



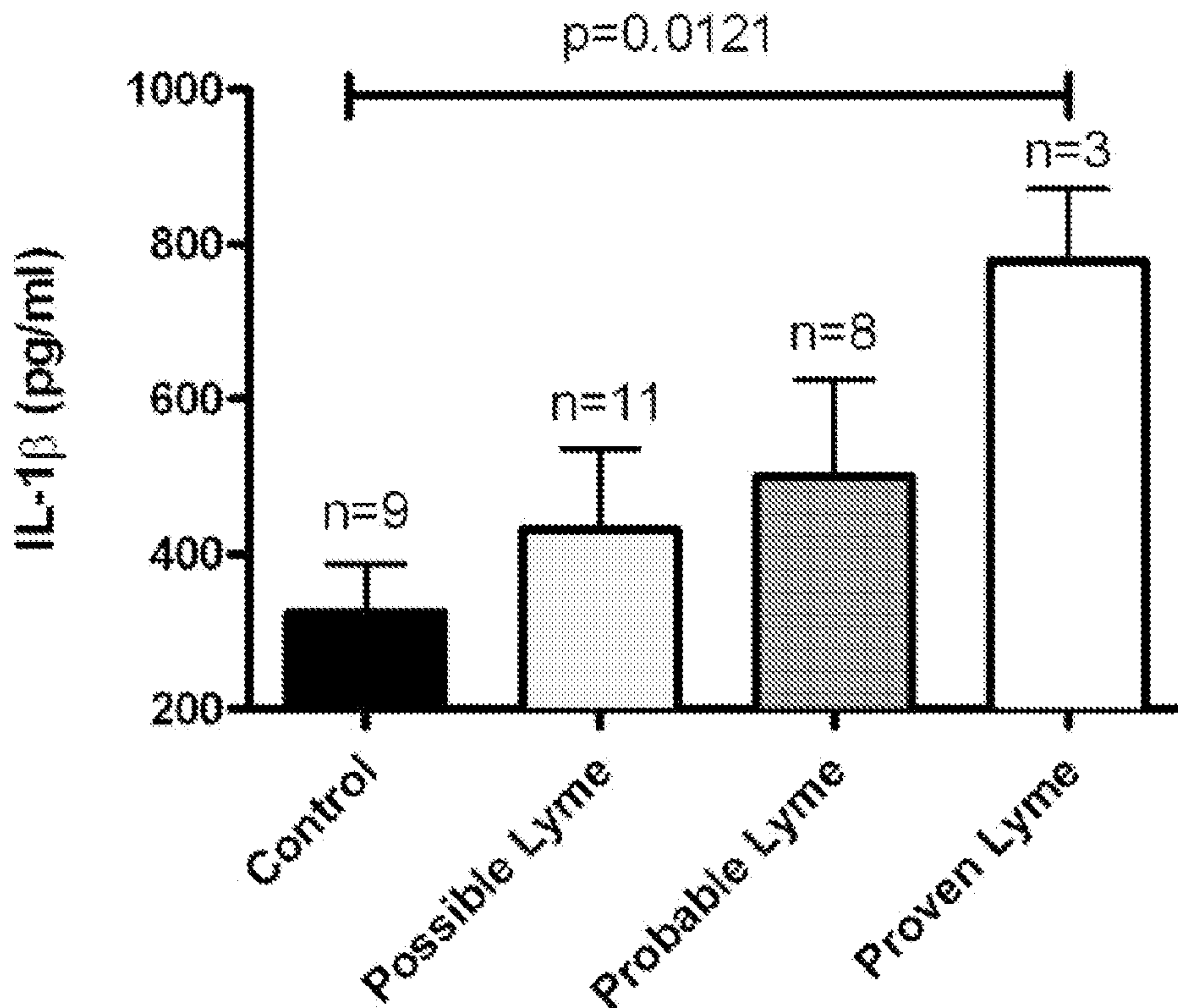
(86) Date de dépôt PCT/PCT Filing Date: 2011/09/21
(87) Date publication PCT/PCT Publication Date: 2012/03/29
(85) Entrée phase nationale/National Entry: 2013/03/19
(86) N° demande PCT/PCT Application No.: NL 2011/050639
(87) N° publication PCT/PCT Publication No.: 2012/039614
(30) Priorités/Priorities: 2010/09/21 (EP10178060.9);
2010/09/21 (US61/384,820)

