BMJ open

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001266
Article Type:	Research
Date Submitted by the Author:	22-Apr-2012
Complete List of Authors:	Vianna, Lucas; UNIFESP, Ophthalmology Martins, Marilaine; Fundação de Medicina Tropical do Amazonas, State University Amazonas, Parasithology Cohen, Jacob; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Cohen, Marcos; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Belfort Junior, Rubens; UNIFESP, Ophthalmology
Primary Subject Heading:	Addiction
Secondary Subject Heading:	Ophthalmology
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, PARASITOLOGY

1) Title Page:

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Authors: Lucas Monferrari Monteiro Vianna (first author)*; Marilaine Martins**; Marcos Jacob Cohen***, Jacob Cohen***, Rubens Belfort Jr*

*Vision Institute and Department of Ophthalmology. Hospital São Paulo - Federal University of São Paulo. Address: Botucatu St, 821 – ZIP: 04023-900 – São Paulo/SP - Brazil

** Fundação de Medicina Tropical do Amazonas, Universidade do Estado do Amazonas. Address: Pedro Teixeira Av., 25 - ZIP: 69040-000 - Manaus/AM - Brazil

*** Universidade Federal do Amazonas (UFAM) / Instituto de Oftalmologia de Manaus (IOM). Address: General Rodrigo Octávio Jordão Ramos Av, 3000, Campus Universitário, Coroado I - 69077-000 - Manaus/AM – Brazil

Keywords: Mansonella, microfilaria, keratitis, microscopy, confocal, biopsy

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010 (lucasmmvianna@yahoo.com.br)

Article Summary

Article Focus

- To describe corneal lesions that can be related to the presence of M. ozzardi microfilaremia.
- To describe corneal confocal microscopy images that can be related to the presence of M. ozzardi microfilaremia.
- To correlate ophthalmologic clinical signs with the presence of microfilaremia in some patients.

Key Messages

- There are some biomicroscopic and corneal confocal microscopy evidences of the presence of *M. ozzardi* microfilariae in the cornea.
- The M. ozzardi microfilaria can be pathogenic to the eye.
- Further studies using ocular tissue PCR and other image techniques can be helpful.

Strengths and Limitations.

- The major strength is that the study describe a very prevalent but not well studied disease in a very difficult access area of Brasil.
- Another strength is that this is the first description of M. ozzardi corneal lesions using confocal microscopy.

- The major limitation is the absence of a similar control group and follow up of the patients due to work field access difficulties.

Relevant Subject Headings: mansonella, microfilaremia, corneal lesions, confocal microscopy.

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Abstract:

Objectives: To characterize and confirm the presence of *M*ansonella *ozzardi* microfilariae in the cornea by biomicroscopy and corneal confocal microscopy.

Design: Cross-sectional study.

Setting: Clinical practice study in the Amazon.

Participants: Two hundred and twelve consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil.

Interventions: Complete eye examination, blood checked for the presence of microfilariae, corneal confocal microscopy (CCME) evaluation and biopsy of ocular tissues.

Primary and secondary outcome measures: Positive correlation between corneal biomicroscopic and confocal lesions with M. ozzardi microfilaremia.

Results*:* Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had suspicious corneal lesions were sent to biomicroscopy and CCME. At biomicroscopy, lesions were divided in quiescent and active. At CCME, they were divided in circular and filiform lesions. The associations between lesions and microfilaremia are shown.

Conclusions*:* We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

Statements:

Data sharing statement: there is no additional data available.

Funding statement: this work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Competing Interests Statement: *w*e state that none of the authors have competing interests.

Contributorship Statement: we state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

2) ***Main Text:***

Introduction

Hundreds of types of filariae have been described and a few can cause infections in humans, being *Mansonella ozzardi* one of them and the first description of *M. ozzardi* associated with corneal disease was published in 1998.[1]

M. ozzardi is a filaria exclusively found in America and one of the causes of mansonelliasis.[2] In Brazil it is found in the states of Roraima, Mato Grosso and Amazonas.[3,4,5] The microfilariae of *M. ozzardi* are found in peripheral blood and can be identified by their morphological characteristics or by molecular biology.[6,7]

The clinical features of mansonelliasis are not well described. Infected individuals are generally asymptomatic or have signs and symptoms common to other infections such as fever, coldness in the legs, joint pain and headache.[8] They may also include skin and ocular lesions[1,9,10] and be associated with *Wolbachiae,* that are bacterial endosymbionts of insects and many filarial nematodes and their products trigger inflammatory responses[10].

Coari City, at Solimões River (04°08'S63°07'W) has a high prevalence of *M. ozzardi* infection. Studies showed average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] Onchocerciasis does not exist in this region of the Amazon.[12]

Materials and Methods

Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil. All patients had the eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. The infection by *M. ozzardi* was determined by light-microscopy examination (200x and 400x) of Giemsa-stained thick smears of peripheral blood obtained by digital puncture with a sterile disposable lancet[6] and by a new identification protocol using the polymerase chain reaction (PCR) (M Martins et al., unpublished observations). Patients with suspicious corneal lesions at the eye exam were submitted to biomicroscopy, fundoscopy and corneal confocal microscopy evaluation (CCME), using the Rostock Cornea Module (RCM) of the Heidelberg Retina Tomograph. Photographs were taken at the slit lamp. CCME was performed in the central cornea and in the corneal regions with the suspected lesions. Two patients underwent biopsy of the limbal conjunctiva adjacent to the suspicious corneal lesions area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

Results

Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had suspicious corneal lesions were sent to biomicroscopy and CCME. The association between microfilaremia and cornea lesions is shown in table 1.

Table 1. Association between microfilaremia and corneal lesions.

Corneal Lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	14 (63,6%)	42 (22%)	56
Negative (-)	8 (33,4%)	148 (88%)	156
Total	22	190	212 (100%)

At biomicroscopy, some lesions were characterized as nummular, mid periphery and anterior stroma corneal opacities, 0.5 to 1.0 mm in diameter with central mottled surrounded by a opaque halo, with marked limit between the white opacity and the clear cornea. Other lesions were characterized by nummular keratitis, also located at mid periphery and anterior stroma .In all cases there was a normal translucent area between the corneal lesions and the limbus and absence of corneal neovascularization or other changes (Picture 1). The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis. The association between nummular keratitis and microfilaremia is shown in table 2.

Table 2. Association between nummular keratitis and microfilaremia.

Keratitis lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	10 (76,9%)	4 (44%)	14
Negative (-)	3 (23,1%)	5 (56%)	8
Total	13	9	22 (100%)

Among the 22 patients with corneal lesions at biomicroscopy, 12 presented suspected related lesions also at the CCME. These confocal suspected lesions was presented in two patterns: circular (increased reflectivity, measuring about 80 micra in diameter, located at the sub-epithelial level, as shown in picture 2) and linear (filiform lesions ranging from 250 to 300 µm in length and 10 to 15 µm wide, with one “C" shape end and the other tapered, as shown in picture 3). Five patients presented only circular lesions, one patient only linear lesions and six patients had both of them. The association between confocal lesions and microfilaremia is shown in table 3.

Table 3. Association between confocal lesions and microfilaremia.

Confocal lesion (horizontal) / Microfilaremia (vertical)	Presence (+) % Circular / Linear / Both	Absence (-)	Total
Positive (+)	2 (25%) / 1 (12,5%) / 5 (62,5%)	6	14
Negative (-)	3 (75%) / 0 / 1 (25%)	4	8
	12	10	22

In two volunteers patients with linear lesions, thick blood smear of the limbal conjunctiva was performed and confirmed the microfilaremia. Picture 4 compares microfilaria of *M. ozzardi* seen at the thick blood smear with the confocal microscopy image.

The study was prospective approved by the UNIFESP Ethical Committee. All participants signed the protocol`s informed consent.

Acknowledgement for Funding/Support to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State) and for technical support for *Mansonella*-Brazil Group (Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T).

Discussion

The prevalence of *M. ozzardi* is high in some riverside communities in Amazon with the increasing occupation of rainforest areas.[2,12] Previous studies in Coari city showed an average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] In the present study, the prevalence rate was of 26,4%.

Presumed keratitis caused by *M. ozzardi* has been described in Brazil by us as well as other authors, with no other defined etiology related.[1,9,10] A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is not onchocerchiasis in that parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only

close to the Venezuela border.[13] None of the patients lived or visited areas where Onchocerca exists.

A study conducted in Pauiní, at the Purus River revealed 20 non-Indian habitants with peripheral corneal opacities among 524 patients examined. No microfilariae were seen in the skin snips and *Mansonella sp* was found in the blood of the 2 patients where it was possible to collect blood.[1] Another study, conducted in São Gabriel da Cachoeira (Negro River) showed a positive association between *M. ozzardi* and corneal lesions with all patients presenting ocular changes having *M. ozzardi* microfilariae in blood samples.[9] *All* the skin biopsies were negative for microfilaria including *Onchocerca*. The association between the keratitis and positive microfilaremia in the Amazon was also related by Cohen et al.[10] In our study, this association was also found, as shown in table 1.

The aspect of corneal lesions was divided by its characteristics in only corneal opacities and keratitis, as shown in picture 1. Among patients positive for microfilaremia, the proportion of keratitis aspect seems to be higher and among patients negative for microfilaria, the proportion of only opacity aspect was higher (table 2). One explanation for the presence of patients with keratitis lesion and negative microfilaremia could be false negatives in the blood tests. Patients with only opacity lesion and positive microfilaremia could be explained by chronic infection.

Like in previous studies,[1,9,10] we could not detect, by biomicroscopy, filariae in any ocular tissue, but corneal lesions similar to previous descriptions were found. Using CCME, we detected not previously described lesions. Among patients positive for microfilaremia, the proportion of confocal lesions was higher (table 3). The confocal

lesions were divided by their aspects in linear and circular. Among patients with lesions identified by CCME, there was a higher proportion of patients positive for microfilaremia and a higher proportion of circular aspect alone among patients negative for microfilaremia (table 3). It could suggest that linear lesions are associated with active disease and circular lesions with inflammatory scars, associated or not with active disease. The size of linear lesions (250-300 microns) on CCME was higher than showed in microbiological studies (149-240 microns)[6]. The microfilariae are probably dead in ocular tissues and could enlarge due to inflammatory reaction. Circular lesions could represent, besides inflammatory reaction surrounding dead microfilariae, cross section of remnants of adult worms (26 to 49 mm in length by 00.7 to 0.15 mm in diameter)[6].

Unaffected patients were not tested due to difficulties of field work. We performed random CCME of some affected patient's fellow eyes with no biomicroscopyc lesions and no similar alterations were detected.

Conclusion

We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

4) *References*:

1. Branco BC, Chamon W, Belfort R et al. Ocular findings in Pauiní (Southwest of the Brazilian Amazon) and possible corneal lesions caused by Mansonella. Arq Bras Oftalmol. 1998; Vol 61(6): 647-82.

2. Martins M, Pessoa FA, de Medeiros MB, et al. Mansonella ozzardi in Amazonas, Brazil: prevalence and distribution in the municipality of Coari, in the middle Solimões River. Mem Inst Oswaldo Cruz. 2010 May;105(3):246-53.

3. Deane MP. Sobre a incidência de filárias humanas em Manaus, estado do Amazonas. *Rev Serv Esp Saude Publ* 1949 *2*: 849-858.

4. Oliveira wr. [Filarial infestation in inhabitants of Vila Pereira, territory of Roraima (Brazil)]. Rev Inst Med Trop Sao Paulo. 1963 Nov-Dec;5:287-8.

5. D'Andretta Jr, Pio da Silva CM, Kameyana F. Ocorrência da mansonelose entre índios do alto Xingu. *Rev Soc Bras Med Trop* 1969. *3*:11.

6. Orihel TC, Eberhard ML. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. Am J Trop Med Hyg. 1982 Nov;31(6):1142-7.

7. Morales-Hojas R, Post RJ, Shelley AJ, et al. Characterisation of nuclear ribosomal DNA sequences from Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea) and development of a PCR-based method for their

detection in skin biopsies. Int J Parasitol. 2001 Feb;31(2):169-77. Erratum in: Int J Parasitol 2001 Jun;31(8):850-1

8. BATISTA D, CERQUEIRA NL, MORAES MA. [Epidemiology of Manson's disease in a locality in the interior of Amazonas]. Rev Assoc Med Bras. 1960 Jun;6:176-84.

9. Garrido C, Campos M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. Cornea. 2000 Nov;19(6):817-9.

10. Cohen JM, Ribeiro JA, Martins M. [Ocular manifestations in mansonelliasis]. Arq Bras Oftalmol. 2008 Mar-Apr;71(2):167-71. Portuguese.

11. Keiser PB, Coulibaly Y, Kubofcik J, et al. Molecular identification of Wolbachia from the filarial nematode Mansonella perstans. Mol Biochem Parasitol. 2008 Aug;160(2):123-8. Epub 2008 May 2. PubMed PMID: 18538871; PubMed Central PMCID: PMC2497444.

12. Medeiros JF, Py-Daniel V, Barbosa UC, et al. [Occurrence of Mansonella ozzardi (Nematoda, Onchocercidae) in riverine communities of the Purus river, Boca do Acre municipality, Amazonas State, Brazil]. Cad Saude Publica. 2009 Jun;25(6):1421-6.

13. Belfort Jr, R e Moraes, M – Oncocercose ocular no Brasil. AMB Rev Assoc Med Bras. 1979 Apr;25(4):123-7.

5) Figure Legends:

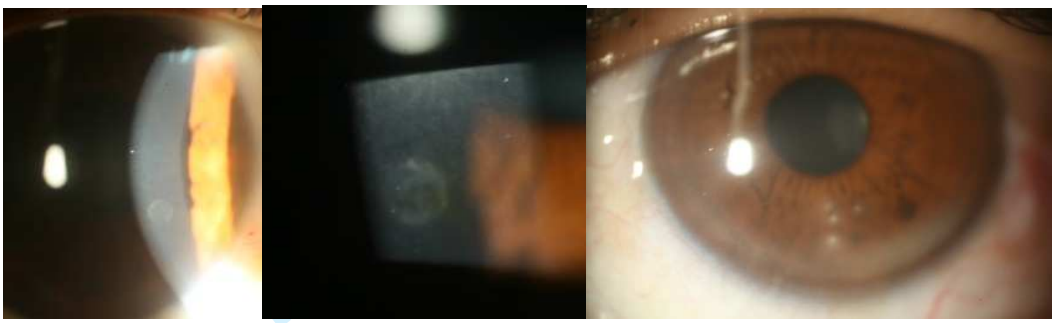
Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.

Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.

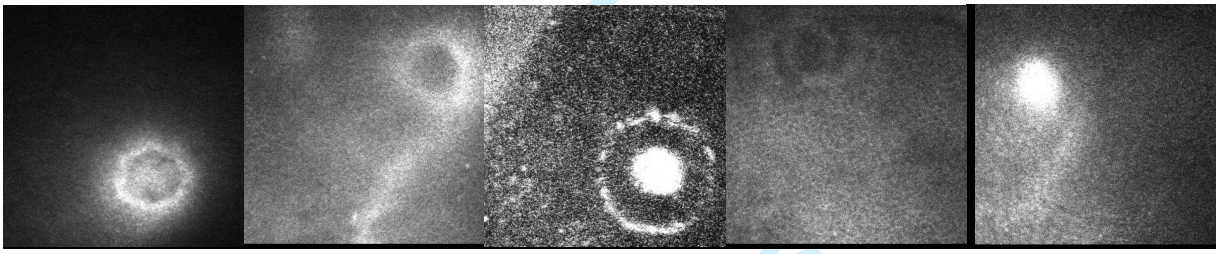
Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.

Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.

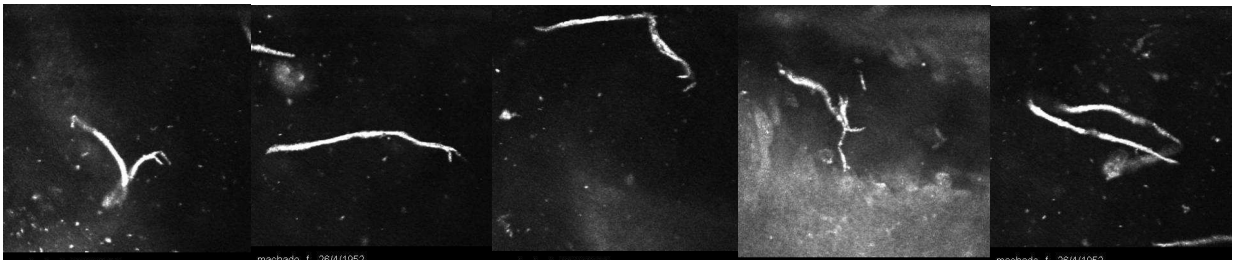
PICTURES:



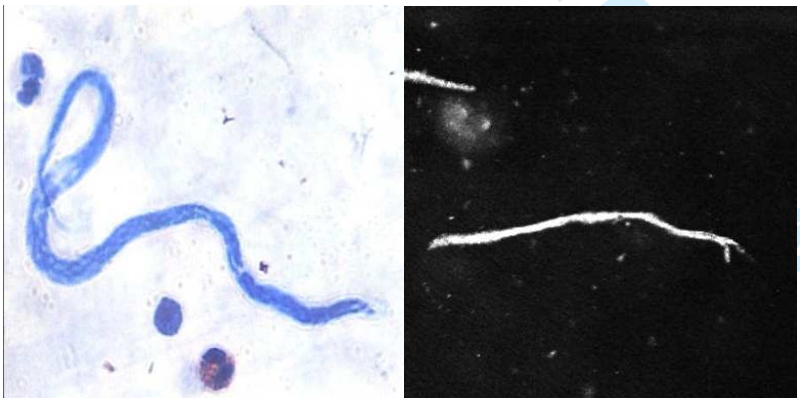
Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.



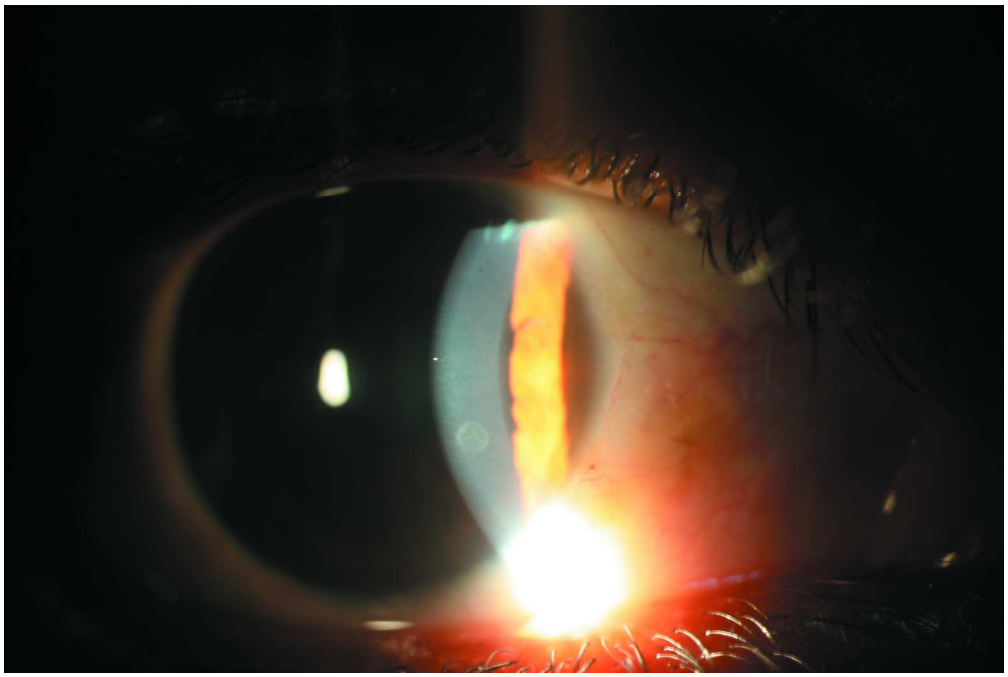
Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.



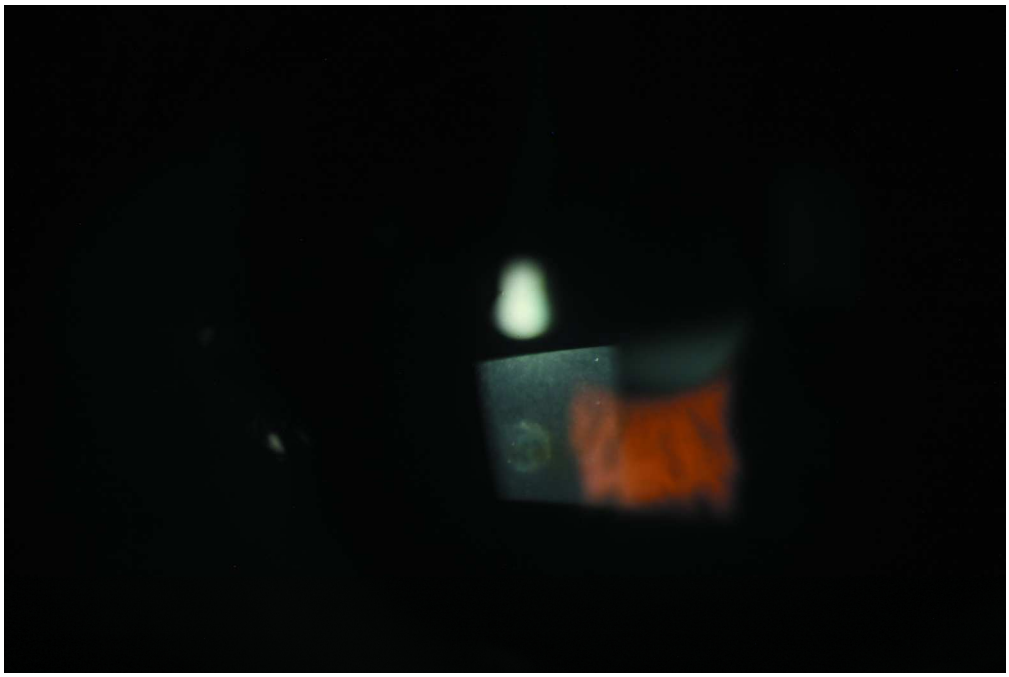
Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.



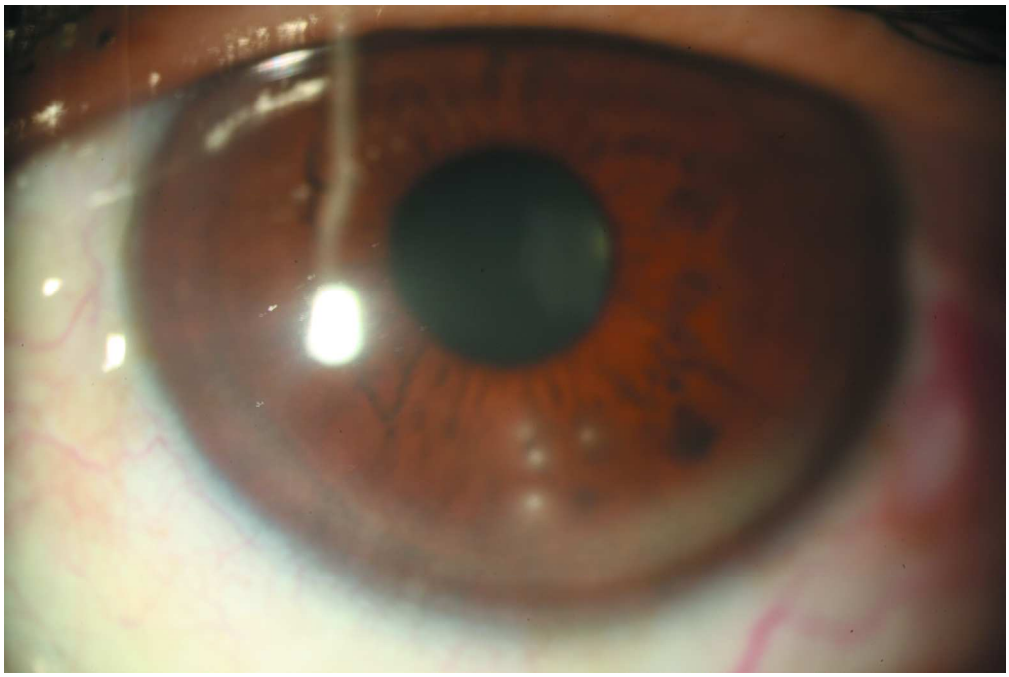
Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.



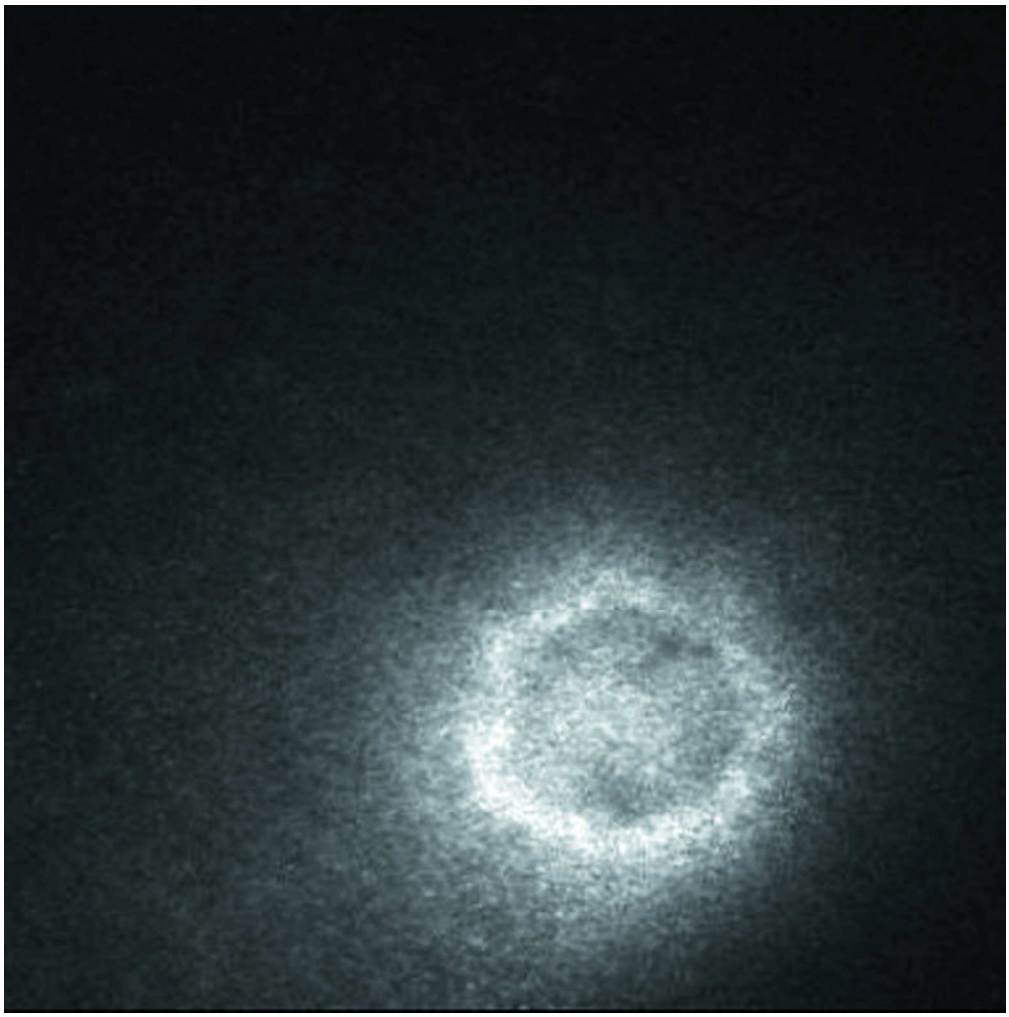
199x133mm (300 x 300 DPI)



