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# Subclinical *Leishmania* infection in patients with rheumatic diseases under biological drugs

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## Abstract

**Purpose** Climate changes and immunosuppression are influencing the spread of leishmaniasis and re-emergence in Northern Italy, respectively. We evaluated the prevalence of subclinical leishmaniasis in patients from a Northern Italian region with chronic inflammatory rheumatism (CIRD) receiving biological drugs (BD) and correlated it to the area of residence.

**Methods** DNA from PBMC of patients affected by CIRD treated with either BD for at least 5 years (Group A) or other immunosuppressive drugs (Group B) was investigated by a qPCR for *Leishmania infantum* kDNA and compared to healthy subjects (Group C). Variables such as sex and age, rural areas, dog ownership, type of BD administered and association between BD and steroids, were evaluated by statistical analysis.

**Results** A higher proportion of *L. infantum* DNA positivity was found in Group A than in Group C ( $p < 0.05$ ), while no parasite DNA was detected in Group B. In Group A, 18/50 patients (36%) had higher rates of parasite DNA (from 1 to 136 to 1.000.000 copies/ml) than Group C (from 1 to 10 copies/ml). 14/18 (77.7%) of positive patients from Group A lived in rural areas, but no statistical differences occurred in relation to dog ownership or BD type ( $p < 0.0003$ ).

**Conclusions** We can speculate that exposure to rural areas appears to be a factor closely linked with the risk of developing *Leishmania* subclinical infection. A screening with molecular methods in patients with CIRD treated with BD living in these areas and monitoring *Leishmania* DNA during such therapies, would be mandatory to prevent delay in diagnosis should VL symptoms appear.

**Keywords** *Leishmania infantum* · Subclinical leishmaniasis · Biologic drugs · Rural areas · Northern Italy · Real-time PCR

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## Abbreviations

VL	Visceral leishmaniasis
CL	Cutaneous leishmaniasis
CIRD	Chronic inflammatory rheumatic diseases
RA	Rheumatoid arthritis
PA	Psoriatic arthritis
AS	Ankylosing spondylitis
BD	Biological drugs
qPCR	Quantitative real-time PCR

## Background

Leishmaniasis is one of the most important human-neglected parasitic diseases and is caused by *Leishmania* species transmitted by insect bites. Approximately, 12 million individuals are currently infected with *Leishmania* spp. with an estimated 2 million new cases occurring every year [1]. The major endemic areas are in the tropics and subtropics;

however, leishmaniasis exclusively due to *Leishmania infantum* is also endemic in large parts of Southern Europe where there is a focal prevalence of latent infection of up to 53% in the adult population [2], but it is a rare disease in other parts of Europe or North and Central Europe where it is diagnosed almost exclusively in travellers or migrants coming from tropical or subtropical countries [3]. In the world, several *Leishmania* spp., in particular *L. donovani/infantum* complex, constitute the predominant group as the ones responsible for causing substantial health problems in up to 400,000 people and 40,000 deaths per year [1, 4, 5].

In Italy, visceral leishmaniasis (VL) the most serious form of the disease to be distinguished from the more common cutaneous leishmaniasis (CL), is an endemic disease present in rural and peri-urban areas of continental and insular regions of Central and South Italy [6]. About 40–70 cases are notified annually, over half of which are from Sicily (data from the Italian Institute of Statistics, ISTAT).

In Emilia-Romagna, a Northern region of Italy, 44 cases were diagnosed in the period 1999–2011 of which 26 were VL; the number of cases rose to 30 in 2013 and the regional capital Bologna, was the most affected city [7].

Although environmental aspects such as climate change, growing urbanisation and socio-economic development are causing important changes in the epidemiology of some infectious diseases including leishmaniasis [8], host immunity is known to represent a key determinant of the clinical manifestation and outcome of this disease. In particular, immunosuppression has been identified as a major contributor to disease re-emergence in Europe other than in AIDS patients, in those with autoimmune rheumatic diseases who live in endemic areas [4, 9].

In this setting, a number of published case reports suggest an association between TNF- $\alpha$  antagonist use and evident leishmaniasis [10, 11].

