the lymph nodes, eventually reaching the blood stream and other organs (Hunter et al 2016). Accordingly, changes in the ability of infected phagocytes to adhere to connective matrix components may play an important role in the clinical presentation of leishmaniasis.

It has been shown that *Leishmania* infection reduces phagocyte adhesion to inflamed connective tissue by modulating Very Late Antigen-4 (VLA-4 [$\alpha_4\beta_1$]) integrin activation (Carvalhal et al. 2004, Pinheiro et al. 2006). VLA-4 belongs to the β1 integrin family, and is highly expressed in leukocytes. This molecule mediates the firm adhesion of leukocytes to other cells, as well as to connective matrix components (Sasaki et al. 2009, Chigaev and Sklar 2012). Similarly to other integrins, VLA-4 is a transmembrane receptor that intermediates interactions between extracellular structures and the leucocyte cytoskeleton. VLA-4 may be present in an inactive state with low affinity to its ligands, or can undergo conformational changes upon activation, thereby acquiring high affinity for its ligands (Plow et al. 2000, Hynes 2002, Humphries et al 2006). *Leishmania* infection decreases the expression of high affinity VLA-4 epitopes on the surfaces of infected monocytes, even upon integrin activation through stimulation (Figueira et al. 2015). Consequently, firm adhesion and cytoplasm spreading over fibronectin-coated surfaces are both inhibited in *Leishmania*-infected phagocytes (Figueira et al. 2015). This may also be responsible for the

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impairment of the directional migration process seen in infected cells.

During the process of integrin-mediated adhesion, structural proteins, such as talin and vinculin, are recruited to the adhesion site and form focal adhesion-complexes. In these complexes, proteins recruit additional components, such as the adaptor proteins paxillin, as well as signaling molecules, including focal adhesion kinase (FAK) and the Scr family of kinases, allowing complex stabilization and connection to the cytoskeleton (Karimi et al. 2018). Besides adhesion complex assembly, integrin clustering is required to enhance cell adhesion (Roca-Cusachs et al. 2009). Vinculin, which is responsible for the functional linkage of Focal Adhesions (FA) to the actin cytoskeleton, regulates integrin dynamics, clustering, and the link to the mechanotransduction machinery (Humphries et al. 2007). Talin is a key molecule in regulating the affinity of the integrin family for its ligand and in the coupling of the integrin with the cytoskeleton. Talin depletion causes defects in integrin activation, maintenance of cell spreading and the assembly of FA in cultured cells (Goult et al. 2009, Lu et al. 2022).

In their activated state, integrins are found in membrane microdomains, also known as detergent-resistant membranes (DRMs), constituted by sphingolipid and cholesterol, which are also referred to as lipid rafts. These microdomains act as platforms for membrane molecule trafficking, FA formation and rearrangement of the actin cytoskeleton through β1 integrin clustering (Leitinger and Hogg 2002, Wang et al. 2013, Sezgin et al. 2017, Santos and Preta 2018). A common approach to investigate lipid raft-dependent mechanisms is to treat the cells with Methyl-β-Cyclodextrin (MβCD), a molecule that causes membrane cholesterol depletion and lipid raft disruption (Zidovetzki and Levitan 2007, Mahammad and Parmryd 2008). One of the strategies used by *Leishmania* to evade the host immune response during its intracellular life cycle is to increase membrane fluidity and disrupt membrane rafts. These changes in the phagocyte membrane impair the cell's capability of antigen presentation (Chakraborty et al. 2005).

The present study aimed to examine the effects of *Leishmania* infection on the phagocyte adhesion complex assembly, integrin clustering, and the role played by lipid rafts in these processes.

Materials and methods

Antibodies

Vybrant™ Alexa Fluor™ 594 Lipid Raft Labeling Kit (Cholera toxin subunit B CT-B Kit, V34404) (Molecular Probes, Invitrogen—USA); Anti-ITGA4 (SAB2900216) (Sigma-Aldrich, USA); Goat Anti-Rabbit IgG Secondary Antibody Alexa Fluor 488 (A11008) (Invitrogen); Goat Anti-Mouse IgG Secondary Antibody Alexa Fluor 594 (A11020) (Invitrogen); Monoclonal Anti-Vinculin produced in mouse (V4505) (Sigma-Aldrich); Monoclonal Anti-Talin produced in mouse (T3287) (Sigma-Aldrich); and Alexa Fluor 488 phalloidin (A12379) (Invitrogen).

J774 cells and Leishmania cultures

Macrophages (lineage J774) were maintained in complete RPMI medium (Gibco, USA)—RPMI containing 10% fetal bovine serum (Gibco), 50 mg/ml gentamicin (Gibco), and 2 mM L-glutamine (Sigma-Aldrich) at 37°C under 5% CO_2. Stationary-phase *Leishmania amazonensis* (MHOM/BR88/BA-125) parasites were expanded in Schneider's insect medium (Sigma-Aldrich) containing 10% fetal bovine serum and 50 mg/ml gentamicin at 24°C.

Cell infection

J774 cells were cultivated in flasks and released when confluence was achieved by washing with cold Ca^{2+}/Mg^{2+}-free HBSS (Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+}; Sigma-Aldrich) for 20 min. J774 cells and *Leishmania* promastigotes were washed three times with HBSS (Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+}; Sigma-Aldrich). J774 cells were then resuspended at 2×10^6/ml in 2 ml of RPMI alone, or medium containing 10 parasites of *L. amazonensis* per cell (2×10^7/ml). The cell suspensions were cultivated for 18–24 h at 35–37°C under 5% CO_2 in nonadherent polypropylene tubes. Cells were then washed with HBSS and used in cholesterol depletion, adhesion, and immunofluorescence assays. Infection rates were estimated morphologically by cytospin (Shandon Cytospin 4; Thermo Scientific—USA) preparations stained with DAPI (Vector Laboratories—USA) under fluorescence microscopy (BX51, Olympus—Japan) using a 100x objective. Acceptable rates of infection and cell viability (trypan blue) were above 50% and 90%, respectively.