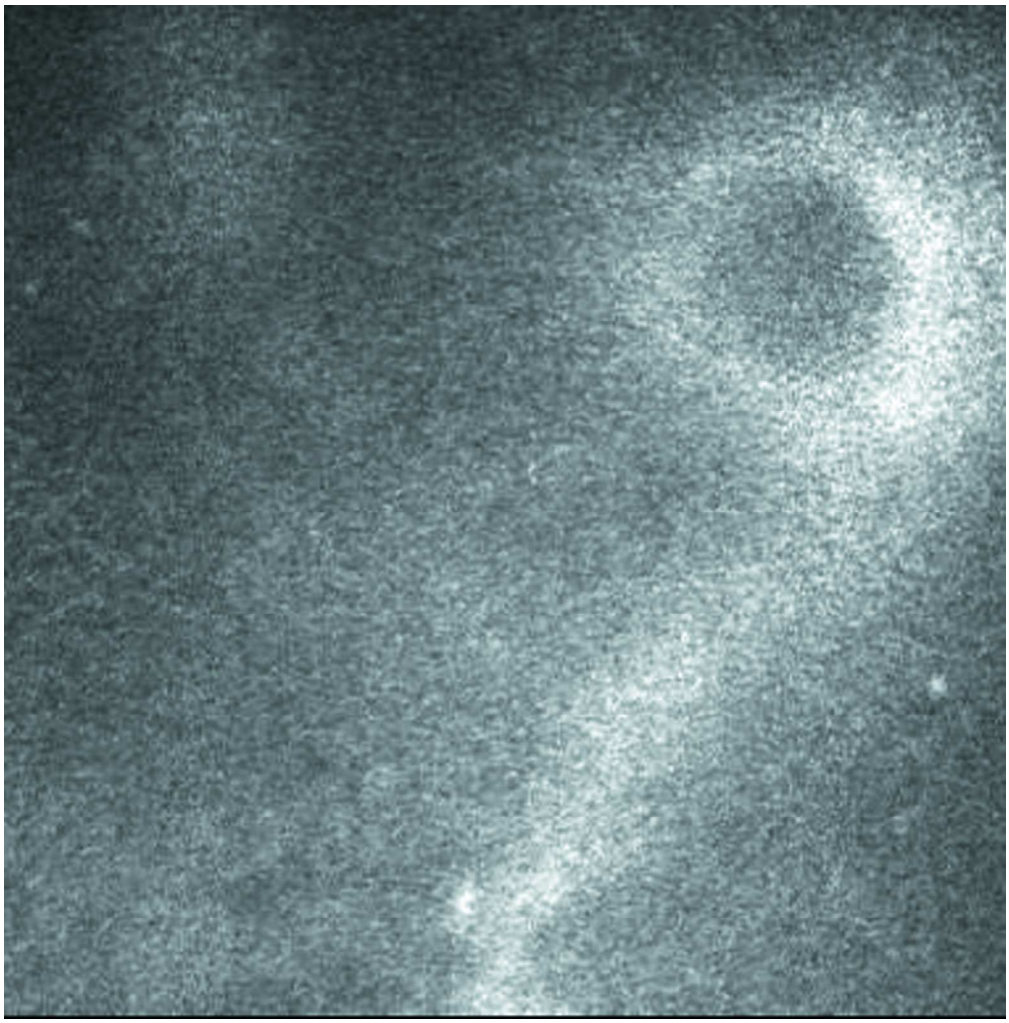
199x133mm (300 x 300 DPI)



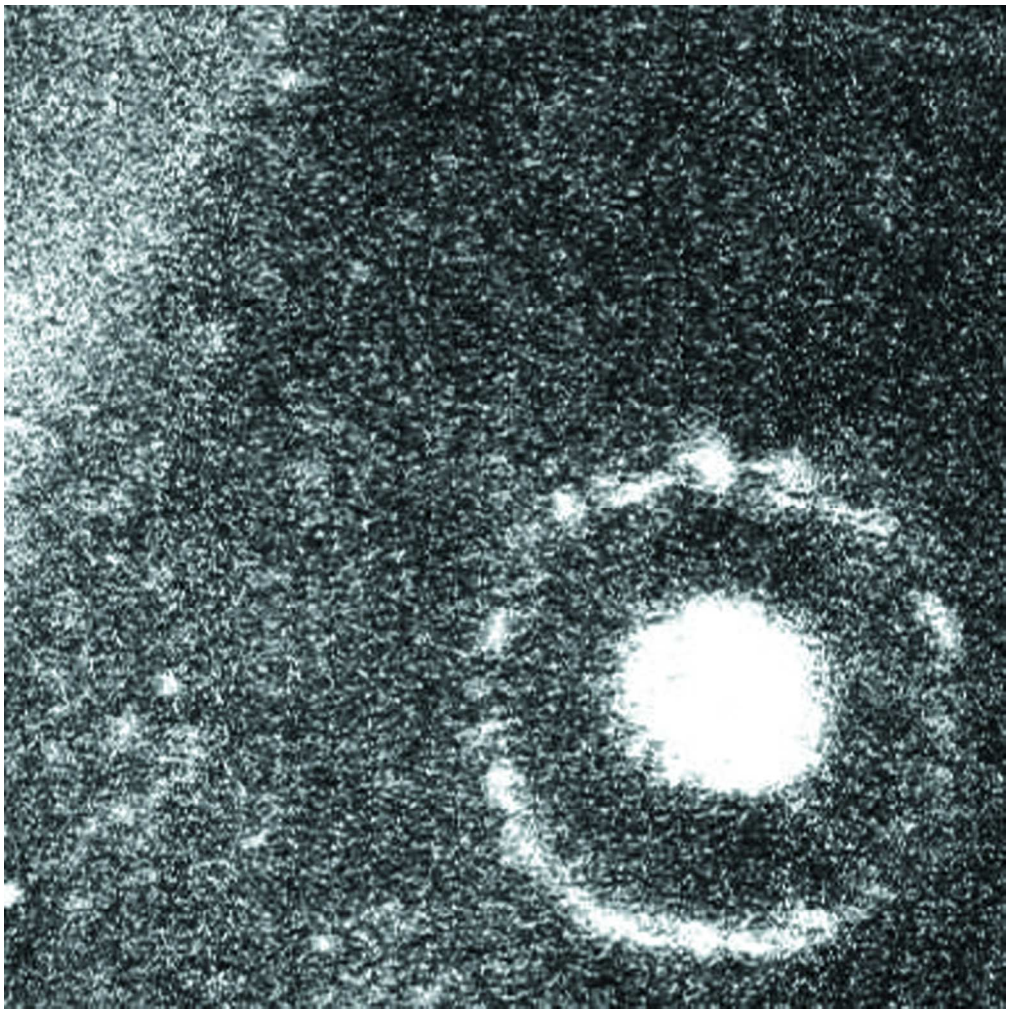
199x133mm (300 x 300 DPI)



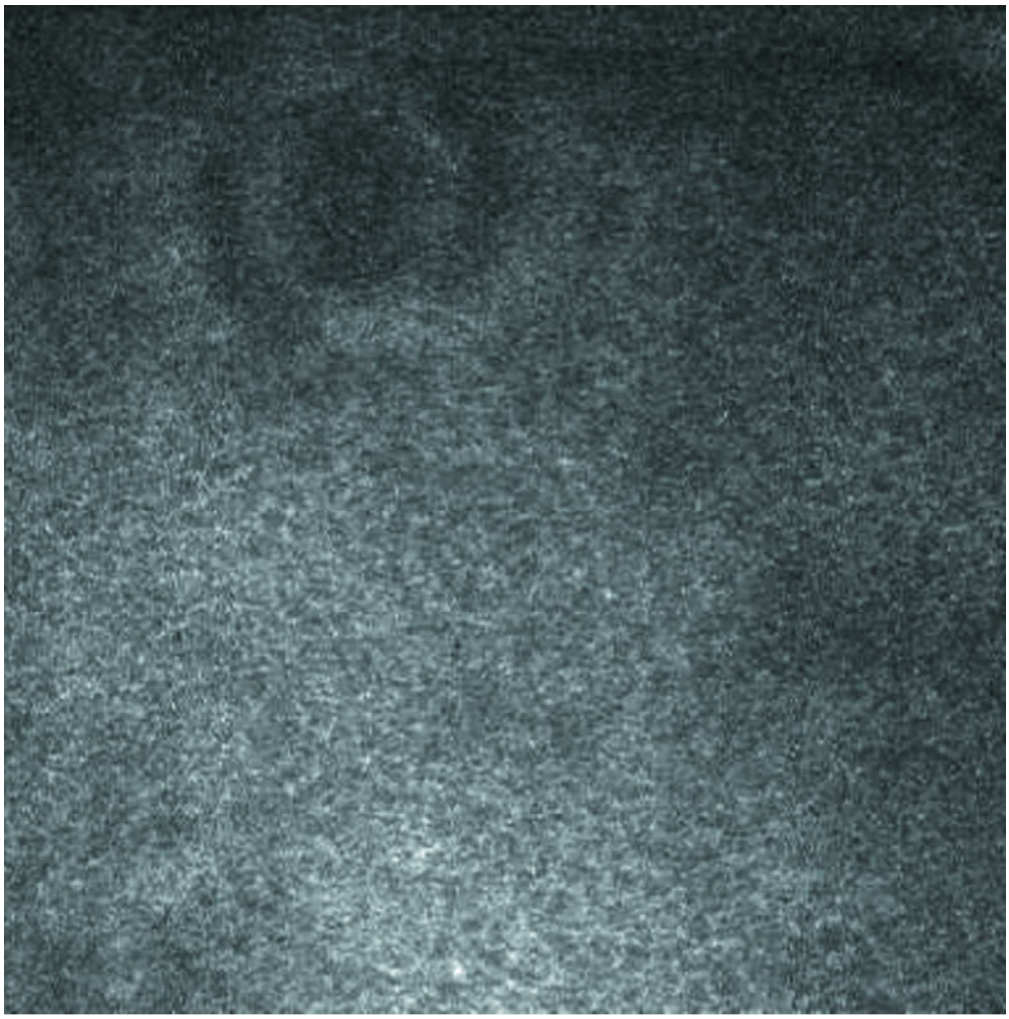
129x130mm (300 x 300 DPI)



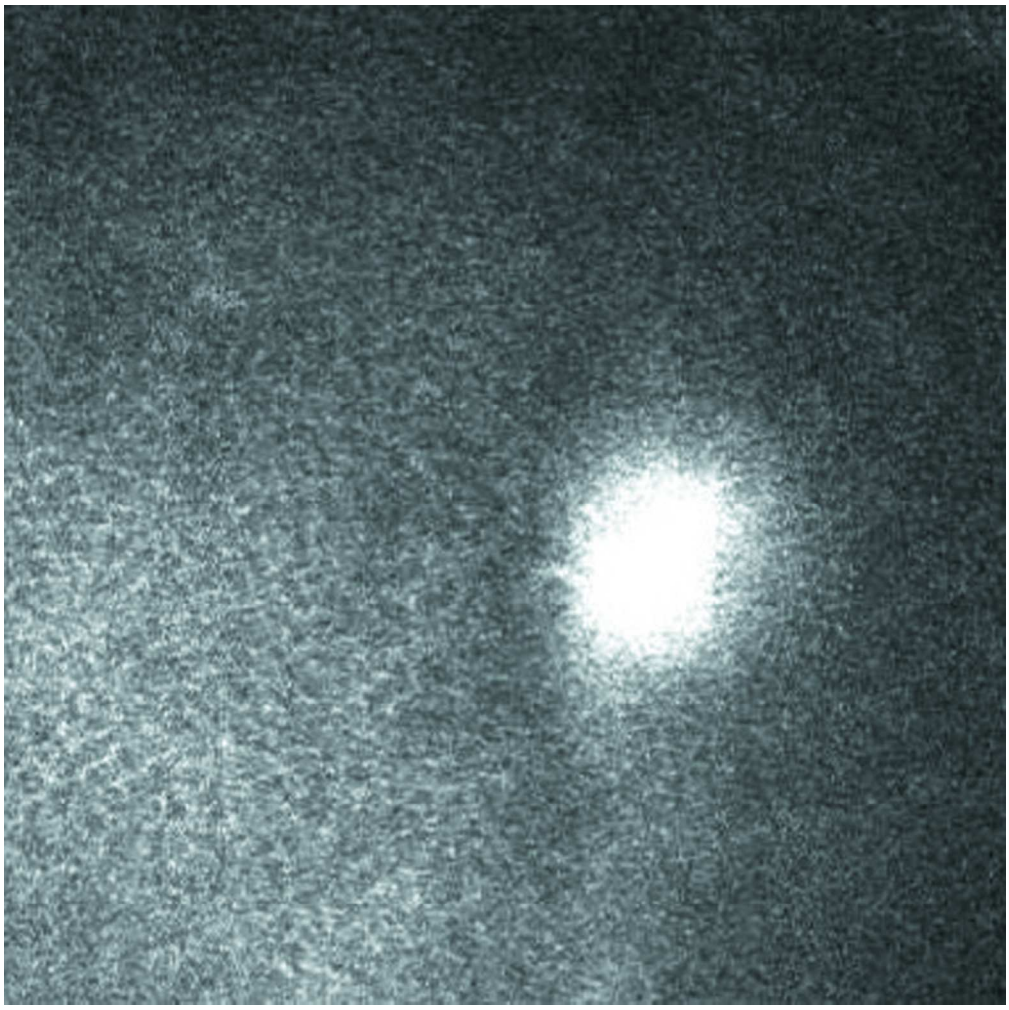
134x135mm (300 x 300 DPI)



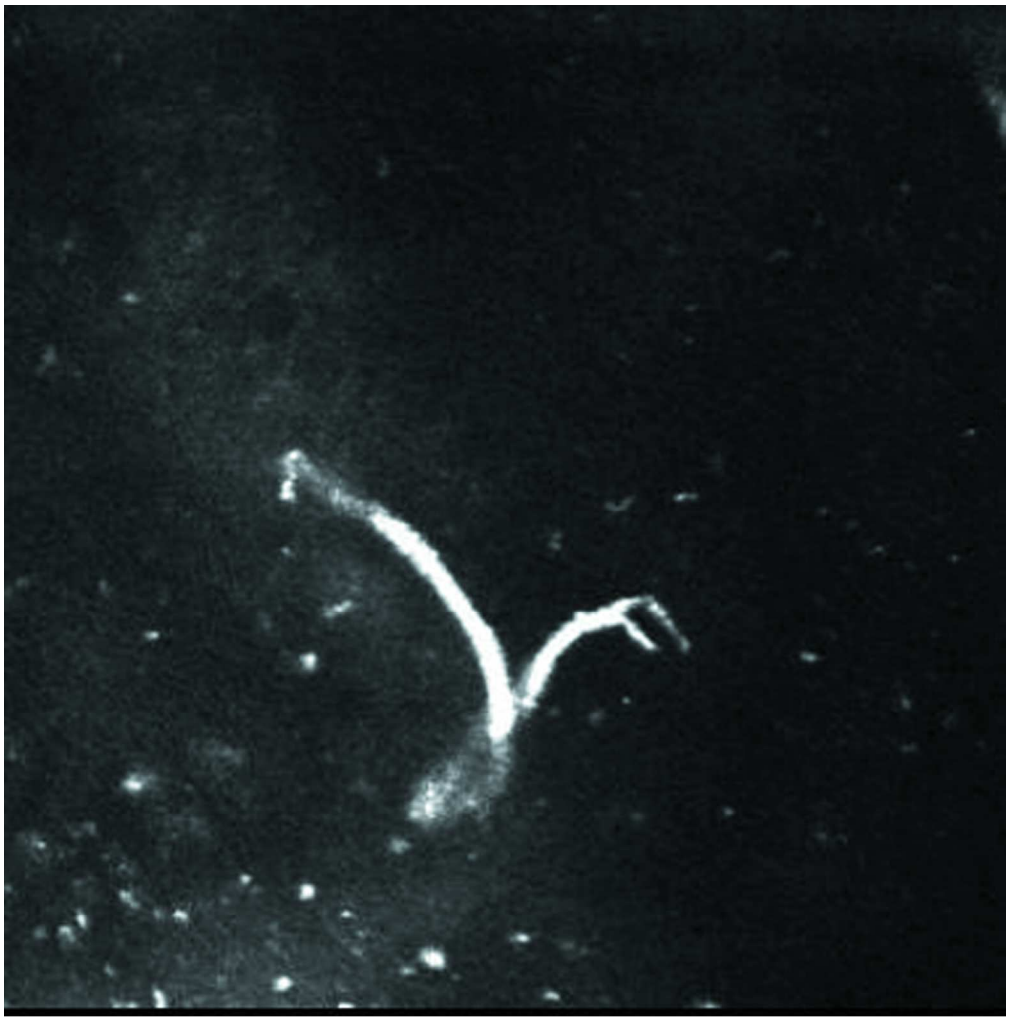
134x135mm (300 x 300 DPI)



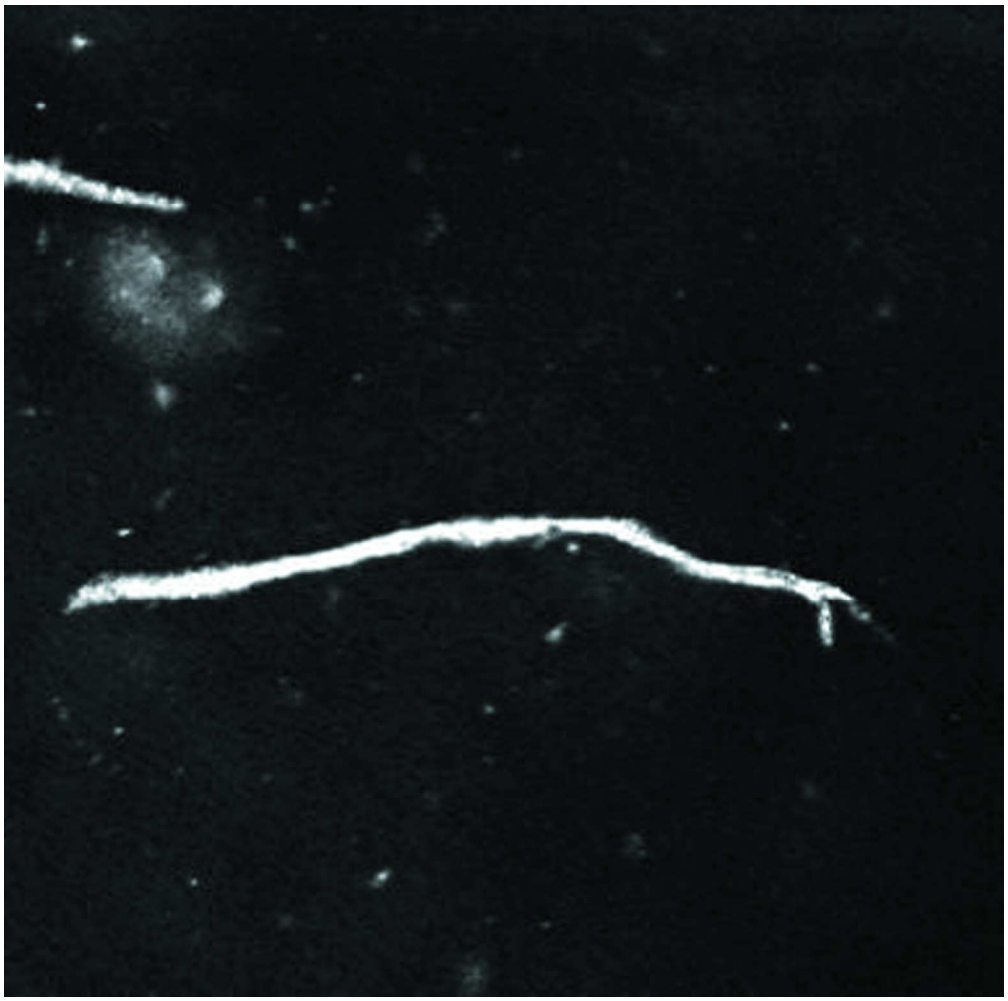
134x135mm (300 x 300 DPI)



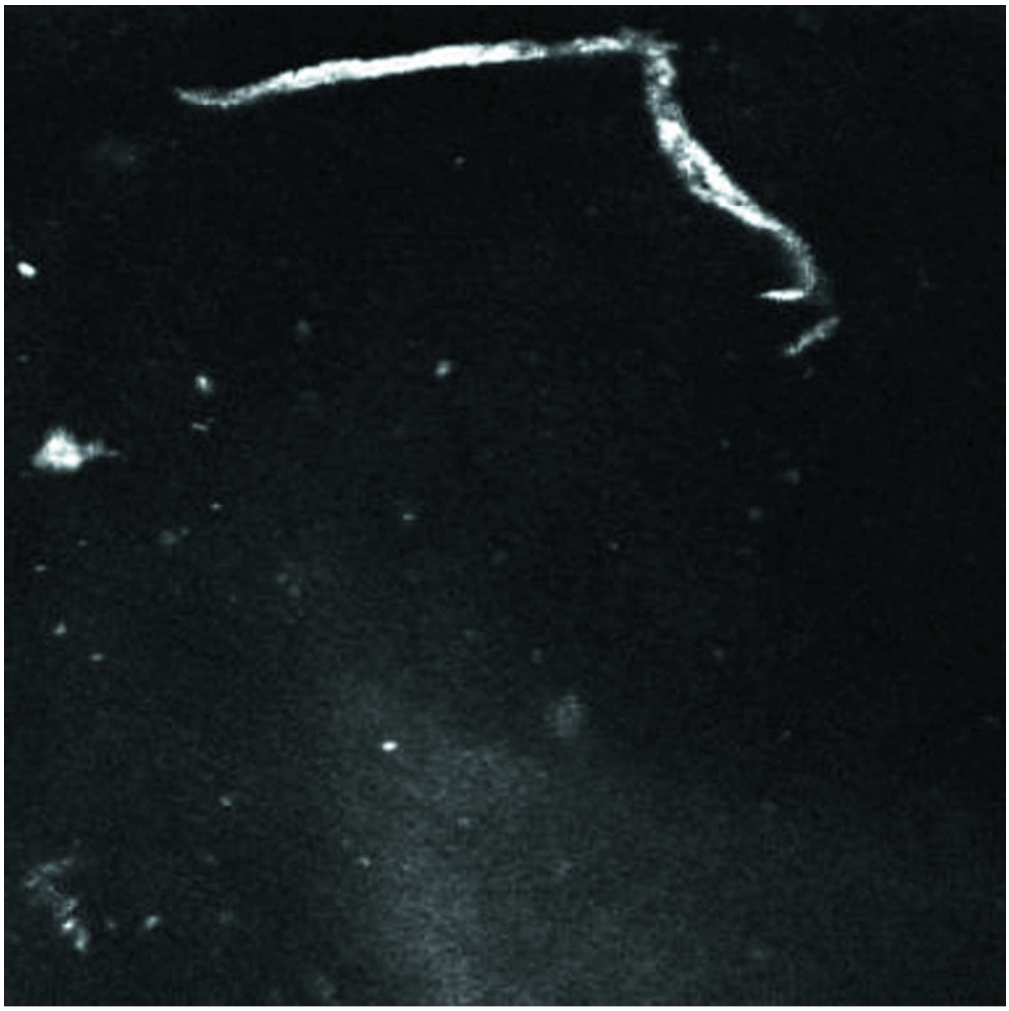
134x133mm (300 x 300 DPI)



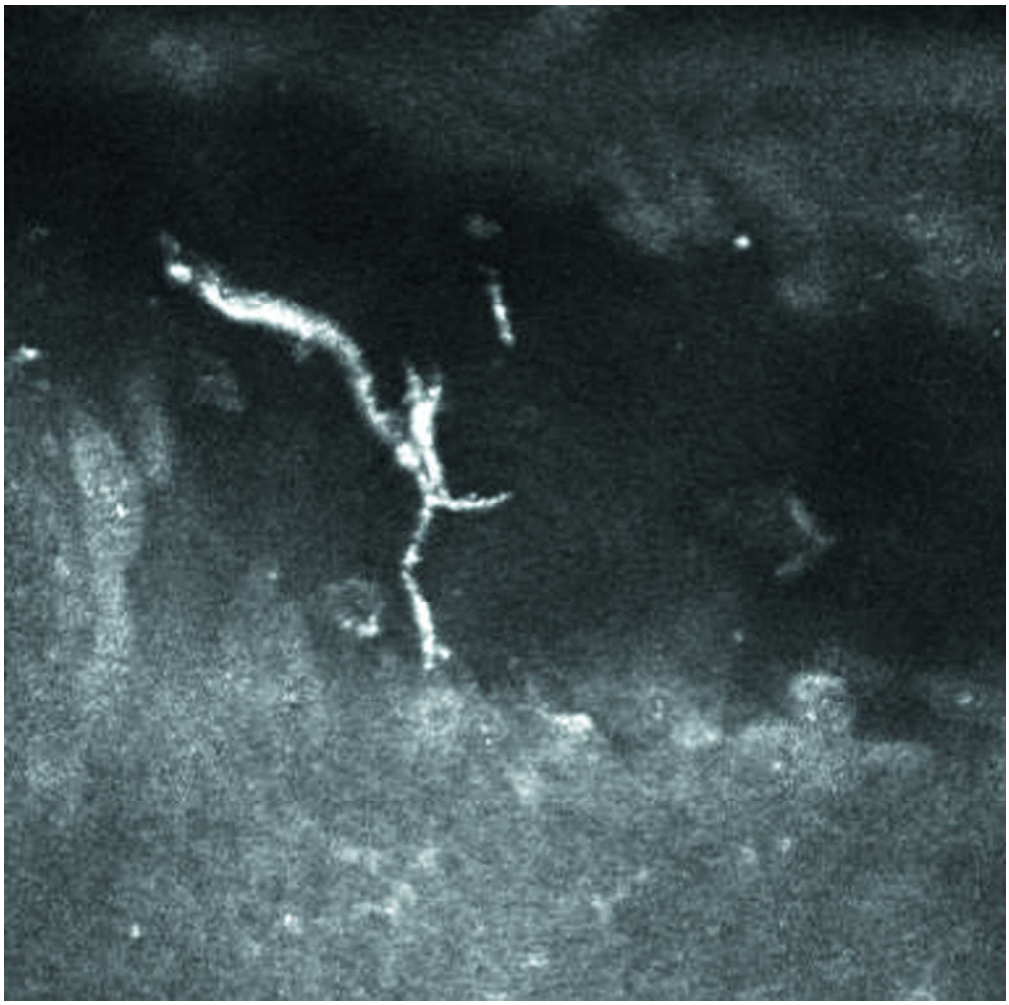
134x135mm (300 x 300 DPI)



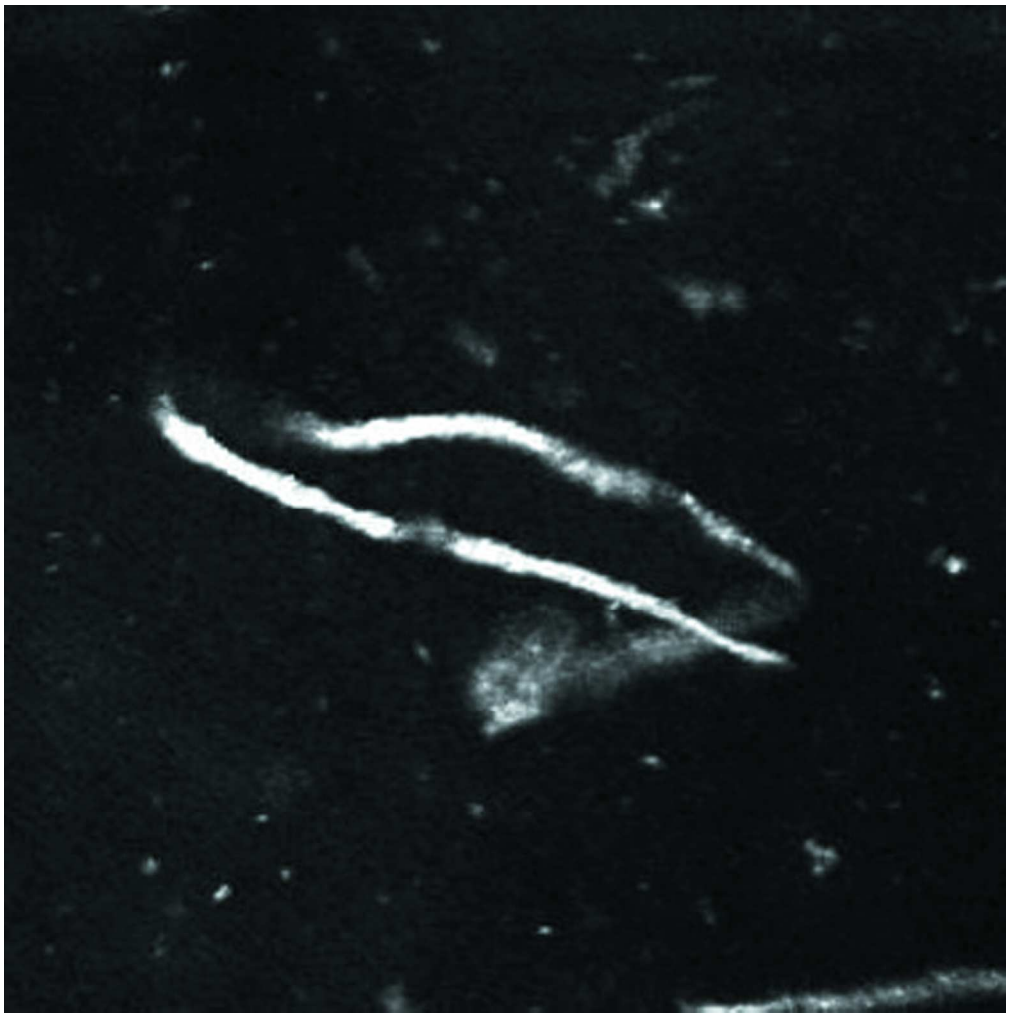
135x134mm (300 x 300 DPI)