One aspect not yet fully defined concerns the start of TNF- $\alpha$  antagonist therapy in patients with autoimmune diseases that require treatment with these drugs, already suffering from a *L. infantum* subclinical infection.

In these last years, the availability of polymerase chain reaction (PCR) assays amplifying leishmanial genetic material, has been a major step forward in improving the diagnosis of leishmanial disease and the response to treatment compared to serological methods which may fail to detect asymptomatic *Leishmania* infections especially in endemic areas [12]. These techniques have also been successfully used to detect follow-up of VL patients [13] and subclinical infections [14–16] and were suggested as marker of *L. infantum* infection in asymptomatic individuals for epidemiological studies in India [13].

Our study aimed to evaluate by a real-time PCR (qPCR) the prevalence of subclinical leishmaniasis in immunocompetent individuals living in some rural and suburban areas of

Emilia-Romagna region suffering from inflammatory rheumatic diseases (CIRD) receiving immunosuppressive treatment with biological drugs (BD) (Group A). This prevalence was compared to that found in two control groups consisting of rheumatologic patients who did not consider biological therapies and healthy individuals (B and C), respectively, matched for age and geographical area according to previous preliminary results [17]. Genomic positivity was related to the area of residence within the region, the dog ownership and, for Group A, to the type of BD administered and the concomitant association with steroids.

## Materials and methods

### Study patients and design

We performed a retrospective observational study enrolling 50 patients affected by CIRDs (rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis) treated with BD for at least 5 years at the Rheumatology Unit of the University of Ferrara (Group A) and two control groups consisting of 50 patients affected by CIRDs treated with drugs other than BD (Group B) and 50 healthy subjects (Group C). The three cohorts were selected from the same regional area.

Demographic and clinical data were collected using standard questionnaires, which included the region of provenance, the province of residence, the location of their home in an urban or rural context, and the ownership of a dog as a family pet.

All the enrolled subjects were negative by serologic screening for hepatitis B and C, HIV and tuberculosis and did not show any symptoms at the moment of the blood sampling. The CD4/CD8 ratio was  $> 2$  in all the enrolled subjects.

### Blood sample collection, PBMC preparation and DNA extraction

The isolation of PBMC was done from 10 ml of EDTA-anti-coagulated blood by density gradient centrifugation over Ficoll-paque plus (Amersham Biosciences Europe GmbH, Milan, Italy), according to the manufacturer's protocol. DNA was extracted from PBMC within 2 h after collection, as previously described [18]. Briefly, each sample was added to an equal volume of lysis buffer containing proteinase K (200 mg/ml), incubated for 1 h at 56 °C and then heated for 10 min at 96 °C. *L. infantum* DNA was extracted by phenol–chloroform–isoamyl alcohol followed by ethanol precipitation, and resuspended in TE buffer. Reference DNA extracted from *Leishmania* promastigotes cultures was provided. Promastigotes of canine *L. infantum* MON-1 (provided by IZSLER, Modena, Italy) were routinely cultured

in RPMI 1640 medium (pH 7.2) (Sigma, Germany) supplemented with 15% heat inactivated fetal calf serum (HIFCS) (Gibco, Paisley, UK), 100 U/ml penicillin, and 100 U/ml streptomycin sulphate in 25 cm<sup>2</sup> non-vented flasks (Corning, USA) [19]. Parasites were then incubated at 25 °C in a humidified incubator under air as a gas phase with medium replacement every 3–4 days. DNA preparations extracted from clinical blood specimens originating from patients with confirmed VL were also included in this study as positive control. In general, one blood sample was collected for each subject/patient.

