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Applications of Quantitative Serum Neopterin Determination in Dogs Affected by Leishmaniasis-Preliminary Study

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Abstract: The aim of the present study was to evaluate possible correlations between Neopterin serum values and Immunofluorescence Antibody Test (IFAT) titers in dogs positive for *Leishmania infantum* and to provide further reference values for Neopterin in seronegative patients. Neopterin has been determined in 74 dogs, 30 of which where clinically healthy, seronegative and without any particular laboratory abnormalities for the tested parameters while the remaining 44 were positive to IFAT. The heterogeneous results obtained for Neopterin values in seronegative dogs in the present study did not allow inferring reference limits in those subjects but some of the correlations between Neopterin and IFAT titers in seropositive ones encourage further studies on that matter, possibly comparing the molecule values with other traditional markers of infection/disease. The present study represents a possible preliminary step towards the use of serum Neopterin values as a biomarker in dogs affected by leishmaniasis.

Key words: Neopterin, dog, leishmania, cellular immune response, disease, patients

INTRODUCTION

Leishmaniasis is a disease caused by *Leishmania* species well known in many countries of the world whose geographic distribution and incidence are continuously growing also in Italy (Otranto *et al*., 2009). Once transmitted to the host, the parasite being an obligate intracellular pathogen, localizes primarily inside macrophages where it starts replication. This leads to activation of lymphocytes T helper (Th) which subsequently differentiate into two subpopulations: Th1, mainly involved in cellular immunity and Th2, responsible for humoral response (Fernandez-Bellon *et al*., 2005; Miranda *et al*., 2007). Interferon γ (IFN-γ) is one of the most important mediators produced by Th1 subpopulation and is in turn, responsible for macrophages activation that could lead to parasite control (Guarga *et al*., 2000; Chamizo *et al*., 2005; Solano-Gallego *et al*., 2009). For this reason the host's response to infection seems to be importantly depending on Th1/Th2 ratio (Choi and Kropf, 2009); at present a protective response against Leishmania involving Th1 cells and mediated by IFN-γ has been suggested (Rosypal *et al*., 2005; Charmoy *et al*., 2010) while the exact role of Th2 subsets is still unclear (Alimohammadian *et al*., 2007) even if a more permissive/non-protective one has been postulated (Fernandez-Bellon *et al*., 2005; Castagnaro *et al*., 2007; Miranda *et al*., 2007; Choi and Kropf, 2009; Goto and Prianti, 2009).

Neopterin (N) derives from Guanosine Triphosphate (GTP) and represents a molecule resulting from biopterin formation, important in the synthesis of some neurotransmitters (Huber *et al*., 1984; Murr *et al*., 2002). Even if the exact role of N is still unclear it seems to be involved in macrophages' Reactive Oxygen Species (ROS) action (Murr *et al*., 2002) and also in bone marrow metabolism (Tsuboi *et al*., 2010). In human medicine it has been shown that the immune system activation leads to serum N increases as an impaired renal excretion of N could also determine; for example infection caused by several virus, bacteria or protozoa have been associated with N increases (Fuchs *et al*., 1992) as well as some autoimmune diseases (i.e., Crohn's disease and rheumatoid arthritis) and neoplasms (Huber *et al*., 1984; Werner *et al*., 1987; Bedarida and Lizioli, 1991; Stang and Koller, 1998; Murr *et al*., 2002; Pingle *et al*., 2008). Furthermore, N has been studied in men affected by leishmaniasis (cutaneous and/or visceral) leading to

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interesting results, hypothesizing a possible role for the molecule in monitoring treated patients (visceral form) (Schriefer *et al*., 1995; Hamerlinck *et al*., 2000).

Additionally, increases of N concentration have also been shown in old and young healthy people (Werner *et al*., 1987). To the researchers' knowledge is known that only few studies regarding N have been performed in dogs (Duch *et al*., 1984; Goldberg and Fuchs, 1996; Stang and Koller, 1998; Strasser *et al*., 2003; Mrljak *et al*., 2004) one of these showed a decrease of N concentration after vaccination (Strasser *et al*., 2003) while another reported the possible increase of N in dogs affected by babesiosis (Mrljak *et al*., 2004).

Since, IFN-γ also secreted by T lymphocytes, represents a stimulus for N production by macrophages (Huber *et al*., 1984; Bedarida and Lizioli, 1991; Mayersbach *et al*., 1994; Stang and Koller, 1998; Weiss *et al*., 1999; Murr *et al*., 2002; Pingle *et al*., 2008), the researchers sought to understand whether such a molecule (N) that represent an indicator for Th1 cells activation (Strasser *et al*., 2003) could be used as a marker in dogs affected by leishmaniasis.