(51) Cl.Int./Int.Cl. *G01N 33/569* (2006.01),
G01N 33/68 (2006.01)
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(54) Titre : NOUVEAU PROCEDE POUR DIAGNOSTIQUER LA MALADIE DE LYME EN UTILISANT UN TEST
IMMUNOLOGIQUE CELLULAIRE

(54) Title: NOVEL METHOD FOR DIAGNOSING LYME DISEASE USING A CELLULAR IMMUNOLOGICAL TEST

Fig. 3a



(57) Abrégé/Abstract:

The present invention relates to a method for diagnosing Lyme disease in a subject, the method comprising the steps of: (a) obtaining a sample from said subject, (b) contacting said sample with a source of Borrelia antigens and (c) determining the expression level of a pro-inflammatory cytokine in said sample at the end of step (b).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
29 March 2012 (29.03.2012)

PCT

(10) International Publication Number
WO 2012/039614 A1(51) International Patent Classification:
G01N 33/569 (2006.01) *G01N 33/68* (2006.01)(74) Agent: **HUTTER, Hans**; Nederlandsch Octrooibureau
N.V., J.W. Frisolaan 13, NL-2517 JS The Hague (NL).(21) International Application Number:
PCT/NL2011/050639(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP,
KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI,
NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU,
RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA,
ZM, ZW.(22) International Filing Date:
21 September 2011 (21.09.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/384,820 21 September 2010 (21.09.2010) US
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bergweg 96, NL-6523 MR Nijmegen (NL).(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

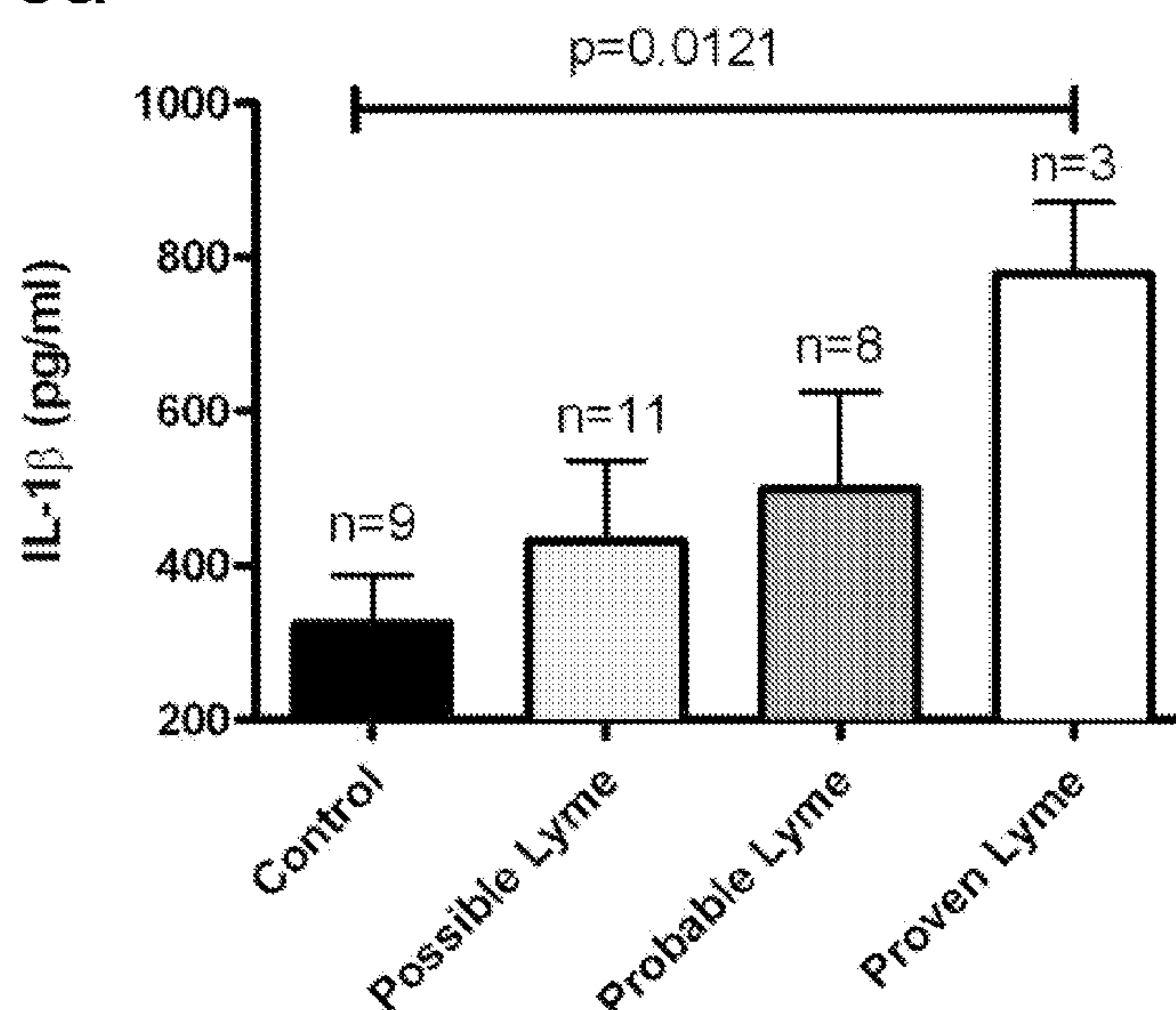
Published:

— with international search report (Art. 21(3))

[Continued on next page]

(54) Title: NOVEL METHOD FOR DIAGNOSING LYME DISEASE USING A CELLULAR IMMUNOLOGICAL TEST

Fig. 3a

(57) Abstract: The present invention relates to a method for diagnosing Lyme disease in a subject, the method comprising the steps of: (a) obtaining a sample from said subject, (b) contacting said sample with a source of *Borrelia* antigens and (c) determining the expression level of a pro-inflammatory cytokine in said sample at the end of step (b).

WO 2012/039614 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))* — *with sequence listing part of description (Rule 5.2(a))*

Novel method for diagnosing Lyme disease using a cellular immunological testField of the invention

The present invention relates to a method for diagnosing Lyme disease in a subject,
5 using the determination of a pro-inflammatory cytokine expression level.

Background of the invention

Lyme disease or Lyme borreliosis is a vector-borne, multisystem inflammatory
bacterial illness transmitted to humans by the bite of ticks (*Ixodes* species) carrying
10 spirochetes of the genus *Borrelia*. The disease presentation varies widely, and may
include rash and flu-like symptoms in its initial stage, and musculoskeletal, arthritic,
neurologic, psychiatric and cardiac manifestations in later stages.

Lyme disease is clinically manifested in three phases as:

- early localized disease with skin inflammation,
- 15 - early disseminated disease (such as heart and nervous system involvement,
including palsies and meningitis)
- late disseminated disease (such as motor and sensory nerve damage and brain
inflammation and arthritis).

Due to the difficulty in culturing the *Borrelia* spirochetes in the laboratory, diagnosis of
20 Lyme disease is typically based on the clinical examination findings and a history of
exposure to endemic Lyme areas. The laboratory tests most widely used are serological
tests based on an Enzyme-linked immunosorbent assay (ELISA) test detecting
antibodies to *Borrelia* species, mostly *B. burgdorferi*, *B. garinii* and *B. afzelii* (see for
example WO 94/19697). This method is unfortunately characterized by a poor
25 sensitivity and specificity, which sometimes provides false-positive results, and very
often is not able to diagnose infected patients. Therefore, humoral immunological tests
based on antibody detection are not a reliable basis for diagnosis of Lyme disease.

Western blot test may also be used to confirm positive results from an ELISA. The
Western blot detects antibodies to several proteins of *Borrelia* species.

30 It is also possible to detect bacterial DNA in fluid drawn from an infected joint or other
body sites using Polymerase chain reaction (PCR). This method is used for people who
may have chronic Lyme arthritis. It may also be used to detect persistent infection in

the cerebrospinal fluid of people who have nervous system symptoms. However, the sensitivity of PCR techniques is also low.

If diagnosed in the early stages, the disease can be cured with antibiotics. If left untreated, complications involving joints, the heart, and the nervous system can occur.

- 5 It is therefore crucial to be able to specifically detect/diagnose Lyme disease in an early stage in order to avoid complications that may develop in later stages.