### Real-time PCR

A real-time PCR (qPCR) for detection and quantification of *L. infantum* DNA was performed according to Lachaud et al. [20]. For accurate sensitivity, kinetoplast DNA was chosen as the molecular target. External primers were derived from RV1 and RV2 as previously described [21]. DNA detection was performed by means of a hybridization probe based on TaqMan chemistry (Applied Biosystems, Courtaboeuf, France) designed with Primer 3 software. A Stratagene (La Jolla, Calif.) MX 4000 system was used for amplification and detection. Optimization experiments led us to use the Stratagene qPCR master mix (catalog number 600549-51), 15 pmol of forward primer (CTTTTCTGGTCC TCCGGGTAGG), 15 pmol of reverse primer (CCACCCGGCCCTATTTTAC ACCAA), and 50 pmol of TaqMan probe (FAM-TTTTTCGCAGAAC GCCCT ACCCGC-TAMRA). Assays were performed with a 25 µl final volume with 1 µl of sample DNA. Two negative controls (DNA extracted from buffy coat obtained from a *Leishmania*-seronegative individual and an aliquot of distilled water, respectively) were included in each PCR run to detect contamination which could lead to false-positive results. The standard curve was established from *Leishmania* DNA extracted from 5 × 10<sup>6</sup> parasites; 1 µl of serial dilutions, ranging from the equivalent of 50,000 to 0.0001 parasites, was introduced into reaction tubes. TaqMan chemistry allowed two-step temperature (94 and 55 °C) cycling over 45 cycles. Linearity of quantification was made by serial dilution from 10 (4) to 10<sup>-1</sup> dilution. Quantification was given in parasite per ml. Within each run, linearity was checked with two dilutions 10 and 10<sup>-1</sup>, according to Mary et al. [22]. All qPCR reactions were performed at Parasitology-Mycology Laboratory of the Archet Hospital, University of Nice, France.

### Statistical analysis

Data analysis was performed using SPSS Statistics for Windows, version 21.0 (SPSS, Inc., Chicago, IL, USA). Normality of distribution for continuous variable was checked by Shapiro–Wilk test. Since the variables were

not normally distributed, group comparisons between controls and patients affected by CIRDS were performed using Mann–Whitney *U* test.

Chi-square tests were used to compare differences in categorical variables. To assess the association of the PCR positivity to *Leishmania* (outcome variable) with explanatory variables such as living in a rural area, having a dog or taking a steroid in association with a biological drug, a binary logistic regression analysis in the positive subgroup of CIRDS subjects was performed. To avoid possible over-correction of the regression models, three different logistic regressions were performed, one for each explanatory variable, including age and sex as confounding factors in each model. Two-tailed probability values < 0.05 were considered statistically significant.

### Ethical considerations

The study was approved by the ethics committee of the University of Ferrara, Italy. Written informed consents were obtained from all participants before their enrolment.

### Results

Demographical and clinical characteristics of the study population (*n* = 150) are summarized in Table 1. Group A patients were older than those from Group C (*p* < 0.001) without any difference with Group B; moreover, no significant difference in sex distribution between the three groups was found. There were no statistically significant differences between A, B and C groups regarding staying in a rural area and owning a dog as a family pet. Finally, Group A showed a statistically significant positivity for *L. infantum* circulant kDNA compared to Group C (*p* < 0.001), but not with Group B, since none of the subjects in this group was found to be

**Table 1** Demographical characteristics of rheumatologic patients receiving BD (A), rheumatologic patients receiving drugs other than BD (B) and healthy controls (C)

Patient's characteristics	A ( <i>n</i> = 50)	B ( <i>n</i> = 50)	C ( <i>n</i> = 50)
Age (years)	61(49–67) <sup>a</sup>	57 (34–72)	55 (31–60)
Gender (females, %)	70	62	70
Rural area (%)	36	38	36
To be owner of a dog (%)	36	38	34
Positivity for circulant <i>Leishmania</i> DNA (%)	36 <sup>b</sup>	0	10

Not-normally distributed variables are expressed as median (interquartile range), discrete variables as percentage

<sup>a</sup>*p* < 0.001, Mann–Whitney vs. Group C

<sup>b</sup>*p* < 0.001,  $\chi^2$  vs. Group C; the Group B was excluded from statistical analyses due to proportion equal to zero

positive and thus was excluded from the analysis. The three cohorts of patients were then analysed separately.

### Group A patients

Of the 50 analysed samples from Group A, 18 (36%; 14 women and 4 men, median age of 58.7), resulted positive by qPCR with the detection of a high parasite burden (1–136 parasites/ml in 4 patients, 1,000–40,000 in 11 patients and over 1,000,000 in 3 patients).

Laboratory features regarding *Leishmania* DNA detected by qPCR analysis and correlation with rural residence are summarized in Table 2.