The aim of the present study was to investigate whether a correlation exists between N and antibody titer in seropositive dog by using an Immunofluorescence Antibody Test (IFAT) as well as to provide further reference values of N in seronegative canine patients.

MATERIALS AND METHODS

In the present study, sera from 74 dogs, clinically healthy or presenting signs of leishmaniasis have been assessed. All samples have been divided into two groups depending on positivity (Leishmaniotic Group (LG)) or negativity (Control Group (CG)) for antibodies anti-*Leishmania infantum* and *L. tropica* tested by IFAT (IFAT-MegaScreen® FLUOLEISH., MegaCor Diagnostik GmbH, Austria; cut-off 1:40). Dogs included in the CG were all clinically healthy and their laboratory exams (complete haematological and biochemical evaluation-Haematology analyser, Cell-Dyn® 3500, Abbott, USA and Automatic analyzer BT 3000 plus®, Biotecnica Instruments, Italy) were within reference ranges for the species. Dogs receiving minor surgery (wounds suture, etc.) at the School of Medical Veterinary Sciences, University of Camerino and whose routine preliminary laboratory exams were normal have also been included in the CG. Sera obtained from blood samples of the 74 patients were stored at -20°C for a period no longer than 3 months and then processed with the competitive immunoenzymatic human kit (Neopterin-MW EIA, DRG® Instruments GmbH, Germany) for quantitative serum Neopterin determination. Resulting values were compared with a reference curve earlier predisposed as indicated by the kit and necessary in order to obtain the real N concentrations (ng mL^{-1}).

Statistical analysis: Mean, median and mode values were calculated for N (always considering N values of <0.5 ng mL^{-1} as equal to 0.5 ng mL^{-1} and values of >100.0 ng mL^{-1} as equal to 100.0 ng mL^{-1}) in seropositive and seronegative patients. Seropositive samples were then grouped according to IFAT titer values and mean, median and mode value of N calculated. Finally, seropositive samples were also sorted in two subgroups based on N values: lower and higher than 0.5 ng mL^{-1} (N median value in seropositives); mean, median and mode of IFAT titers for the two subgroups were then calculated.

Results were further elaborated by χ^2-test with Yates' correction factor when necessary. The level of significance was established at $p<0.05$ for all the tests.

RESULTS AND DISCUSSION

Of the 74 samples investigated 44 resulted to be positive at IFAT (with titers ranging from 1:40-1:1280) while the remaining 30 were negative (Table 1). N values were ranging from <0.5 ng mL^{-1} (the minimum detectable) to >100 ng mL^{-1} (the maximum detectable) (Table 1) with mean, median and mode values of respectively 11.37-1.3 and 0.5 ng mL^{-1} in seronegative patients and of respectively 8.65-0.5 and 0.5 ng mL^{-1} in seropositive ones (Table 2 and 3). Seropositive canine patients were then divided depending on IFAT titers. Mean N values ranged from <0.5 ng mL^{-1}, the only patient with a titer of 1:1280-17.31 ng mL^{-1} in patients with titers of 1:80 (median and mode values of respectively 0.85 and 0.5 ng mL^{-1}). Mean N values in patients with titers of 1:40 and 1:160 were rather low (0.73 and 0.74 ng mL^{-1} respectively; median and mode values always equal to 0.5 ng mL^{-1} for both titers) while mean N values in 1:320 positive dogs where higher (7.19 ng mL^{-1}; median and mode values equal to 0.5 ng mL^{-1}).

When dividing seropositive patients depending on N values, mean, median and mode of IFAT titers were found to be higher in those patients with N <0.5 ng mL^{-1} (242.96-160 and 320, respectively) than in those with N >0.5 ng mL^{-1} (176.47-160 and 80, respectively).