The significant disadvantages (poor sensitivity and specificity) of humoral immunity tests (ELISA and Western-blot) and PCR lead to a significant medical need for better
10 diagnostic tests for diagnosing Lyme disease.

Description of the figures

Figure 1.

- 15 Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers (C) and patients with Lyme disease (P). 1.10^6 cells/ml were stimulated for 24h with *Borrelia* species (1.10^5 /ml). Thereafter IL- 1β was determined by ELISA.

- Figure 2** Peripheral blood mononuclear cells (PBMC) were isolated from healthy
20 volunteers (C) and patients with Lyme disease (P). 1.10^6 cells/ml were stimulated for 48h with *Borrelia* species (1.10^5 /ml). Thereafter IFN γ was determined by ELISA.

Figure 3

- Peripheral blood mononuclear cells (PBMC) were isolated from control, possible,
25 probable and proven Lyme patients and stimulated with 1.10^6 *Borrelia burgdoferi* cells/ml for 24h. Thereafter IFN γ or IL- 1β was determined by ELISA.

Description of the invention

30 Diagnosis method

In a first aspect, the invention relates to a method for diagnosing Lyme disease in a subject, the method comprising the steps of:

- (a) obtaining a sample from said subject,

(b) contacting said sample with a source of *Borrelia* antigens and

(c) determining the expression level of a pro-inflammatory cytokine in said sample at the end of step (b).

5 In the context of the invention, “diagnosing Lyme disease” preferably means that a diagnosis is reached in at least the following cases presented below:

The Lyme disease may be diagnosed at an early stage before a classical test will diagnose it.

10 In this context “early stage” preferably means shortly after the clinical apparition of symptoms of local skin inflammation (EM) or before the stage wherein the disease has disseminated into the heart and/or nervous system or early disseminated into the heart and/or nervous system and/or before the stage of a disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring motor and sensory nerve damage
15 and brain inflammation and arthritis as defined later herein.

Local skin inflammation is not always easily recognized or is not always easily recognizable (i.e. atypic EM) by the physician. The method of the invention could provide a specific diagnostic of the Lyme disease at an early stage in these cases. In this context “early stage” preferably means before the stage wherein the disease has
20 disseminated into the heart and/or nervous system or early disseminated into the heart and/or nervous system and/or before the stage of a disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis as defined later herein.

25

Alternatively some 20% or 30% of the Lyme patients will not develop EM. For these patients, the method of the invention also provides a specific diagnostic of the Lyme disease, preferably at an early stage preferably before the stage wherein the disease has disseminated into the heart and/or nervous system or early disseminated
30 into the heart and/or nervous system and/or before the stage of a disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis as defined later herein.

The disease may also be diagnosed using a method of the invention in a later stage in case the patient will consult a physician in a later stage. A later stage is preferably before the stage wherein the disease has disseminated into the heart and/or nervous system or early disseminated into the heart and/or nervous system and/or before the stage of a disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis as defined later herein. In a later stage of the disease the method of the invention is also attractive compared to classical methods since at this later stage classical methods may not give a positive response or may give a positive response. However, classical methods often give false positive responses.

Alternatively, the disease may be diagnosed when the disease has disseminated into the heart and/or nervous system and/or at the stage of a disseminated disease involving nerve damage, brain inflammation, or arthritis also called a late disseminated disease involving nerve damage, brain inflammation, or arthritis and/or at the stage wherein the disease may become persistent or refractory to a 2 weeks antibiotic treatment. The clinical apparition of symptoms of local skin inflammation may be assessed by the physician. Local skin inflammation is usually characterized by an Erythema migrans (EM) that occurs at the site of the tick bite. The rash is usually salmon to red-colored; the color may cover the entire lesion or may have an area in the centre that is flesh-colored. In some cases, the rash consists of multiple rings, which give it a "bull's eye" appearance.

Preferably, the invention relates to a method for diagnosing Lyme disease shortly after the clinical apparition of symptoms of local skin inflammation (EM) in an subject, the method comprising the steps of:

- (a) obtaining a sample from said subject,
- (b) contacting said sample with a source of *Borrelia* antigens and
- (c) determining the expression level of a pro-inflammatory cytokine in said sample at the end of step (b).