Group A patients were then divided into negative and positive according to the detection of *Leishmania* DNA (Table 3).

All positive patients presented a long history of rheumatic disease begun between 8 and 29 years, when patients experienced symptoms, with a mean value of  $20.3 \pm 5.6$ . Of these, 12/18 (77.8%) suffered from rheumatoid arthritis (RA), 3/18 (11.1%) from ankylosing spondylitis (AS) and psoriatic arthritis (PA). 14/18 (66.6%) lived in rural areas as defined by the Organization for Cooperation and Economic Development (OECD; <http://www.oecd.org/regional/>). To have a dog did not influence the positivity to *Leishmania*.

**Table 3** Negative and positive patients from Group A according to detection of *Leishmania* DNA by qPCR analysis

Group A	qPCR	
Patient's characteristics	Negative (n = 32)	Positive (n = 18)
Age (years)	58.4 ± 13.2	58.9 ± 9.5
Disease duration (years)	22.0 ± 5.7	20.3 ± 5.6
Therapy duration (years)	10.7 ± 2.7	10.6 ± 3.4
Rural area (% , yes)	21.8	66.6 <sup>a</sup>
To be owner of a dog (% , yes)	31.3	50.0
Rheumatologic disease (%)		
RA	61.7	77.8
PA	6.0	11.1
AS	32.3	11.1
BD treatment (%)		
Anti-TNF-α	81.4	55.6
T Lymphocyte modulator	9.3	22.2
Anti-IL-6 receptor	9.3	22.2
Steroid drug (% , yes)	25.0	61.1 <sup>b</sup>

Continue variables are expressed as mean ± SD, discrete variables as percentage. RA: rheumatoid arthritis

PA psoriatic arthritis, AS ankylosing spondylitis

<sup>a</sup>p < 0.001,  $\chi^2$  vs. negative

<sup>b</sup>p < 0.05  $\chi^2$  vs. negative

**Table 2** *L. infantum* kDNA load (copies/ml) in Group A and Group C and correlation with rural residence

Membership group of the studied population	No. of rheumatologic patients positive to <i>L. infantum</i> circulant DNA	Rural areas	Parasite DNA load (copies/ml)
Group A	1	No	136
	2	Yes	21.666
	3	Yes	27.266
	4	No	15.000
	5	Yes	5.956
	6	No	0.2
	7	Yes	28.066
	8	Yes	24.366
	9	Yes	39.400
	10	Yes	6.5
	11	No	1.650
	12	Yes	1.960.000
	13	Yes	3.056
	14	Yes	1.286.666
	15	Yes	2.423.000
	16	Yes	7.610
	17	Yes	100
	Group C	18	Yes
1		Yes	10
2		Yes	1
3		Yes	1
	4	Yes	1

In Table 3 patients positive to *L. infantum* DNA are grouped, according to the pharmacological action of the BD into TNF- $\alpha$  antagonists, modulators of T lymphocyte activity and anti-IL-6 receptor antibodies. The possible influence of each type of drug on the molecular positivity for *L. infantum* circulant kDNA was assessed through a Chi-square test. No statistical difference among the different administered treatments [ $\chi^2(2) = 3.37, p = 0.185$ ] was found, although the percentage of positive subjects to qPCR was higher when under therapy with modulators of T lymphocyte activity and anti-IL-6 receptor antibodies compared to TNF- $\alpha$  antagonist therapy (57.1%, 50.0% and 26.3%, respectively). A further statistical analysis was performed on the basis of the concurrent, or not, administration of steroids together with the BD.

Interestingly, patients that were taking a steroid in association with the BD showed a higher proportion of *Leishmania* DNA positivity than those assuming the BD only [ $\chi^2(1) = 6.18, p < 0.05$ ; 55.6% vs. 21.2%]. Finally, by binary logistic regression we evaluated whether the kind of living area, to be owner of a dog or taking a steroid drug were associated to an increased risk of being positive to molecular method. As shown in Table 4, the fact of living in a rural area or taking a steroid associated to the assumption of a biological therapy, were factors strongly linked to an increased risk of being positive to *L. infantum* kDNA [O.R. (95%CI): 8.74 (2.24–34.05) and 5.52 (1.40–21.78), respectively], independently from confounding factors such as sex and age.

