Research Article

Serological and Molecular Evaluation of *Leishmania infantum* Infection in Stray Cats in a Nonendemic Area in Northern Italy

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Infection by *Leishmania* species is increasing worldwide. It was hypothesized recently that cats act as a secondary reservoir for *Leishmania* infection. The aim of the present study was to assess the prevalence of *Leishmania infantum* antibodies and DNA in blood samples collected in a sample of stray cats in metropolitan area of Milan in northern Italy, which is a nonendemic area for leishmaniasis. An indirect immunofluorescence antibody test for *L. infantum* showed that 59 of 233 cats (25.3%) were seroreactive, 38 samples (16.3%) had antibody titers of 1:40, 15 (6.4%) had antibody titers of 1:80, and 6 (2.6%) had antibody titers of 1:160. Feline immunodeficiency virus (FIV) seropositive status was statistically associated with seroreactivity to *L. infantum* (\(P = 0.01\)) as shown by univariate and multivariate logistic regression (\(P = 0.0098; \text{OR} = 7.34\)). All blood samples that were tested using real-time PCR were negative for parasite DNA. These results were surprising, since no autochthonous human or canine cases of leishmaniasis have ever been reported in this region of northern Italy. It is possible that this high seroreactivity to *L. infantum* could be due to cross-reaction with antigens from other parasites. Additional studies that include parasite isolation are needed to clarify our findings on feline leishmaniasis in this region.

1. Introduction

Leishmaniasis in the Old World is caused by the protozoa *Leishmania infantum*. It is prevalent in countries in the Mediterranean basin, and dogs are the main reservoir of the parasite in that region [1]. In recent years, autochthonous cases of human and canine disease have been recorded at higher latitudes, namely, in Germany [2, 3], The Netherlands [4], and North America [5]. Infections have also been reported in species other than dogs and humans, including horses [6] and cows [7]. There have been numerous reports of feline leishmaniasis (FeL), mostly in cats living in known endemic areas [8–10]; some of the cats had concurrent immunosuppressive infections [9–11]. In countries in southern Europe, where canine leishmaniasis (CanL) is endemic, serological investigations of feline populations have revealed seroprevalence rates ranging from less than 1% to more than 60% [9–21]. Given the diffusion of *Leishmania* infection and the lack of information regarding infection rates in cats in the Milan metropolitan area in northern Italy, the aim of the present study was to assess the prevalence of leishmaniasis in a large representative sample of stray cats from this nonendemic area. A secondary aim was to analyze the results according to clinical, laboratory and infectious data.

2. Materials and Methods

2.1. Feline Population. During a 2-year period (January 2008 to January 2010), blood samples were collected from 233 European shorthair stray cats from urban colonies in Milan, northern Italy, during a trap-neuter-release (TNR) program that was approved by the local authority of the city council. The program was conducted as described previously [22].
2.2. Data Collection. The following data were recorded: sex 
 \((n = 233)\), age \((n = 233)\), body condition score (BCS) 
 \((n = 215)\), area of colony of provenance, that is, one 
of the seven municipalities of Milan \((n = 233)\), health status 
based on physical examination \((n = 233)\), and dermatological 
evaluation \((n = 121)\). Cats were classified as healthy or 
unhealthy depending on the clinical findings (Table 1).

2.3. Sample Collection. Whole blood samples were collected 
by cephalic or jugular venipuncture into tubes with EDTA 
anticoagulant for complete blood cell (CBC) count and 
polymerase chain reaction (PCR) testing and into empty 
tubes for serology. All samples used for serology and PCR 
were stored at −20°C until use.

2.4. Hematological and Serological Examination. Within 24 
hours of sample collection, a CBC count was performed on 
whole blood \((n = 127)\) using an ADVIA 120 System (Siemens 
Healthcare Diagnostics, Milan, Italy). Cats were categorized 
as having alterations in the CBC as shown in Table 1.

