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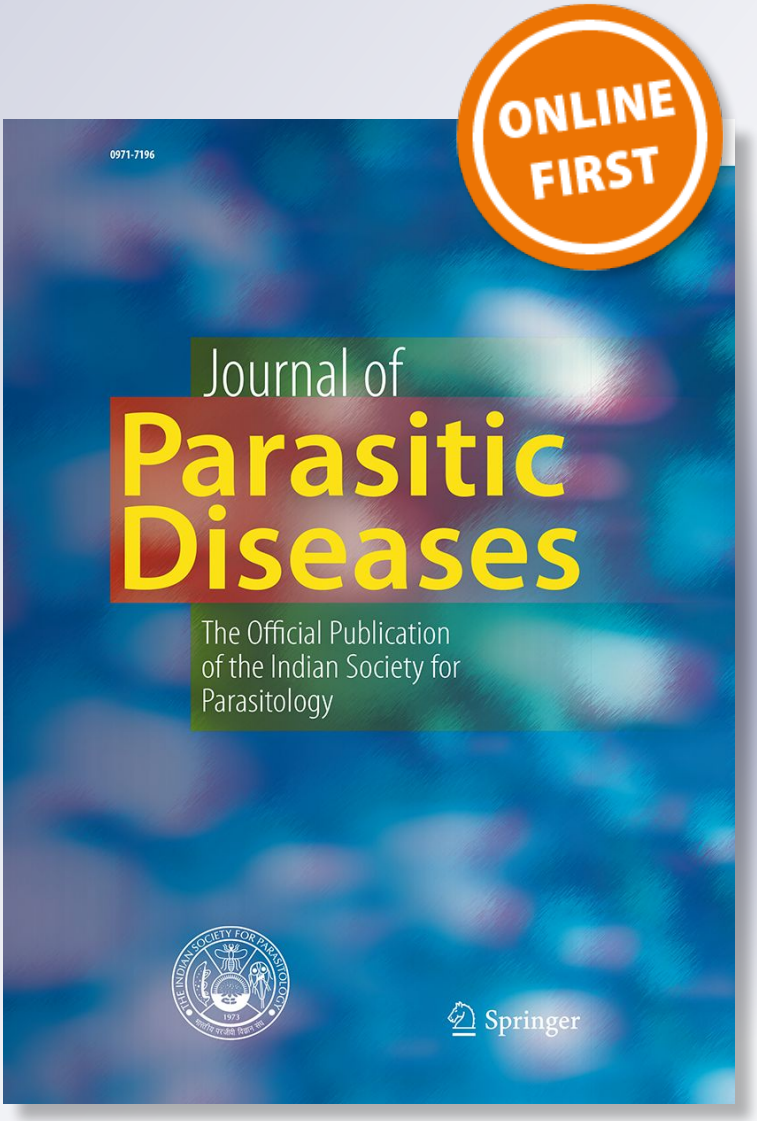
Journal of Parasitic Diseases

ISSN 0971-7196

J Parasit Dis
DOI 10.1007/s12639-019-01118-2



Springer

J Parasit Dis
https://doi.org/10.1007/s12639-019-01118-2

ORIGINAL ARTICLE

Evaluation of the effect of 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol in comparison with metronidazole in an in vitro culture of *Blastocystis* in samples of patients with irritable bowel syndrome

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Received: 1 October 2018 / Accepted: 23 April 2019

Abstract Metronidazole is the most-used pharmaceutical for the treatment of infection by *Blastocystis*. However, studies have reported resistance of the microorganism towards this pharmaceutical. In Mexico, studies concerning the prevalence of this parasite and its relationship to Irritable Bowel Syndrome have been carried out. To evaluate the in vitro effect of metronidazole and the compound 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol over *Blastocystis*, as well as the prevalence of *Blastocystis* in patients with Irritable Bowel Syndrome. A prospective, transversal design study (April 2016–April 2017) of 51 samples of patients with Irritable Bowel Syndrome, obtained from the ISSEMyM Medical Center in Toluca, Mexico. For the identification of *Blastocystis* was done in three serial stool samples through direct microscopic examination and the Ritchie technique. The in vitro susceptibility test towards metronidazole and the triazolic compound was done through a microculture in concentrations of 1 to 1000 µg/mL, each one in triplicate. A 31.3% prevalence of *Blastocystis* was observed in the population, with greater prevalence in women (30.2%) than in men (25%). In the susceptibility test, a CL_{50} of 64 µg/mL was obtained for metronidazole, in comparison to the CL_{50} of 250 µg/mL for 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol. This molecule in development has an effect for the treatment of infection by *Blastocystis* in vitro in patients with IBS and therefore, more studies should be performed.