## Patients from Group B and C

No sample among those analysed from Group B was positive by qPCR. In the Group C, 4 positive (8%) samples were detected by qPCR (10 parasites/ml in 1 patient and 1 parasite/ml in 3 patients) (Table 2). Of the healthy subjects who resulted to be positive, only one owned a dog.

## Discussion

Global warming and man-made risk factors such as travel, migration, trade of animals and environmental modifications, are changing the temporal–spatial evolution of leishmaniasis in Europe which is hypoendemic especially in Mediterranean countries and remains in most cases asymptomatic, although immunosuppression can predispose to clinical disease [23–25].

In Northern Italy, leishmaniasis has been spreading in part because of climatic conditions that favour the development of the vector *Phlebotomus perniciosus* [26, 27], but also for the increased mobility of dogs to and from the traditional VL endemic areas [27], although the risk for human disease does exist but it is moderate, as the intensity of transmission seems to be lower than in traditional settings of Mediterranean VL.

Moreover, immunosuppressive drugs, in particular BD, have contributed to expand the number of reported cases in this area. In fact, the commercialization of the BD (such as TNF- $\alpha$  antagonists) has become increasingly frequent since the end of the 1990s and has been proven to be

**Table 4** Binary logistic regression for the risk of being positive to *Leishmania* DNA in subjects living in rural areas, having a dog, and taking steroid drugs in association with the BD

	B (SE)	Odds ratio (95% CI)
Model 1: rural area		
Intercept	−2.52 (2.00)	
Rural area (yes/no)	2.18 (0.69)**	8.74 (2.24–34.05)
Model 2: to be owner of a dog		
Intercept	−1.952 (1.78)	
Dog (yes/no)	0.948 (0.65)	2.58 (0.73–9.18)
Model 3: steroids		
Intercept	−0.27 (1.64)	
Steroids (yes/no)	1.71 (0.70)*	5.53 (1.40–21.78)

For Model 1:  $R^2 = 0.199$  (Cox & Snell), 0.278 (Nagelkerke)

For Model 2:  $R^2 = 0.044$  (Cox & Snell), 0.061 (Nagelkerke)

For Model 3:  $R^2 = 0.121$  (Cox & Snell), 0.168 (Nagelkerke)

B: unstandardized regression coefficient; SE: standard error; 95% CI: 95% confidence interval

Both models were corrected for possible confounders including age and sex, with living in a rural area, having a dog and assumption of steroid drugs in association with the biological therapy as predictors, and positivity to *Leishmania* as dependent variable

\* $p < 0.05$ , \*\* $p < 0.001$

effective in the management of several immune diseases, including CIRDS, RA, AS and psoriasis [28]. Their use, however, is not free of side effects especially for the onset of opportunistic infections, including leishmaniasis [29].

We aimed to investigate asymptomatic *L. infantum* human infection in Emilia-Romagna, an Italian region not traditionally considered as an area of endemicity, characterized by continental climate and by a low incidence of officially reported VL cases, but recently switched to “phlebotomus risk region” because of repeated and uninterrupted contacts with the vector and the animal reservoirs, as some coastal and hilly regions of the middle Adriatic and those of pre-Alpine Northern Italy. Canine leishmaniasis, has indeed been increasing in Northern Italy, spreading to rural and peri-urban areas as recently demonstrated [27].

Our preliminary results have demonstrated a strikingly higher prevalence of parasite DNA in Group A than in Group B and C ( $p < 0.001$ ,  $\chi^2$  vs. controls) in a sample consisting of 50 patients with CIRDS (Group A) treated with BD for at least 5 years compared to 50 rheumatologic patients assuming drugs other than BD (Group B) and 50 healthy controls randomized from the apparently health population (Group C). A large percentage of the positive patients of Group A (18; 66.6%), resided in rural areas where *L. infantum* is considered to be autochthonous and *P. perfliewi* is the predominant vector, as recently reported [8]. Theoretically, current global warming in all Mediterranean area may enhance leishmaniasis distribution due to the effect of temperature on parasite development and vector spread [7].