Cholesterol depletion, lipid raft fluorescence staining, and cell plating

Uninfected cells were treated with MβCD (Sigma-Aldrich) (10 mM) using 1×10^6 cells in 2 ml of complete RPMI for 30–40 min at 37°C under 5% CO_2. After incubation, cells were washed three times with PBS 1x (Phosphate Buffered Saline, Sigma-Aldrich). Uninfected cells treated or not with MβCD, and *Leishmania*-infected cell lipid rafts were labeled using a CT-B 594 kit, following the manufacturer's protocol. After labeling, a suspension of 1.5×10^5 cells in 500 μl of medium was distributed over 12-mm diameter glass coverslips previously coated with 20 μg/ml of fibronectin (Sigma-Aldrich) placed at the bottom of wells on a 24-well plate. Cells were incubated for 1 h at 37°C under 5% CO_2. Next, the supernatant was collected and adhered cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature (RT). Fixed macrophages were submitted to immunostaining reactions to label VLA-4 and complex adhesion proteins.

Immunofluorescence assay

Adhered cells were washed three times with PBS 1x and incubated with detergent solution [PBS 1x + 5% BSA (Sigma-Aldrich) + 0.1% Trinton-x-100 (Sigma-Aldrich)] for 5 min. The cells were then incubated with an ammonium chloride solution (50 mM) for 30 min at RT and blocked with BSA (PBS 1x + 5% BSA) for 30 min at RT. To label VLA-4 integrin, antibody α-ITGA4 (1:200) was applied for a 2 h incubation period at 37°C under 5% CO_2, followed by the addition of secondary antibody Alexa Fluor 488 (1:200) for 1 h at RT. To label intracellular adhesion complex proteins, ammonium chloride was used followed by blocking and permeabilization with a detergent solution for 30 min. In addition, the antibodies α-talin (1:50), α-vinculin (1:50) and, for actin labeling, phalloidin-488 (1:200), were applied for 2 h at 37°C under 5% CO2. To label both α-talin and α-vinculin, Alexa Fluor 594 (1:200) was applied for 1 h at RT as a secondary antibody.

Cell adhesion assay

The adhesion assay was performed on 96-well plates coated with fibronectin and collagen as previously described (Pinheiro et al. 2006). Then to placing 150 μl of complete RPMI containing 3×10^4 phagocytes (previously cultured for 18–24 h) in each well, one of the following was added: (i) medium containing phagocytes alone (Control); (ii) *L. amazonensis*-infected phagocytes (promastigotes per cell–10:1) (Infected); (iii) 10 mM MβCD-treated phagocytes

(MβCD); (iv) 5 μm latex beads-incubated phagocytes (beads per cell–10:1) (Latex); and (v) 10 mM MβCD-treated and 5 μm latex beads-incubated phagocytes (Latex MβCD). The cells were left to adhere for 1 h at 37°C under 5% CO_2. Next, cells were washed four times with HBSS, fixed in 1% glutaraldehyde (Sigma-Aldrich) diluted in warm HBSS for 1 h at RT, and finally stained with hematoxylin. Snapshots of three fields, representing three areas: right side, middle, and left side (excluding edges), were taken for each well using an inverted microscope (DMi8, Leica—Germany) with a 40x objective. Cells were manually counted using ImageJ software (v1.53c, NIH). Results were expressed in terms of absolute cell density [count/image size × 1 × 10^6 (mm^2)]. The experiments were performed three times with three or five replicates per group.

Fluorescence image capture, processing, and analysis

Cells were visualized via a laser confocal scanning microscope (LCSM—TCS SP8 Leica) using a 63x objective. Images were acquired in z-stack mode to capture the entire cell, from top to bottom. Cell fluorescence was studied as total cell fluorescence (TCF) and base cell fluorescence (BCF). Both was calculated using the following equation [corrected total cell fluorescence (CTCF)]: CTCF = *IDF*—(Acell x Xbf); where *IDF* = the integrated density of fluorescence emitted from cell planes, Acell = cell area and Xbf = mean background reading fluorescence (Measuring cell fluorescence using ImageJ; The Open Lab Book 2014, McCloy et al. 2014). BCF measurements were obtained using the largest cell-spreading plane as a reference, including the range from two planes above to two planes below the spreading plane. Using ImageJ software, fluorescence measurements were obtained for individual cells, considering an average of 40 cells/well, by cell delimitation using the selection tool. In the infected group, only cells in which the presence of *Leishmania* was confirmed by DAPI labeling were measured. VLA-4 mobilization was calculated as the TCF/BCF ratio, while VLA-4 cluster formation was analyzed using the 'find maxima' tool provided by ImageJ.

Statistical analysis

Numeric data were represented graphically using absolute numbers, means with standard deviation (SD) or medians with first and third quartiles. Percentage differences (experimental group means—control means/control means) were used to represent differences between experimental groups and controls. For two groups with non-normal distribution, comparisons were performed using the Mann–Whitney test, while for more than two groups, the Kruskal–Wallis test was applied, followed by Dunn's post-test. Likewise, for more than two groups with normal Gaussian distribution, comparisons were performed using one-way ANOVA testing, followed by Dunnett's test. For correlation analysis, Spearman's coefficient was used. The level of significance was established at $P < .05$.