Serological assessment was performed to determine the 
presence of the following antibodies: to the feline immuno- 
deficiency virus (FIV) relative to the gp40 and p24 FIV 
antigens, the feline leukemia virus (FeLV) p27 antigen (Snap 
FeLV/FIV Combo Plus Test, Idexx Laboratories, Hoofddorp, 
The Netherlands) \((n = 137)\), and Toxoplasma gondii IgG 
antibodies (IFAT, Fuller Laboratories, Fullerton, CA, USA) 
\((n = 79)\). The results of these serological tests have already 
been published [22] and were reanalyzed with the present 
results.

For various technical reasons, not all data were available 
for all 233 cats.

2.5. Indirect Immunofluorescence Antibody Test. The pres-
ence of anti-\(L. \infantum\) antibodies was measured by an 
indirect immunofluorescence antibody test (IFAT) per-
fomed according to the recommendations of OIE [23] 
using MHOM/IT/80/IPT1 as a whole-parasite antigen fixed 
on multipot slides (Bio Merieux Spa, Florence, Italy) 
and fluorescently-labeled antifeline gamma globulin (Sigma 
Aldrich, Milan, Italy) as conjugate. Positive sera were diluted 
serially and tested to establish the maximum reaction titer, 
starting at a dilution of 1:40. Positive and negative controls 
were included on each slide.

2.6. PCR. \(L. \infantum\) DNA was amplified from 200 \(\mu L\) 
of whole blood by real-time PCR using the Illustra Blood 
genomicPrep Mini Spin kit (GE Healthcare, Milan, Italy) 
following the manufacturer’s instructions. The target for 
amplification was a 116-bp fragment in the constant region 
of the \(k\)DNA minicircle of \(L. \infantum\). This is one of the \(k\)DNA 
minicircle families that is used to identify the \(Leishmania\) 
genus. The primers used were QLK2-UP \(5’-\text{GGCGTTCTG-} \)
\(\text{CGAAACCCG-3’}\) and QLK2-DOWN \(5’-\text{AAATGGCCA-} \)
\(\text{TTTCGGGCCC-3’}\); the TaqMan probes were Q Leish Probe 
2 and \(5’-\text{FAM TGGGTGCAGAATCCCGTTCAGG-3’-Black Hole.}\)

2.7. Statistical Analysis. Univariate analysis of the categorical 
data was performed using the chi-square test or Fisher’s exact 
test. Any parameters statistically linked to IFAT seroreactivity 
for \(L. \infantum\) or to the presence of \(L. \infantum\) DNA as 
detected by PCR were used in a logistic regression model to 
test for independent risk factors associated with the \(L. \infan-
tum\) positivity. Associations were considered statistically sig-
nificant when \(P < 0.05\); both the \(P\) value and odds ratio (OR) 
are reported. Data were analyzed using MedCalc Software 
(version 12.3.0; Mariakerke, Belgium).

3. Results

The characteristics of the feline study population are summa-
rized in Table 1. The serology test for \(L. \infantum\) showed that 
25.3\% \((59/233)\) of the cats had \(L. \infantum\) seroreactivity, 38 
(16.3\%) had antibody titers of 1:40, 15 (6.4\%) had titers of 
1:80, and 6 (2.6\%) had antibody titers of 1:160. All blood 
samples tested using real-time PCR were negative for the 
presence of \(L. \infantum\) DNA. Standard curve and amplifi-
cation curve of real-time PCR were reported in Figures I and 
2, respectively.

No statistical association was found between seroreactiv-
ity to \(L. \infantum\) and age, sex, BCS, municipality of prove-
ance, clinical finding, dermatological findings or FeLV, and 
\(T. \text{gondii}\) serology. In contrast, in terms of CBC, neutrophilia 
was statistically associated with seroreactivity to \(L. \infan-
tum\) \((P = 0.01)\) in univariate analysis, but this association 
was not confirmed using multivariate logistic regression 
\((P = 0.57)\). In terms of serology for the retrovirus, FIV seropositive 
status was statistically associated with seroreactivity to \(L. \infan-
tum\) \((P = 0.01)\). This association was confirmed by 
multivariate logistic regression: \(P = 0.0098\) and OR \(= 7.34\) 
\((95\% CI = 1.96 \text{ to 27.59})\). The distribution of the parameters 
that were evaluated and compared in \(L. \infantum\) seropositive 
and seronegative cats is shown in Table 1.