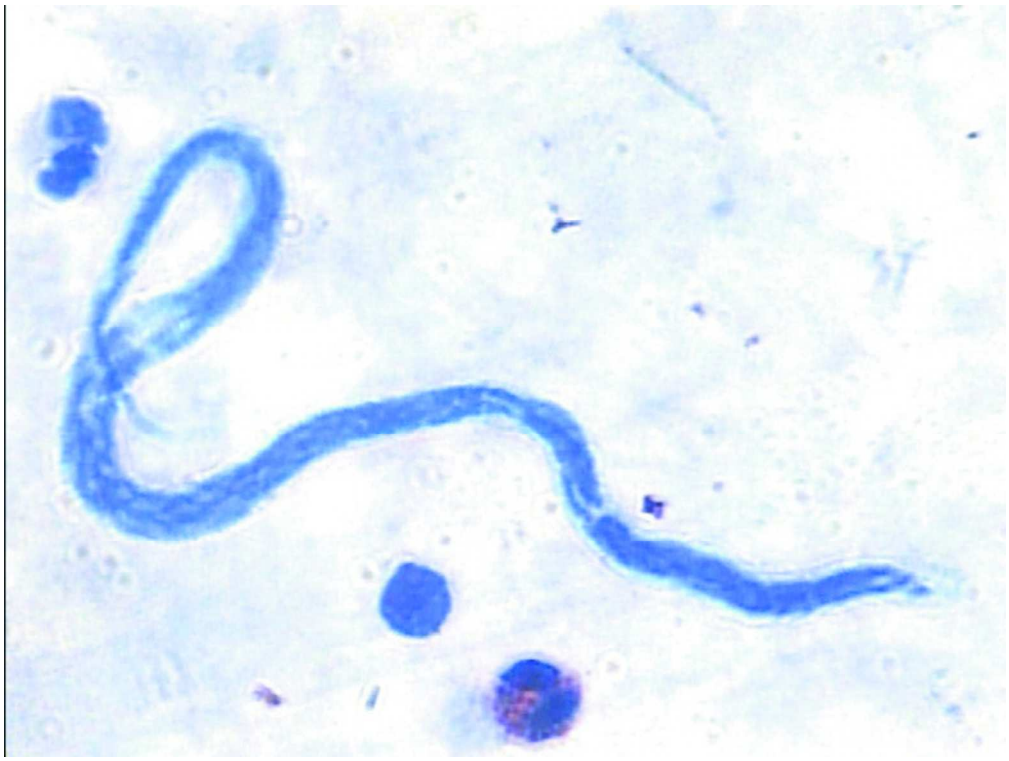
134x134mm (300 x 300 DPI)



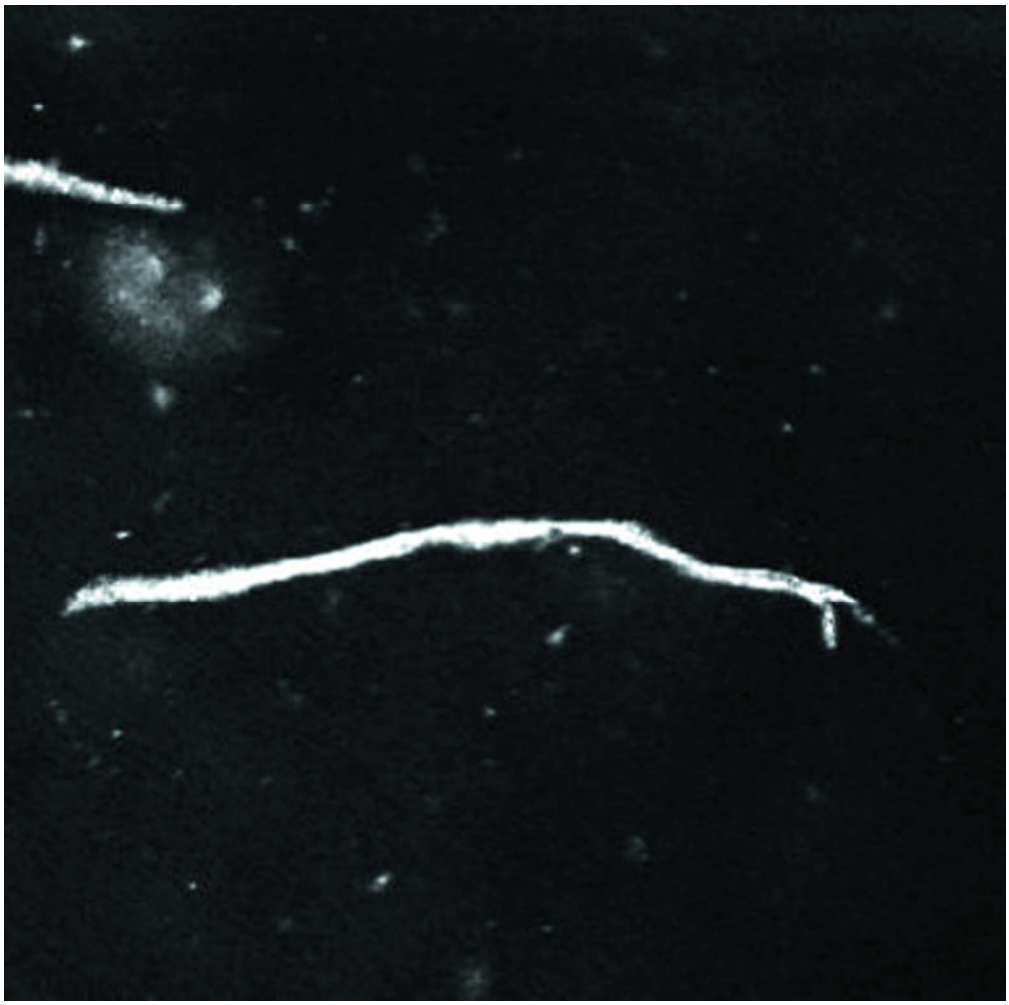
134x134mm (300 x 300 DPI)



132x133mm (300 x 300 DPI)



150x112mm (300 x 300 DPI)



134x134mm (300 x 300 DPI)

Competing Interests and Contributorship Statements:

***Article: Mansonella ozzardi* corneal lesions in the Amazon**

Authors: Lucas Monferrari Monteiro Vianna, Marilaine Martins, Marcos Jacob Cohen, Jacob Cohen, Rubens Belfort Jr

Statetment:

We state that none of us have competing interests.

We also state that all of us contributed in the conception and design of the study, acquisition, analysis and interpretation of data, drafting and revising of the article and the final approval of the version to be published.

BMJ open

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001266.R1
Article Type:	Research
Date Submitted by the Author:	23-Sep-2012
Complete List of Authors:	Vianna, Lucas; UNIFESP, Ophthalmology Martins, Marilaine; Fundação de Medicina Tropical do Amazonas, State University Amazonas, Parasithology Cohen, Jacob; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Cohen, Marcos; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Belfort Junior, Rubens; UNIFESP, Ophthalmology
Primary Subject Heading:	Addiction
Secondary Subject Heading:	Ophthalmology
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, PARASITOLOGY

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1) Title Page:

***Mansonella ozzardi* corneal lesions in the Amazon**

Authors: Lucas Monferrari Monteiro Vianna (first author)*; Marilaine Martins**; Marcos Jacob Cohen***, Jacob Cohen***, Rubens Belfort Jr*

*Vision Institute and Department of Ophthalmology. Hospital São Paulo - Federal University of São Paulo. Address: Botucatu St, 821 – ZIP: 04023-900 – São Paulo/SP - Brazil

** Fundação de Medicina Tropical do Amazonas, Universidade do Estado do Amazonas. Address: Pedro Teixeira Av., 25 - ZIP: 69040-000 - Manaus/AM - Brazil

*** Universidade Federal do Amazonas (UFAM) / Instituto de Oftalmologia de Manaus (IOM). Address: General Rodrigo Octávio Jordão Ramos Av, 3000, Campus Universitário, Coroado I - 69077-000 - Manaus/AM – Brazil

Keywords: Mansonella, microfilaria, keratitis, microscopy, confocal, biopsy

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010 (lucasmmvianna@yahoo.com.br)

***Mansonella ozzardi* corneal lesions in the Amazon**

Abstract:

Purpose: To characterize and confirm the presence of *M*ansonella *ozzardi* microfilariae in the cornea by biomicroscopy and corneal confocal microscopy.

Methods*:* Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil. All patients had the eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. Patients with suspicious corneal lesions (characterized as nummular keratitis) at the eye exam were submitted to biomicroscopy, fundoscopy and corneal confocal microscopy (CCME) evaluation. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

Results*:* Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. At biomicroscopy, lesions were divided in quiescent and active. At CCME, they were divided in circular and filiform lesions. The associations between lesions and microfilaremia are shown.

Conclusions*:* We presented some evidences of the presence of *M*. *ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

2) ***Main Text:***

Introduction

Hundreds of types of filariae have been described and a few can cause infections in humans, *Mansonella ozzardi* being one of them and the first description of *M. ozzardi* associated with corneal disease was published in 1998.[1]

M. ozzardi is a filaria exclusively found in America and one of the causes of mansonelliasis.[2] In Brazil it is found in the states of Roraima, Mato Grosso and Amazonas.[3,4,5] The microfilariae of *M. ozzardi* are found in peripheral blood and can be identified by their morphological characteristics or by molecular biology.[6,7]

The clinical features of mansonelliasis are not well described. Infected individuals are generally asymptomatic or have signs and symptoms common to other infections such as fever, coldness in the legs, joint pain and headache.[8] They may also include skin and ocular lesions[1,9,10] and be associated with *Wolbachiae,* that are bacterial endosymbionts of insects and many filarial nematodes and their products trigger inflammatory responses[10].

Coari City, at Solimões River (04°08'S63°07'W) has a high prevalence of *M. ozzardi* infection. Studies showed average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] Onchocerciasis does not exist in this region of the Amazon.[12]

Materials and Methods

Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil. All patients had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. The peripheral blood infection by *M. ozzardi* was determined by light-microscopy examination (200x and 400x) of Giemsa-stained thick smears of peripheral blood obtained by digital puncture with a sterile disposable lancet[6], Knott and polycarbonate membrane filtration and by a new identification protocol using the polymerase chain reaction (PCR) (M Martins et al., unpublished observations). Patients with nummular keratitis, similar to those previously described in the literature[1,9,10], at the eye examination were subjected to biomicroscopy, fundoscopy and corneal confocal microscopy evaluation (CCME), using the Rostock Cornea Module (RCM) of the Heidelberg Retina Tomograph. Photographs were taken at the slit lamp. The CCME was performed in the central cornea and in the corneal regions with the nummular keratitis. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

The study was prospective and approved by the UNIFESP Ethical Committee. All participants signed the protocol`s informed consent.

Data were presented in contingency tables and Fisher exact test was used to compare proportions. P-values less than 0.05 were considered statistically signficant. Analyses were done in Stata v.11 (College Station, Texas).

We have no competing interests.

Results

Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Four of them had bilateral lesions. The association between microfilaremia and cornea lesions is shown in table 1, with statistically significant finding.

At biomicroscopy, some lesions were characterized as nummular, mid periphery and anterior stroma corneal opacities, 0.5 to 1.0 mm in diameter with central mottled surrounded by a opaque halo, with marked limit between the white opacity and the clear cornea. Other lesions were characterized by nummular keratitis, also located at mid periphery and anterior stroma .In all cases there was a normal translucent area between the corneal lesions and the limbus and absence of corneal neovascularization or other changes (Picture 1). The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis. The association between nummular keratitis and microfilaremia is shown in table 2.

Among the 22 patients with corneal lesions at biomicroscopy, 12 presented suspected related lesions also at the CCME. These confocal suspected lesions was presented in two patterns: circular (increased reflectivity, measuring about 80 micra in diameter, located at the sub-epithelial level, as shown in picture 2) and linear (filiform lesions ranging from 250 to 300 µm in length and 10 to 15 µm wide, with one “C" shape end and the other tapered, as shown in picture 3). Five patients presented only circular

lesions, one patient only linear lesions and six patients had both of them. The association between confocal lesions and microfilaremia is shown in table 3.

In two volunteers patients with linear lesions, thick blood smear of the limbal conjunctiva was performed and confirmed the microfilaremia. Picture 4 compares microfilaria of *M. ozzardi* seen at the thick blood smear with the confocal microscopy image.

Acknowledgement for Funding/Support to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State) and for technical support for *Mansonella*-Brazil Group (Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T).

Discussion

The prevalence of *M. ozzardi* is high in some riverside communities in Amazon with the increasing occupation of rainforest areas.[2,12] Previous studies in Coari city showed an average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] In the present study, the prevalence was of 26,4%.

Presumed keratitis caused by *M. ozzardi* has been described in Brazil by us as well as other authors, with no other defined etiology related.[1,9,10] A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is no onchocerchiasis in those parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only

close to the Venezuela border, in the Yanomami Indian Reservation.[13,14] None of the patients lived or visited areas where Onchocerca exist.

A study conducted in Pauiní, at the Purus River revealed 20 non-Indian habitants with peripheral corneal opacities among 524 patients examined. No microfilariae were seen in the skin snips and *Mansonella sp* was found in the blood of the 2 patients where it was possible to collect blood.[1] Another study, conducted in São Gabriel da Cachoeira (Negro River) showed a positive association between *M. ozzardi* and corneal lesions with all patients presenting ocular changes having *M. ozzardi* microfilariae in blood samples.[9] *All* the skin biopsies were negative for microfilaria including *Onchocerca*. The association between the keratitis and positive microfilaremia in the Amazon was also related by Cohen et al.[10] In our study, this association was also found, with statistically significant finding, as shown in table 1.

The aspect of corneal lesions was divided by its characteristics in only corneal opacities and keratitis, as shown in picture 1. Among patients positive for microfilaremia, the proportion of keratitis aspect seems to be higher and among patients negative for microfilaria, the proportion of only opacity aspect was higher (table 2). One explanation for the presence of patients with keratitis lesion and negative microfilaremia could be false negatives in the blood tests. Patients with only opacity lesion and positive microfilaremia could be explained by chronic infection.

Like in previous studies,[1,9,10] we could not detect, by biomicroscopy, filariae in any ocular tissue, but corneal lesions similar to previous descriptions were found. Using CCME, we detected not previously described lesions. Among patients positive for microfilaremia, the proportion of confocal lesions was higher (table 3). The confocal

lesions were divided by their aspects in linear and circular. Among patients with lesions identified by CCME, there was a higher proportion of patients positive for microfilaremia and a higher proportion of circular aspect alone among patients negative for microfilaremia (table 3). It could suggest that linear lesions are associated with active disease and circular lesions with inflammatory scars, associated or not with active disease. The size of linear lesions (250-300 microns) on CCME was higher than showed in microbiological studies (149-240 microns)[6]. The microfilariae are probably dead in ocular tissues and could enlarge due to inflammatory reaction. Circular lesions could represent, besides inflammatory reaction surrounding dead microfilariae, cross section of remnants of adult worms (26 to 49 mm in length by 00.7 to 0.15 mm in diameter)[6].

Unaffected patients were not tested due to difficulties of field work. We performed confocal microscopy in both eyes of all the 22 patients with keratitis. In the 18 with unilateral keratitis, the non affected fellow eye never showed alterations in the confocal microscopy.

Conclusion

We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

4) Tables:

Table 1. Association between microfilaremia and corneal lesions.

Corneal Lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	14 (63,6%)	42 (22%)	56
Negative (-)	8 (33,4%)	148 (88%)	156
Total	22	190	212 (100%)

p-value: 0.0001

Table 2. Association between nummular keratitis and microfilaremia.

Keratitis lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	10 (76,9%)	4 (44%)	14
Negative (-)	3 (23,1%)	5 (56%)	8
Total	13	9	22 (100%)

p-value: 0.1347

Table 3. Association between confocal lesions and microfilaremia.

Confocal lesion (horizontal)	Presence (+)	Absence	Total
Microfilaremia (vertical)	**% Circular / Linear / Both**	**(-)**	
Positive (+)	2 (25%) / 1 (12,5%) / 5 (62,5%)	6	14
Negative (-)	3 (75%) / 0 / 1 (25%)	4	8
	12	10	22

p-value: 0.4266

5) Acknowledgments:

Funding/Support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Mansonella-Brazil Group: Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T

6) **Competing Interests Statement:**

We state that none of the authors have competing interests.

7) Contributorship Statement:

We state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

8) *References*:

1. Branco BC, Chamon W, Belfort R et al. Ocular findings in Pauiní (Southwest of the Brazilian Amazon) and possible corneal lesions caused by Mansonella. Arq Bras Oftalmol. 1998; Vol 61(6): 647-82.

2. Martins M, Pessoa FA, de Medeiros MB, et al. Mansonella ozzardi in Amazonas, Brazil: prevalence and distribution in the municipality of Coari, in the middle Solimões River. Mem Inst Oswaldo Cruz. 2010 May;105(3):246-53.

3. Deane MP. Sobre a incidência de filárias humanas em Manaus, estado do Amazonas. *Rev Serv Esp Saude Publ* 1949 *2*: 849-858.

4. Oliveira wr. [Filarial infestation in inhabitants of Vila Pereira, territory of Roraima (Brazil)]. Rev Inst Med Trop Sao Paulo. 1963 Nov-Dec;5:287-8.

5. D'Andretta Jr, Pio da Silva CM, Kameyana F. Ocorrência da mansonelose entre índios do alto Xingu. *Rev Soc Bras Med Trop* 1969. *3*:11.

6. Orihel TC, Eberhard ML. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. Am J Trop Med Hyg. 1982 Nov;31(6):1142-7.

7. Morales-Hojas R, Post RJ, Shelley AJ, et al. Characterisation of nuclear ribosomal DNA sequences from Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea) and development of a PCR-based method for their

detection in skin biopsies. Int J Parasitol. 2001 Feb;31(2):169-77. Erratum in: Int J Parasitol 2001 Jun;31(8):850-1

8. BATISTA D, CERQUEIRA NL, MORAES MA. [Epidemiology of Manson's disease in a locality in the interior of Amazonas]. Rev Assoc Med Bras. 1960 Jun;6:176-84.

9. Garrido C, Campos M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. Cornea. 2000 Nov;19(6):817-9.

10. Cohen JM, Ribeiro JA, Martins M. [Ocular manifestations in mansonelliasis]. Arq Bras Oftalmol. 2008 Mar-Apr;71(2):167-71. Portuguese.

11. Keiser PB, Coulibaly Y, Kubofcik J, et al. Molecular identification of Wolbachia from the filarial nematode Mansonella perstans. Mol Biochem Parasitol. 2008 Aug;160(2):123-8. Epub 2008 May 2. PubMed PMID: 18538871; PubMed Central PMCID: PMC2497444.

12. Medeiros JF, Py-Daniel V, Barbosa UC, et al. [Occurrence of Mansonella ozzardi (Nematoda, Onchocercidae) in riverine communities of the Purus river, Boca do Acre municipality, Amazonas State, Brazil]. Cad Saude Publica. 2009 Jun;25(6):1421-6.

13. Belfort Jr, R e Moraes, M – Oncocercose ocular no Brasil. AMB Rev Assoc Med Bras. 1979 Apr;25(4):123-7.

14. The Onchocerciasis Elimination Program Sauerbrey for the Americas (OEPA) M. Annals of Tropical Medicine & Parasitology, Vol 102, Supplement No. 1 , S25-S29 (2008)

9) Figure Legends:

Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.

Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.

Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.

Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.

1) Title Page:

Mansonella ozzardi corneal lesions in the Amazon

Authors: Lucas Monferrari Monteiro Vianna (first author)*; Marilaine Martins**; Marcos Jacob Cohen***, Jacob Cohen***, Rubens Belfort Jr*

*Vision Institute and Department of Ophthalmology. Hospital São Paulo - Federal University of São Paulo. Address: Botucatu St, 821 – ZIP: 04023-900 – São Paulo/SP - Brazil

** Fundação de Medicina Tropical do Amazonas, Universidade do Estado do Amazonas. Address: Pedro Teixeira Av., 25 - ZIP: 69040-000 - Manaus/AM - Brazil

*** Universidade Federal do Amazonas (UFAM) / Instituto de Oftalmologia de Manaus (IOM). Address: General Rodrigo Octávio Jordão Ramos Av, 3000, Campus Universitário, Coroado I - 69077-000 - Manaus/AM – Brazil

Keywords: Mansonella, microfilaria, keratitis, microscopy, confocal, biopsy

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010 (lucasmmvianna@yahoo.com.br)

***Mansonella ozzardi* corneal lesions in the Amazon**

Abstract:

Purpose: To characterize and confirm the presence of *M*ansonella *ozzardi* microfilariae in the cornea by biomicroscopy and corneal confocal microscopy.

Methods*:* Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil. All patients had the eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. Patients with suspicious corneal lesions (characterized as nummular keratitis) at the eye exam were submitted to biomicroscopy, fundoscopy and corneal confocal microscopy (CCME) evaluation. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

Results*:* Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. At biomicroscopy, lesions were divided in quiescent and active. At CCME, they were divided in circular and filiform lesions. The associations between lesions and microfilaremia are shown.

Conclusions*:* We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

2) ***Main Text:***

Introduction

Hundreds of types of filariae have been described and a few can cause infections in humans, *Mansonella ozzardi* being one of them and the first description of *M. ozzardi* associated with corneal disease was published in 1998.[1]

M. ozzardi is a filaria exclusively found in America and one of the causes of mansonelliasis.[2] In Brazil it is found in the states of Roraima, Mato Grosso and Amazonas.[3,4,5] The microfilariae of *M. ozzardi* are found in peripheral blood and can be identified by their morphological characteristics or by molecular biology.[6,7]

The clinical features of mansonelliasis are not well described. Infected individuals are generally asymptomatic or have signs and symptoms common to other infections such as fever, coldness in the legs, joint pain and headache.[8] They may also include skin and ocular lesions[1,9,10] and be associated with *Wolbachiae,* that are bacterial endosymbionts of insects and many filarial nematodes and their products trigger inflammatory responses[10].

Coari City, at Solimões River (04°08'S63°07'W) has a high prevalence of *M. ozzardi* infection. Studies showed average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] Onchocerciasis does not exist in this region of the Amazon.[12]

Materials and Methods

Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil. All patients had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. The peripheral blood infection by *M. ozzardi* was determined by light-microscopy examination (200x and 400x) of Giemsa-stained thick smears of peripheral blood obtained by digital puncture with a sterile disposable lancet[6], Knott and polycarbonate membrane filtration and by a new identification protocol using the polymerase chain reaction (PCR) (M Martins et al., unpublished observations). Patients with nummular keratitis, similar to those previously described in the literature[1,9,10], at the eye examination were subjected to biomicroscopy, fundoscopy and corneal confocal microscopy evaluation (CCME), using the Rostock Cornea Module (RCM) of the Heidelberg Retina Tomograph. Photographs were taken at the slit lamp. The CCME was performed in the central cornea and in the corneal regions with the nummular keratitis. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

The study was prospective and approved by the UNIFESP Ethical Committee. All participants signed the protocol`s informed consent.

Data were presented in contingency tables and Fisher exact test was used to compare proportions. P-values less than 0.05 were considered statistically signficant. Analyses were done in Stata v.11 (College Station, Texas).

We have no competing interests.

Results

Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Four of them had bilateral lesions. The association between microfilaremia and cornea lesions is shown in table 1, with statistically significant finding.

At biomicroscopy, some lesions were characterized as nummular, mid periphery and anterior stroma corneal opacities, 0.5 to 1.0 mm in diameter with central mottled surrounded by a opaque halo, with marked limit between the white opacity and the clear cornea. Other lesions were characterized by nummular keratitis, also located at mid periphery and anterior stroma .In all cases there was a normal translucent area between the corneal lesions and the limbus and absence of corneal neovascularization or other changes (Picture 1). The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis. The association between nummular keratitis and microfilaremia is shown in table 2.

Among the 22 patients with corneal lesions at biomicroscopy, 12 presented suspected related lesions also at the CCME. These confocal suspected lesions was presented in two patterns: circular (increased reflectivity, measuring about 80 micra in diameter, located at the sub-epithelial level, as shown in picture 2) and linear (filiform lesions ranging from 250 to 300 µm in length and 10 to 15 µm wide, with one “C" shape end and the other tapered, as shown in picture 3). Five patients presented only circular

lesions, one patient only linear lesions and six patients had both of them. The association between confocal lesions and microfilaremia is shown in table 3.

In two volunteers patients with linear lesions, thick blood smear of the limbal conjunctiva was performed and confirmed the microfilaremia. Picture 4 compares microfilaria of *M. ozzardi* seen at the thick blood smear with the confocal microscopy image.

Acknowledgement for Funding/Support to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State) and for technical support for *Mansonella*-Brazil Group (Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T).

Discussion

The prevalence of *M. ozzardi* is high in some riverside communities in Amazon with the increasing occupation of rainforest areas.[2,12] Previous studies in Coari city showed an average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2, 10] In the present study, the prevalence was of 26,4%.

Presumed keratitis caused by *M. ozzardi* has been described in Brazil by us as well as other authors, with no other defined etiology related.[1,9,10] A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is no onchocerchiasis in those parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only

close to the Venezuela border, in the Yanomami Indian Reservation.[13,14] None of the patients lived or visited areas where Onchocerca exist.

A study conducted in Pauiní, at the Purus River revealed 20 non-Indian habitants with peripheral corneal opacities among 524 patients examined. No microfilariae were seen in the skin snips and *Mansonella sp* was found in the blood of the 2 patients where it was possible to collect blood.[1] Another study, conducted in São Gabriel da Cachoeira (Negro River) showed a positive association between *M. ozzardi* and corneal lesions with all patients presenting ocular changes having *M. ozzardi* microfilariae in blood samples.[9] *All* the skin biopsies were negative for microfilaria including *Onchocerca*. The association between the keratitis and positive microfilaremia in the Amazon was also related by Cohen et al.[10] In our study, this association was also found, with statistically significant finding, as shown in table 1.

The aspect of corneal lesions was divided by its characteristics in only corneal opacities and keratitis, as shown in picture 1. Among patients positive for microfilaremia, the proportion of keratitis aspect seems to be higher and among patients negative for microfilaria, the proportion of only opacity aspect was higher (table 2). One explanation for the presence of patients with keratitis lesion and negative microfilaremia could be false negatives in the blood tests. Patients with only opacity lesion and positive microfilaremia could be explained by chronic infection.

Like in previous studies,[1,9,10] we could not detect, by biomicroscopy, filariae in any ocular tissue, but corneal lesions similar to previous descriptions were found. Using CCME, we detected not previously described lesions. Among patients positive for microfilaremia, the proportion of confocal lesions was higher (table 3). The confocal

lesions were divided by their aspects in linear and circular. Among patients with lesions identified by CCME, there was a higher proportion of patients positive for microfilaremia and a higher proportion of circular aspect alone among patients negative for microfilaremia (table 3). It could suggest that linear lesions are associated with active disease and circular lesions with inflammatory scars, associated or not with active disease. The size of linear lesions (250-300 microns) on CCME was higher than showed in microbiological studies (149-240 microns)[6]. The microfilariae are probably dead in ocular tissues and could enlarge due to inflammatory reaction. Circular lesions could represent, besides inflammatory reaction surrounding dead microfilariae, cross section of remnants of adult worms (26 to 49 mm in length by 00.7 to 0.15 mm in diameter)[6].

Unaffected patients were not tested due to difficulties of field work. We performed confocal microscopy in both eyes of all the 22 patients with keratitis. In the 18 with unilateral keratitis, the non affected fellow eye never showed alterations in the confocal microscopy.

Conclusion

We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

4) Tables:

Table 1. Association between microfilaremia and corneal lesions.

Corneal Lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	14 (63,6%)	42 (22%)	56
Negative (-)	8 (33,4%)	148 (88%)	156
Total	22	190	212 (100%)

p-value: 0.0001

Table 2. Association between nummular keratitis and microfilaremia.

Keratitis lesions (horizontal) / Microfilaremia (vertical)	Presence (+)	Absence (-)	Total
Positive (+)	10 (76,9%)	4 (44%)	14
Negative (-)	3 (23,1%)	5 (56%)	8
Total	13	9	22 (100%)

p-value: 0.1347

Table 3. Association between confocal lesions and microfilaremia.

Confocal lesion (horizontal)	Presence (+)	Absence	Total
Microfilaremia (vertical)	**% Circular / Linear / Both**	**(-)**	
Positive (+)	2 (25%) / 1 (12,5%) / 5 (62,5%)	6	14
Negative (-)	3 (75%) / 0 / 1 (25%)	4	8
	12	10	22

p-value: 0.4266

5) Acknowledgments:

Funding/Support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Mansonella-Brazil Group: Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T

6) **Competing Interests Statement:**

We state that none of the authors have competing interests.

7) Contributorship Statement:

We state that all authors contributed in the:

1) Conception and design of the study;

2) Acquisition, analysis and interpretation of data;

3) Drafting and revising of the article;

4) Final approval of the version to be published.

8) *References*:

1. Branco BC, Chamon W, Belfort R et al. Ocular findings in Pauiní (Southwest of the Brazilian Amazon) and possible corneal lesions caused by Mansonella. Arq Bras Oftalmol. 1998; Vol 61(6): 647-82.

2. Martins M, Pessoa FA, de Medeiros MB, et al. Mansonella ozzardi in Amazonas, Brazil: prevalence and distribution in the municipality of Coari, in the middle Solimões River. Mem Inst Oswaldo Cruz. 2010 May;105(3):246-53.

3. Deane MP. Sobre a incidência de filárias humanas em Manaus, estado do Amazonas. *Rev Serv Esp Saude Publ* 1949 *2*: 849-858.

4. Oliveira wr. [Filarial infestation in inhabitants of Vila Pereira, territory of Roraima (Brazil)]. Rev Inst Med Trop Sao Paulo. 1963 Nov-Dec;5:287-8.

5. D'Andretta Jr, Pio da Silva CM, Kameyana F. Ocorrência da mansonelose entre índios do alto Xingu. *Rev Soc Bras Med Trop* 1969. *3*:11.