The assessment that the disease has already reached a stage wherein it has disseminated into the heart and/or nervous system may be assessed using serology, which has a poor sensitivity and specificity.

The assessment that the disease has already reached a disseminated stage with, among others, featuring motor and sensory nerve damage and brain inflammation and arthritis, also called a late disseminated stage with, among others, featuring motor and sensory nerve damage and brain inflammation and arthritis may be assessed using PCR to detect bacterial DNA in these organs/tissues. However, organ biopsies are very seldom available for testing in patients with chronic Lyme disease.

10 In this context, “shortly after the clinical apparition of symptoms of local skin inflammation (EM)” preferably means at least one day, at least two days, at least three days, at least four days, at least five days, at least six days, at least seven days, at least eight days, at least nine days, at least ten days at least 15 days, at least 20 days, at least 25 days, at least 30 days or more after the clinical apparition of symptoms of local skin
15 inflammation.

In this context, “shortly after the clinical apparition of symptoms of local skin inflammation (EM)” preferably means before the stage wherein the disease has disseminated into the heart and/or nervous system or early disseminated into the heart and/or nervous system and/or before the stage of a disseminated disease involving
20 featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis. In this context, “before” preferably means at least one day, at least two days, at least three days, at least four days, at least five days, at least six days, at least seven days, at least eight days, at least nine days, at least ten days
25 at least 15 days, at least 20 days, at least 25 days, at least 30 days or more before the stage wherein the disease has disseminated into the heart and/or nervous system or early disseminated into the heart and/or nervous system and/or before the stage of a disseminated disease involving featuring motor and sensory nerve damage and brain inflammation and arthritis also called a late disseminated disease involving featuring
30 motor and sensory nerve damage and brain inflammation and arthritis.

In the context of the invention, diagnosis preferably means a predictive risk assessment of the subsequent development of Lyme disease in a subject.

In the context of the invention, a subject may be an animal or a human being. The diagnosis method may be applied as often as necessary in a subject. Preferably, a subject diagnosed is a subject suspected to have a high risk of having or developing chronic Lyme disease, due for example to the fact that this subject lives in a region
5 wherein the Lyme disease is common, this subject spends a lot of time outdoors, including a subject who works outdoors, gardens, or participates in outdoor activities such as hunting or hiking or subject has a pet. Preferably, a subject is a human being. In a preferred method, the Lyme disease is diagnosed when step (c) leads to the finding of a detectable expression level or an increase of the expression level of a pro-
10 inflammatory cytokine.

A pro-inflammatory cytokine is a cytokine that is able to promote systemic inflammation.

A pro-inflammatory cytokine is preferably selected from the group consisting of IL-1 β , or IFN γ , IL-6, and IL-17. Good results were obtained using IL-1 β . IL-1 β is therefore a
15 preferred pro-inflammatory cytokine in this context.

Optionally in a method of the invention, one may compare the expression level of a pro-inflammatory cytokine as determined in step (c) with a reference value for said expression level, the reference value preferably being the average value for said expression level in a control sample. In the context of the invention, "a reference value"
20 for the expression level of a pro-inflammatory cytokine is preferably the average value for said expression level in a control sample. A control sample may be derived from a control subject or from control subjects or from the medium or culture medium used for step (b). A control subject may be a subject who do not live in a region at risk or who does not spend a lot of time outdoors. A pro-inflammatory cytokine tested may be not
25 detectable in "a reference value". Said reference value may therefore be 0 or not detectable in an assay as defined herein. In other words, said cytokine may not be detectable in a control sample.

The assessment of the expression level of the respective pro-inflammatory cytokine may be directly realised at the protein expression level (quantifying the
30 amount of the cytokine), and/or indirectly by quantifying the amount of a nucleotide sequence encoding the respective cytokine (the value from a subject wherein the method is being carried out and optionally the reference value from a control sample). A nucleotide acid sequence encoding a human IL-1 β , IFN γ , IL-6, IL-17 is given as

SEQ ID NO:1, 2, 3, 4 respectively. A corresponding amino acid sequence of human IL-1 β , IFN γ , IL-6, IL-17 is given as SEQ ID NO:5, 6, 7, 8 respectively. The skilled person will understand that it is possible to isolate multiple isoforms of each of the identified pro-inflammatory cytokine depending on the subject to be tested.