4. Discussion

This study is the first epidemiological investigation of feline 
\(Leishmania\) infection in the metropolitan area of Milan, 
which is a nonendemic area for leishmaniasis. We found 
seroreactivity to \(L. \infantum\) by IFAT in 59 of the 233 
(25.3\%) stray cats that we examined. These results were 
surprising, since no autochthonous human or canine cases 
of leishmaniasis have ever been reported in this region in 
northern Italy. In countries in southern Europe where leish-
maniasis is endemic, serological investigations performed in 
feline populations using different techniques have revealed 
prevalence rates that range from less than 1\% to more than 60\% [9–21]. In particular, the seroprevalence in Italy ranges 
from 0.9\% to 68\% [9–21, 13], in Spain from 3.7\% to 60\% 
[14–16], and in Portugal from 0.6\% to 2.8\% [18–20]. In 
Greece, the seroprevalence is 3.9\% [17] and in France it is 
12.4\% [12]. These results in \(L. \infantum\) endemic geographical 
regions may reflect differences in the serological techniques 
used, in the cut-off values or positive thresholds and in 
the populations of cats that were tested. As here, previous
Table 1: Characteristics of a population of stray cats in northern Italy and a comparison of characteristics in *Leishmania infantum* seropositive versus seronegative cats as determined using an indirect immunofluorescence antibody test.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Category</th>
<th>Total population</th>
<th>Sero positive</th>
<th>Sero negative</th>
<th>Univariate P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Young (&lt;6 months)</td>
<td>106 (45.5%)</td>
<td>24 (40.7%)</td>
<td>82 (47.1%)</td>
<td>0.4788</td>
</tr>
<tr>
<td></td>
<td>Adult (&gt;6 months)</td>
<td>127 (54.5%)</td>
<td>35 (59.3%)</td>
<td>92 (52.9%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>153 (65.7%)</td>
<td>38 (64.4%)</td>
<td>115 (66.1%)</td>
<td>0.9387</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>80 (34.3%)</td>
<td>21 (35.6%)</td>
<td>59 (33.9%)</td>
<td></td>
</tr>
<tr>
<td>BCS</td>
<td>Scarce (&lt;3/9)</td>
<td>19 (8.8%)</td>
<td>4 (7.4%)</td>
<td>15 (9.3%)</td>
<td>0.8802</td>
</tr>
<tr>
<td></td>
<td>Good (&gt;3/9)</td>
<td>196 (91.2%)</td>
<td>50 (92.6%)</td>
<td>146 (90.7%)</td>
<td></td>
</tr>
<tr>
<td>Colony of origin</td>
<td>Zone 2</td>
<td>11 (4.7%)</td>
<td>2 (3.4%)</td>
<td>9 (5.2%)</td>
<td>0.0825</td>
</tr>
<tr>
<td></td>
<td>Zone 4</td>
<td>95 (40.8%)</td>
<td>18 (30.5%)</td>
<td>77 (44.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zone 5</td>
<td>9 (3.9%)</td>
<td>0 (0.0%)</td>
<td>9 (5.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zone 6</td>
<td>23 (9.9%)</td>
<td>8 (13.6%)</td>
<td>15 (8.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zone 7</td>
<td>53 (22.7%)</td>
<td>17 (28.8%)</td>
<td>36 (20.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zone 8</td>
<td>21 (9.0%)</td>
<td>5 (8.5%)</td>
<td>16 (9.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zone 9</td>
<td>21 (9.0%)</td>
<td>9 (15.2%)</td>
<td>12 (6.9%)</td>
<td></td>
</tr>
<tr>
<td>Clinical examination</td>
<td>Healthy</td>
<td>49 (21.0%)</td>
<td>12 (20.3%)</td>
<td>37 (21.3%)</td>
<td>0.9728</td>
</tr>
<tr>
<td></td>
<td>Unhealthy</td>
<td>184 (79.0%)</td>
<td>47 (79.7%)</td>
<td>137 (78.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stomatitis</td>
<td>92 (39.5%)</td>
<td>17 (28.8%)</td>
<td>75 (43.1%)</td>
<td>0.0740</td>
</tr>
<tr>
<td></td>
<td>Ocular discharge</td>
<td>35 (15.0%)</td>
<td>10 (16.9%)</td>
<td>25 (14.4%)</td>
<td>0.7881</td>
</tr>
<tr>
<td></td>
<td>Nasal discharge</td>
<td>21 (9.0%)</td>
<td>5 (8.5%)</td>
<td>16 (9.2%)</td>
<td>0.9236</td>
</tr>
<tr>
<td></td>
<td>Pale mucous membranes</td>
<td>12 (5.2%)</td>