Keywords *Blastocystis* · Irritable bowel syndrome · Triazole · Metronidazole · Susceptibility

Introduction

Blastocystis is a eukaryotic, anaerobic, chromist parasite that infects a great variety of vertebrates, including humans. *Blastocystis* is a polymorphic protozoan, and four major forms have been described in the literature. In reality, *Blastocystis spp.* can present with a bewildering array of forms within a single culture, and it may be difficult to assign a specific form to the cell in question. The multivacuolar and avacuolar form are exclusive to the in vitro culture (Tan 2008). Its transmission mechanism is fecal–oral, in the form of a cyst, when ingesting contaminated water and food. Its prevalence is higher in developing countries (60%) than in developed countries (10%) (Khoshnood et al. 2015) and tropical and subtropical climates (Duda et al. 2015). The most common clinical manifestations associated to a *Blastocystis* infection are a variety of gastrointestinal disorders, including diarrhea, abdominal spasms, fatigue, constipation, flatulence, nauseas, cutaneous allergies (Ramirez et al. 2010; Shawky et al. 2011; Stark et al. 2007; Rajic et al. 2015) and, in recent years, Irritable Bowel Syndrome, characterized by inflammation, abdominal distension, presence of recurrent abdominal pain associated to alterations of the depositional rhythm, be this in the form of constipation, diarrhea or both (constipation with diarrhea) (Mearin et al. 2016; Longstreth et al. 2006; Sinagra et al. 2016; Occhipinti and Smith

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Published online: 02 May 2019

2012). This association is attributed to the infection, given that forms of *Blastocystis* have been found in stool samples of patients with IBS in comparison to the control group (Das et al. 2016).

The treatments that has been used for infection by *Blastocystis* have been pharmaceuticals such as metronidazole, nitazoxanide, trimethoprim-sulfamethoxazole, paromomycin, iodoquinol, ketoconazole, secnidazole, emetine, tinidazole and the probiotic Saccharomyces boulardii, of which metronidazole is the pharmaceutical of choice to treat the infection (Sekar and Shanthi 2013). Metronidazole belongs to the nitroimidazoles and is used to treat infections by anaerobic microorganisms, such as parasites and bacteria. It possesses a variety of functional groups and two hydrogen atoms coordinated to the imidazole ring (Quinlivan et al. 2015).

Besides its high stability, bistriazole is also attractive because this compounds is prepared only in two synthetic steps, (1) which is shorter than other synthetic methodologies for metronidazole and analogue compounds, (2) as CuAAC key reaction is a modular chemical process, (3) diverse functional groups can be introduces to triazole moiety with a subsequent modification of biological activity which can be used for increase antibiotic/antiprotozoal activity or to minimize secondary undesired effects (González et al. 2011).

Triazoles, on the other hand, are molecules with a triazole core and are composed by a pair of isomeric chemical compounds with the molecular formula $C_2H_3N_3$, with a 2-carbon, 3-nitrogen ring. The importance of these triazolic compounds is given by its therapeutic properties as an: antifungal, antiviral, anti-inflammatory, anticarcinogenic, anticonvulsive, antidepressant, antitubercular, antihypertensive, hypoglycemic, antiparasitic, enzyme inhibitor, herbicide, insecticide and antibacterial agent. Because of such properties, they are considered "privileged" molecules and are used frequently in the pharmaceutical industry (Keri et al. 2015; Miceli and Kauffman 2015; Ye et al. 2017).

The purpose of this work was to evaluate the effect of 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol and compare it with metronidazole, in regard to the in vitro culture of *Blastocystis* in samples of patients with irritable bowel syndrome.