- 5 In a preferred embodiment, a pro-inflammatory cytokine to be quantified has:
- at least 60% (or at least 65%, at least 70%, at least 75%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or more or 100%) identity or similarity with SEQ ID NO:5, 6, 7, or 8 and/or
 - is encoded by a nucleotide acid sequence which has at least 60% (or at least
- 10 65%, at least 70%, at least 75%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or more or 100%) identity or similarity with SEQ ID NO:1, 2, 3, or 4.

In another preferred embodiment, a nucleotide acid sequence encoding the respective pro-inflammatory cytokine to be quantified has:

- 15 - at least 60% (or at least 65%, at least 70%, at least 75%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or more or 100%) identity or similarity with SEQ ID NO:1, 2, 3 or 4 and/or
- encodes an amino acid sequence of the pro-inflammatory cytokine (e.g. IL-1 β , IFN γ , IL-6, IL-17) that has at least 60% (or at least 65%, at least 70%, at least
- 20 75%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or more or 100%) identity or similarity with an amino acid sequence encoded by SEQ ID NO:1, 2, 3 or 4.

Identity and similarity are later herein defined. The quantification of the amount of a nucleotide sequence encoding a pro-inflammatory cytokine is preferably performed

25 using classical molecular biology techniques such as (real time) PCR, arrays or northern analysis. In this embodiment, a nucleotide sequence encoding a pro-inflammatory cytokine means a messenger RNA (mRNA). Alternatively, according to another preferred embodiment, in a diagnosis method the expression level of a pro-inflammatory cytokine is determined directly by quantifying the amount of said pro-

30 inflammatory cytokine. Quantifying a polypeptide amount may be carried out by any known technique. Preferably, a polypeptide amount is quantified using a molecule which specifically binds to said pro-inflammatory cytokine. Preferred binding molecules are selected from: an antibody, which has been specifically raised for

recognizing said pro-inflammatory cytokine, any other molecule which is known to specifically bind said pro-inflammatory cytokine. Such antibody could be used in any immunoassay known to the skilled person such as western blotting, or ELISA (Enzyme-Linked Immuno Sorbent Assay) or FACS (Fluorescence Activated Cell
5 Sorting) using latex beads. The preparation of an antibody is known to those skilled in the art. A short explanation of methods that could be used to prepare antibodies is later herein given. Suitable antibodies are commercially available. For example antibodies from R&D systems could be used to assess IL-1 β , IL-6, IL-17 or IFN γ . Such antibodies could be used for assessing such cytokine by ELISA (duo-set). In the context of the
10 invention, any other molecule known to bind the cytokine tested may be a nucleic acid, e.g. a DNA regulatory region, a polypeptide, a metabolite, a substrate, a regulatory element, a structural component, a chaperone (transport) molecule, a peptide mimetic, a non-peptide mimetic, or any other type of ligand. Mimetic is later herein defined. Examples of molecules known to bind a pro-inflammatory cytokine, include a receptor
15 for said pro-inflammatory cytokine, an antibody directed against said pro-inflammatory cytokine. In case, IL-1 β is chosen as a pro-inflammatory cytokine, a preferred anti-IL1 β antibody is the IL-1F2 (from R&D systems) antibody. Binding of a pro-inflammatory cytokine to a second binding molecule may be detected by any standard methods known to those skilled in the art. Suitable methods include affinity
20 chromatography co-electrophoresis (ACE) assays and ELISA. The skilled person will understand that alternatively or in combination with the quantification of a nucleic acid sequence encoding a pro-inflammatory cytokine tested and/or a corresponding polypeptide, the quantification of a substrate of a corresponding polypeptide or of any compound known to be associated with a function or activity of a corresponding
25 polypeptide or the quantification of a function or activity of a corresponding polypeptide using a specific assay is encompassed within the scope of the diagnosis method of the invention. For example, trans-activation of a target gene of a pro-inflammatory cytokine or a molecule known to bind a pro-inflammatory cytokine can be determined and quantified, e.g., in a transient transfection assay in which the
30 promoter of the target gene is linked to a reporter gene, e.g., P-galactosidase or luciferase. Such evaluations can be done *in vitro* or *in vivo* or *ex vivo*.
A method of the invention may encompass determining the expression level of at least one, or two or three or four pro-inflammatory cytokines in a sample at the end of step

(b). For example IL-1 β and IL-6 or IFN γ and IL-17 could be assessed. It is expected that IL-1 β and IL-6 are produced within 48 hours of culture, whereas IFN γ and IL-17 are expected to be produced within 7 days of culture.