<td>4 (6.8%)</td>
<td>8 (4.6%)</td>
<td>0.7532</td>
</tr>
<tr>
<td></td>
<td>Lymphadenomegaly</td>
<td>117 (50.2%)</td>
<td>30 (50.8%)</td>
<td>87 (50%)</td>
<td>0.9696</td>
</tr>
<tr>
<td>Dermatological examination</td>
<td>Absence of lesions</td>
<td>83 (68.6%)</td>
<td>17 (54.8%)</td>
<td>66 (73.3%)</td>
<td>0.0912</td>
</tr>
<tr>
<td></td>
<td>Presence of lesions</td>
<td>38 (31.4%)</td>
<td>14 (45.2%)</td>
<td>24 (26.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crusted dermatitis</td>
<td>22 (18.2%)</td>
<td>7 (22.6%)</td>
<td>15 (16.7%)</td>
<td>0.6410</td>
</tr>
<tr>
<td></td>
<td>Scaling</td>
<td>5 (4.1%)</td>
<td>1 (3.2%)</td>
<td>4 (4.4%)</td>
<td>0.8188</td>
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<tr>
<td></td>
<td>Nodular dermatitis</td>
<td>3 (2.5%)</td>
<td>2 (6.5%)</td>
<td>1 (1.1%)</td>
<td>0.3273</td>
</tr>
<tr>
<td></td>
<td>Alopecia</td>
<td>18 (14.9%)</td>
<td>8 (25.8%)</td>
<td>10 (11.1%)</td>
<td>0.0910</td>
</tr>
<tr>
<td></td>
<td>Ectoparasites</td>
<td>27 (22.3%)</td>
<td>6 (22.2%)</td>
<td>21 (77.8%)</td>
<td>0.8346</td>
</tr>
<tr>
<td></td>
<td>Dermatophytosis</td>
<td>9 (7.4%)</td>
<td>1 (3.2%)</td>
<td>8 (8.9%)</td>
<td>0.5225</td>
</tr>
<tr>
<td>CBC results</td>
<td>Absence of anemia</td>
<td>29 (22.8%)</td>
<td>5 (16.7%)</td>
<td>24 (24.7%)</td>
<td>0.5015</td>
</tr>
<tr>
<td></td>
<td>Presence of anemia</td>
<td>98 (77.2%)</td>
<td>25 (83.3%)</td>
<td>73 (75.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased Ht</td>
<td>97 (76.4%)</td>
<td>25 (83.3%)</td>
<td>72 (74.2%)</td>
<td>0.4352</td>
</tr>
<tr>
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<td>Decreased Hb</td>
<td>23 (18.1%)</td>
<td>7 (23.3%)</td>
<td>16 (16.5%)</td>
<td>0.5627</td>
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<tr>
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<td>Decreased RBC</td>
<td>41 (32.3%)</td>
<td>11 (36.7%)</td>
<td>30 (30.9%)</td>
<td>0.7158</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia</td>
<td>10 (7.9%)</td>
<td>2 (7.7%)</td>
<td>8 (8.2%)</td>
<td>0.9149</td>
</tr>
<tr>
<td></td>
<td>Leukocytosis</td>
<td>5 (3.9%)</td>
<td>2 (6.7%)</td>
<td>3 (3.1%)</td>
<td>0.7319</td>
</tr>
<tr>
<td></td>
<td>Leukopenia</td>
<td>15 (11.8%)</td>
<td>3 (10.0%)</td>
<td>12 (12.4%)</td>
<td>0.9776</td>
</tr>
<tr>
<td></td>
<td>Neutrophilia</td>
<td>15 (11.8%)</td>
<td>8 (26.7%)</td>
<td>7 (7.2%)</td>
<td>0.01 (0.57)*</td>
</tr>
<tr>
<td></td>
<td>Neutropenia</td>
<td>2 (1.6%)</td>
<td>0 (0.0%)</td>
<td>2 (2.1%)</td>
<td>0.9631</td>
</tr>
<tr>
<td></td>
<td>Lymphocytosis</td>
<td>2 (1.6%)</td>
<td>0 (0.0%)</td>
<td>2 (2.1%)</td>
<td>0.9631</td>
</tr>
<tr>
<td></td>
<td>Lymphopenia</td>
<td>33 (26.0%)</td>
<td>12 (40.0%)</td>
<td>21 (21.6%)</td>
<td>0.0776</td>
</tr>
<tr>
<td></td>
<td>Eosinophilia</td>
<td>12 (9.4%)</td>
<td>4 (13.3%)</td>
<td>8 (8.2%)</td>
<td>0.6346</td>
</tr>
<tr>
<td></td>
<td>Eosinopenia</td>
<td>33 (26.0%)</td>
<td>9 (30.0%)</td>
<td>24 (24.7%)</td>
<td>0.7371</td>
</tr>
<tr>
<td>FIV status</td>
<td>Positive</td>
<td>12 (8.8%)</td>
<td>7 (21.2%)</td>
<td>5 (4.8%)</td>
<td>0.01 (0.0098)*</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>125 (91.2%)</td>
<td>26 (78.8%)</td>
<td>99 (95.2%)</td>
<td>OR = 7.34 (95% CI 1.96–27.59)</td>
</tr>
</tbody>
</table>
epidemiological studies have used IFAT to detect antibodies to *Leishmania* spp. in cats. An important concern is that there is no standardized IFAT method for serological evaluation of antibodies to *Leishmania* spp. in cats; accordingly, there is no universally accepted antibody titer cut-off value that corresponds to active infection. Cut-off titers validated in dogs are used frequently for cats, but the immune response could be different in cats than in dogs.