The high detection rate of circulant *L. infantum* DNA in a region where the pathogen is not considered endemic suggests that a significant local transmission of *L. infantum* to humans has been ongoing for years in these areas; in fact, *Leishmania* positivity in both study groups appears to be significantly related with residence in the rural rather than in urban context, highlighting the importance of a repeated and uninterrupted contact with the responsible vector and the animal reservoir [30].

So far, findings demonstrating higher rates of positivity in rural than in urban regions were recently reported in HIV positive patients in Morocco, where the risk of clinical VL is increased due to immunosuppression that may reactivate latent infections by *L. infantum* [31]. In other settings, travelling to an endemic area as well as the increasing international tourism and the influx of immigrants from endemic regions to non-endemic area can also be considered as significant risk factors for VL [32].

Four (8%) out of 50 healthy controls from Group C resulted to be positive by qPCR. This result was not surprising in accordance with other reports [14, 33, 34]. As for Group A, the reasons for the recent expansion of VL cases in these areas are almost unknown.

The absence of DNA positivity in Group B is difficult to explain since data regarding subclinical infections in patients receiving steroid treatments are lacking. As reported, these therapies could cause reactivation of serious opportunistic infections in immunocompromised patients with lupus, pemphigus, transplant recipients [28] and may increase the risk for *Leishmania*-infected people to develop VL by affecting the effector, suppressor, and cytotoxic T cells functions through the blockade of cytokine expression, increasing susceptibility to intracellular pathogens, including *Leishmania* [35]. The absence of *L. infantum* circulant DNA in this group, despite the intake of steroid therapy, could be also due to the fact that only 19 (38%) out of 50 patients resided in a rural context from some regional areas not fully involved by the circulation of the parasite and the competent vectors, with the consequence that less than half was exposed to *Leishmania* infection. This finding seems to support the hypothesis that only some regional areas are involved in the circulation of the parasite and the competent vector although the reasons for this phenomenon are not fully understood.

On the contrary, the detection of a very high parasitaemia in Group A, is an important finding that should be monitored as a potential indicator of a VL. BD therapies, by altering the inflammatory and immune balance, can impair the ability of host immune system to better control latent infections including leishmaniasis. Furthermore, it is likely that these patients did not develop clinical symptoms, despite the high level of parasitaemia and normal CD4<sup>+</sup> lymphocyte count. In this regard, no change of T-helper and regulatory cells activity was observed also in cirrhotic patients with unusual fever from areas endemic for *Leishmania*, later diagnosed as VL after careful specific investigations [36].

In contrast to the *Leishmania* DNA parasitemia found in blood donors infected with *Leishmania* or in multiply transfused patients and in persons from highly endemic countries for *L. infantum* [37, 38], we are not aware of any data on *Leishmania* parasitemia in completely asymptomatic individuals taking BD in whom the host's immune response has not demonstrated to be changed or downregulated towards a Th-2 cell-associated immune response against *Leishmania*. Probably, the exposure to rural areas appears to be a factor closely linked with the risk of developing *Leishmania* subclinical infection. In this regard, identification by qPCR of *Leishmania* DNA from asymptomatic cases, would allow new perspectives in *Leishmania* control and management. Although the presented data do not allow to make an interpretation on a parasitaemia dependent on the dose of the BD, we believe that in absence of literature data in this setting, the most appropriate choice in these individuals is to monitor DNA parasitemia throughout the period of treatment with BD. Moreover, since incubation period of VL in patients treated with TNF-alpha antagonists is perhaps much longer than in non-immunosuppressed patients, as demonstrated in



patients who have developed CL after such treatment [39], discontinuing TNF- $\alpha$  antagonist therapy should be done only when a diagnosis of leishmaniasis is established, and anti-leishmanial treatment should be started.