Results

Cholesterol depletion or *Leishmania* infection similarly decrease cell adhesion to fibronectin and collagen

To test whether the depletion of cholesterol-enriched domains of the cell membrane would affect phagocyte adhesion to connective matrix components to the same extent as inhibited by *Leishmania* infection, we treated macrophages with MβCD, a cholesterol depleting agent, or infected leukocytes with *Leishmania* to perform adhesion assays. The level of cell adhesion in the control group was determined at 827.9 ± 472.7 cells (Fig. 1A and F). Both *Leishmania*-infected and MβCD-treated macrophages presented similar decreases (46.5% and 47.5%, respectively) in adhesion to fibronectin and collagen, compared to uninfected control macrophages (Fig. 1B, C, and F). To confirm that the adhesion-impairing effect was specifically caused by *Leishmania* and not exclusively due to phagocytosis, macrophages were also incubated with inert latex particles of similar size to *Leishmania* amastigotes. Our results showed that latex phagocytosis had no effect on the adhesion of untreated macrophages (590.5 ± 240.5 cells) (Fig. 1D and F), whereas the MβCD treatment of latex-incubated cells resulted in a significant decrease (312.2 ± 157.3 cells) (Fig. 1E and F).

Leishmania infection and MβCD treatment impairs VLA-4 mobilization and clustering in the cell adhesion plane

To evaluate whether both *Leishmania* infection and cholesterol depletion would similarly affect VLA-4 recruitment to the cell adhesion plane, VLA-4 was immunolabelled using a fluorescent marker and the distribution of this molecule was analyzed in macrophages infected with *L. amazonensis* or treated with MβCD. VLA-4-associated BCF was lower (28.4%) in infected cells (9.4 ± 4.4 × 10^3 BCF, $P < .05$) and exhibited a decreasing trend in MβCD-treated cells (9.8 ± 4.7 × 10^3 BCF, $P = .0712$) compared to uninfected controls (13.1 ± 8.8 × 10^3 BCF) (data not shown). However, the VLA-4-associated BCF/TCF ratio was lower in infected (17 ± 3.0%) cells, as well as in cells treated with MβCD (20 ± 5.1%) compared to uninfected cells (24 ± 6.2%) (Fig. 2A and B). As expected, no differences in VLA-4-associated TCF were observed between groups (Fig. 2A and C).

The mobilization of integrin to the cell base, as well as molecular clustering and FA complex formation are important steps in the cell adhesion process (Roca-Cusachs et al. 2009). Accordingly, we estimated the number of VLA-4 clusters by counting the points of fluorescence at the adhesion site. A decrease in number of VLA-4 clusters was observed at the base of phagocytes treated with MβCD (16.2 ± 6.8 clusters) and those infected with *Leishmania* (12.6 ± 5.4 clusters), in comparison to uninfected control phagocytes (26.3 ± 14.7 clusters) (Fig. 2A and D).

Does *Leishmania* infection disrupt lipid rafts aggregates at the base of J774 cells?

Since *L. amazonensis*-infection and cholesterol depletion treatment resulted in similar effects on leukocyte adhesion and in cell surface integrin mobilization, we investigated whether lipid raft disruption occurred in *Leishmania*-infected cells using a fluorescence probe to stain GM1, a lipid raft component. We observed a trend towards decreased lipid raft-associated expression as estimated by TCF, or BFC values relative to TFC in cells treated with MβCD or infected with *Leishmania*. However, our results presented inconsistency among experimental replicates. (Fig. 1A–D; Figure S1, Supporting Information).

Leishmania infection reduces talin expression and impairs adhesion complex assembly at the fibronectin contact plane

Leishmania infection reduces adhesion, as well as the cytoplasmic spreading of monocytes. These effects are associated with the inhibition of integrin activation observed in *Leishmania*-infected cells (Carvalhal et al. 2004, Figueira et al. 2015). Here, we