None of the peripheral blood samples we examined using real-time PCR were positive for parasite DNA. PCR has been used previously by others, either alone or in combination with serology, as in our study, to assess the prevalence of feline *Leishmania* infection [9, 11, 14, 24, 25]. Blood is not the best specimen for PCR diagnosis of leishmaniasis. Specifically, PCR performed on canine blood has lower sensitivity, specificity, and positive and negative predictive values compared to PCR performed on canine lymph node aspirates [26], and this may be true for samples from cats as well. However, blood sampling is less invasive and is easy to perform, particularly for epidemiological studies involving numerous subjects, as in our survey.

Although dogs have been universally regarded as the domestic reservoir hosts of zoonotic visceral leishmaniasis caused by *L. infantum*, some researchers have hypothesized that cats may also act as a secondary reservoir host of leishmaniasis rather than simply as an accidental host [9, 14, 15]. Differences in immune response, vector host preference, or innate resistance in cats to vector-borne diseases could account for the observed differences in the prevalence of infection in canine versus feline populations in endemic areas. Immunosuppressive agents, such as FIV or FeLV, or disease and stress, can induce immunological dysfunction and impair the cellular immune response. This allows active multiplication of the parasite and widespread visceral dissemination of the protozoa [27]. In our survey, FIV infection was statistically associated with seroreactivity to *L. infantum* by IFAT, and FIV-positive cats were 7.3 times more likely to be *L. infantum* seroreactive than FIV-negative cats (*P* = 0.0098). This association has also been found in previous studies performed in endemic area of Southern Italy [9, 11].