Materials and methods

Population and sample

A transversal design of patients with IBS was carried out over a year (April 2016–April 2017), in the gastroenterology department of the State of Mexico Institute of Social Security Medical Center, in the city of Toluca, where 321 patients were received during the sampling period, were diagnosed based on the Rome IV criteria (2016). The patients diagnosed with IBS were selected under the following inclusion criteria: patients with IBS, exclusion criteria: patients under treatment with metronidazole or another antiparasitic agent (automedication), elimination criteria: patients with no diagnosis of Irritable Bowel Syndrome and patients that present IBS but would not accept to participate in the study. Of the sample size (321 patients), only 51 patients participated in the study and signed a letter of informed consent for the donation of three serial stool samples (See Fig. 1).

Rome IV criteria used for the diagnosis of IBS

Diagnosis of Irritable Bowel Syndrome. It is diagnosed by the presence of recurrent abdominal pain that must be present at least 1 day a week, with two or more of the following characteristics: (a) associated to defecating,

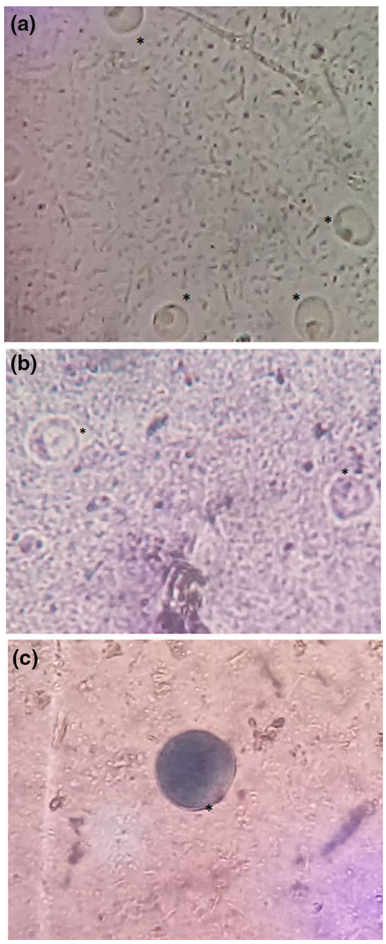

Fig. 1 **a**, **b** show extraction of *Blastocystis* spp viable, **c** non viable with trypan blue in which the concentrate that was obtained from the samples for the inoculation of the microculture can be seen

(b) related to a change in the frequency of deposition, (c) related to a change in the consistency of the depositions. It can be predominantly constipation, where more than a 25% of the depositions are Bristol type 1 or 2 feces and less than 25% are Bristol type 6 or 7 feces. On the other hand, there are those associated predominantly to diarrhea, in which more than 25% of the intestinal evacuations are Bristol type 6 or 7 and less than 25% are Bristol type 1 and 2. The criteria must be met at least during the first 3 months and the symptoms must have initiated at least 6 months prior to diagnosis. As for the requirements of duration of the symptoms, it must be considered that the criteria must be met during the first 3 months and must have started at least 6 months before diagnosis.

The diagnostic criteria for irritable bowel syndrome (IBS) have recently been updated from Rome III to Rome IV. Whereas in Rome III a diagnosis of IBS entailed chronic abdominal pain or discomfort at least 3 days per month, in Rome IV the term discomfort has been removed and the frequency of abdominal pain increased to at least 1 day per week in this hospital the physician used IV IBS criteria with the objective to increase the sensibility and specificity at diagnostic.

Detection of Blastocystis in stool samples

A total of 51 samples were analyzed through direct microscopic examination, previously described by Alarcón et al. (2007), Barassa and Bueno (2000) and concentrated through the Ritchie sedimentation method, as indicated in the Laboratory Procedure Manual for the Diagnosis of Intestinal Parasites in Man (No. 37). The samples were observed through an electronic NIKON microscope, with a 40X objective. The analyses were carried out at the Zamser Clinical Analysis Laboratory (sanitary license: 19782).