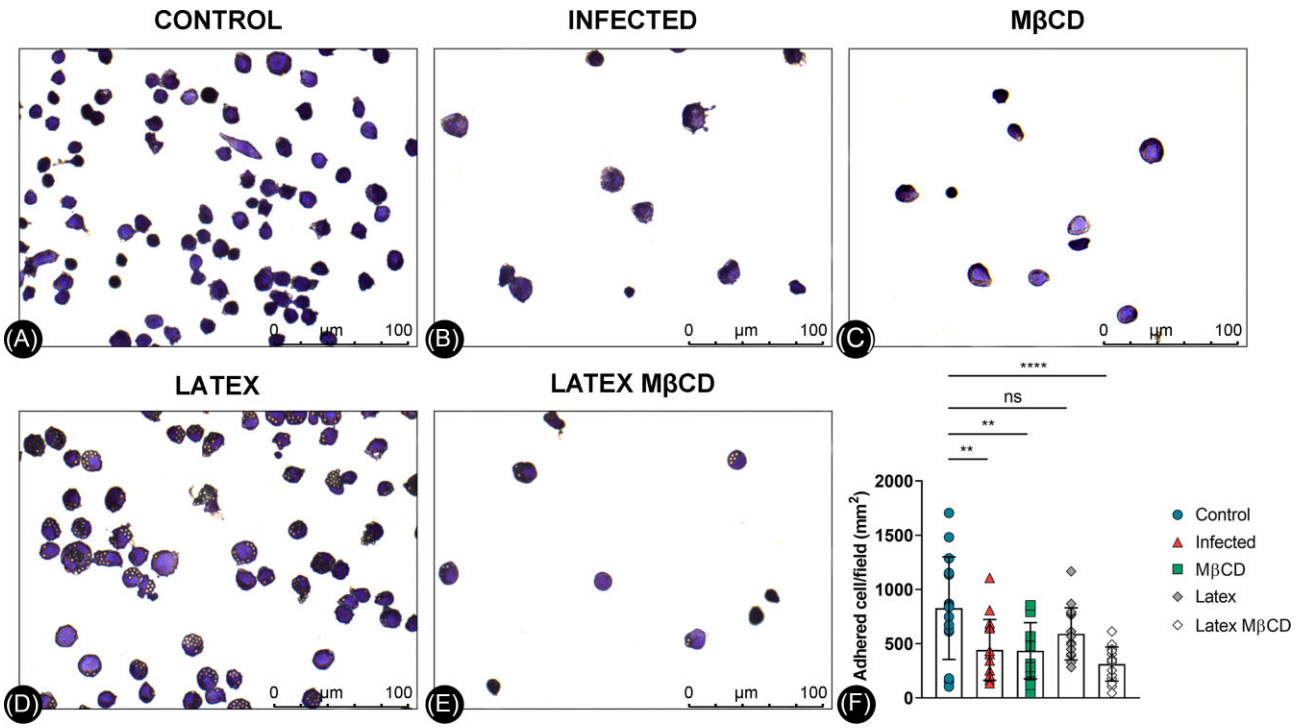

Figure 1. Adhesion assay: J774 cells cultured under different conditions were allowed to adhere to surfaces covered with collagen and fibronectin (Scale bar—100 μm). **(A)** J774 cells incubated with medium alone. **(B)** J774 cells incubated with *L. amazonensis* for 18–24 h. **(C)** J774 cells treated with 10 mM MβCD for 40 min. **(D)** J774 cells incubated with latex beads for 18–24 h. **(E)** J774 cells incubated with latex beads for 18–24 h and treated with 10 mM MβCD for 40 min. **(F)** The bar chart details cell counts in each group (three fields per well in five replicates), with bars representing mean ± SD. Decreased adhesion capacity was observed in *Leishmania*-infected cells ($P = .0030$) and MβCD-treated cells ($P = .0024$ for MβCD, $P < .0001$ for Latex MβCD), but not in cells incubated exclusively with latex beads (One-way ANOVA, Dunnett's post-test). Data are representative of three independent experiments performed in triplicate or quintuplicate. ns—no significance.

investigated whether the adhesion complex proteins talin and vinculin were differentially expressed, or whether these proteins were not recruited to the adhesion site in *Leishmania*-infected cells. Decreased talin TCF was observed in infected cells ($11.5 \pm 6.3 \times 10^4$) compared to uninfected cells ($13.1 \pm 4.8 \times 10^4$) (Fig. 3A and B), as well as reduced talin recruitment to site of FA in *L. amazonensis*-infected macrophages, as evidenced by low BCF/TCF values (Fig. 3A and C). Moreover, the observed reduction in talin-associated TCF correlated with a decrease in VLA-4-associated BCF (Fig. 2A; Figure S2, Supporting Information).

No changes were observed in vinculin-associated TCF following phagocyte infection (Fig. 4A and B). However, similarly to talin, vinculin recruitment was also affected following *Leishmania* infection, as evidenced by lower BCF/TCF ratios in cells expressing vinculin (Fig 4A and C). Furthermore, despite the absence of differences in vinculin TCF expression between infected and uninfected cells, a correlation was observed with respect to decreases in VLA-4 BCF (Fig. 2B; Figure S2, Supporting Information).

Talin depletion by *Leishmania* infection is correlated with impaired cell spreading

Reductions in talin may be associated with the impairment of integrin clustering, which can impact the cell spreading observed after *Leishmania*-infection. To explore this aspect, a double immunofluorescence assay for actin by way of phalloidin with talin staining was performed in both uninfected and infected cells adhered to fibronectin. Extensive cytoplasm spreading was observed in uninfected macrophages, while infected macrophages presented less spreading and a more rounded shape (Fig. 5A). Infection by *L. amazonensis* did not alter actin expression (Fig. 5A and B). The estimated cell area in Phalloidin-stained macrophages was smaller in *Leishmania*-infected cells (345 ± 129 μm^2) compared to uninfected controls (446 ± 211 μm^2, Fig. 5A and C). In addition, reduced talin expression was correlated with an impairment in cytoplasm spreading (Fig 5A and D). Similarly, lower vinculin values were found to be correlated with less cytoplasm spreading (Fig. 5A and E).

Discussion

Previous studies have demonstrated that *Leishmania* infection impairs macrophage adherence to extracellular matrix components (Carvalhal et al. 2004), cell spreading over a fibronectin-coated surface (Figueira et al. 2015) and chemokine-induced directional movement (de Menezes et al. 2017). Studies have also shown that these changes are associated with decreased integrin affinity to the substrate, and impaired formation of cell adhesion complexes (Figueira et al. 2015, de Menezes et al. 2017). Here, we expand on previous investigations by observing two phenomena: VLA-4 clustering and redistribution of this integrin to FA contacts at the cell base membrane. Our results indicate that *Leishmania* infection impairs VLA-4 recruitment to the cell–matrix contact region, which is accompanied by reduced FA complex formation and decreased talin expression.

Under normal conditions, integrin–ligand binding leads to the recruitment of these molecules, originally dissipated around the cell at the basal aspect of the cell membrane. This redistribution promotes integrin aggregation at the contact points of cell-substrate, where these molecules mediate FA (Roca-Cusachs et al. 2009, Karimi et al. 2018). Integrin clustering depends on