6. Orihel TC, Eberhard ML. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. Am J Trop Med Hyg. 1982 Nov;31(6):1142-7.

7. Morales-Hojas R, Post RJ, Shelley AJ, et al. Characterisation of nuclear ribosomal DNA sequences from Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea) and development of a PCR-based method for their

detection in skin biopsies. Int J Parasitol. 2001 Feb;31(2):169-77. Erratum in: Int J Parasitol 2001 Jun;31(8):850-1

8. BATISTA D, CERQUEIRA NL, MORAES MA. [Epidemiology of Manson's disease in a locality in the interior of Amazonas]. Rev Assoc Med Bras. 1960 Jun;6:176-84.

9. Garrido C, Campos M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. Cornea. 2000 Nov;19(6):817-9.

10. Cohen JM, Ribeiro JA, Martins M. [Ocular manifestations in mansonelliasis]. Arq Bras Oftalmol. 2008 Mar-Apr;71(2):167-71. Portuguese.

11. Keiser PB, Coulibaly Y, Kubofcik J, et al. Molecular identification of Wolbachia from the filarial nematode Mansonella perstans. Mol Biochem Parasitol. 2008 Aug;160(2):123-8. Epub 2008 May 2. PubMed PMID: 18538871; PubMed Central PMCID: PMC2497444.

12. Medeiros JF, Py-Daniel V, Barbosa UC, et al. [Occurrence of Mansonella ozzardi (Nematoda, Onchocercidae) in riverine communities of the Purus river, Boca do Acre municipality, Amazonas State, Brazil]. Cad Saude Publica. 2009 Jun;25(6):1421-6.

13. Belfort Jr, R e Moraes, M – Oncocercose ocular no Brasil. AMB Rev Assoc Med Bras. 1979 Apr;25(4):123-7.

14. The Onchocerciasis Elimination Program Sauerbrey for the Americas (OEPA) M. Annals of Tropical Medicine & Parasitology, Vol 102, Supplement No. 1 , S25-S29 (2008)

9) Figure Legends:

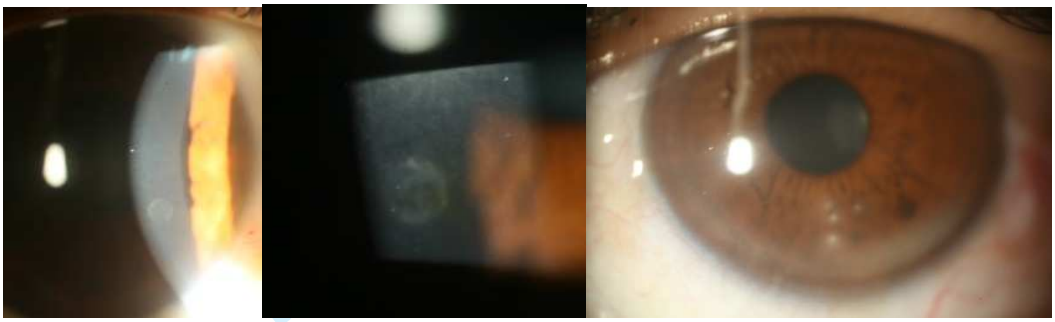
Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.

Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.

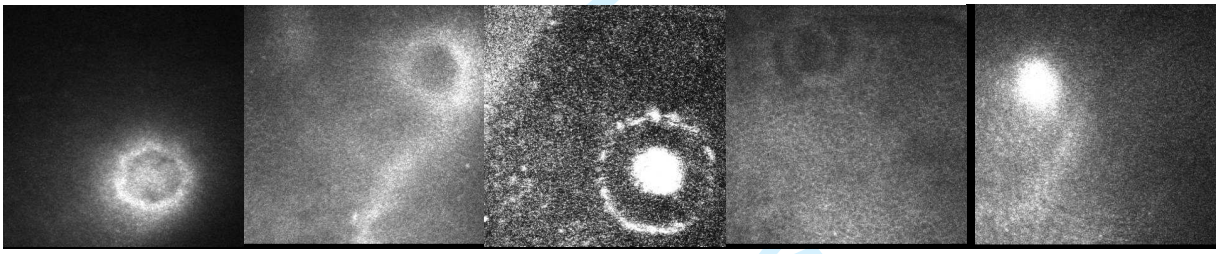
Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.

Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.

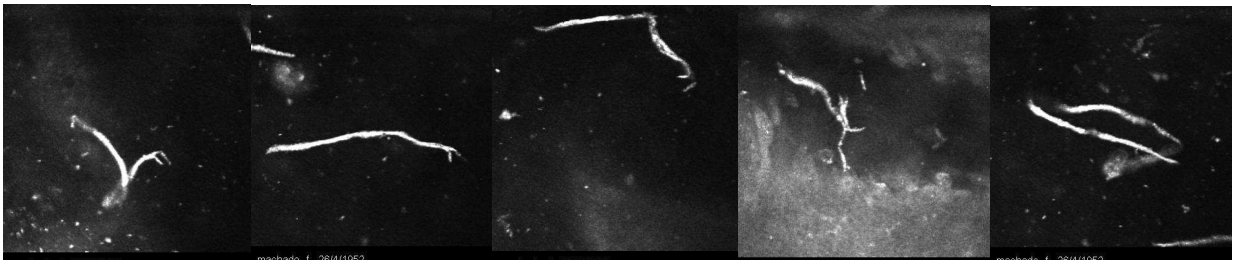
PICTURES:



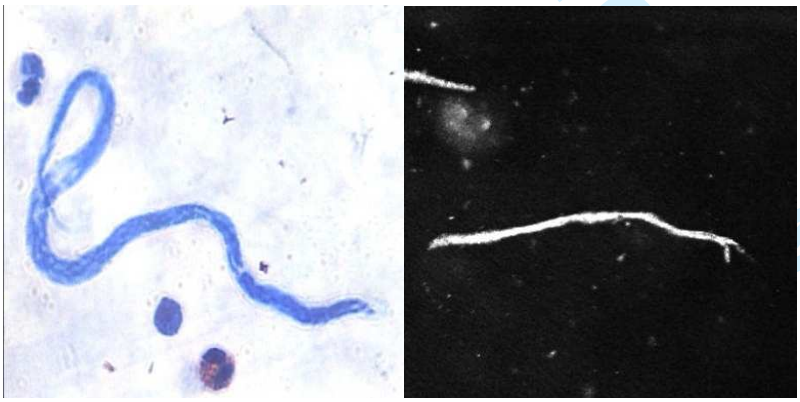
Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.



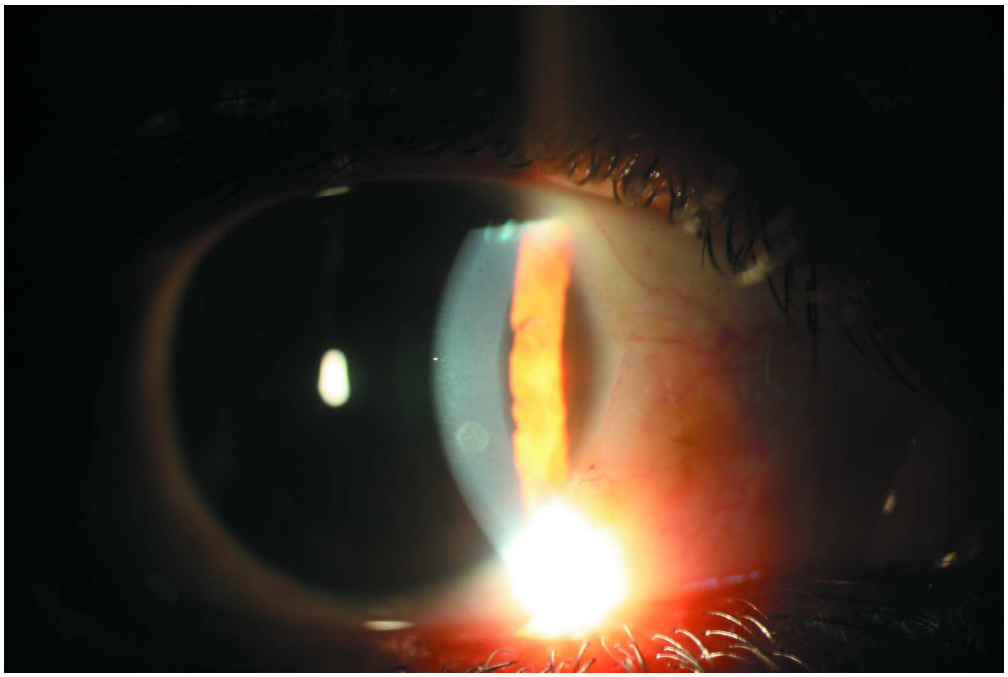
Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.



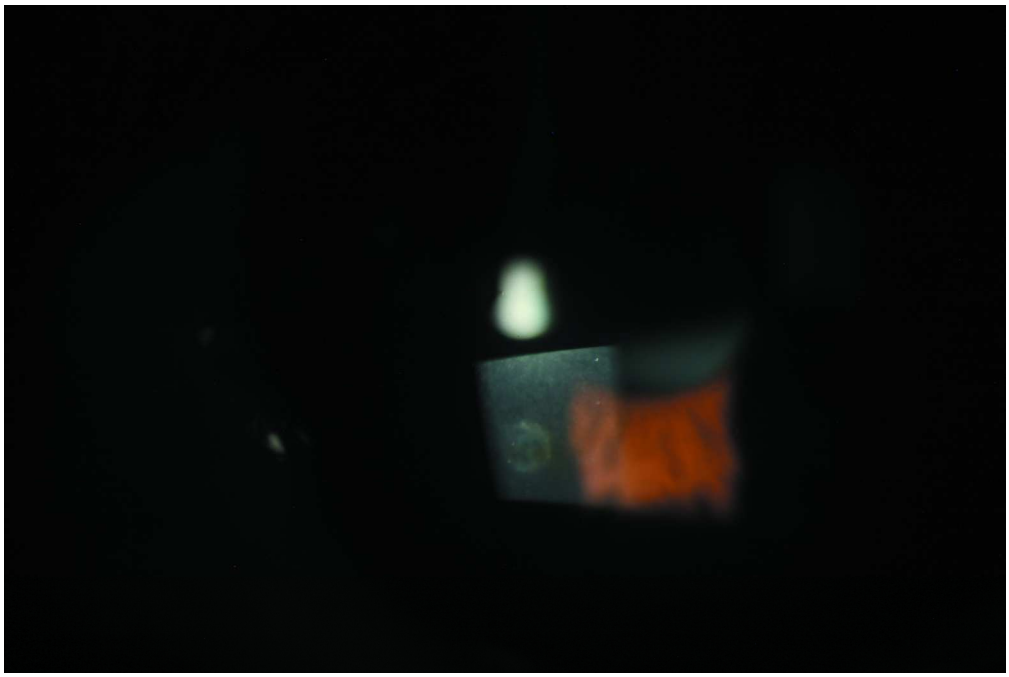
Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.



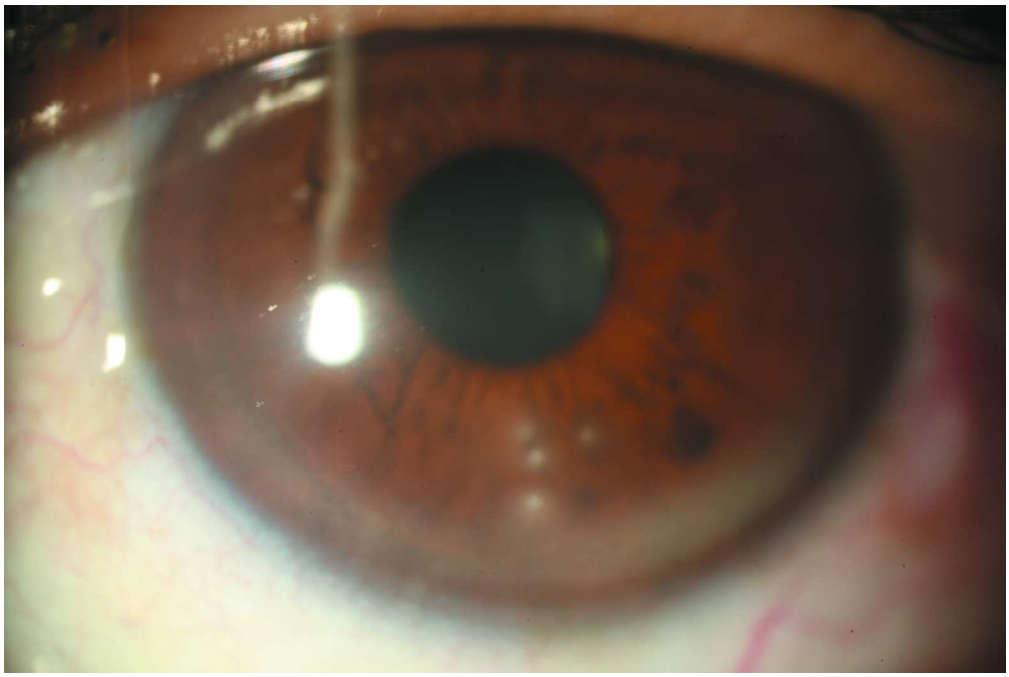
Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.



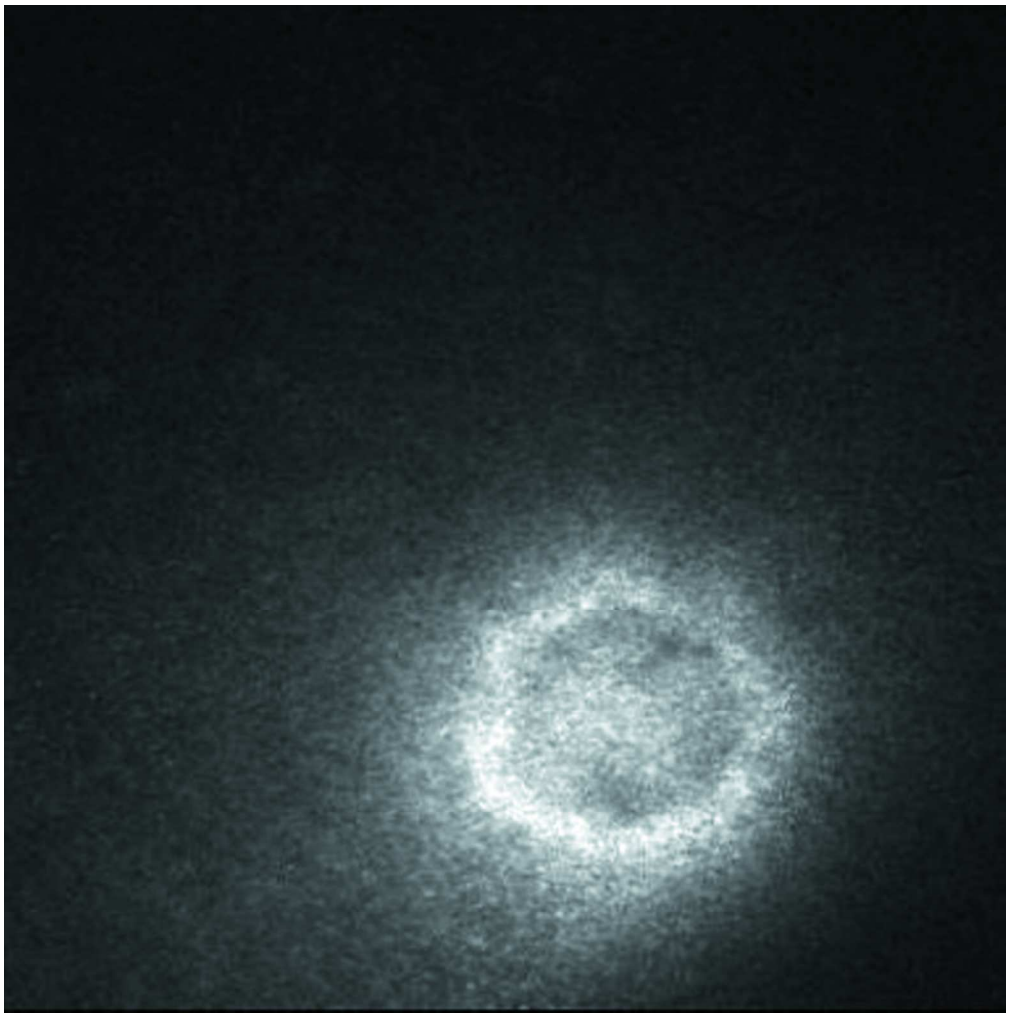
199x133mm (300 x 300 DPI)



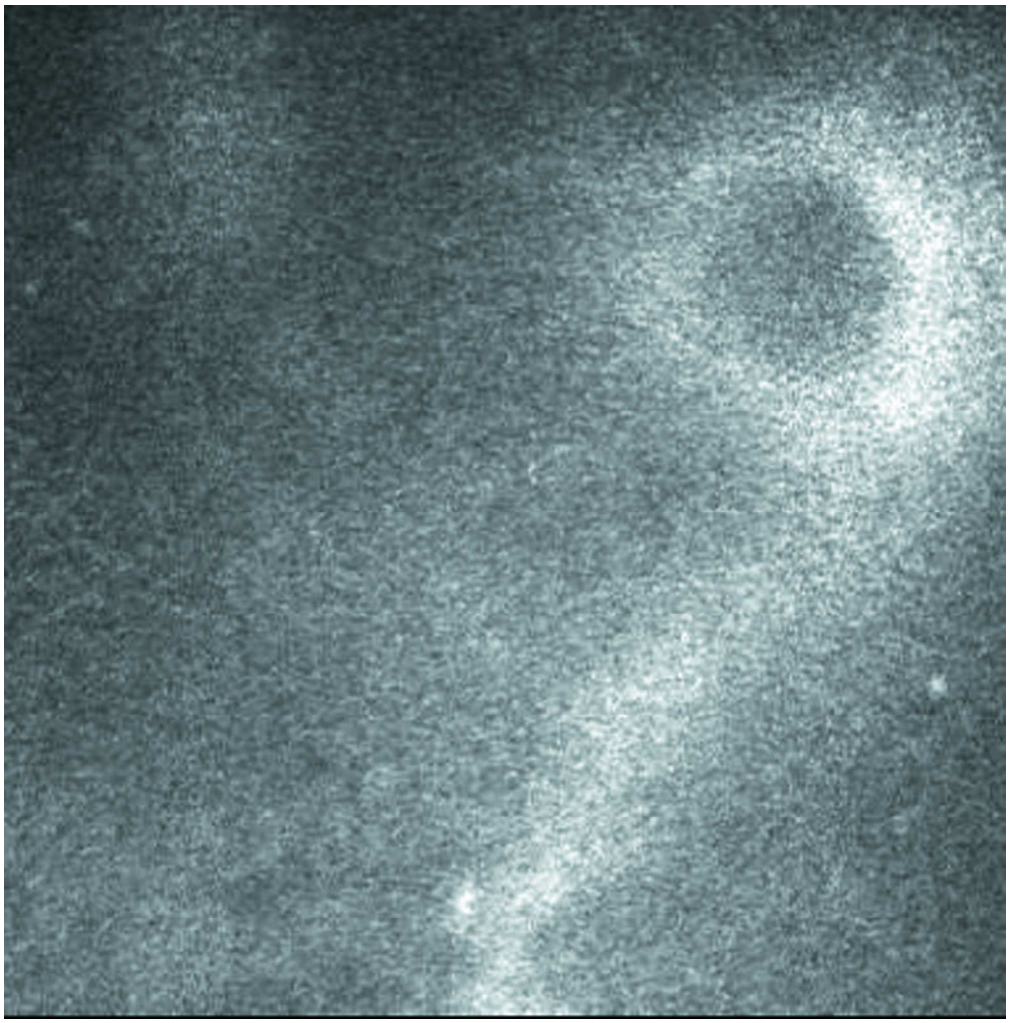
199x133mm (300 x 300 DPI)



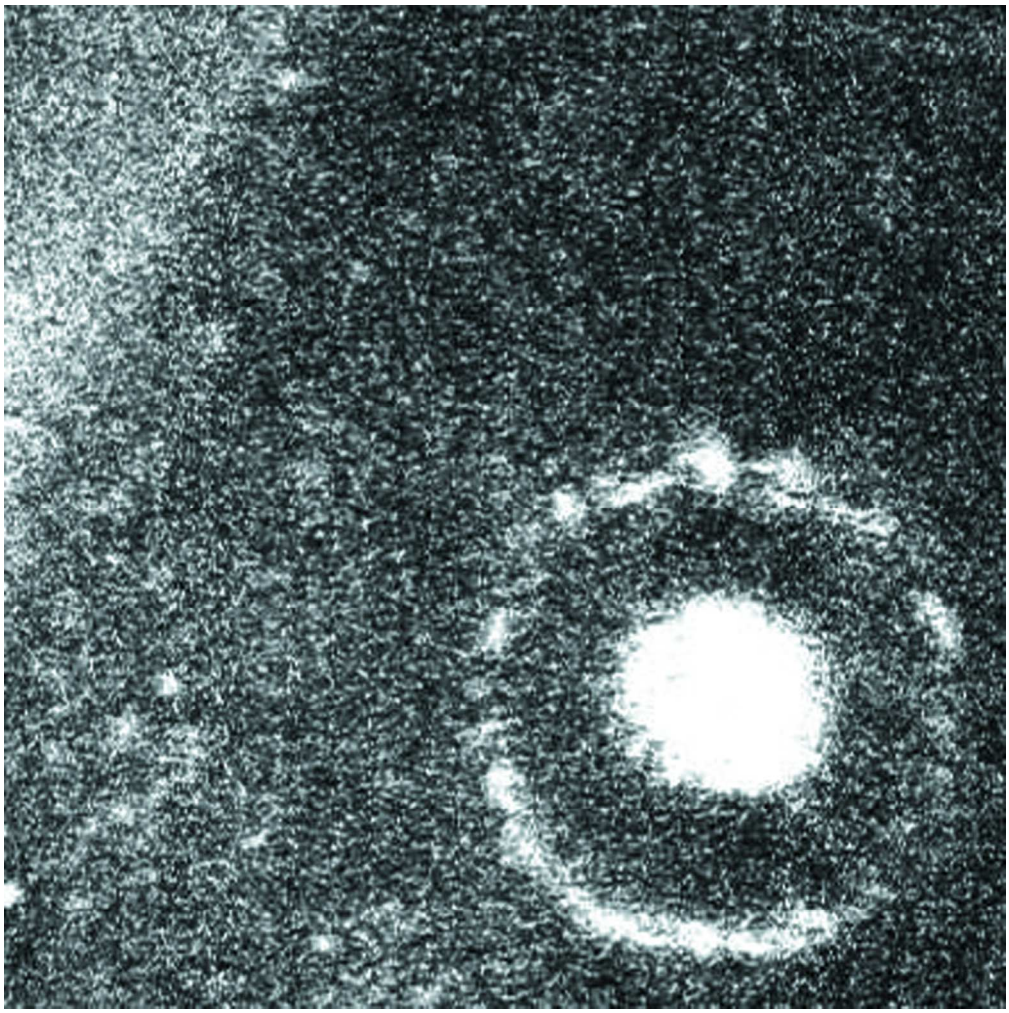
199x133mm (300 x 300 DPI)



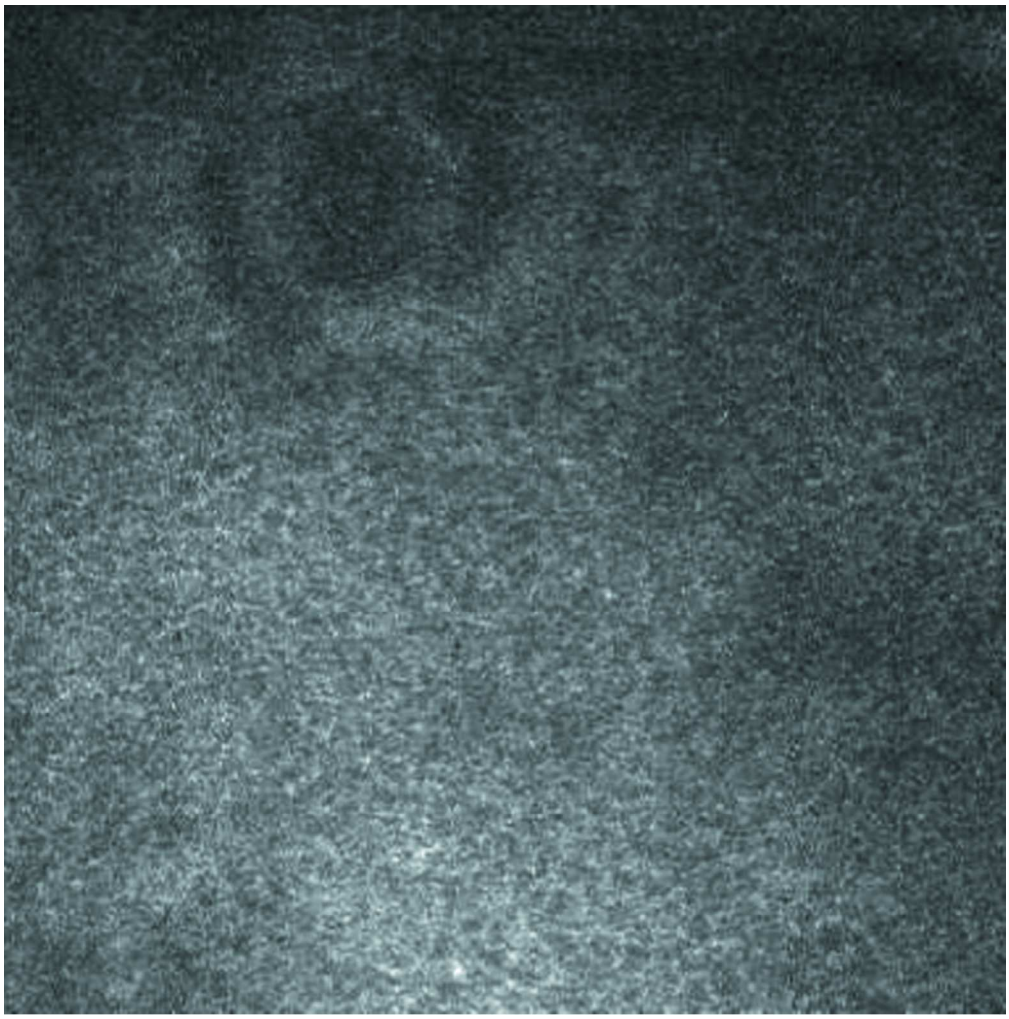
129x130mm (300 x 300 DPI)



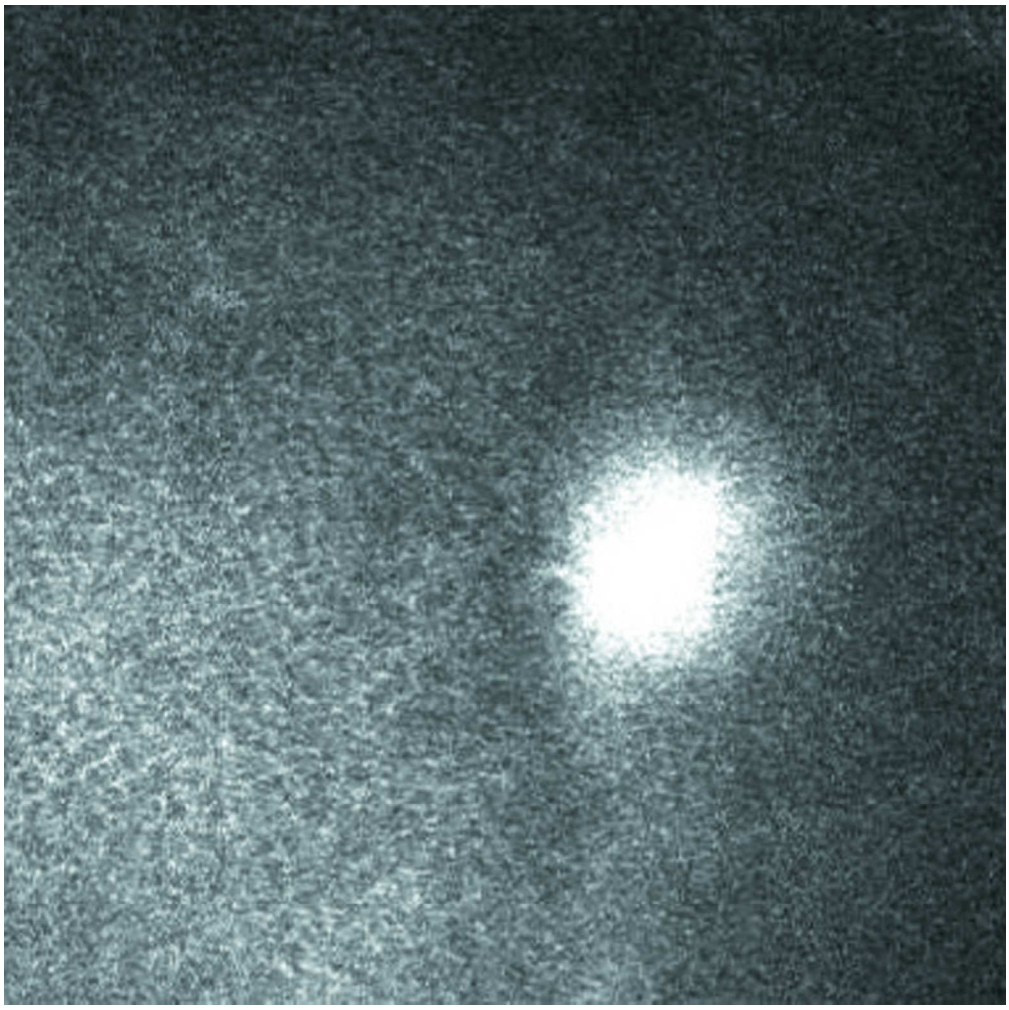
134x135mm (300 x 300 DPI)