In our study, the decision to employ molecular methods for the diagnosis of asymptomatic *Leishmania* infection was supported by the fact that serology testing is quite specific and sensitive in diagnosing active VL in immunocompetent subjects [40, 41], although serological titers decline more or less rapidly in a significant portion of patients after the resolution of the disease [41]. Moreover, the above test frequently gives false-negative results in immunocompromised patients even in the presence of positive qPCR amplification [42] making their use unsatisfactory for detecting asymptomatic *Leishmania* infection, characterized by low or intermittent parasitaemia. Finally, the choice to extract and test DNA from PBMCs, instead of whole blood, has favoured the finding of high parasitic burdens, since PBMCs are target cells for the intracellular parasite [14].

Our data also showed that dog ownership was not correlated with *Leishmania* DNA positivity as domestic dogs play only a minor role in the transmission cycle of the parasite [43–45] even if the neighbour's dogs as well as the stray dogs present in large amounts in Northern Italy known to be highly seropositive for *L. infantum* [27], can be potential vectors of disease transmission. Moreover, rates of infection of sandflies fed with blood from VL patients are not dissimilar to those fed with infected dogs [43] and parasite prevalence in wild canines is often as high as in domestic dogs [45]. In this regard, to perform a canine serology near a patient's homes as a kind of sentinel for exposure to *Leishmania* would be of great interest.

With regard to the type of immunosuppressive treatment, we have not found statistically significant differences in relation to the BD administered (TNF- $\alpha$  antagonists, modulators of T lymphocyte activity and anti-IL-6 receptor antibodies) and to its possible influence on the molecular positivity for qPCR ( $\chi^2$  (2) = 3.37,  $p$  = 0.185), although the percentage of the subjects positive to parasite DNA were higher when under therapy with modulators of T lymphocyte activity and anti-IL-6 receptor antibodies, compared to TNF- $\alpha$  antagonist treatment (Table 3, 57.1%, 50.0% and 26.3%, respectively). This result, however, although interesting and worthy of further investigations, has not allowed to draw any statistical significance in this group for the small sample size.

Several literature data suggest that TNF- $\alpha$  antagonists may increase susceptibility to opportunistic infections, including leishmaniasis [10, 11, 46].

Interestingly, the proportion of positive patients to qPCR (n. 18) statistically increases among those receiving the BD in combination with steroids (Table 3,  $\chi^2$  (1) = 6.18,  $p$  < 0.05; 55.6 vs. 21.2%). This finding supports several reports regarding cases of *Leishmania* reactivation in patients receiving

chronic steroid treatment for different indications, including asthma, sarcoidosis, and myasthenia gravis [29].

Our study was limited by the small sample size and the lack of seroprevalence survey in property and neighbours' dogs. However, considering that Emilia-Romagna is a well-known region with high canine seroprevalence for *L. infantum* (2%, according data published in 2014) [47], living in a rural area or taking a steroid drug in addition to a biological therapy, were demonstrated (Table 4) to be factors strongly associated to an increased risk of being positive to *Leishmania* independently from sex and age [O.R. (95% CI): 8.74 (2.24–34.05) and 5.52 (1.40–21.78), respectively]. This original finding seems to be relevant since current literature lacks sufficient data to fully explain the spread of leishmaniasis in endemic and hypo-endemic rural areas such as Emilia-Romagna region.

## Conclusion

To our knowledge, the present study represents a first survey on *Leishmania* subclinical infection in rheumatologic patients receiving BD in an Italian region not traditionally considered endemic.

Since exposure to rural areas has been shown to be closely linked to the risk of developing a subclinical leishmanial infection that CIRD patients under BD seem to control less efficiently compared to healthy controls, screening and monitoring *Leishmania* DNA using molecular methods in candidates treated with BD living in these areas would be mandatory to prevent delay in diagnosis should VL symptoms appear.

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**Author contribution** MM and CC equally contributed to the design of the study and the writing of the manuscript. MM CC and AT analysed and interpreted the patient's data. GM and AT performed the statistical analysis. PM, SA, SH and TB contributed to the revision of the article. MG contributed to the collection of patient's data. All authors read and approved the final version of the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Ethics approval** The study was approved by the ethics committee of the University of Ferrara, Italy (protocol no. 150494).

**Informed consent** Written informed consents were obtained from all participants before their enrolment.

**Consent for publication** All the enrolled subjects had signed a written informed consent for publication.

**Data availability** All the data included in the manuscript are available from the authors upon reasonable request.

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