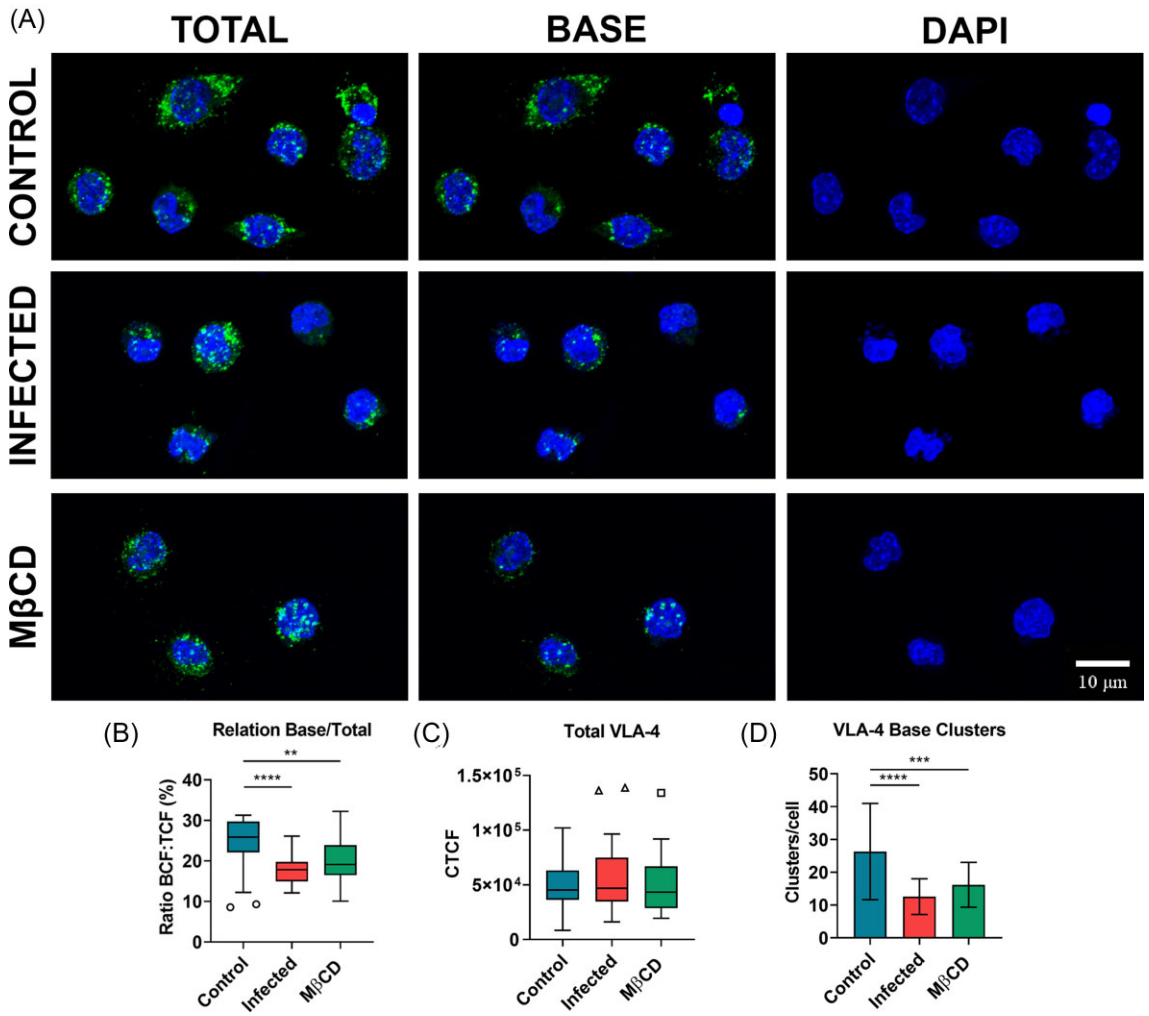

Figure 2. Integrin distribution: Immunostaining for VLA-4 (α-ITGA4/Alexa488) in uninfected J774 cells, *L. amazonensis*-infected J774 cells and J774 treated with MβCD. (A) VLA-4 fluorescence in the entire cell (Total—TCF) or at the cell base (BCF) and DAPI staining (Scale bar—10 μm). (B) Box plot illustrating the ratio of BCF to TCF, in which controls exhibited higher proportions than infected cells ($P < .0001$) or cells treated with MβCD ($P = .0022$). (C) No differences were observed in VLA-4 TCF among the groups. (D) Bars and SD representative of integrin cluster counts at the cell base. Fewer clusters were identified in infected cells ($P < .0001$) and the MβCD group ($P = .0004$). Points in (A) and (B) shown outside the box plot are outliers by Tukey's test. Values shown in (B) are representative of mean fluorescence quantification by two observers. Statistical analysis performed using Kruskal–Wallis, followed by Dunn's post-test. Data are representative of five independent experiments performed in one or two replicates.

integrin mobilization, integrin activation and the assembly of adhesion complex proteins. Our data, as well as that of previous studies, show that all three of these steps are affected in the context of *Leishmania* infection (Figueira et al. 2015, de Menezes et al. 2017). An inhibitory effect on the formation of clusters of VLA-4 and LFA-1 integrins was described in *Toxoplasma gondii*-infected monocytes, indicating that this parasite is capable of interfering with the activity of these integrins and modulating the dynamics of human monocyte adhesion to the vascular endothelium under fluid shear stress (Harker et al. 2013, Cook et al 2018).

Here, we observed impairment in VLA-4-clustering at the base of infected cells. However, decreased overall leukocyte migration to the internal organs has not been observed in the context of *Leishmania* infection. Our group previously showed the permanence of migratory capacity of both *L. amazonensis*-infected monocytes and neutrophils from inflamed tissue to the draining lymph nodes in mice; at the same time, dendritic cells exhibited reduced migration capability (Hermida et al. 2014). Likewise, in a murine model of *Leishmania donovani* infection, the blockade of VLA-4/VCAM-1-depedent adhesion mechanisms did not alter leukocyte migration to the liver, nor that of lymphocytes and dendritic cells to the spleens of mice (Stanley et al. 2008). Instead, VLA-4-modulation and loss of firm adhesion were shown to contribute to prolonged phagocyte rolling movement and reaching greater distances under fluid shear stress (Harker et al. 2013, Figueira et al. 2015, Cook et al 2018), which likely facilitates the bloodstream dissemination of infected cells without affecting migration to organs.