No correlation was found between IFAT positivity/negativity or IFAT titers and N values even if

Table 1: Serum Neopterin values and IFAT titers of patients included in the study

No. of sample	Neopterin values (ng mL^{-1})	IFAT titers-Leishmania	No. of sample	Neopterin values (ng mL^{-1})	IFAT titers-Leishmania
1	0.60	1:160	38	<0.50	NEG.
2	<0.50	1:320	39	<0.50	NEG.
3	<0.50	1:80	40	<0.50	NEG.
4	<0.50	1:80	41	<0.50	NEG.
5	1.80	1:320	42	<0.50	NEG.
6	4.60	1:80	43	100.00	1:320
7	<0.50	1:320	44	<0.50	NEG.
8	1.95	1:320	45	5.75	NEG.
9	2.60	1:80	46	1.20	1:80
10	100.00	1:80	47	0.80	NEG.
11	11.00	1:320	48	4.60	NEG.
12	<0.50	1:320	49	<0.50	1:320
13	<0.50	1:80	50	<0.50	NEG.
14	<0.50	1:320	51	<0.50	1:320
15	<0.50	1:160	52	1.20	NEG.
16	<0.50	1:320	53	<0.50	1:1280
17	0.60	1:160	54	<0.50	NEG.
18	2.25	1:160	55	<0.50	NEG.
19	1.20	1:40	56	<0.50	1:320
20	<0.50	1:40	57	62.00	NEG.
21	<0.50	1:160	58	<0.50	1:80
22	<0.50	1:160	59	<0.50	NEG.
23	<0.50	1:40	60	4.70	1:320
24	<0.50	1:160	61	3.50	NEG.
25	<0.50	1:160	62	5.50	1:80
26	<0.50	1:320	63	12.00	NEG.
27	<0.50	1:320	64	2.60	NEG.
28	100.00	1:80	65	1.40	NEG.
29	<0.50	1:80	66	>100.00	NEG.
30	<0.50	1:80	67	10.00	NEG.
31	<0.50	1:320	68	2.00	NEG.
32	<0.50	1:80	69	6.00	NEG.
33	<0.50	1:320	70	1.20	NEG.
34	25.00	1:80	71	4.00	1:320
35	<0.50	NEG.	72	14.00	NEG.
36	>100.00	NEG.	73	2.60	NEG.
37	<0.50	NEG.	74	5.40	NEG.

Table 2: Serum Neopterin values in patients seronegative for Leishmania

No. of sample	Neopterin values (ng mL^{-1})	IFAT titers- Leishmania
35	<0.50	NEG.
36	>100.00	NEG.
37	<0.50	NEG.
38	<0.50	NEG.
39	<0.50	NEG.
40	<0.50	NEG.
41	<0.50	NEG.
42	<0.50	NEG.
44	<0.50	NEG.
45	5.75	NEG.
47	0.80	NEG.
48	4.60	NEG.
50	<0.50	NEG.
52	1.20	NEG.
54	<0.50	NEG.
55	<0.50	NEG.
57	62.00	NEG.
59	<0.50	NEG.
61	3.50	NEG.
63	12.00	NEG.
64	2.60	NEG.
65	1.40	NEG.
66	>100.00	NEG.
67	10.00	NEG.
68	2.00	NEG.
69	6.00	NEG.
70	1.20	NEG.
72	14.00	NEG.
73	2.60	NEG.
74	5.40	NEG.

Mean±SD = 11.37±26.63; Median = 1.3; Mode = 0.5

a relationship with p-value close to statistical significance ($p = 0.07$) was found between IFAT positivity/negativity and the presence of a detectable amount of N (values >0.5 ng mL^{-1}).

Data obtained in the present study suggest the possibility of dosing N in dogs by using a human kit and even if they are not able to provide an obvious correlation between N values and IFAT titers in seropositive patients they are however providing many cues of interest. Patients with positive IFAT (Table 3), considered dogs exposed to the parasite were found to present contextually different values of N that could be correlated to a variable Th1 cells activation. In case of low IFAT titers it is possible that low values of N (e.g., patient No. 23) could be traced back to a mixed response (Th1/Th2) possibly consequently to a low parasitic load while high values of N (e.g., No. 10) could be instead the consequence of a prevalent Th1 subsets response or being the effect of a concomitant disease leading to increases of that molecule as demonstrated in humans and previously reported (Murr *et al*., 2002). Also, among dogs with higher IFAT titers we found patients with both low and high N concentration. In the former case (e.g., No. 56) it is possible to presume a prevalent response of Th2 cells whereas in the latter one (e.g., No. 43) is supposable a mixed response possibly consequently to an elevated protozoan concentration or to a contextual pathological condition promoting elevated N values. Moreover, it is interesting to underline that also patients with negative IFAT (Table 2), considered not exposed to the parasite (or exposed but still in absence of seroconversion) showed different values of N. When such values were lower than the minimum detectable (0.5 ng mL^{-1}) (e.g., No. 35) is reasonable that patients were not exposed while when N was higher than that limit (e.g., No. 36) patients were possibly affected by other diseases leading to N increases; alternatively, they could have been exposed to the parasite, resulting in a predominant Th1 cells activation but were not yet presenting seroconversion.