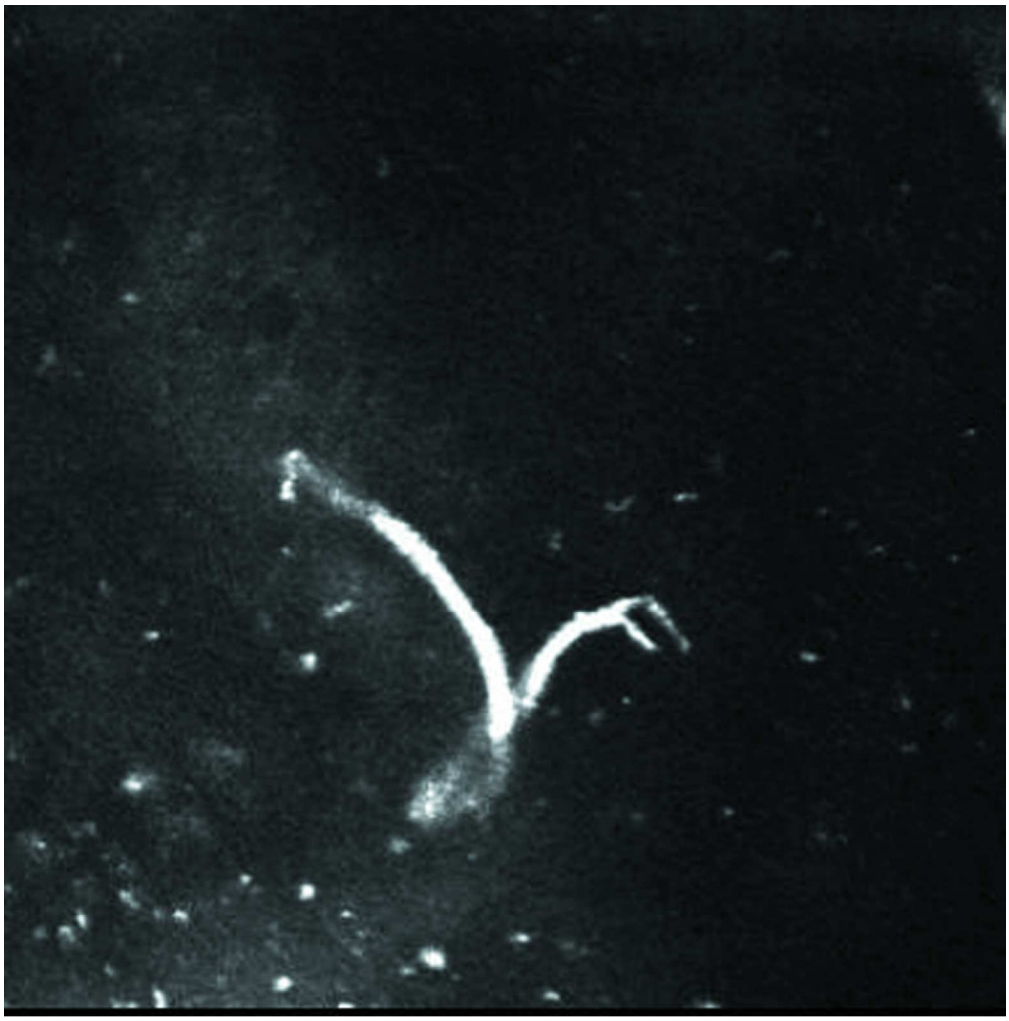
134x135mm (300 x 300 DPI)



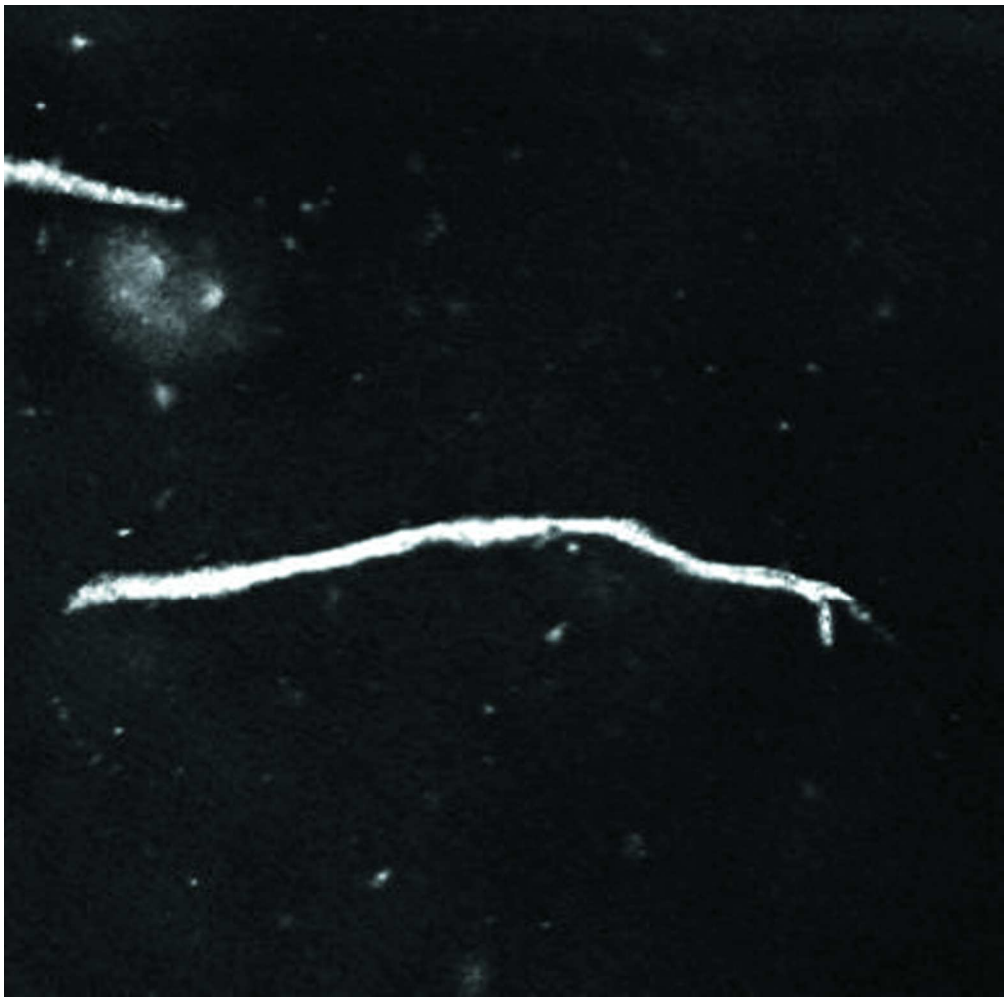
134x135mm (300 x 300 DPI)



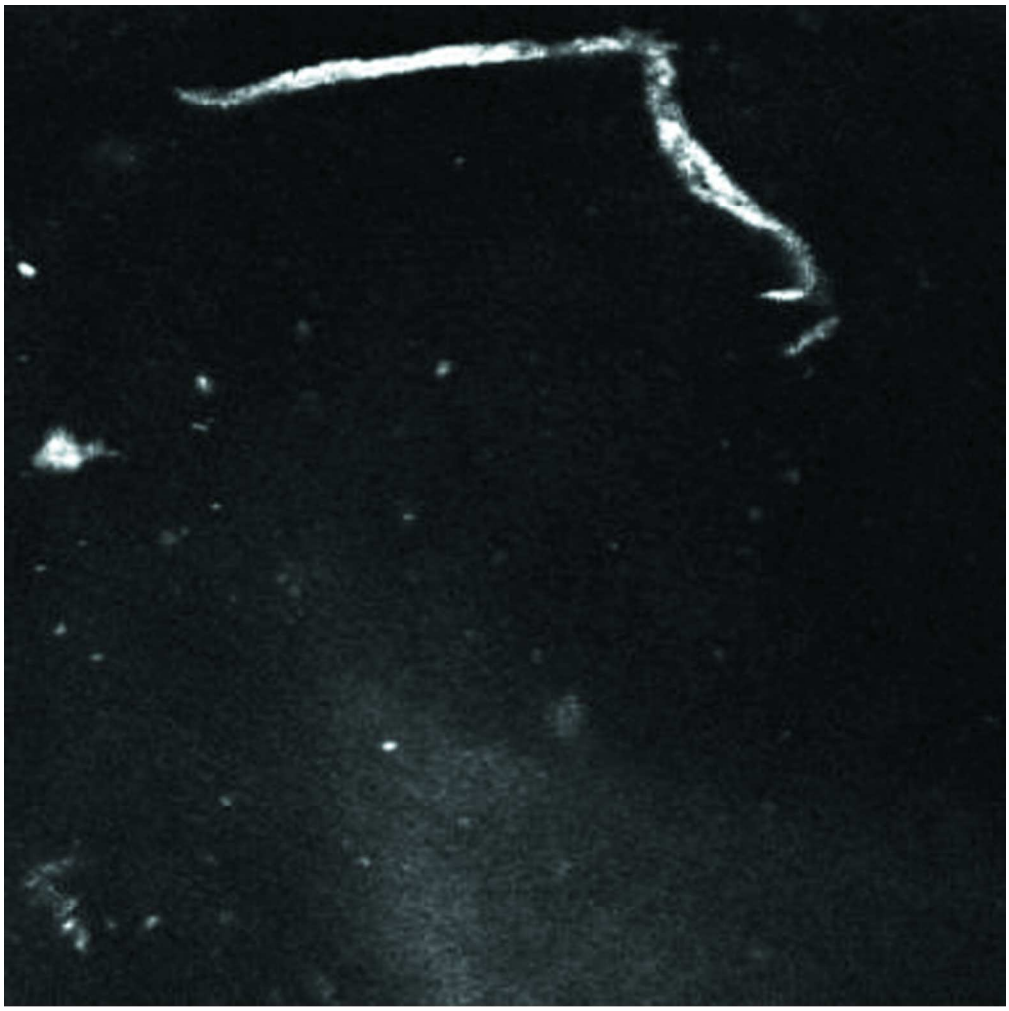
134x133mm (300 x 300 DPI)



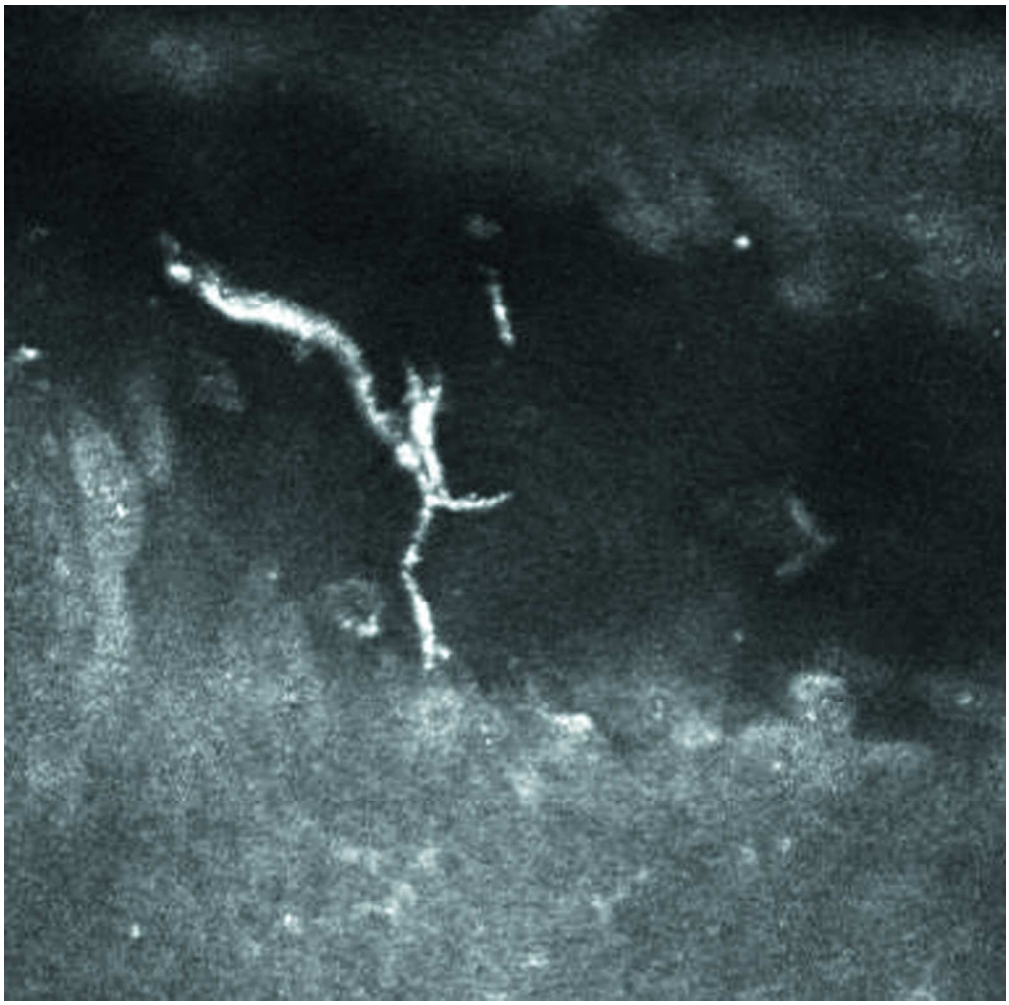
134x135mm (300 x 300 DPI)



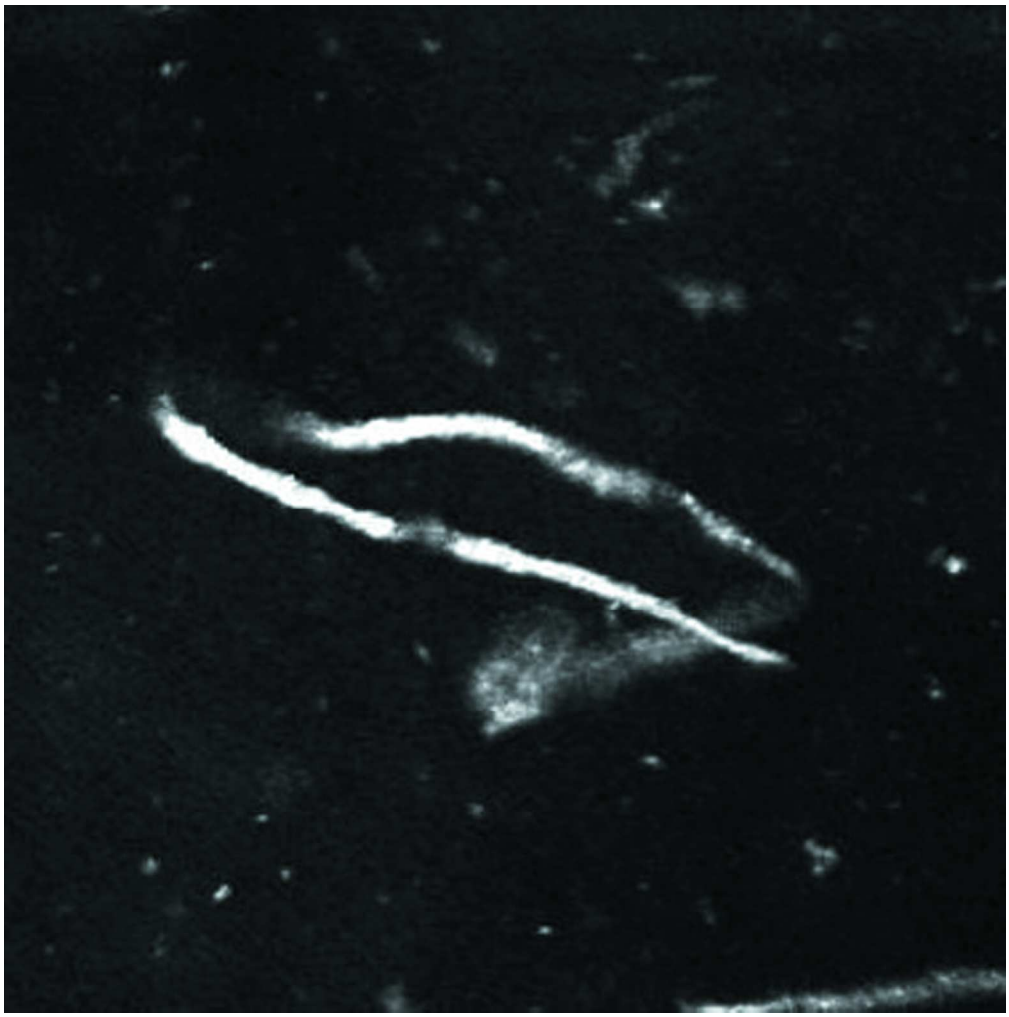
135x134mm (300 x 300 DPI)



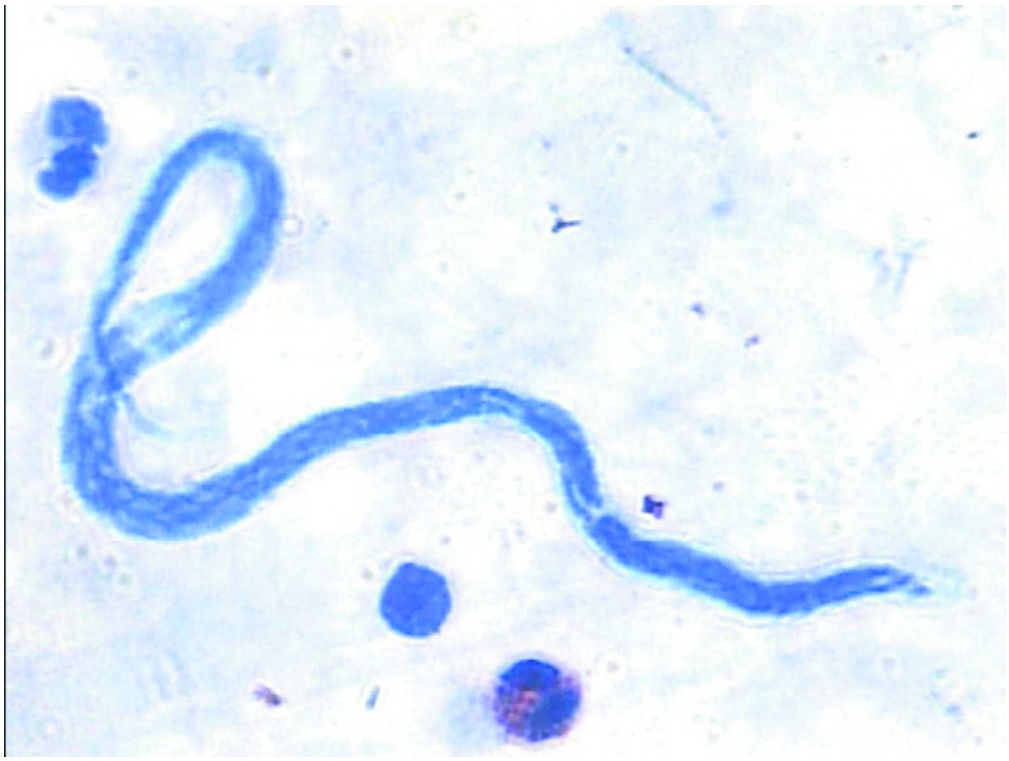
134x134mm (300 x 300 DPI)



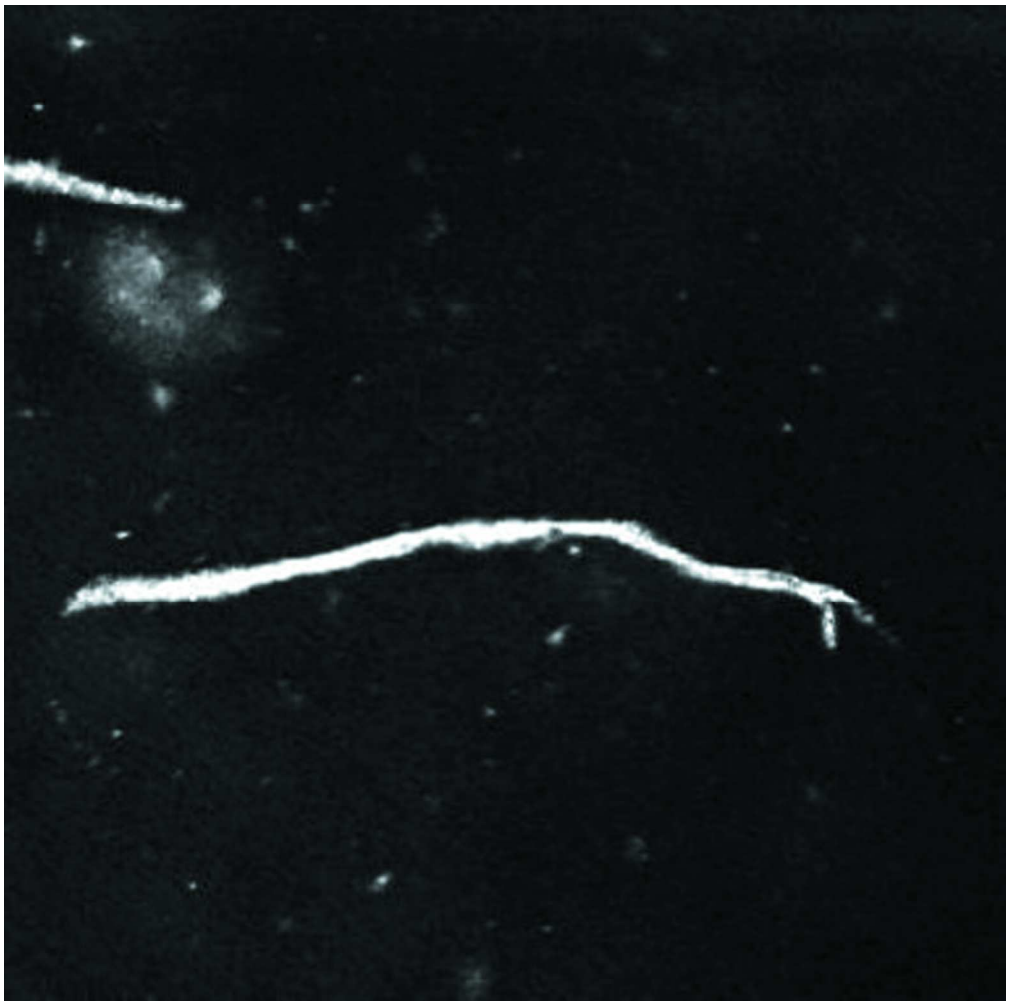
134x134mm (300 x 300 DPI)



132x133mm (300 x 300 DPI)



150x112mm (300 x 300 DPI)



134x134mm (300 x 300 DPI)

Competing Interests and Contributorship Statements:

***Article: Mansonella ozzardi* corneal lesions in the Amazon**

Authors: Lucas Monferrari Monteiro Vianna, Marilaine Martins, Marcos Jacob Cohen, Jacob Cohen, Rubens Belfort Jr

Statetment:

We state that none of us have competing interests.

We also state that all of us contributed in the conception and design of the study, acquisition, analysis and interpretation of data, drafting and revising of the article and the final approval of the version to be published.

Response (highlighted) to the reviewers comments:

Firstly, I would like to thank for the deadline extension and for comments. We consider all consistent and we'll try to answer them below:

***Reviewer 1*: Luís Marcelo Aranha Camargo**
Professor, Medical Doctor, PhD
University of São Paulo
In head of the Rondônia Advanced Research Unit-Amazonia-Brazil

1) "No statistical method described or used to detect statistical correlation between microfilaremia x ocular lesions." We are sorry for this mistake. Statistical analyzes were included in the text (highlighted).

2) "The sensitivity of the blood thick smear examination is not reliable. There maybe some patients with microfilaremia in the non affected group. They should better use the blood filtration with policarbonate membrane plus microscopic examination with Giemsa stain and/or PCR for microfilaremia detection in all blood samples." We were really not clear in our methods section. In all patients thick smear, Knott and polycarbonate membrane filtration and PCR were performed by the co-author Marilaine Martins. This information was added to the text.

3) "It is not clear where the PCR was used." PCR was used to confirm if the species of microfilariae were Mansonella ozzardi in peripheral blood of all the patients.

4) The absence of a control group also affects the reliability of the study. We performed confocal microscopy in both eyes of all the 22 patients with keratitis. All of them had unilateral keratitis. We observed that the non affected fellow eye never showed alterations in the confocal microscopy.

5) "It´s an original article and aims to produce more information about clinical manifestations of a high prevalent neglected Amazonian disease. Unfortunately the methodology is biased." This is mainly due to conditions of the work field. We visited several coastal communities (distant travel hours or days of each other), traveling by boat and examination of patients I such places were very difficult because they had to come aboard for this.

***Reviewer 2*: Dr Fatima Kyari**
Research Degree Fellow
International Centre for Eye Health (ICEH),
London School of Hygiene and Tropical Medicine (LSHTM)
London, United Kingdom

I have no competing interests.

1) "The term "suspicious corneal lesions" frequently used needs to be described/defined." We call it "suspicious corneal lesions" the ones similar to those described in previous articles cited in our references. It is, basically, nummular keratitis. The description and this information were added to the revised text.

2) "How did the investigators distinguish between corneal lesions due to M. ozzardi and those from other causes?" In the original text we wrote in the Results section **"The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis."** and in the Discussion section **"A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is no onchocerchiasis in those parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only close to the Venezuela border. None of the patients lived or visited areas where Onchocerca exist."** Besides this, until today, onchocercose is limited to the region of the Yanomami Indians. (The Onchocerciasis Elimination Program Sauerbrey for the Americas (OEPA) M. Annals of Tropical Medicine & Parasitology, Vol 102, Supplement No. 1 , S25-S29 (2008). This information was added to the text.

3) "Tables 1, 2 and 3 showed associations between the presence of microfilaremia and corneal lesions assessed by the different examinations. However, no statistical tests were described to give the significance of association." Statistical analyzes were included in the text (highlighted).

4) "The standard of written English is acceptable. However, there are some minor corrections needed."

We apologize. All suggested changes listed above were made in the review.

page 6, line 11 ...in humans, M.ozzardi BEING one of them. The first description....

page 7, line 11 .. al patients had THEIR eyes examined..

page 7, line 25 ...patients with suspicious corneal lesions at the eye examination were SUBJECTED to biomicroscopy..

page 7, line 29 THE CCME...

page 10, line 42 use "prevalence", and not "prevalence rate"

page 10, line 48 ...but there is NO onchocerciasis in THOSE parts of Brazil..

page 11, line 6 ... onchocerca EXIST.

page 12, line 34 ... eyes with no BIOMICROSCOPIC lesions..

5) “With the information provided and manuscript, I am not able to make a definite statement on plagiarism, fabrication, and undeclared conflict of interests.” A declaration of no competing interests was made in Methods Section.

***Reviewer 3*: Thomas B. Nutman, MD**

Head, Helminth Immunology Section

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

National Institutes of Health

1) “What is actually missing here are data demonstrating the statistical validity of their findings. The

authors need to spell out the types of statistical methods they will perform and then actually perform them on their data. I actually performed statistics on the data presented in Tables 1-3; for only data in Table 1 was their a statistically significant finding." Statistical analyzes were included in the text (highlighted).

2) "Given that the authors are using PCR in corroborate their microscopy data, the details of this must be performed. Also, given that biopsies were performed in 2 patients, PCR should be applied to the tissue in order to demonstrate definitively the relationship between the ocular disease and the presence of microfilariae in the eye proper." The PCR was not applied to the ocular tissue because it was not available. It will be a part of our next study.

3) "A native English speaker should edit the manuscript to make the English a bit more colloquial and correct a few mistakes in syntax." We revised it again including the suggestions made by one of the reviewers.

4) "As mentioned above, both the statistical validity of their findings and a definitive speciation of the ocular microfilariae would make this much more compelling, particularly because of number of the pictures of microfilariae (particularly the ones with the forked tail) are not characteristic of Mansonella ozzardi."

Statistical analyzes were included in the text (highlighted). The ideal confirmation of the presence and what kind of microfilaria would be through corneal biopsy, but this is difficult, especially in the case of asymptomatic patients. This will be the subject of future studies. The intention of the article is to produce more evidence of the association between microfilaraemia by M. ozzrdi and lion eye.

5) There is no statement about ethical approval nor about informed consent in the manuscript. The following statement was already in the Results, but now its in the Methods section: "The study was

prospective approved by the UNIFESP Ethical Committee. All participants signed the protocol`s informed consent."

We hope we're able to answer the comments. Thank you for them.