Leishmania amazonensis infection was also shown to reduce FA complex assembly at the cell base through decreased talin expression, as well as reductions in talin and vinculin mobilization to the adhesion plane. Talin promotes integrin activation via inside-out signaling and is required for integrin clustering (Calderwood et al. 1999, Tadokoro et al. 2003, Ellis et al. 2014, Lu et al. 2022). The expression of vinculin, another adaptor protein that regulates integrin dynamics, was not observed to change considering total cellular expression; however, we observed reduced mobilization to FA sites. As vinculin is known to enhance integrin activation by way of a talin-dependent mechanism (Humphries et al. 2007, Nanda et al. 2014), talin depletion may end up affecting macrophage

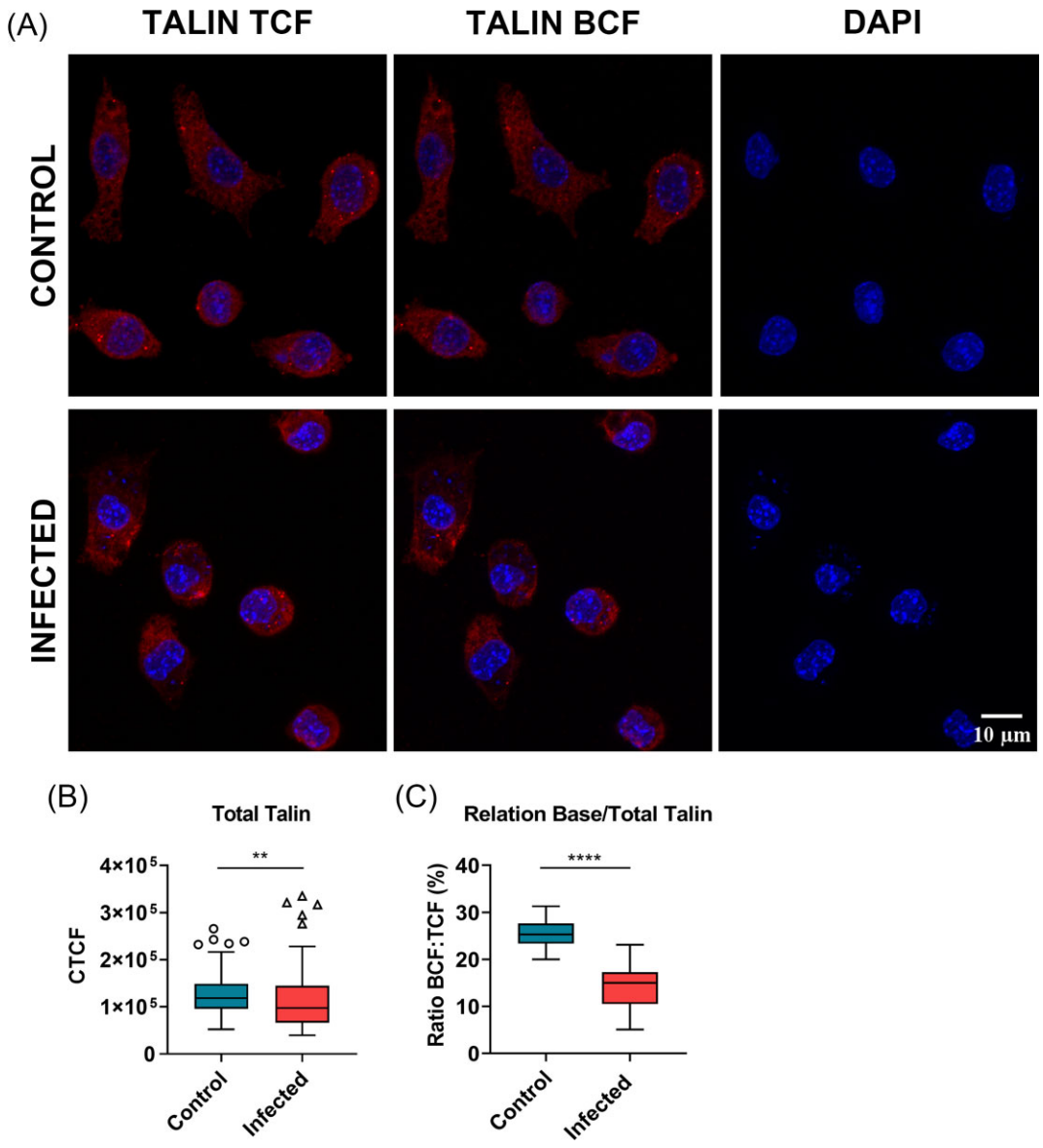

Figure 3. Talin expression and adhesion complex assembly: Immunostaining for talin (α-Talin/Alexa594) was performed in uninfected J774 cells and cells incubated with *L. amazonensis*. **(A)** Confocal microscopy image of talin expression in the entire cell (Total—TCF) or at the adhesion site (Base—BCF) and DAPI staining (Scale bar–10 μm). **(B)** *Leishmania amazonensis*-infected cells presented decreased talin TCF ($P = .0039$, Mann–Whitney). **(C)** Infected cells showed reduced talin mobilization to the cell base ($P < .0001$, Mann–Whitney). Points outside box plots represent outliers by Tukey's test. Data representative of a single experiment performed in duplicate.

adhesion 2-fold. In fact, the recruitment of these structural proteins that form FA complexes requires interaction with the cell plasma membrane though Phosphatidylinositol 4,5-bisphosphate (PIP2) (Gilmore and Burridge 1996, Chinthalapudi et al 2018), which is present in lipid raft domains (Myeong et al. 2021). In addition, our results demonstrate that the decreased talin and vinculin recruitment to the cell base was also related to diminished VLA-4 concentrations in the adhesion plane.

A study evaluating the mobility of macrophages infected with *L. amazonensis* observed that the infection reduces FAK and paxillin expression, which are also part of the adhesion complex, leading to abnormalities in the formation of adhesion complex signaling and macrophage motility (de Menezes et al. 2017). Similar changes in components of the FA complex have also been observed in macrophage infection by *T. gondii*, as well as in cardiomyocytes infected with *Trypanosoma cruzi* (Cook et al 2018, Melo et al. 2019).