Other peculiar remarks originate if analysing N values of <0.5 ng mL^{-1} as equal to 0.5 ng mL^{-1} and values of >100.0 ng mL^{-1} as equal to 100.0 ng mL^{-1}. Dividing patients into two groups depending on IFAT (negative or positive, respectively Table 2 and 3) we observed that the seropositive group had lower mean and median values of N compared to the seronegative one; such data are

Table 3: Serum Neopterin values in patients seropositive for Leishmania

No. of sample	Neopterin values (ng mL^{-1})	IFAT titers-Leishmania
1	0.60	1:160
2	<0.50	1:320
3	<0.50	1:80
4	<0.50	1:80
5	1.80	1:320
6	4.60	1:80
7	<0.50	1:320
8	1.95	1:320
9	2.60	1:80
10	100.00	1:80
11	11.00	1:320
12	<0.50	1:320
13	<0.50	1:80
14	<0.50	1:320
15	<0.50	1:160
16	<0.50	1:320
17	0.60	1:160
18	2.25	1:160
19	1.20	1:40
20	<0.50	1:40
21	<0.50	1:160
22	<0.50	1:160
23	<0.50	1:40
24	<0.50	1:160
25	<0.50	1:160
26	<0.50	1:320
27	<0.50	1:320
28	100.00	1:80
29	<0.50	1:80
30	<0.50	1:80
31	<0.50	1:320
32	<0.50	1:80
33	<0.50	1:320
34	25.00	1:80
43	100.00	1:320
46	1.20	1:80
49	<0.50	1:320
51	<0.50	1:320
53	<0.50	1:1280
56	<0.50	1:320
58	<0.50	1:80
60	4.70	1:320
62	5.50	1:80
71	4.00	1:320

Mean±SD = 8.65±25.32; Median = 0.5; Mode = 0.5

interesting, since considering the increase of N as a sign of the organism's response it would have been expected higher values of N in seropositives.

Furthermore, dividing the seropositive dogs in groups based on their IFAT titer (1:40; 1:80; 1:160; 1:320; 1:1280) we noticed that the highest mean and median values of N were found in one of the lowest IFAT titer group (1:80), supporting the idea that elevated values of N might correlate to a higher protective Th1 cells response. Finally, the highest IFAT titer (1:1280) was found in a patient with one of the lowest values of N (<0.5 ng mL^{-1}), corroborating the hypothesis that patients with low concentration of N could be less protected against Leishmania infection because of a reduced Th1 cells response.

Dividing seropositive patients into two groups depending on N values above or below 0.5 ng mL^{-1} (which is the N median value in the group of seropositives) we observed that the mean value of IFAT titers and the most represented one (mode) are considerably lower in those patients with N >0.5 ng mL^{-1} (176.47 and 80 instead of 242.96 and 320). These findings further indicate that higher values of N might correlate to lower IFAT titers because of a prevalent Th1 lymphocytes activation in parasitized organisms, possibly resulting in a minor sensibility to the infection.

Compared to results of statistical analysis, the weak correlation reported suggested that negativity to IFAT is most likely associated with higher values of N, representing a datum that necessitate of further investigations and apparently in contrast with what initially expected.

CONCLUSION

Even in the absence of a statistically significant correlation between N and IFAT, this study lets the Researchers consider N as a molecule with possible interesting prognostic potentialities in a parasitic disease (leishmaniasis) that still presents unclear aspects particularly in patient management over time. In the present article, the absence of a subdivision of patients based on their sex, breed and, especially, age (to which have been correlated variations of N baseline values in humans) (Werner *et al*., 1987) in addition to the irregular N values found in seronegative patients, did not allow the Researchers to achieve reference limits for N concentrations in such patients, stimulating the research in that sense in future studies. The researchers hypothesize that serial N determinations in leishmaniotic patients, before during and after treatment could lead to interesting acquisitions about the possibility of predicting patients' response to infection/disease and then, the evolution, similarly to what reported in men (Schriefer *et al*., 1995; Hamerlinck *et al*., 2000). Interesting could also be the comparison with other traditional exams (e.g., cytology and PCR) performed in leishmaniasis (infection/disease). Currently the direct survey of lymphocyte subsets quantified by flow cytometric analysis and cytokine determination are excellent methods for studying the organism response to certain noxae. If the potentialities of serum N determination will be confirmed, it could support the above mentioned methods by providing useful indirect data collected on cellular immune response in leishmaniotic patients with lower expenses and higher simplicity of implementation, especially if tested contextually on more samples.

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