At your disposal,

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010

(lucasmmvianna@yahoo.com.br)mailto:presidente@spdm.org.br

BMJ open

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001266.R2
Article Type:	Research
Date Submitted by the Author:	08-Oct-2012
Complete List of Authors:	Vianna, Lucas; UNIFESP, Ophthalmology Martins, Marilaine; Fundação de Medicina Tropical do Amazonas, State University Amazonas, Parasithology Cohen, Jacob; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Cohen, Marcos; Federal University of Amazonas / Instituto de Oftalmologia de Manaus, Ophthalmology Belfort Junior, Rubens; UNIFESP, Ophthalmology
Primary Subject Heading:	Addiction
Secondary Subject Heading:	Ophthalmology
Keywords:	OPHTHALMOLOGY, Corneal and external diseases < OPHTHALMOLOGY, PARASITOLOGY

1) TITLE PAGE:

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Authors: Lucas Monferrari Monteiro Vianna (first author)*; Marilaine Martins**; Marcos Jacob Cohen***, Jacob Cohen***, Rubens Belfort Jr*

*Vision Institute and Department of Ophthalmology. Hospital São Paulo - Federal University of São Paulo. Address: Botucatu St, 821 – ZIP: 04023-900 – São Paulo/SP - Brazil

** Fundação de Medicina Tropical do Amazonas, Universidade do Estado do Amazonas. Address: Pedro Teixeira Av., 25 - ZIP: 69040-000 - Manaus/AM - Brazil

*** Universidade Federal do Amazonas (UFAM) / Instituto de Oftalmologia de Manaus (IOM). Address: General Rodrigo Octávio Jordão Ramos Av, 3000, Campus Universitário, Coroado I - 69077-000 - Manaus/AM – Brazil

Keywords: Mansonella, microfilaria, keratitis, microscopy, confocal, biopsy

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010 (lucasmmvianna@yahoo.com.br)

***Mansonella ozzardi* corneal lesions in the Amazon: a cross-sectional study**

2) ABSTRACT:

Objectives: To characterize and confirm the presence of *M*ansonella *ozzardi* microfilariae in the cornea by biomicroscopy and corneal confocal microscopy.

Design: Cross-sectional study.

Settings: Clinical practice study in patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil.

Participants: Two hundred and twelve consecutive volunteers patients that had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. Patients with suspicious corneal lesions (characterized as nummular keratitis) at the eye exam were submitted to biomicroscopy, fundoscopy and corneal confocal microscopy (CCME) evaluation. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

Primary and secondary outcome measures: Positive correlation between corneal biomicroscopic and confocal lesions with M. ozzardi microfilaremia.

Results*:* Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Corneal lesions were positive correlated to microfilaremia (P=0,0001). At biomicroscopy, lesions were divided in quiescent and active. At CCME, they were

divided in circular and filiform lesions. The associations between corneal lesions, CCME findings and microfilaremia are shown.

Conclusions*:* We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

Ethical Comitee Registration: UNIFESP Ethical Committee (number 0767/10).

ARTICLE SUMMARY

Article Focus

- To describe corneal lesions that can be related to the presence of M. ozzardi microfilaremia.
- To describe corneal confocal microscopy images that can be related to the presence of *M. ozzardi* microfilaremia.
- To correlate ophthalmologic clinical signs with the presence of microfilaremia in some patients.

Key Messages

- There are some biomicroscopic and corneal confocal microscopy evidences of the presence of *M. ozzardi* microfilariae in the cornea.
- The *M. ozzardi* microfilaria can be pathogenic to the eye.
- Further studies using ocular tissue PCR and other image techniques can be helpful.

Strengths and Limitations

- The major strength is that the study describes a very prevalent but not well studied disease in a very difficult access area of Brazil.
- Another strength is that this is the first description of *M. ozzardi* corneal lesions using confocal microscopy.
- The major limitation is the absence of a similar control group and follow up of the patients due to work field access difficulties.

Statements:

Data sharing statement: there is no additional data available.

Funding statement: this work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Competing Interests Statement: *we* state that none of the authors have competing interests.

Contributorship Statement: we state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

3) MAIN TEXT:

Introduction

Hundreds of types of filariae have been described and a few can cause infections in humans, *Mansonella ozzardi* being one of them. The first description of *M. ozzardi* associated with corneal disease was published in 1998.[1]

M. ozzardi is a filaria exclusively found in the America continent and one of the causes of mansonelliasis.[2] In Brazil it is found in the states of Roraima, Mato Grosso and Amazonas.[3,4,5] The microfilariae of *M. ozzardi* are found in peripheral blood and can be identified by their morphological characteristics or by molecular biology.[6-11]

The clinical features of mansonelliasis are not well described. Infected individuals are generally asymptomatic or have signs and symptoms common to other infections such as fever, coldness in the legs, joint pain and headache.[12] They may also include skin and ocular lesions[1,13,14] and be associated with *Wolbachiae,* that are bacterial endosymbionts of insects and many filarial nematodes and their products trigger inflammatory responses[14].

Coari City, at Solimões River (04°08'S, 63°07'W) has a high prevalence of *M. ozzardi* infection. Studies showed average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2,14] Other studies conducted in non-endemic areas shows no infection by *M. ozzardi*.[15] Onchocerciasis does not exist in this region of the Amazon.[16]

The objective of the present study is to characterize and confirm the presence of *Mansonella ozzardi* microfilariae in the cornea and to show the positive correlation between corneal biomicroscopic and confocal lesions with *M. ozzardi* microfilaremia.

Methods

Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil, examined in August, 2010. All patients had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. The peripheral blood infection by *M. ozzardi* was determined by light-microscopy examination (200x and 400x) of Giemsa-stained thick smears of peripheral blood obtained by digital puncture with a sterile disposable lancet[6], Knott and polycarbonate membrane filtration and by a new identification protocol using the polymerase chain reaction (PCR) (M Martins et al., unpublished observations).

Patients with nummular keratitis, similar to those previously described in the literature[1,13,14] at the one or both eye examination were subjected to bilateral biomicroscopy, fundoscopy and corneal confocal microscopy evaluation (CCME), using the Rostock Cornea Module (RCM) of the Heidelberg Retina Tomograph. Photographs were taken at the slit lamp. The CCME was performed in the central cornea of both eyes and in the corneal regions with the nummular keratitis in the eyes they were present.

Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

The inclusion criteria were being volunteer to *M. ozzardi* microfilaremia blood check and to sign the informed consent or have it signed by the legal responsible. The exclusion criteria were not being volunteer or able to sign the informed consent.

The study was prospective and approved by the UNIFESP Ethical Committee (number 0767/10). All participants signed the protocol`s informed consent.

Data were presented in contingency tables and Fisher exact test was used to compare proportions. P-values less than 0.05 were considered statistically significant. Analyses were done in Stata v.11 (College Station, Texas).

Results

Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Four of them had bilateral lesions. The association between microfilaremia and cornea lesions is shown in table 1, with statistically significant finding (p=0,0001).

At biomicroscopy, some lesions were characterized as nummular, mid periphery and anterior stromal corneal opacities, 0·5 to 1·0 mm in diameter with central mottled surrounded by a opaque halo, with marked limit between the white opacity and the clear cornea. Other lesions were characterized by nummular keratitis, also located at mid periphery and anterior stromal .In all cases there was a normal translucent area between the corneal lesions and the limbus and absence of corneal neovascularization

or other changes (Picture 1). The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis. The association between nummular keratitis and microfilaremia is shown in table 2 (p=0.1347).

Among the 22 patients with corneal lesions at biomicroscopy, 12 presented suspected related lesions also at the CCME. These confocal suspected lesions were presented in two patterns: circular (increased reflectivity, measuring about 80 micra in diameter, located at the sub-epithelial level, as shown in picture 2) and linear (filiform lesions ranging from 250 to 300 µm in length and 10 to 15 µm wide, with one “C" shape end and the other tapered, as shown in picture 3). Five patients presented only circular lesions, one patient only linear lesions and six patients had both of them. The association between confocal lesions and microfilaremia is shown in table 3 (p=0.4266).

In two volunteers patients with linear lesions, thick blood smear of the limbal conjunctiva was performed and confirmed the microfilaremia. Picture 4 compares microfilaria of *M. ozzardi* seen at the thick blood smear with the confocal microscopy image.

Acknowledgement for Funding/Support to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State) and for technical support for *Mansonella*-Brazil Group (Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T).

Discussion

The prevalence of *M. ozzardi* is high in some riverside communities in Amazon with the increasing occupation of rainforest areas.[2,16] Previous studies in Coari city showed an average infection rate between 13.3% and 18.9%, with higher prevalence in rural areas and patient aging.[2,14] Other studies conducted in non-endemic areas shows no infection by *M. ozzardi*.[15] In the present study, the prevalence was of 26,4%.

Presumed keratitis caused by *M. ozzardi* has been described in Brazil by us as well as other authors, with no other defined etiology related.[1,13,14] A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is no onchocerchiasis in those parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only close to the Venezuela border, in the Yanomami Indian Reservation.[17,18] None of the patients lived or visited areas where Onchocerca exist.

A study conducted in Pauiní, at the Purus River revealed 20 non-Indian habitants with peripheral corneal opacities among 524 patients examined. No microfilariae were seen in the skin snips and *Mansonella sp* was found in the blood of the 2 patients where it was possible to collect blood.[1] Another study, conducted in São Gabriel da Cachoeira (Negro River) showed a positive association between *M. ozzardi* and corneal lesions with all patients presenting ocular changes having *M. ozzardi* microfilariae in blood samples.[13] *All* the skin biopsies were negative for microfilaria including *Onchocerca*. The association between the keratitis and positive microfilaremia in the Amazon was also related by Cohen et al.[14] In our study, this association was also found, with statistically significant finding, as shown in table 1 (p=0,0001).

The aspect of corneal lesions was divided by its characteristics in only corneal opacities and keratitis, as shown in picture 1. Among patients positive for microfilaremia, the proportion of keratitis aspect seems to be higher and among patients negative for microfilaria, the proportion of only opacity aspect was higher, although there was no statistical significance (table 2, p=0,1347). One explanation for the presence of patients with keratitis lesion and negative microfilaremia could be false negatives in the blood tests. Patients with only opacity lesion and positive microfilaremia could be explained by chronic infection.

Like in previous studies,[1,13,14] we could not detect, by biomicroscopy, filariae in any ocular tissue, but corneal lesions similar to previous descriptions were found. Using CCME, we detected not previously described lesions. Among patients positive for microfilaremia, the proportion of confocal lesions was higher (table 3, p=0,4266). The confocal lesions were divided by their aspects in linear and circular. Among patients with lesions identified by CCME, there were a higher proportion of patients positive for microfilaremia and a higher proportion of circular aspect alone among patients negative for microfilaremia, although there was no statistical significance (table 3, p=0,4266). It could suggest that linear lesions are associated with active disease and circular lesions with inflammatory scars, associated or not with active disease. The size of linear lesions (250-300 microns) on CCME was higher than showed in microbiological studies (149-240 microns)[6]. The microfilariae are probably dead in ocular tissues and could enlarge due to inflammatory reaction. Circular lesions could represent, besides inflammatory reaction surrounding dead microfilariae, cross section of remnants of adult worms (26 to 49 mm in length by 00.7 to 0.15 mm in diameter)[6].

Unaffected patients were not tested due to difficulties of fieldwork. We performed confocal microscopy in both eyes of all the 22 patients with keratitis. In the 18 with unilateral keratitis, the non affected fellow eye never showed alterations in the confocal microscopy.

New molecular biology techniques for *M ozzardi* has been used[6-11] and can be helpfull in future studies of ocular tissues PCR, helping to confirm the association between microfilaremia and ocular lesions.

Conclusion

We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

4) Tables:

Table 1. Association between corneal lesions and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Corneal Lesions (horizontal) / **Microfilaremia (vertical)**	**Presence (+)**	**Absence (-)**	**Total**
Positive (+)	14 (63,6%)	42 (22%)	56
Negative (-)	8 (33,4%)	148 (88%)	156
Total	22	190	212 (100%)

p-value: 0.0001

Table 2. Association between nummular keratitis and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Keratitis lesions (horizontal) / **Microfilaremia (vertical)**	**Presence (+)**	**Absence (-)**	**Total**
Positive (+)	10 (76,9%)	4 (44%)	14
Negative (-)	3 (23,1%)	5 (56%)	8
Total	13	9	22 (100%)

p-value: 0.1347

Table 3. Association between confocal lesions and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Confocal lesion (horizontal) / **Microfilaremia (vertical)**	**Presence (+)** % Circular / Linear / Both	**Absence (-)**	**Total**
Positive (+)	2 (25%) / 1 (12,5%) / 5 (62,5%)	6	14
Negative (-)	3 (75%) / 0 / 1 (25%)	4	8
	12	10	22

p-value: 0.4266

5) ACKNOWLEDGMENTS, COMPETING INTERESTS, FUNDING:

Acknowledgments: *Mansonella-Brazil Group*: Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T

Competing Interests Statement: We state that none of the authors have competing interests.

Funding/Support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Contributorship Statement: We state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

6) ***REFERENCE LIST*:**

1. Branco BC, Chamon W, Belfort R et al. Ocular findings in Pauiní (Southwest of the Brazilian Amazon) and possible corneal lesions caused by Mansonella. Arq Bras Oftalmol. 1998; Vol 61(6): 647-82.

2. Martins M, Pessoa FA, de Medeiros MB, et al. Mansonella ozzardi in Amazonas, Brazil: prevalence and distribution in the municipality of Coari, in the middle Solimões River. Mem Inst Oswaldo Cruz. 2010 May;105(3):246-53.

3. Deane MP. Sobre a incidência de filárias humanas em Manaus, estado do Amazonas. *Rev Serv Esp Saude Publ* 1949 *2*: 849-858.

4. Oliveira wr. [Filarial infestation in inhabitants of Vila Pereira, territory of Roraima (Brazil)]. Rev Inst Med Trop Sao Paulo. 1963 Nov-Dec;5:287-8.

5. D'Andretta Jr, Pio da Silva CM, Kameyana F. Ocorrência da mansonelose entre índios do alto Xingu. *Rev Soc Bras Med Trop* 1969. *3*:11.

6. Orihel TC, Eberhard ML. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. Am J Trop Med Hyg. 1982 Nov;31(6):1142-7.

7. Morales-Hojas R, Post RJ, Shelley AJ, et al. Characterisation of nuclear ribosomal DNA sequences from Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea) and development of a PCR-based method for their

detection in skin biopsies. Int J Parasitol. 2001 Feb;31(2):169-77. Erratum in: Int J Parasitol 2001 Jun;31(8):850-1

8. Vera LJ, Basano S de A, Camargo J de S, et al. Improvement of a PCR test to diagnose infection by Mansonella ozzardi. Rev Soc Bras Med Trop. 2011 May-Jun;44(3):380-2.

9. Tang TH, López-Vélez R, Lanza M, et al. Nested PCR to detect and distinguish the sympatric filarial species Onchocerca volvulus, Mansonella ozzardi and Mansonella perstans in the Amazon Region. Mem Inst Oswaldo Cruz. 2010 Sep;105(6):823-8.

10. Degese MF, Cabrera MG, Krivokapich SJ, et al.Contribution of the PCR assay to the diagnosis of Mansonella ozzardi in endemic areas of Argentina. Rev Argent Microbiol. 2012 Apr-Jun;44(2):97-100. Spanish.Genetic

11. Marcos LA, Arrospide N, Recuenco S, et al. Genetic Characterization of Atypical Mansonella (Mansonella) ozzardi Microfilariae in Human Blood Samples from Northeastern Peru. Am J Trop Med Hyg. 2012 Sep;87(3):491-4. Epub 2012 Jul 23.

12. BATISTA D, CERQUEIRA NL, MORAES MA. [Epidemiology of Manson's disease in a locality in the interior of Amazonas]. Rev Assoc Med Bras. 1960 Jun;6:176-84.

13. Garrido C, Campos M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. Cornea. 2000 Nov;19(6):817-9.

14. Cohen JM, Ribeiro JA, Martins M. Ocular manifestations in mansonelliasis. Arq Bras Oftalmol. 2008 Mar-Apr;71(2):167-71. Portuguese.

15. Basano S de A, Camargo J de S, Vera LJ, Velasques SN, et al. Investigation of the occurrence of Mansonella ozzardi in the State of Rondônia, Western Amazonia, Brazil. Rev Soc Bras Med Trop. 2011 Oct;44(5):600-3. Epub 2011 Aug 26.

16. Medeiros JF, Py-Daniel V, Barbosa UC, et al. [Occurrence of Mansonella ozzardi (Nematoda, Onchocercidae) in riverine communities of the Purus river, Boca do Acre municipality, Amazonas State, Brazil]. Cad Saude Publica. 2009 Jun;25(6):1421-6.

17. Belfort Jr, R e Moraes, M – Oncocercose ocular no Brasil. AMB Rev Assoc Med Bras. 1979 Apr;25(4):123-7.

18. The Onchocerciasis Elimination Program Sauerbrey for the Americas (OEPA) M. Annals of Tropical Medicine & Parasitology, Vol 102, Supplement No. 1 , S25-S29 (2008).

7) FIGURE LEGENDS:

Picture 1. Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.

Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.

Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.

Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.

1) TITLE PAGE:

Mansonella ozzardi corneal lesions in the Amazon: a cross-sectional study

Authors: Lucas Monferrari Monteiro Vianna (first author)*; Marilaine Martins**; Marcos Jacob Cohen***, Jacob Cohen***, Rubens Belfort Jr*

*Vision Institute and Department of Ophthalmology. Hospital São Paulo - Federal University of São Paulo. Address: Botucatu St, 821 – ZIP: 04023-900 – São Paulo/SP - Brazil

** Fundação de Medicina Tropical do Amazonas, Universidade do Estado do Amazonas. Address: Pedro Teixeira Av., 25 - ZIP: 69040-000 - Manaus/AM - Brazil

*** Universidade Federal do Amazonas (UFAM) / Instituto de Oftalmologia de Manaus (IOM). Address: General Rodrigo Octávio Jordão Ramos Av, 3000, Campus Universitário, Coroado I - 69077-000 - Manaus/AM – Brazil

Keywords: Mansonella, microfilaria, keratitis, microscopy, confocal, biopsy

Lucas Monferrari Monteiro Vianna, MD (Corresponding Author)

Department of Ophthalmology / Federal University of Sao Paulo, Sao Paulo, Brazil

Phone: 55-11-7493-8000 - Fax: 55-11-5085-2010 (lucasmmvianna@yahoo.com.br)

***Mansonella ozzardi* corneal lesions in the Amazon: a cross-sectional study**

2) ABSTRACT:

Objectives: To characterize and confirm the presence of *M*ansonella *ozzardi* microfilariae in the cornea by biomicroscopy and corneal confocal microscopy.

Design: Cross-sectional study.

Settings: Clinical practice study in patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil.

Participants: Two hundred and twelve consecutive volunteers patients that had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. Patients with suspicious corneal lesions (characterized as nummular keratitis) at the eye exam were submitted to biomicroscopy, fundoscopy and corneal confocal microscopy (CCME) evaluation. Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

Primary and secondary outcome measures: Positive correlation between corneal biomicroscopic and confocal lesions with M. ozzardi microfilaremia.

Results*:* Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Corneal lesions were positive correlated to microfilaremia (P=0,0001). At biomicroscopy, lesions were divided in quiescent and active. At CCME, they were

divided in circular and filiform lesions. The associations between corneal lesions, CCME findings and microfilaremia are shown.

Conclusions*:* We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

Ethical Comitee Registration: UNIFESP Ethical Committee (number 0767/10).

ARTICLE SUMMARY

Article Focus

- To describe corneal lesions that can be related to the presence of M. ozzardi microfilaremia.
- To describe corneal confocal microscopy images that can be related to the presence of *M. ozzardi* microfilaremia.
- To correlate ophthalmologic clinical signs with the presence of microfilaremia in some patients.

Key Messages

- There are some biomicroscopic and corneal confocal microscopy evidences of the presence of *M. ozzardi* microfilariae in the cornea.
- The *M. ozzardi* microfilaria can be pathogenic to the eye.
- Further studies using ocular tissue PCR and other image techniques can be helpful.

Strengths and Limitations

- The major strength is that the study describes a very prevalent but not well studied disease in a very difficult access area of Brazil.
- Another strength is that this is the first description of *M. ozzardi* corneal lesions using confocal microscopy.
- The major limitation is the absence of a similar control group and follow up of the patients due to work field access difficulties.

Statements:

Data sharing statement: there is no additional data available.

Funding statement: this work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Competing Interests Statement: *we* state that none of the authors have competing interests.

Contributorship Statement: we state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

3) MAIN TEXT:

Introduction

Hundreds of types of filariae have been described and a few can cause infections in humans, *Mansonella ozzardi* being one of them. The first description of *M. ozzardi* associated with corneal disease was published in 1998.[1]

M. ozzardi is a filaria exclusively found in the America continent and one of the causes of mansonelliasis.[2] In Brazil it is found in the states of Roraima, Mato Grosso and Amazonas.[3,4,5] The microfilariae of *M. ozzardi* are found in peripheral blood and can be identified by their morphological characteristics or by molecular biology.[6-11]

The clinical features of mansonelliasis are not well described. Infected individuals are generally asymptomatic or have signs and symptoms common to other infections such as fever, coldness in the legs, joint pain and headache.[12] They may also include skin and ocular lesions[1,13,14] and be associated with *Wolbachiae,* that are bacterial endosymbionts of insects and many filarial nematodes and their products trigger inflammatory responses[14].

Coari City, at Solimões River (04°08'S, 63°07'W) has a high prevalence of *M. ozzardi* infection. Studies showed average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2,14] Other studies conducted in non-endemic areas shows no infection by *M. ozzardi*.[15] Onchocerciasis does not exist in this region of the Amazon.[16]

The objective of the present study is to characterize and confirm the presence of *Mansonella ozzardi* microfilariae in the cornea and to show the positive correlation between corneal biomicroscopic and confocal lesions with *M. ozzardi* microfilaremia.

Methods

Cross-sectional, clinical practice study of 212 consecutive volunteers patients from communities of the rural area of Coari City in the Solimões River, Amazonas State, Brazil, examined in August, 2010. All patients had their eyes examined using a flash light and had blood checked for the presence of microfilariae by expert microscopist. The peripheral blood infection by *M. ozzardi* was determined by light-microscopy examination (200x and 400x) of Giemsa-stained thick smears of peripheral blood obtained by digital puncture with a sterile disposable lancet[6], Knott and polycarbonate membrane filtration and by a new identification protocol using the polymerase chain reaction (PCR) (M Martins et al., unpublished observations).

Patients with nummular keratitis, similar to those previously described in the literature[1,13,14] at the one or both eye examination were subjected to bilateral biomicroscopy, fundoscopy and corneal confocal microscopy evaluation (CCME), using the Rostock Cornea Module (RCM) of the Heidelberg Retina Tomograph. Photographs were taken at the slit lamp. The CCME was performed in the central cornea of both eyes and in the corneal regions with the nummular keratitis in the eyes they were present.

Two patients underwent biopsy of the limbal conjunctiva adjacent to the nummular keratitis area and blood was collected from the surgical wound for microfilariae investigation by thick blood examination.

The inclusion criteria were being volunteer to *M. ozzardi* microfilaremia blood check and to sign the informed consent or have it signed by the legal responsible. The exclusion criteria were not being volunteer or able to sign the informed consent.

The study was prospective and approved by the UNIFESP Ethical Committee (number 0767/10). All participants signed the protocol`s informed consent.

Data were presented in contingency tables and Fisher exact test was used to compare proportions. P-values less than 0.05 were considered statistically significant. Analyses were done in Stata v.11 (College Station, Texas).

Results

Of the 212 patients, 56 (26,4%) were positive for microfilaremia. By flash light examination, 22 patients that had nummular keratitis were sent to biomicroscopy and CCME. Four of them had bilateral lesions. The association between microfilaremia and cornea lesions is shown in table 1, with statistically significant finding (p=0,0001).

At biomicroscopy, some lesions were characterized as nummular, mid periphery and anterior stromal corneal opacities, 0·5 to 1·0 mm in diameter with central mottled surrounded by a opaque halo, with marked limit between the white opacity and the clear cornea. Other lesions were characterized by nummular keratitis, also located at mid periphery and anterior stromal .In all cases there was a normal translucent area between the corneal lesions and the limbus and absence of corneal neovascularization

or other changes (Picture 1). The clinical characteristics of the lesions as well as the high prevalence exclude the possibility of traumas and other known causes for the differential diagnosis, specifically Onchocercosis. The association between nummular keratitis and microfilaremia is shown in table 2 (p=0.1347).

Among the 22 patients with corneal lesions at biomicroscopy, 12 presented suspected related lesions also at the CCME. These confocal suspected lesions were presented in two patterns: circular (increased reflectivity, measuring about 80 micra in diameter, located at the sub-epithelial level, as shown in picture 2) and linear (filiform lesions ranging from 250 to 300 µm in length and 10 to 15 µm wide, with one “C" shape end and the other tapered, as shown in picture 3). Five patients presented only circular lesions, one patient only linear lesions and six patients had both of them. The association between confocal lesions and microfilaremia is shown in table 3 (p=0.4266).

In two volunteers patients with linear lesions, thick blood smear of the limbal conjunctiva was performed and confirmed the microfilaremia. Picture 4 compares microfilaria of *M. ozzardi* seen at the thick blood smear with the confocal microscopy image.

Acknowledgement for Funding/Support to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State) and for technical support for *Mansonella*-Brazil Group (Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T).

Discussion

The prevalence of *M. ozzardi* is high in some riverside communities in Amazon with the increasing occupation of rainforest areas.[2,16] Previous studies in Coari city showed an average infection rate between 13·3% and 18·9%, with higher prevalence in rural areas and patient aging.[2,14] Other studies conducted in non-endemic areas shows no infection by *M. ozzardi*.[15] In the present study, the prevalence was of 26,4%.

Presumed keratitis caused by *M. ozzardi* has been described in Brazil by us as well as other authors, with no other defined etiology related.[1,13,14] A comparison with other filarial diseases shows some similarities with onchocerchiasis but there is no onchocerchiasis in those parts of Brazil and also the clinical picture is different. Onchocerciasis has been excluded by the characteristics of the microfilaria tail and because it exists in Brazil only close to the Venezuela border, in the Yanomami Indian Reservation.[17,18] None of the patients lived or visited areas where Onchocerca exist.

A study conducted in Pauiní, at the Purus River revealed 20 non-Indian habitants with peripheral corneal opacities among 524 patients examined. No microfilariae were seen in the skin snips and *Mansonella sp* was found in the blood of the 2 patients where it was possible to collect blood.[1] Another study, conducted in São Gabriel da Cachoeira (Negro River) showed a positive association between *M. ozzardi* and corneal lesions with all patients presenting ocular changes having *M. ozzardi* microfilariae in blood samples.[13] *All* the skin biopsies were negative for microfilaria including *Onchocerca*. The association between the keratitis and positive microfilaremia in the Amazon was also related by Cohen et al.[14] In our study, this association was also found, with statistically significant finding, as shown in table 1 (p=0,0001).

The aspect of corneal lesions was divided by its characteristics in only corneal opacities and keratitis, as shown in picture 1. Among patients positive for microfilaremia, the proportion of keratitis aspect seems to be higher and among patients negative for microfilaria, the proportion of only opacity aspect was higher, although there was no statistical significance (table 2, p=0,1347). One explanation for the presence of patients with keratitis lesion and negative microfilaremia could be false negatives in the blood tests. Patients with only opacity lesion and positive microfilaremia could be explained by chronic infection.

Like in previous studies,[1,13,14] we could not detect, by biomicroscopy, filariae in any ocular tissue, but corneal lesions similar to previous descriptions were found. Using CCME, we detected not previously described lesions. Among patients positive for microfilaremia, the proportion of confocal lesions was higher (table 3, p=0,4266). The confocal lesions were divided by their aspects in linear and circular. Among patients with lesions identified by CCME, there were a higher proportion of patients positive for microfilaremia and a higher proportion of circular aspect alone among patients negative for microfilaremia, although there was no statistical significance (table 3, p=0,4266). It could suggest that linear lesions are associated with active disease and circular lesions with inflammatory scars, associated or not with active disease. The size of linear lesions (250-300 microns) on CCME was higher than showed in microbiological studies (149-240 microns)[6]. The microfilariae are probably dead in ocular tissues and could enlarge due to inflammatory reaction. Circular lesions could represent, besides inflammatory reaction surrounding dead microfilariae, cross section of remnants of adult worms (26 to 49 mm in length by 00.7 to 0.15 mm in diameter)[6].

Unaffected patients were not tested due to difficulties of fieldwork. We performed confocal microscopy in both eyes of all the 22 patients with keratitis. In the 18 with unilateral keratitis, the non affected fellow eye never showed alterations in the confocal microscopy.

New molecular biology techniques for *M ozzardi* has been used[6-11] and can be helpfull in future studies of ocular tissues PCR, helping to confirm the association between microfilaremia and ocular lesions.

Conclusion

We presented some evidences of the presence of *M. ozzardi* microfilariae in the cornea and its pathogenicity to the eye. Further studies using ocular tissue PCR and other image techniques can be helpful.