Most *Leishmania*-infected macrophages exhibited a rounded cell shape and limited cell spreading, which was associated with reductions in talin and vinculin. Talin depletion did not affect the initial spreading over the extracellular matrix, but was necessary for more widespread dissemination in later phases, provoking a rounded form in the cells adherent to fibronectin (Zhang et al. 2008, Roca-Cusachs et al. 2009). Similarly, vinculin was also found to be required for cell spreading (Jannie et al. 2015). Finally, limitations in cell-spreading were found to be associated with decreased β1 integrin cluster formation at the cell base (Harker et al. 2013).

The transit of receptors, such as integrins, along the plasmatic membrane occurs through the complexation of these molecules in lipid rafts. It was observed that integrins in an inactive confor-

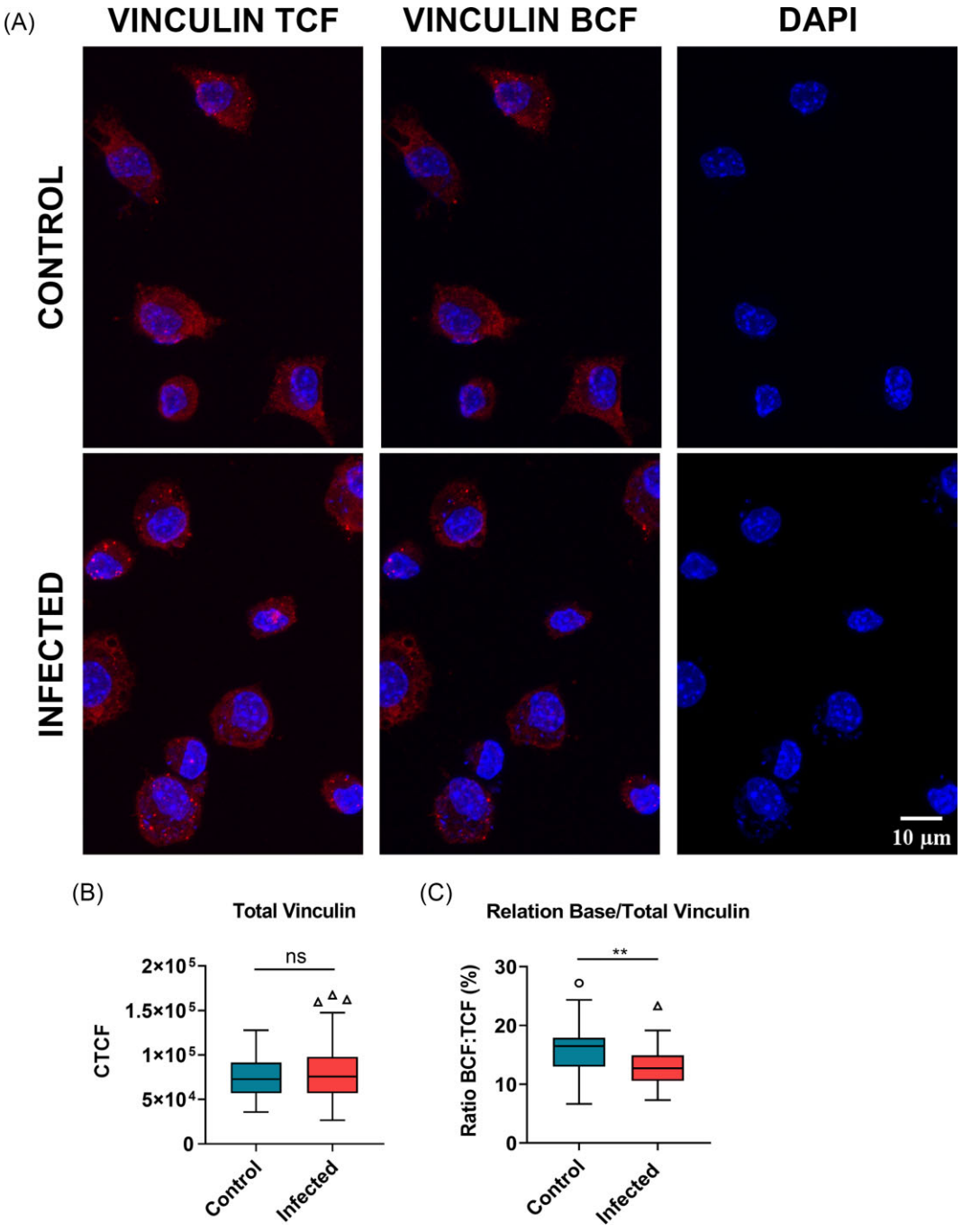

Figure 4. Vinculin expression and adhesion complex assembly: Immunostaining for vinculin (α-Vinculin/Alexa594) was performed in uninfected J774 cells and cells incubated with *L. amazonensis*. **(A)** Confocal microscopy image of vinculin expression in the entire cell (Total—TCF) or at the adhesion site (Base—BCF) and DAPI staining (Scale bar–10 μm). **(B)** No differences evidenced in vinculin TCF between the groups. **(C)** Infected cells exhibited reduced vinculin mobilization to the cell base ($P = .0010$, Mann–Whitney). Points outside box plots represent outliers by Tukey's test. Data representative of a single experiment performed in duplicate. ns—no significance.

mational state are restricted to the nonraft portion of the membrane. Following activation, they then migrate to the raft domains, which allows their mobilization (Leitinger and Hogg 2002). Some authors have already demonstrated that *Leishmania* promotes the disruption of lipid rafts in *L.donovani*-infected macrophages, leading to repercussions in antigen presentation capability and macrophagic activation induced by IFN-γ (Chakraborty et al. 2005, Sen et al. 2011).

We evaluated whether reduced VLA-4 cluster formation was associated with lipid raft disruption in infected cells. Although similar reductions in phagocyte adhesion were obtained by *Leishmania* infection and lipid raft disruption (using MβCD), our experiments attempting to demonstrate a reduction in lipid rafts in infected cells or in MβCD-treated cells produced inconsistent results. This inconsistency may be explained by the technique adopted for measuring lipid raft disruption, which may not be sensitive or specific enough to demonstrate differences. In fact, CT-B may bind to other sugar membrane structures, such as galactose, or to other non-GM1 ligands in B cells, which could decrease its specific signal vs. background during the analysis (Uesaka et al.