Extraction of Blastocystis

For the extraction of *Blastocystis*, a 10-mL saline solution suspension of 5 g of stool was prepared, and was then centrifuged at 3500 rpm. To extract it, the layer that formed in the sediment where *Blastocystis* concentrated, was taken. The extract was afterwards placed in Locke solution within a capped test tube in order to conserve the conditions of anaerobiosis. The entire process aforementioned was repeated until the supernatant became transparent. Posteriorly, the tube was incubated at 36 °C in a Rios Rotcha incubator in anaerobic conditions until inoculation.

Susceptibility of Blastocystis

Inoculation of the parasite A microculture was prepared using Boeck-Drbohlav media in a sterile 96-well microplate. Each well was inoculated with 100 µL of media with a concentration of 325 parasites/10µL (M1), 312.5 parasites/10µL (M2), 150 parasites/10µL (M3), 300 parasites/10µL (M4), 212.5 parasites/10µL (M5) and 100 µL of Locke solution for each one of these, with concentrations of 1, 2, 4, 8, 16, 32, 64, 250, 500 and 1000 µg/mL, prepared through double dilution of metronidazole acquired from Sigma-Aldrich and 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol (synthesized by Dr. Erick Cuevas Yañez). The analyses was carried out in triplicate (Phillips and Zierdt 1976).

As a control, a fourth column was filled, in which the same quantity of parasites was placed for each of the sample without any of the test compounds. The parasites were incubated at 36 °C in the Rios Rotcha incubator in anaerobic conditions during 48 h and were evaluated each 12 h.

Viability of the cells

In order to determine the viability of the parasites, the trypan blue technique was used (Strober 2001). 10 mL of the media were taken, stirring the sediment in order to obtain a uniform distribution, to which 10 µL of trypan blue solution were added. The *Blastocystis* count was carried out using haemocytometer chamber (Improved Neubauer, Hausser Scientific) with 0.4% trypan blue dye exclusion (Sigma-Aldrich Corp. USA) as viability indicator. The parasite count was determined daily in cultures until it became non-viable. Only viable cells that did not take up trypan blue stain were counted and carried out each 12 h.

Statistical analysis

In order to obtain the prevalence of *Blastocystis*, the number of patients with presence of *Blastocystis*, divided by the total of patients analyzed, multiplied by one-hundred percent, was calculated. To determine if there was a difference in the prevalence of parasites found, a Student's t test and the Mann–Whitney U test were performed.

Analysis was performed with the Probit model and, according to calculations for the point of 50% of dead of all forms, the letal concentration 50 (CL_{50}), with a confidence interval (CI) of 95%. The results were analyzed with the statistical program IBM SPSS, version 22.

Results

Characteristics of the population

Of the sample size (321 patients), an n = 51 patients was obtained, which complied with the Rome IV criteria, as shown in Table 1 and was obtained, IBS was more present in women (84.3%) than in men (15.7%). Likewise, IBS-C is the most prevalent form of IBS (58.8%) in men (50%) as well as women (60.5%), in comparison with IBS-M (7.9%) for both genders. with data related to the diagnosis of IBS. The characteristics in case of patients with IBS-C, more than 25% are Bristol type 1 or 2 feces (30) and less than 25% Bristol Type 6 or 7 (30). For IBS–D more than 25% of intestinal evacuations are Bristol type 6 or 7 (17) and less than 25% Bristol Type 1 or 2 (17). For IBS- M more than 25% are Bristol type 1 or 2 feces (4) and less than 25% Bristol Type 6 or 7 (4). It is important to mention that in the evaluation of patients with IBS, some are very difficult to classify, so they were considered as indeterminate IBS-I.

Diagnosis of *Blastocystis*

Patients with Irritable Bowel Syndrome were analyzed, in which *Blastocystis*, *Entamoeba coli*, *Endolimax nana*, *Ascaris lumbricoides* and *Giardia lamblia* were identified, as shown in Prevalence of *Blastocystis* and other parasites found Table 2.

For determination of the minimum inhibitory concentration, for each of the samples, 15 mL of *Blastocystis* concentrate was extracted, with an average of 325 parasites/10 µL (M1), 312.5 parasites/10 µL (M2), 150 parasites/10 µL (M3), 300 parasites/10 µL (M4) and 212.5 parasites/10 µL (M5) Table 3.