4) Tables:

Table 1. Association between corneal lesions and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Corneal Lesions (horizontal) / **Microfilaremia (vertical)**	**Presence (+)**	**Absence (-)**	**Total**
Positive (+)	14 (63,6%)	42 (22%)	56
Negative (-)	8 (33,4%)	148 (88%)	156
Total	22	190	212 (100%)

p-value: 0.0001

Table 2. Association between nummular keratitis and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Keratitis lesions (horizontal) / **Microfilaremia (vertical)**	**Presence (+)**	**Absence (-)**	**Total**
Positive (+)	10 (76,9%)	4 (44%)	14
Negative (-)	3 (23,1%)	5 (56%)	8
Total	13	9	22 (100%)

p-value: 0.1347

Table 3. Association between confocal lesions and microfilaremia (detected by any of the diagnose methods: Giemsa staining, Knott and polycarbonate membrane filtration and/or PCR).

Confocal lesion (horizontal)	**Presence (+)**	**Absence**	**Total**
Microfilaremia (vertical)	**% Circular / Linear / Both**	**(-)**	
Positive (+)	2 (25%) / 1 (12,5%) / 5 (62,5%)	6	14
Negative (-)	3 (75%) / 0 / 1 (25%)	4	8
	12	10	22

p-value: 0.4266

5) ACKNOWLEDGMENTS, COMPETING INTERESTS, FUNDING:

Acknowledgments: *Mansonella-Brazil Group*: Belfort Jr RB, Borborema M, Cohen J, Cohen MJ, Cunha P, Fontes G, Martins M, Medeiros JF, Pessoa, FAC, Vianna LMM, Zanelato T

Competing Interests Statement: We state that none of the authors have competing interests.

Funding/Support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo or Foundation for Research Support of São Paulo State).

Contributorship Statement: We state that all authors contributed in the:

1) Conception and design of the study;
2) Acquisition, analysis and interpretation of data;
3) Drafting and revising of the article;
4) Final approval of the version to be published.

6) ***REFERENCE LIST*:**

1. Branco BC, Chamon W, Belfort R et al. Ocular findings in Pauiní (Southwest of the Brazilian Amazon) and possible corneal lesions caused by Mansonella. Arq Bras Oftalmol. 1998; Vol 61(6): 647-82.

2. Martins M, Pessoa FA, de Medeiros MB, et al. Mansonella ozzardi in Amazonas, Brazil: prevalence and distribution in the municipality of Coari, in the middle Solimões River. Mem Inst Oswaldo Cruz. 2010 May;105(3):246-53.

3. Deane MP. Sobre a incidência de filárias humanas em Manaus, estado do Amazonas. *Rev Serv Esp Saude Publ* 1949 *2*: 849-858.

4. Oliveira wr. [Filarial infestation in inhabitants of Vila Pereira, territory of Roraima (Brazil)]. Rev Inst Med Trop Sao Paulo. 1963 Nov-Dec;5:287-8.

5. D'Andretta Jr, Pio da Silva CM, Kameyana F. Ocorrência da mansonelose entre índios do alto Xingu. *Rev Soc Bras Med Trop* 1969. *3*:11.

6. Orihel TC, Eberhard ML. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. Am J Trop Med Hyg. 1982 Nov;31(6):1142-7.

7. Morales-Hojas R, Post RJ, Shelley AJ, et al. Characterisation of nuclear ribosomal DNA sequences from Onchocerca volvulus and Mansonella ozzardi (Nematoda: Filarioidea) and development of a PCR-based method for their

detection in skin biopsies. Int J Parasitol. 2001 Feb;31(2):169-77. Erratum in: Int J Parasitol 2001 Jun;31(8):850-1

8. Vera LJ, Basano S de A, Camargo J de S, et al. Improvement of a PCR test to diagnose infection by Mansonella ozzardi. Rev Soc Bras Med Trop. 2011 May-Jun;44(3):380-2.

9. Tang TH, López-Vélez R, Lanza M, et al. Nested PCR to detect and distinguish the sympatric filarial species Onchocerca volvulus, Mansonella ozzardi and Mansonella perstans in the Amazon Region. Mem Inst Oswaldo Cruz. 2010 Sep;105(6):823-8.

10. Degese MF, Cabrera MG, Krivokapich SJ, et al.Contribution of the PCR assay to the diagnosis of Mansonella ozzardi in endemic areas of Argentina. Rev Argent Microbiol. 2012 Apr-Jun;44(2):97-100. Spanish.Genetic

11. Marcos LA, Arrospide N, Recuenco S, et al. Genetic Characterization of Atypical Mansonella (Mansonella) ozzardi Microfilariae in Human Blood Samples from Northeastern Peru. Am J Trop Med Hyg. 2012 Sep;87(3):491-4. Epub 2012 Jul 23.

12. BATISTA D, CERQUEIRA NL, MORAES MA. [Epidemiology of Manson's disease in a locality in the interior of Amazonas]. Rev Assoc Med Bras. 1960 Jun;6:176-84.

13. Garrido C, Campos M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. Cornea. 2000 Nov;19(6):817-9.

14. Cohen JM, Ribeiro JA, Martins M. Ocular manifestations in mansonelliasis. Arq Bras Oftalmol. 2008 Mar-Apr;71(2):167-71. Portuguese.

15. Basano S de A, Camargo J de S, Vera LJ, Velasques SN, et al. Investigation of the occurrence of Mansonella ozzardi in the State of Rondônia, Western Amazonia, Brazil. Rev Soc Bras Med Trop. 2011 Oct;44(5):600-3. Epub 2011 Aug 26.

16. Medeiros JF, Py-Daniel V, Barbosa UC, et al. [Occurrence of Mansonella ozzardi (Nematoda, Onchocercidae) in riverine communities of the Purus river, Boca do Acre municipality, Amazonas State, Brazil]. Cad Saude Publica. 2009 Jun;25(6):1421-6.

17. Belfort Jr, R e Moraes, M – Oncocercose ocular no Brasil. AMB Rev Assoc Med Bras. 1979 Apr;25(4):123-7.

18. The Onchocerciasis Elimination Program Sauerbrey for the Americas (OEPA) M. Annals of Tropical Medicine & Parasitology, Vol 102, Supplement No. 1 , S25-S29 (2008).

7) FIGURE LEGENDS:

Picture 1. Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.

Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.

Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.

Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.

STROBE 2007 (v4) checklist of items to be included in reports of observational studies in epidemiology*

Checklist for cohort, case-control, and cross-sectional studies (combined)

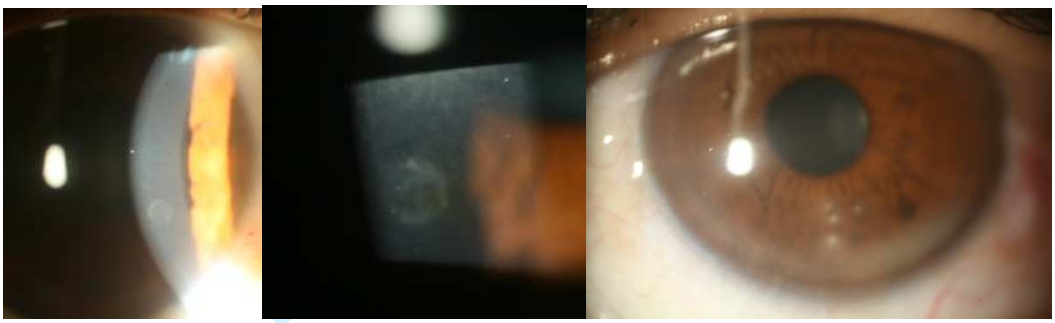
Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2-4
Introduction			**5**
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	3	State specific objectives, including any pre-specified hypotheses	6
Methods			**6, 7**
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	6, 7
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6, 7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*6, 7*
Bias	9	Describe any efforts to address potential sources of bias	6, 7
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed	6, 7

		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	6, 7
Results			7, 8
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	7
		(b) Give reasons for non-participation at each stage	7, 8
		(c) Consider use of a flow diagram	7, 8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	7, 8
		(b) Indicate number of participants with missing data for each variable of interest	7, 8
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	7, 8
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7, 8
		(*b*) Report category boundaries when continuous variables were categorized	7, 8
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	7, 8
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	7, 8
Discussion			9-11
Key results	18	Summarise key results with reference to study objectives	11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9-11
Generalisability	21	Discuss the generalisability (external validity) of the study results	9-11
Other information			14
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

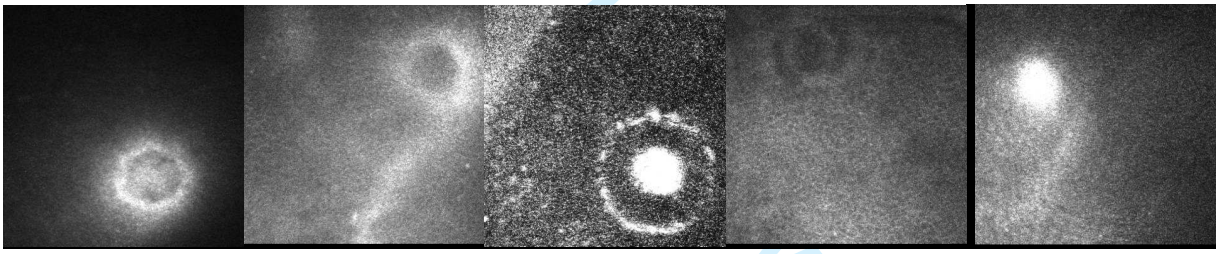
*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

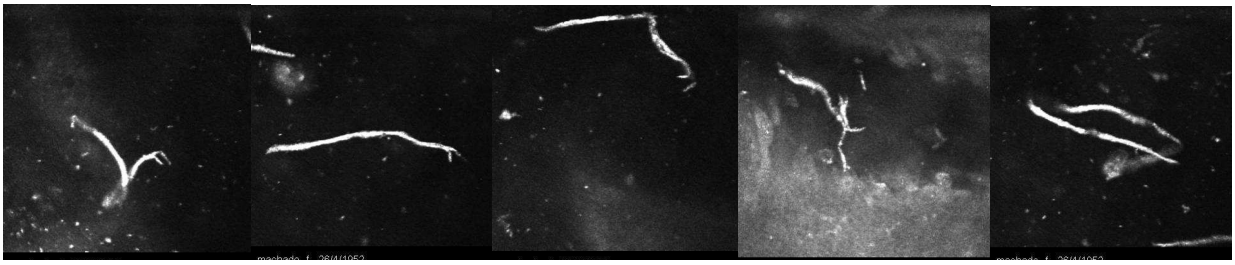
PICTURES:



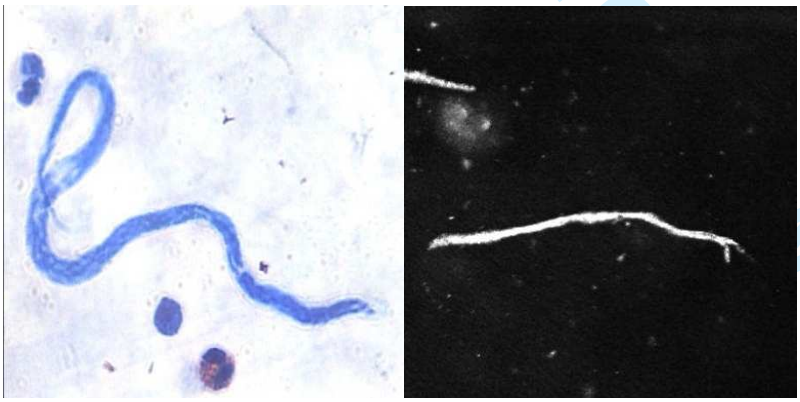
Picture 1: Clinical features. (Left and bottom) Central irregular “mottled” surrounded by a opaque halo opacitie; (Right) Four typical keratitis lesions. All of them had normal translucent area between the lesions and the limbus, without the presence of corneal neovascularization.



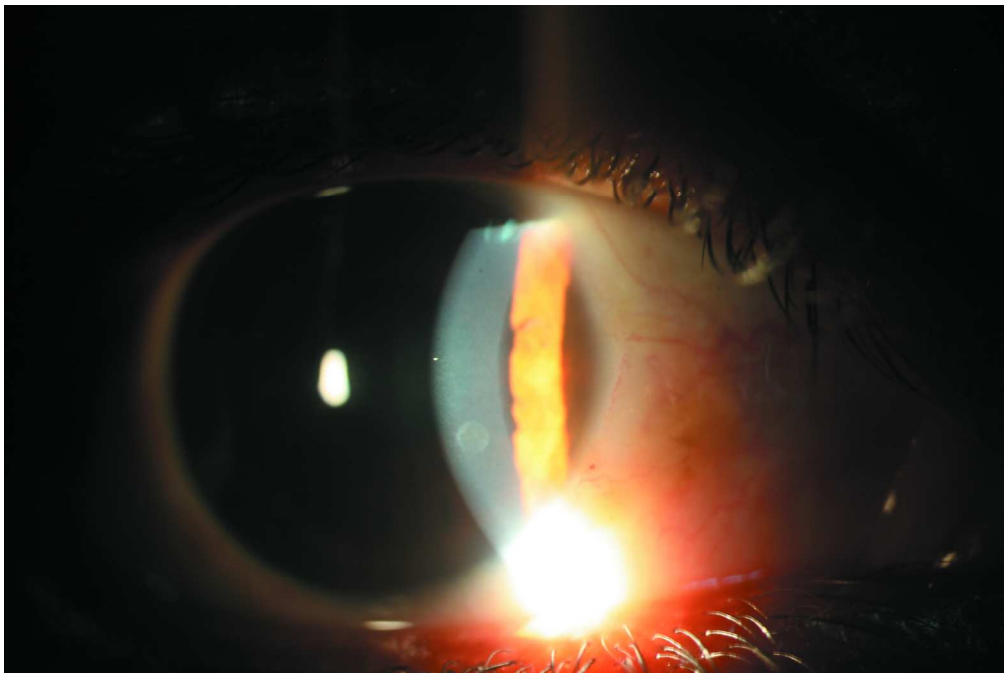
Picture 2. Five different patterns of circular lesions measuring about 80 µm in diameter, located sub-epithelial, in different patients. The figures are 400 µm x 400 µm.



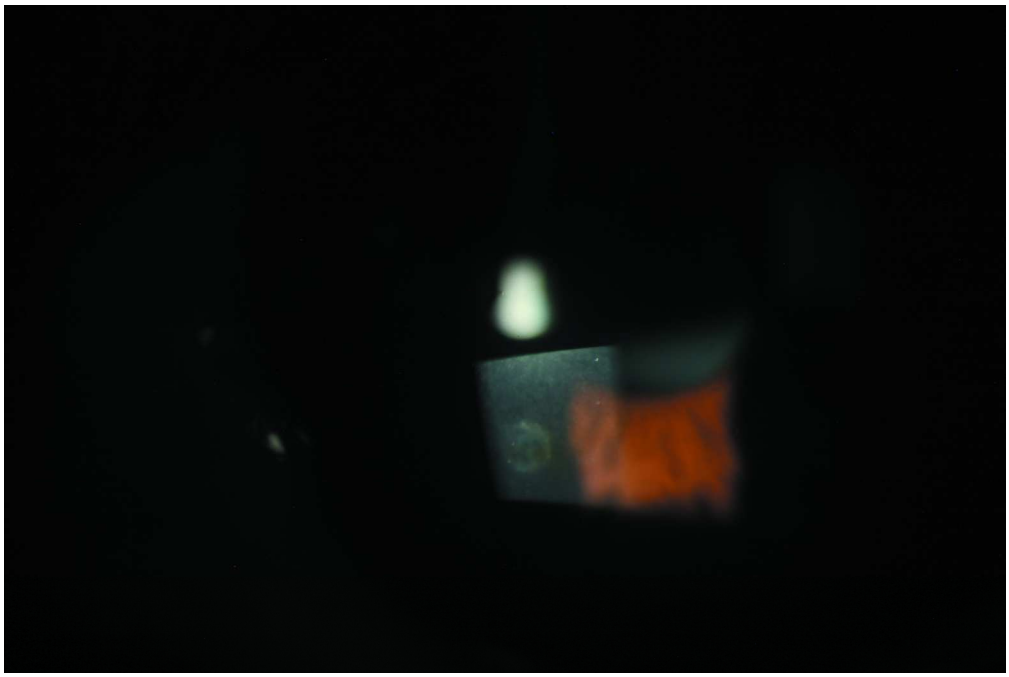
Picture 3. Filiform lesions located above the epithelium basal layer about 300 µm long and 10 µm wide, with one dichotomized end into a "C" shape and the other tapered, all in the same patient, who had limbal conjunctiva thick blood smear positive for *Mansonella ozzardi*. The figures are 400 µm x 400 µm.



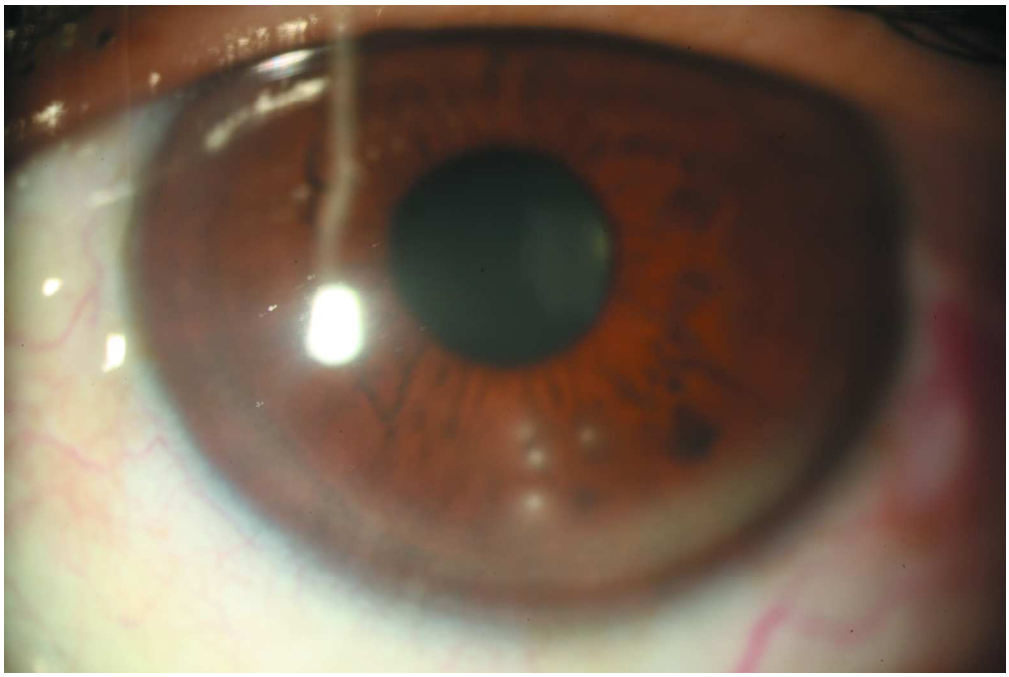
Picture 4. Microfilaria as detected by thick blood smear and corneal confocal image from a patient who had both tests performed.



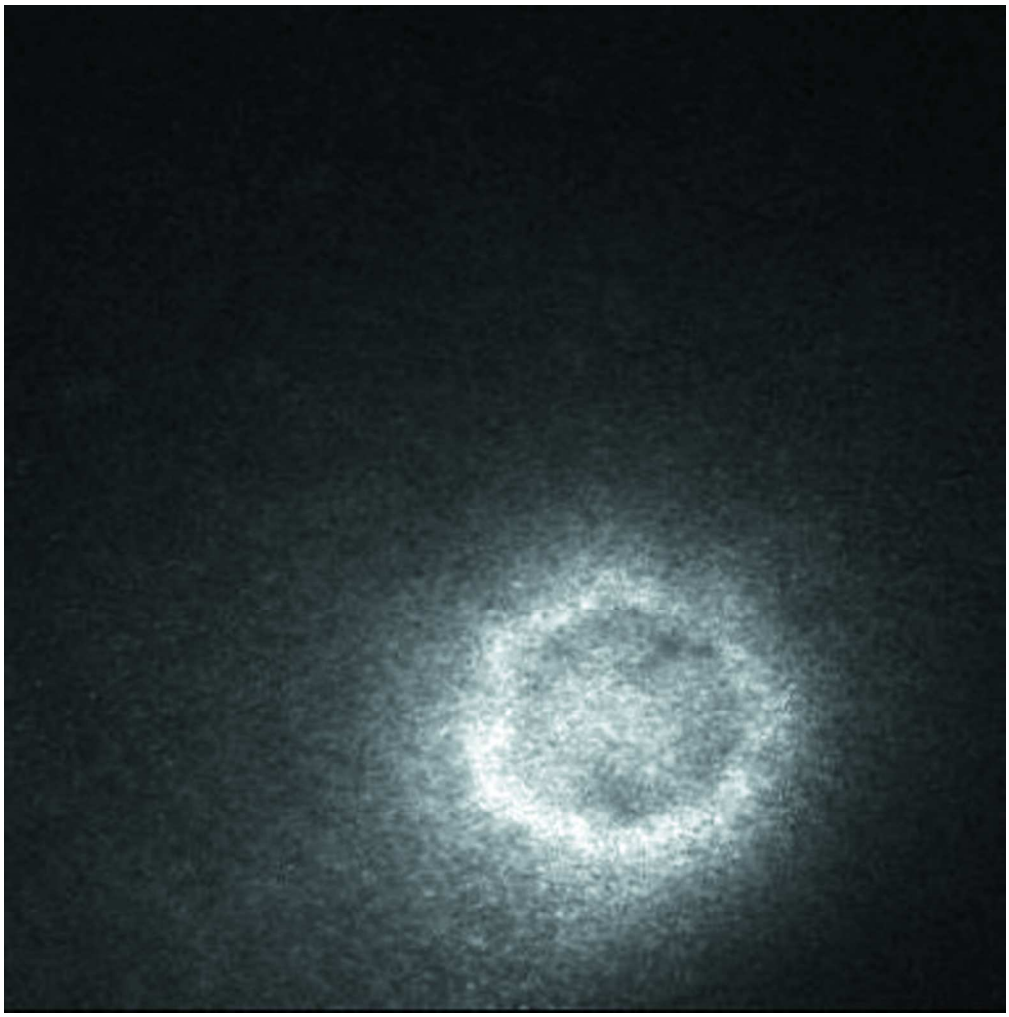
199x133mm (300 x 300 DPI)



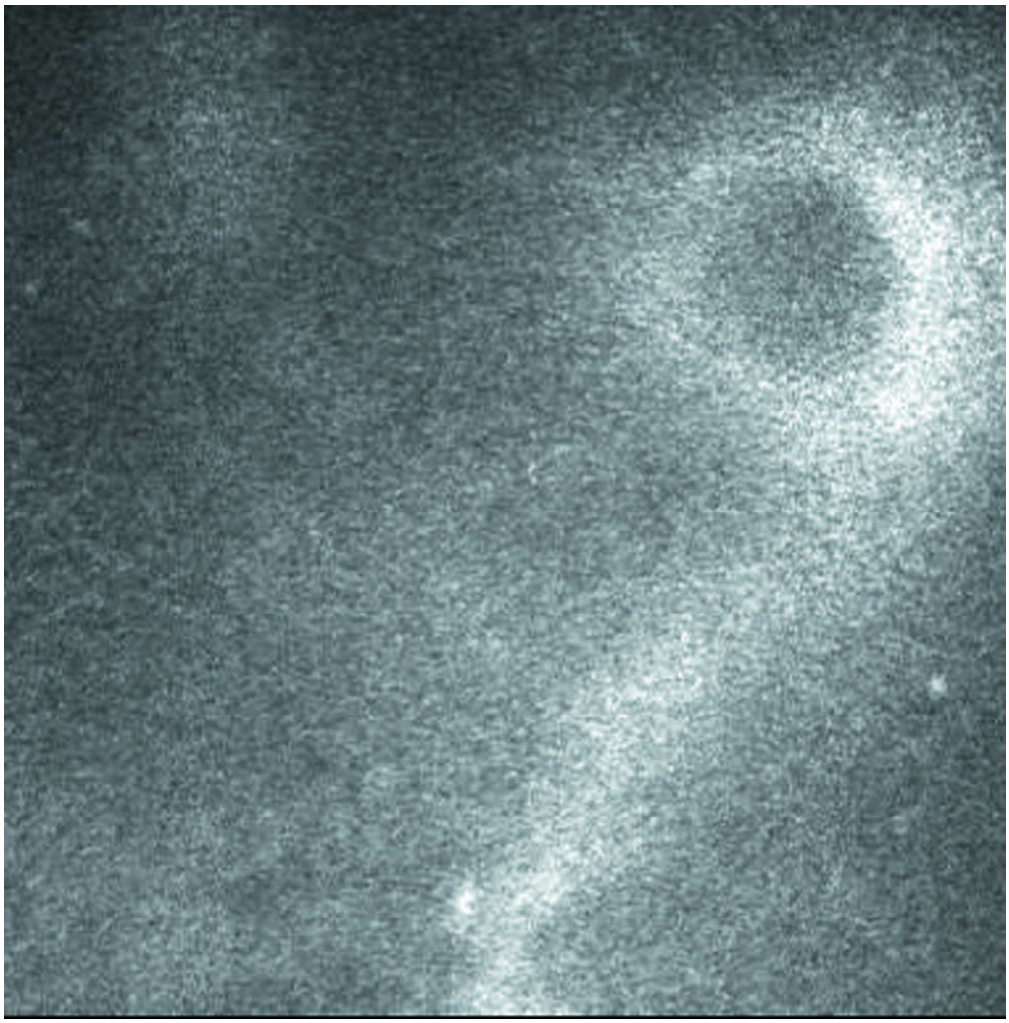
199x133mm (300 x 300 DPI)



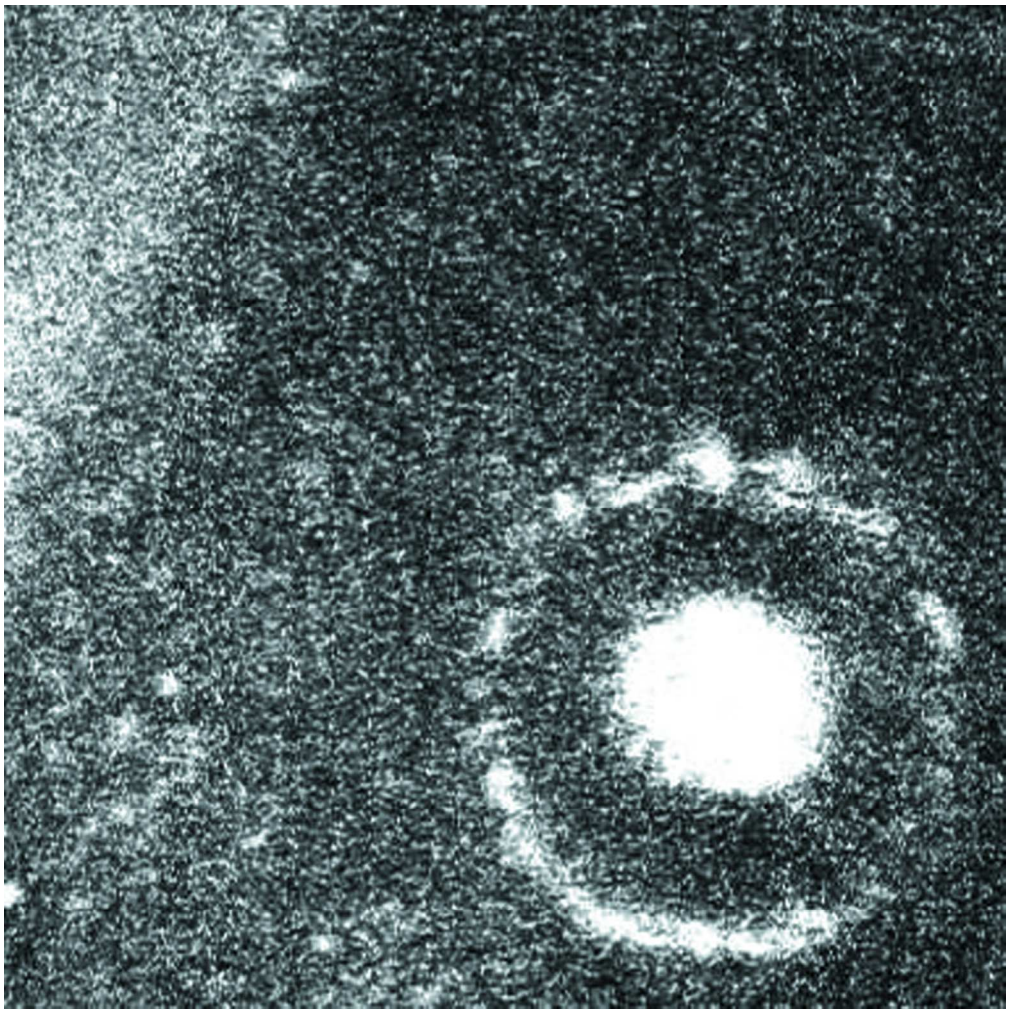
199x133mm (300 x 300 DPI)