Based on results from a recent survey, continental northern Italy is now focally endemic for leishmaniasis, but no sand-flies (vector) or autochthonous cases of human and canine leishmaniasis have been identified in Milan or its suburbs [28]. Cases of CanL are commonly diagnosed in the area where we performed our study, but the histories of the affected dogs always reveal that they have lived or travelled in areas that are endemic for CanL [29, 30]. A canine epidemiological survey of 313 dogs in a public animal shelter that were tested for *L. infantum* by IFAT more than 10 years ago (2002-2003) in the urban area of Milan found a seroprevalence of 3.4% [31] Although the history of dogs in animal shelters is often unknown, some of these dogs may have come from areas that are endemic for *L. infantum* infection. In contrast, it is unlikely that all of the *Leishmania* seropositive cats found in our study population were infected in endemic areas. In the present study, the canine seroprevalence for *L. infantum* was much higher than the canine seroprevalence found 10 years previously in a canine population in an animal shelter in the same area. Notably, this area is still considered non endemic for leishmaniasis. We speculate that the serology results for leishmaniasis in our survey may be an overestimation due to the possibility of IFAT cross-reactivity between *L. infantum* and other pathogens. Cross-reactivity with other pathogens is possible on some serologic tests, especially those that use a whole-parasite antigen, as we did here. There was no significant correlation between *T. gondii* positivity and *L. infantum* positivity in our study. This may suggest a lack of cross-reactivity with *Toxoplasma* parasites. New vector-borne parasites have been found that affect cats, such as *Ehrlichia* spp., *Rickettsia felis*, *Anaplasma phagocytophilum*, and *Babesia* spp. (according to the vector-borne disease ESCCAP guidelines) [32] that might be able to cross-react with *Leishmania*. This has been demonstrated in dogs in that IFAT cross-reactivity has been reported for *L. infantum* and *Trypanosoma cruzi*, *Leishmania braziliensis*, and *Ehrlichia canis* infection [33].

### 5. Conclusions

Our results demonstrate high levels of seroreactivity to *L. infantum* in cats in an area of northern Italy that has traditionally been considered to be free of leishmaniasis and non endemic for this infection in dogs. Possible IFAT cross-reactivity and a lack of a validated serological method for feline specie could explain our unexpectedly high seroprevalence. Additional studies that include parasite isolation are needed to clarify our findings on feline leishmaniasis in this geographic area.
**Standard curve**

Log starting quantity

<table>
<thead>
<tr>
<th>Standard</th>
<th>Unknown</th>
<th>FAM</th>
</tr>
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<tbody>
<tr>
<td>40</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>25</td>
<td>1 2 3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>Cq</td>
<td>E = 82.9% $R^2 = 0.992$ slope = −3.814 y-int = 43.93840</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1:** Standard curve in logarithmic scale.

**Quantification**

- Step number: 4
- Analysis mode: fluorophore
- Cq determination: single threshold
- Baseline method: FAM: autocalculated
- Threshold setting: FAM: 64.05, autocalculated

**Amplification**

<table>
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</tr>
<tr>
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<td>400</td>
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<td>200</td>
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**Figure 2:** Amplification curve: amplification of the standards (from $10^6$ Leish/mL to 100 Leish/mL). Below the threshold the nonamplified samples (negative).

**References**


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**Conflict of Interests**

All the authors (E. Spada, D. Proverbio, A. Migliazzo, A. Della Pepa, R. Perego, and G. Bagnagatti De Giorgi) declare that they have no conflict of interests.