Discussion

The results obtained is corroborated with that reported by Schmulson and Drossman in 2017 (Schmulson and Drossman 2017), who described that Irritable Bowel Syndrome (IBS) is the gastrointestinal disorder with the highest incidence in the human population, in which Irritable Bowel Syndrome associated to constipation (IBS-C) is the most predominant in patients with IBS. Also, IBS can be present in people ages of 28–76 years, with an average age of 56 years.

Blastocystis is the parasite with the highest prevalence in patients with Irritable Bowel Syndrome (IBS), when compared to the control group (Yakoob et al. 2004; Ustün and Turgay 2006). In a study carried out in patients with IBS, the prevalence of *Blastocystis* was of 13.6% (Tungtrongchitr et al. 2004). In Mexico, it has been reported that *Blastocystis* has a prevalence of 31% in patients with IBS (Jiménez et al. 2012). In the present work, the prevalence of *Blastocystis* was of 31.3% in patients with IBS, in comparison with other intestinal parasites such as *Entamoeba coli* (5.8%), *Endolimax nana* (5.8%), *Ascaris lumbricoides* (1.9%) and *Giardia lamblia* (1.9%). Likewise, *Blastocystis* was more present in women (30.23%) than in men (25%) (see Table 1), and therefore, more studies should be performed comparing the same number of men and women because in this study women are greater. Taking into account the prevalence presented in both studies, it can be observed that it is very similar. It has been described that the high prevalence of *Blastocystis* is linked to the coexistence with animals, lack of hygiene and consumption of contaminated water and food (Beyham et al. 2015; Mostafa et al. 2015). When compared to the prevalence seen in developing countries (30–60%) (Khoshnood et al. 2015), it can be observed that the prevalence of *Blastocystis* is within that previously established. However, based on the results obtained, it is the parasite with the highest prevalence in patients with IBS.

As for the extraction of *Blastocystis*, there are no specialized methods of extraction for this parasite. However, there are specialized methods for its identification, such as polymerase chain reaction (PCR) (Stensvold et al. 2006), immunofluorescence assay, lugol stain, direct observation and trichrome stain (Dogruman et al. 2010). In the present work, the extraction was done in order to obtain a greater concentration in the number of microorganisms and have a cleaner microculture. This process was carried out through repeated rinses and centrifugations of the sample, and

Table 1 Shows the characteristics that were observed in the studied population, in which IBS-C is the most predominant in patients with IBS and women have the highest incidence of IBS

Diagnosis de IBS	n = 51 patients	Prevalence (%)	Femenine n = 43 (%)	Masculine n = 8 (%)	Average	±
IBS-C	28	54.9	49.0	5.8	14	15.6
IBS-D	13	25.4	21.5	3.9	2	0
IBS-M	4	7.8	3.9	3.9	2	0
IBS-I	6	11.7	9.8	1.9	6	7.1

Table 2 Prevalence of *Blastocystis* and other intestinal parasites found in patients with Irritable Bowel Syndrome. It can be observed that *Blastocystis* is the parasite with the highest prevalence in comparison with the other parasites found. It can also be noted that women present higher incidence of infection by *Blastocystis*

Species of parasite	n	Prevalence (%)	Femenine (%)	Masculine (%)	*p*
Blastocystis	16	31.3	30.23	25	0.0001
E.nana	3	5.8	6.9	0	0.0001
E. coli	3	5.8	6.9	0	0.0001
G. lamblia	1	1.9	0	12.5	0.0001
A. lumbricoides	1	1.9	0	12.5	0.0001

Prevalence of *Blastocystis* and other parasites found

Table 3 Determination of the MIC, in which an MIC of 64 μg/mL was obtained for metronidazole in majority of the samples, except in M3; the MIC for 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol was of 250 μg/mL. However, in M1 there was no inhibition

Determination of the MIC					
Pharmaceutical	M1	M2 (μg/mL)	M3 (μg/mL)	M4 (μg/mL)	M5 (μg/mL)
Metronidazole	64 μg/mL	64	32	64	64
1,3-Bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol	–	250	250	250	250

allowed for the omission of a previous culture in order to obtain a greater number of microorganisms. This technique facilitated the acquisition of a *Blastocystis* culture that does not have to be prepared directly from the sample and thus reduces the risk of culture contamination.