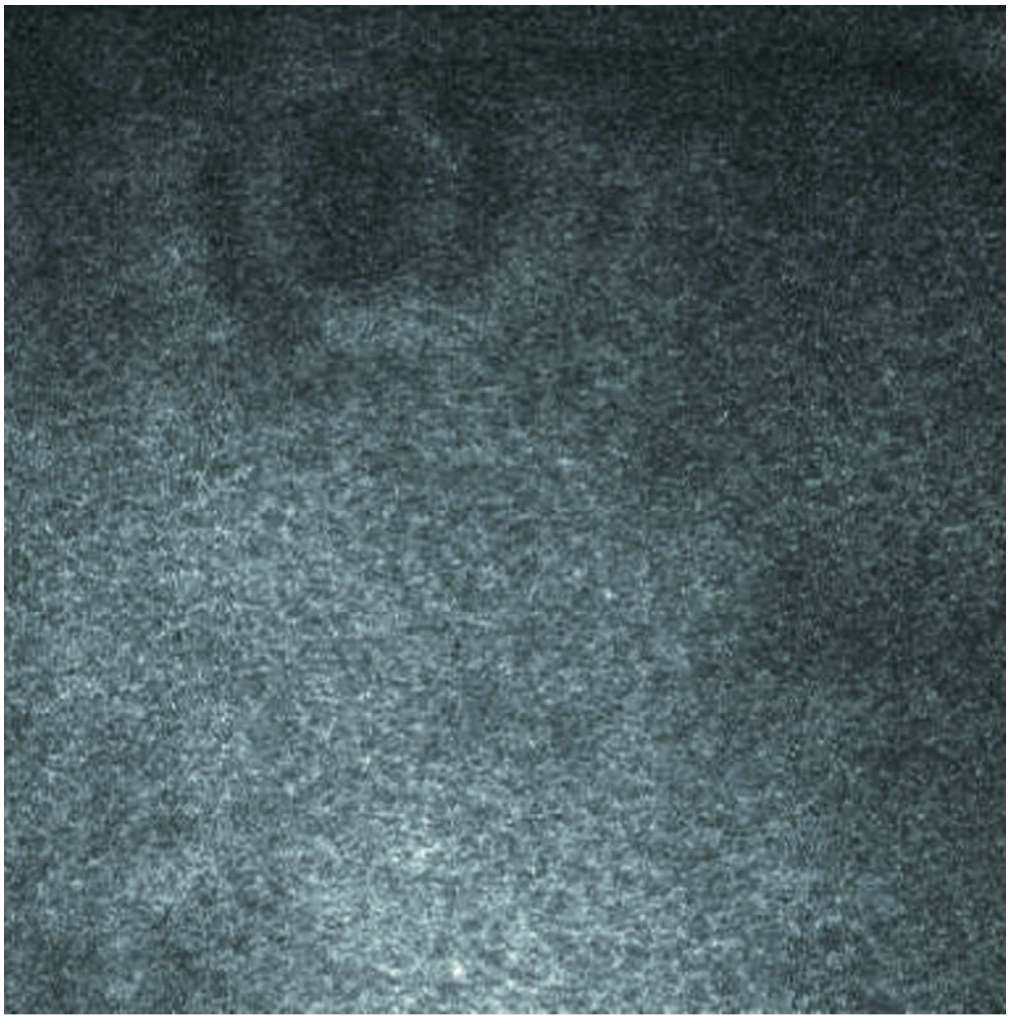
129x130mm (300 x 300 DPI)



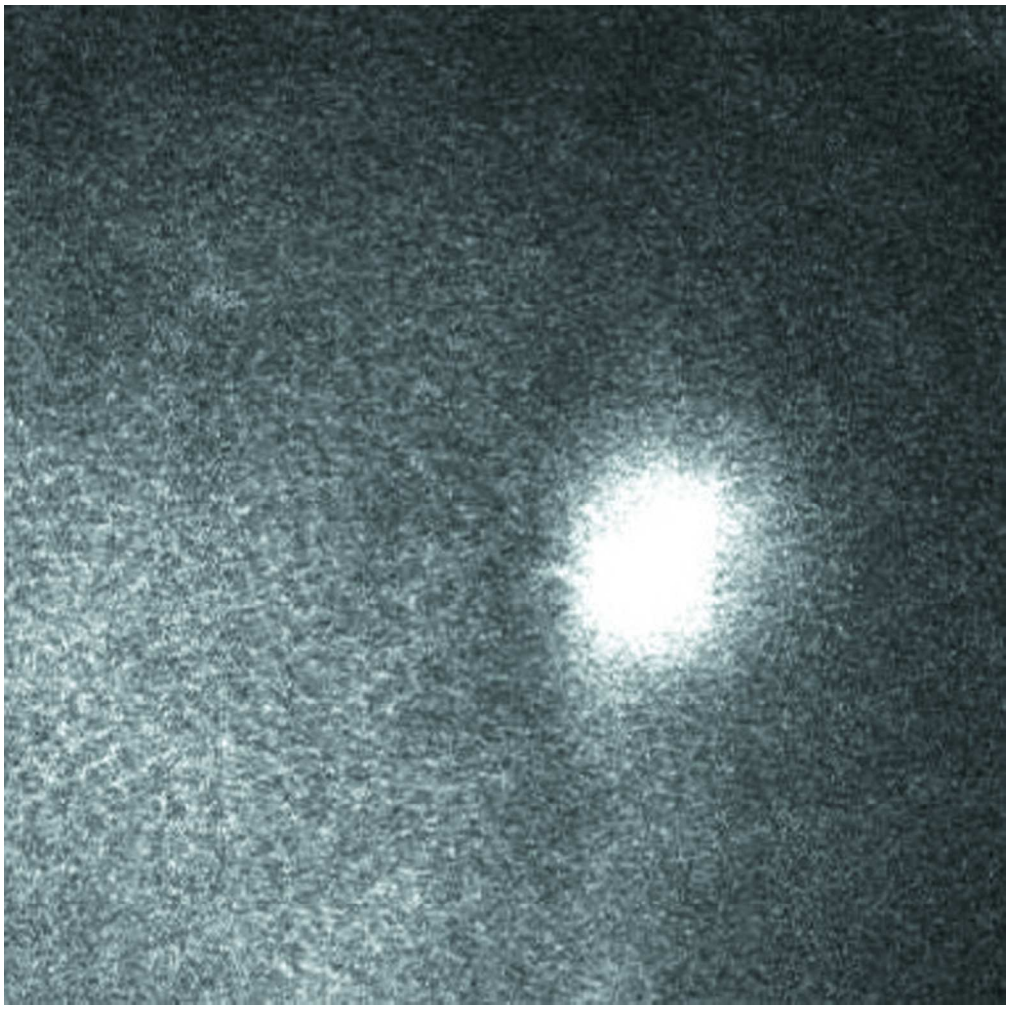
134x135mm (300 x 300 DPI)



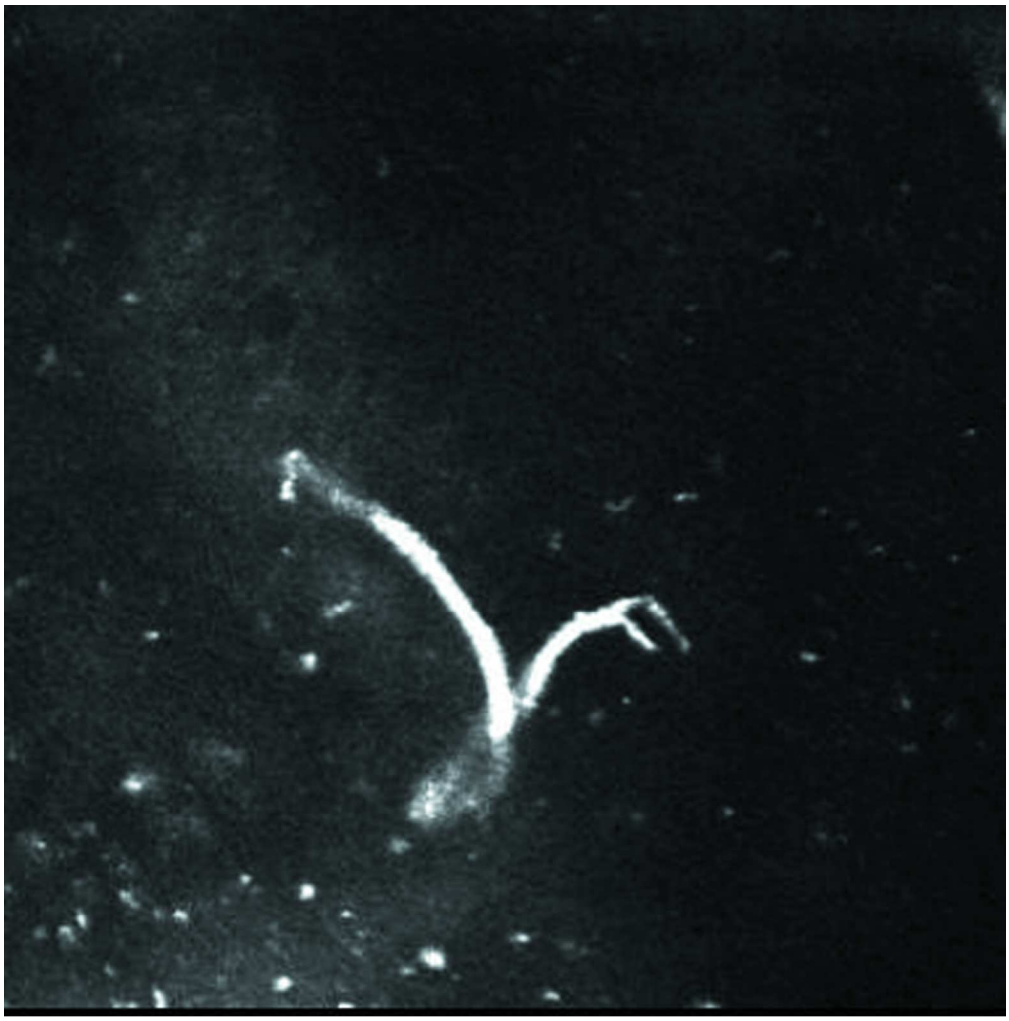
134x135mm (300 x 300 DPI)



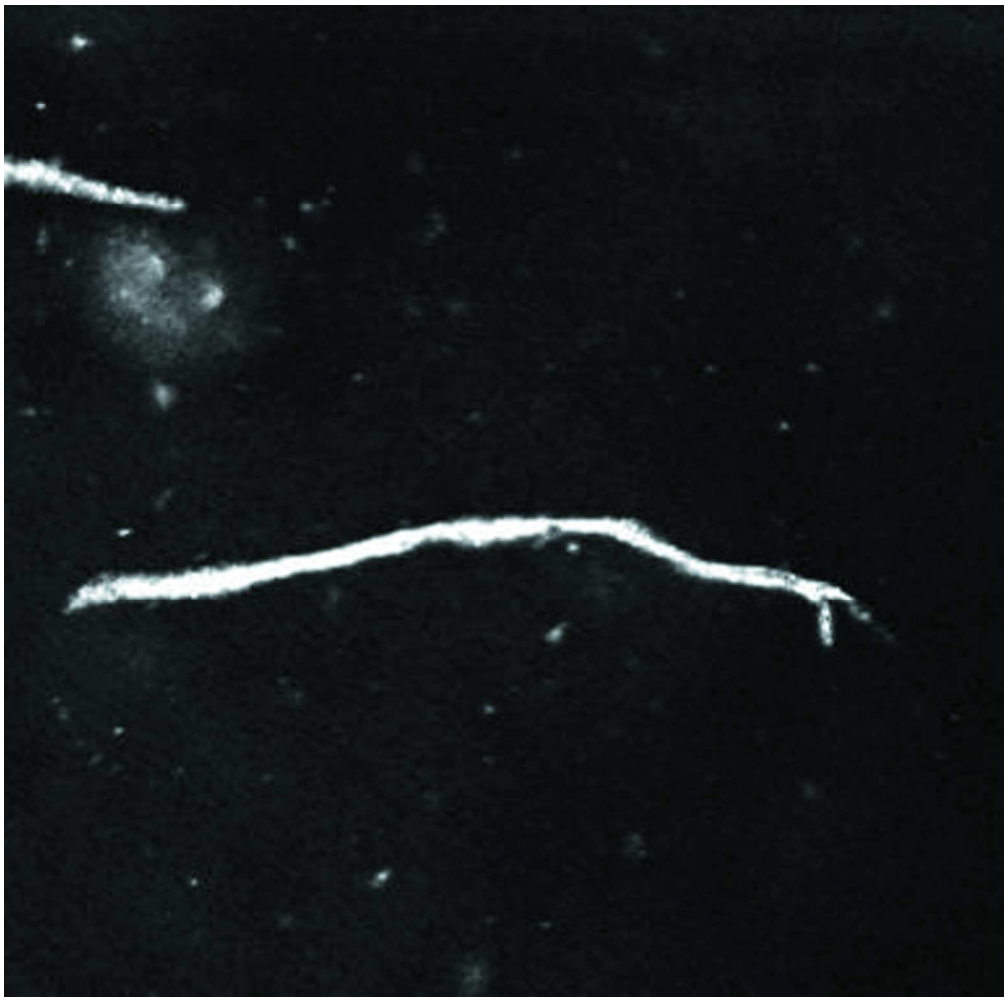
134x135mm (300 x 300 DPI)



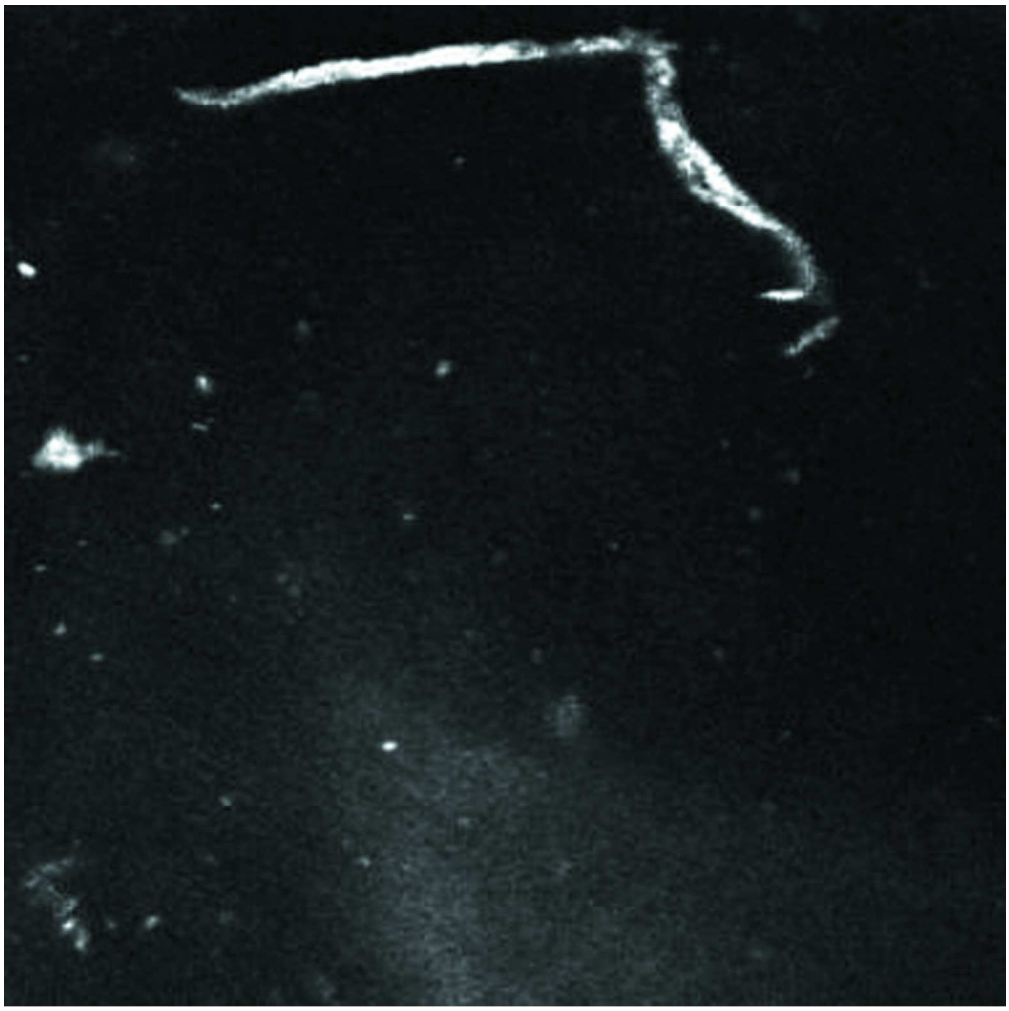
134x133mm (300 x 300 DPI)



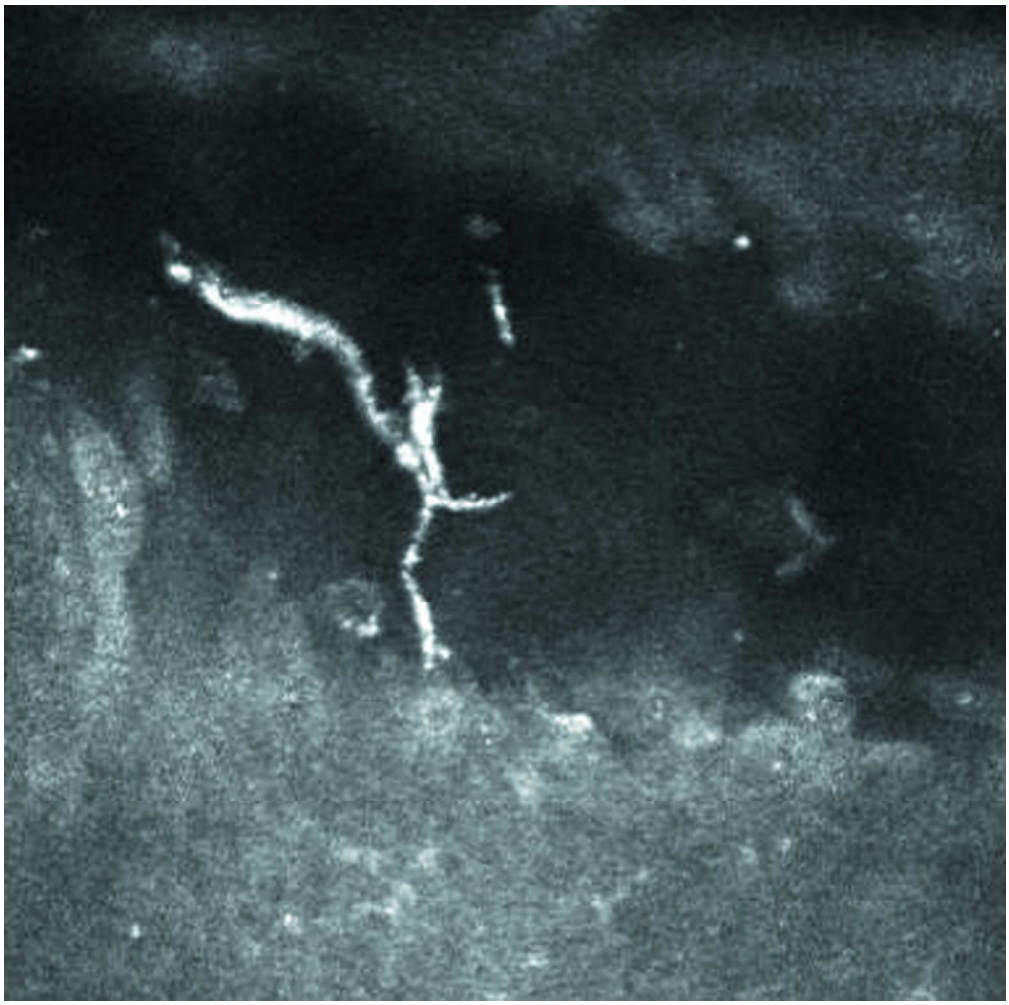
134x135mm (300 x 300 DPI)



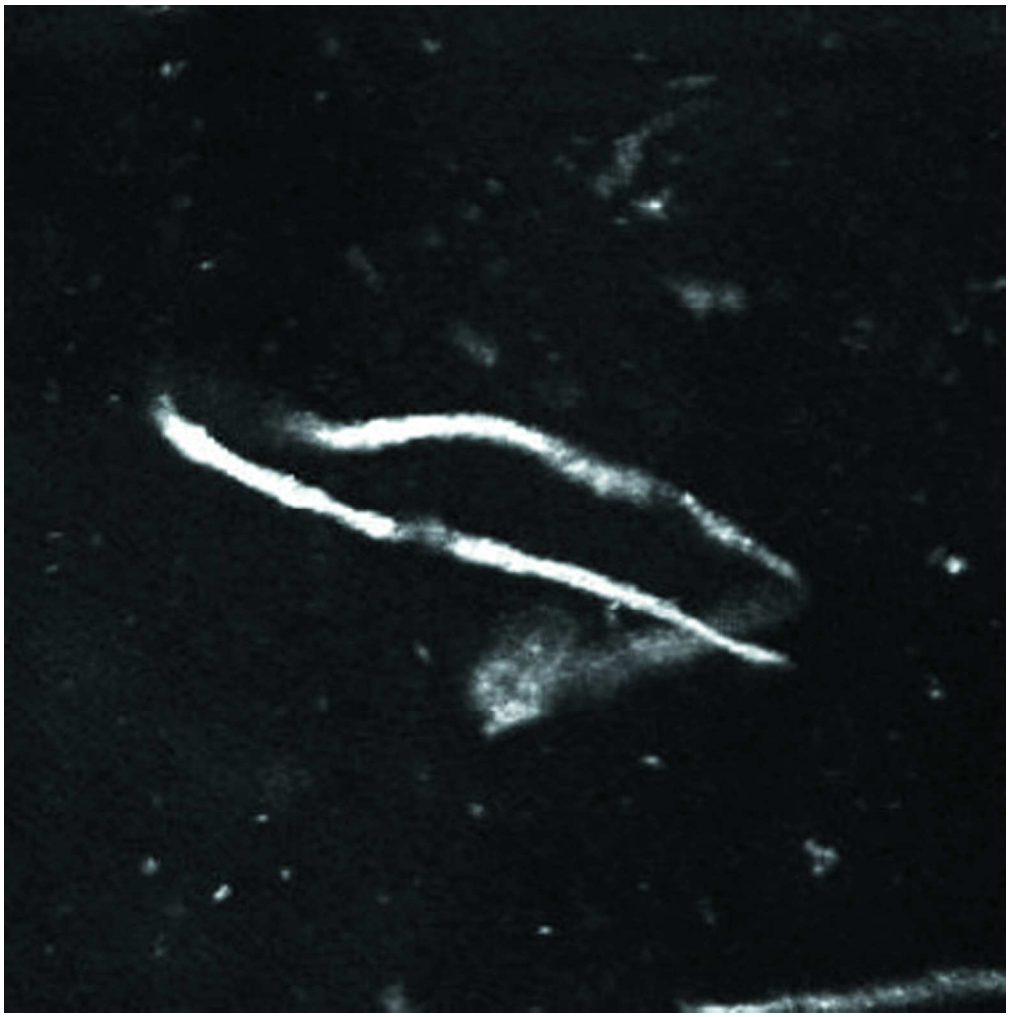
135x134mm (300 x 300 DPI)



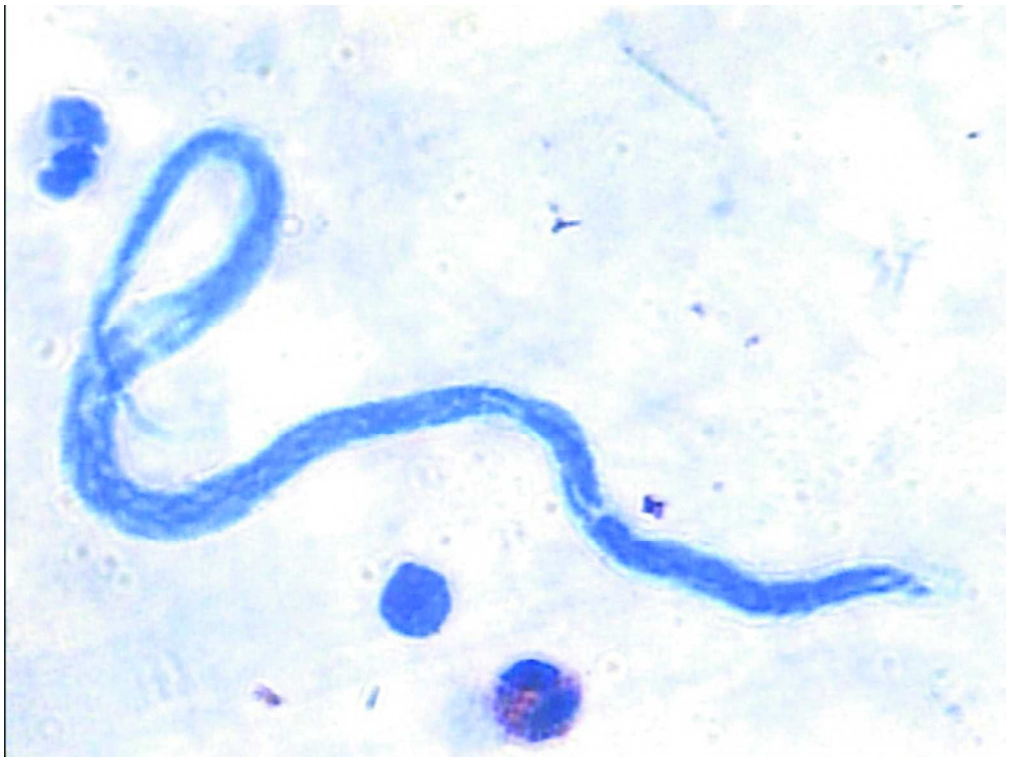
134x134mm (300 x 300 DPI)



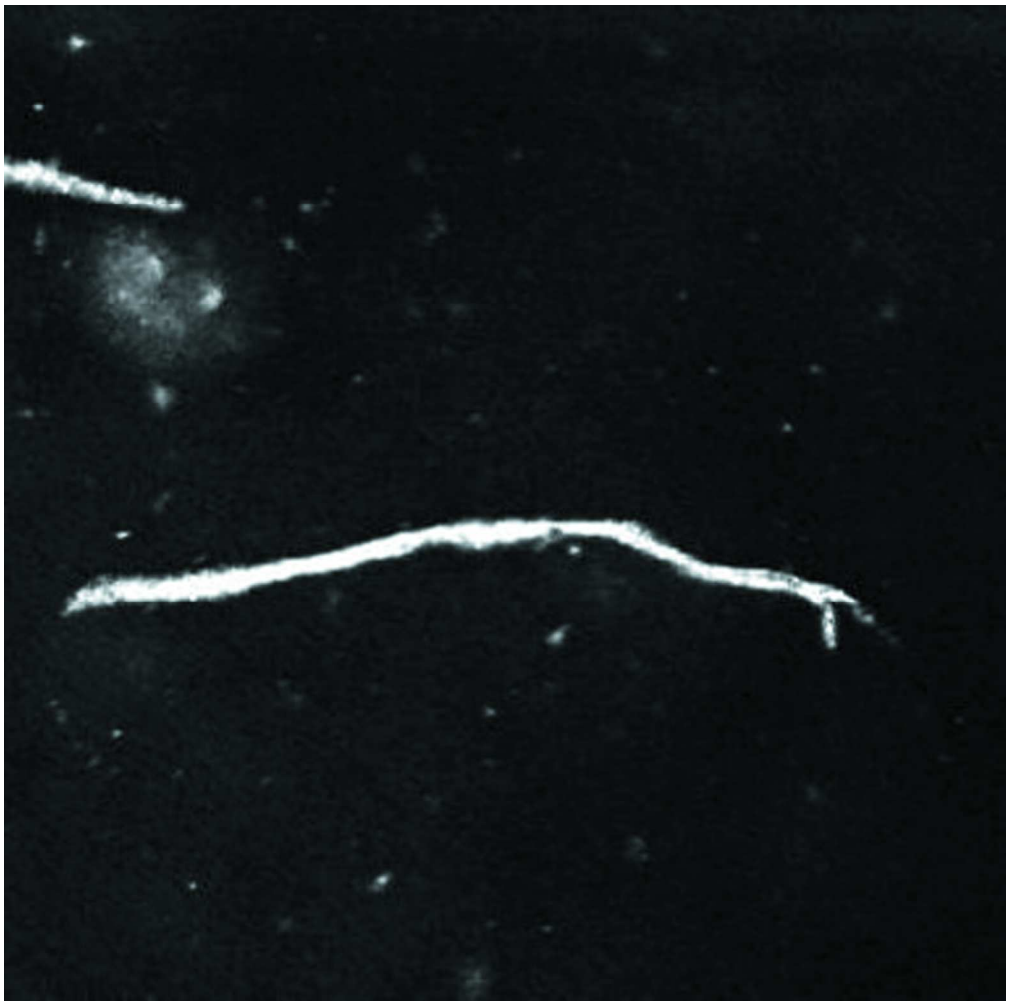
134x134mm (300 x 300 DPI)



132x133mm (300 x 300 DPI)



150x112mm (300 x 300 DPI)



134x134mm (300 x 300 DPI)

Competing Interests and Contributorship Statements:

***Article: Mansonella ozzardi* corneal lesions in the Amazon**

Authors: Lucas Monferrari Monteiro Vianna, Marilaine Martins, Marcos Jacob Cohen, Jacob Cohen, Rubens Belfort Jr

Statetment:

We state that none of us have competing interests.

We also state that all of us contributed in the conception and design of the study, acquisition, analysis and interpretation of data, drafting and revising of the article and the final approval of the version to be published.