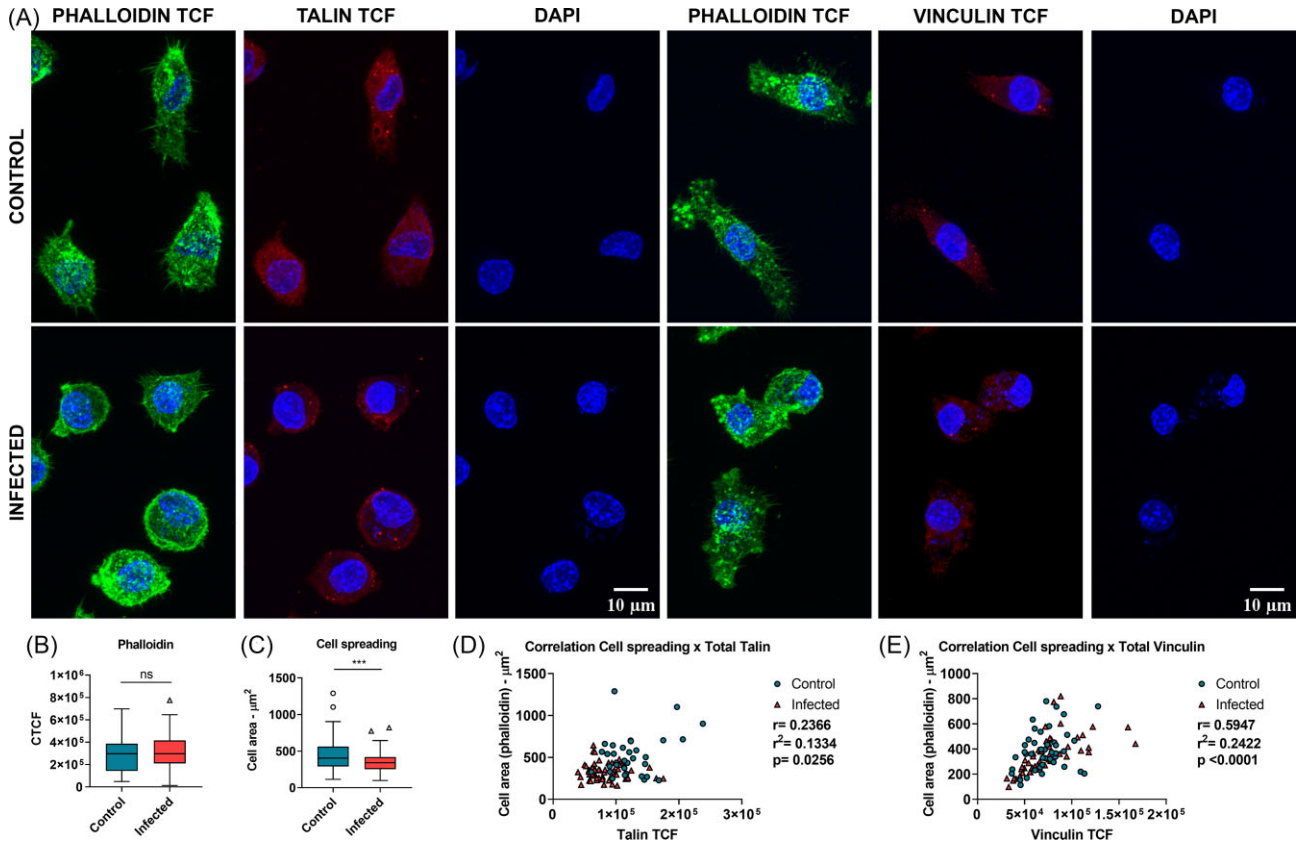

Figure 5. Cell spreading: fluorescence staining for actin (Phalloidin-488) with talin (α-Talin/Alexa594) or vinculin (α-Vinculin/Alexa594) was performed in uninfected J774 cells and cells incubated with *L. amazonensis*. **(A)** Confocal microscopy image of phalloidin and talin or vinculin fluorescence in the entire cell and DAPI staining (Scale bar—10 μm). **(B)** Box plot representing fluorescence quantification of phalloidin staining, with no differences between groups. **(C)** Area measurement of phalloidin labelling, demonstrating decreased cell spreading in infected cells ($P = .0007$, Mann–Whitney). Points outside box plots represent outliers by Tukey's test. **(D)** Positive correlation between talin TCF and cell area ($P = .0256$, $r = 0.2366$). **(E)** Positive correlation between vinculin TCF and cell area ($P < .0001$, $r = 0.5947$). Correlations determined by Spearman's coefficient testing. Data representative of a single experiment performed in duplicate. ns—no significance.

1994, Aman et al. 2001). In addition, it was unfortunately not possible to validate the present results regarding talin depletion using an alternative method, such as western blotting, which would be necessary to confirm this finding.

Based on our findings, we hypothesize that *Leishmania* infection may selectively modulate later stages of cell adhesion, thusly impairing cell spreading during firm adhesion through reduced adhesion complex assembly by way of reduced protein mobilization and the modulation of integrin clustering. These changes may further be associated with lipid raft disruption in *Leishmania*-infected cells.

Authors' contributions

Reginaldo Brito (Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing), Erina Masayo Hassegawa (Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing), Patrick Camardelli (Formal analysis, Investigation, Methodology), Kalene Elpídio (Formal analysis), Juliana de Menezes (Formal analysis, Methodology), Cláudio Pereira Figueira (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing), and Washington LC dos-Santos (Conceptualization, Funding acquisition, Supervision, Writing – review & editing)

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Supplementary data

Supplementary data is available at *FEMSPD* online.

Conflict of interest statement. The authors declare no conflicts of interest. The images have been processed to enhance their quality for publication.

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