Metronidazole is the antibiotic most widely accepted for the treatment of an infection by *Blastocystis* (Roberts et al. 2014). Is considered a first-line treatment for this infection; however, there has been increasing evidence for the lack of efficacy of this treatment. Treatment failure has been reported in several clinical cases, and recent in vitro studies have suggested the occurrence of metronidazole-resistant strains. The eradication of *Blastocystis* is essential in some cases where it is the only infectious agent and patient is suffering from some symptoms. In such cases, metronidazole is the drug of choice but its efficacy is relatively low in some cases (Roberts et al. 2015; Kurt et al. 2016). Clinical reports have shown that at least 80% of the patients treated with metronidazole has achieved the elimination of the parasite in stool samples 6 months later (Aguilar and Lucia 2009). However, in patients with IBS, 60% presented resistance to 0.1 mg/mL of metronidazole (in Indonesia, a resistance to 1.0 mg/mL was observed) (Sekar and Shanthi 2013). In the study carried out, an MIC of 64 μg/mL was obtained for metronidazole at 48 h (see Table 3), but in sample 3, there was an MIC from 32 μg/mL onwards. Likewise, it was observed that in low concentrations of 1–2 μg/mL, there were not adverse effects over *Blastocystis*. With the compound 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol, an MIC of 250 μg/mL was obtained for M2, M3, M4, M5 and for M1, no susceptibility in *Blastocystis* was observed. It is possible that this is a result of resistance to the compound. It has been described that this parasite has presented resistance to some pharmaceuticals such as metronidazole (Batista et al. 2017; Tamalee et al. 2014) and that it should not be the first therapeutic choice, due to the fact that there is not a complete eradication of the parasite (Tamalee et al. 2015). This parasite has demonstrated resistance in some cystic and ameboid forms, to 0.01 mg/mL of metronidazole (Yakoob et al. 2011). Reports on the in vitro susceptibility of *Blastocystis* have described that the resistance towards metronidazole is generated in accordance to the subtype. These reports mention that the ST7 subtype is the most pathogenic and resistant to metronidazole, while ST4 has demonstrated to be susceptible (Mirza et al. 2011; Mirza and Tan 2014; Wu et al. 2014). In the results obtained, comparing both compounds, for 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol, resistance was observed with M1, while with metronidazole, an MIC was obtained for all samples. Thus, comparing both compounds, it can be stated that metronidazole continues to be the better option for the treatment of an infection by *Blastocystis*, with an MIC of 64 μg/mL. However, studies of subtypification are necessary in order to determine which subtypes were those that present susceptibility towards both compounds. It is likewise necessary to carry out in vivo studies in order to

evaluate toxicity and pharmacokinetics, as well as any adverse effects of the novel compound.

Conclusion

Blastocystis is the most prevalent parasite in patients with IBS in Mexico and metronidazole continues to be the best option as a treatment for an infection by *Blastocystis*, when compared with the compound 1,3-bis-(4-phenyl-[1,2,3] triazole-1-il)2-propanol. However, studies of subtypification are still needed in order to determine which subtypes present susceptibility towards both compounds. Likewise, in vivo studies are recommended in order to analyze of the effects of the novel compound in greater detail.

Acknowledgements To CONACyT, chemist Zamudio-Chávez of HGI Valentín Gómez Farías, to the Zamser Clinical Analysis Laboratory of Ixtlahuaca and to Caballero-Vásquez, P.MD, of the ISSEMyM Medical Center of Toluca, Mexico.

Authors' contributions GF-L, ZC-S and SB-JG collected the samples and performed the coprological assays. GF-L, performed inoculation parasites, viability of the cells and determination of MIC, CV-P performed the Rome IV criteria used for the diagnosis of IBS. CY-E performed the Synthesis of the molecule. SB-JG and MA-E formulated the idea and MA-E, contributed with critical comments. GF-L and SB-JG obtained the authorizations. All authors participated during the discussion and writing of the manuscript and approved its final version.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethics approval The Ethics and Research Committee of the Medical Center ISSEMyM approved the study Reference Number 015/16.

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