5 In a method of the invention, a sample from a subject is used. A method of the invention is therefore an in vitro or ex vivo method. A sample preferably comprises or consists of a fluid obtained from a subject. More preferably, a fluid comprises or consists of or is selected from: urine, blood, spinal cord fluid, saliva, semen, or bronchoalveolar lavage. A preferred fluid is, comprises, consists of or is derived from
10 blood. Blood may be diluted before being further used. The dilution may be 1:4, 1:5 or 1:6. The dilution is preferably carried out in a medium, preferably a culture medium such as RPMI 1640 or a buffered solution.

In a method of the invention, said obtained sample of step (a) is subsequently contacted
15 with a source of *Borrelia* antigens. The contacting step may have a duration of at least 1, 2, 3, 4, 5, 6, 7, 8 up to 24 hours, or longer. Preferably the contact has a duration of 4-96 hours. This contact step may be a culture step in a culture medium such as RPMI 1640. At least 10⁴ and up to 10⁶ bacteria may be inoculated at the start of the contacting step. Preferred species of *Borrelia* as a source of *Borrelia* antigens include:
20 *B. burgdorferi*, more preferably the strain ATCC 35210, *B. garinii*, more preferably the strain ATCC 51383 and *B. afzelii*, more preferably the strain ATCC 51567. Therefore, in step (b) the *Borrelia* antigen is preferably from a species of *Borrelia* selected from: *B. burgdorferi*, *B. garinii* and *B. afzelii*. These strains are preferred since they are the most widely present in Europe and in America.

25

A source of a *Borrelia* antigen may mean that a whole *Borrelia* cell or a *Borrelia* cell is being used. In a preferred embodiment, a whole *Borrelia* cell or a *Borrelia* cell is heat-inactivated or formalin fixated. Heat-inactivated could be replaced by heat-killed. The skilled person knows how to obtain heat-inactivated or formalin fixated *Borrelia*
30 cells. Heat-inactivated cells are preferably prepared by heating at 52, 53, 54, 55 or 56 for 20, 25 or 30 minutes. More preferably heat-inactivated cells are prepared by heating at 52°C for 30 minutes. Formalin fixated cells may be obtained by contacting the cells with formaldehyde for 40, 50, 60 minutes. More preferably, cells are contacted

or transferred to 4% formaldehyde for one hour. Subsequently cells are washed several times with a saline buffer such as PBS (Phosphate Buffered Saline) buffer.

Alternatively, part of a *Borrelia* cell may be used. A part of a *Borrelia* cell is preferably an antigenic part thereof; it comprises or consists of an antigen. An antigen may be a
5 protein, a digest of the protein and/or a fragment thereof, which may be in a purified form or may be comprised within a crude composition, preferably of biological origin, such as a lysate, sonicate or fixate of a *Borrelia*. Alternatively, an antigen may be chemically synthesized or enzymatically produced *in vitro*. The source of a protein, or fragment thereof as antigen, may also be a nucleic acid encoding said, or fragment
10 thereof, from an RNA or DNA template. In a preferred embodiment, a source of a *Borrelia* antigen is a whole *Borrelia* cell or an antigen from said cell.

The use of a whole *Borrelia* cell is attractive and preferred above the use of a part of a cell for at least two reasons. The use of a whole cell is easier and cheaper for the skilled person. There is no need to identify and subsequently synthesize suitable parts (i.e.
15 antigenic parts) of a cell. In addition, by using a whole cell, all potential suitable part (i.e. all antigens) of said cell are present. The diagnostic method is therefore expected to be far more sensitive and efficient than a corresponding diagnostic method carried out using a given antigen.

20 In a preferred method wherein in step (b) several species of *Borrelia* are used as a source of a *Borrelia* antigens, the sample of step (a) is divided into several sub-samples, each sub-sample being contacted with a source of antigens from one species of *Borrelia*. This preferred method may allow us to identify the *Borrelia* species that infected a given subject.

25 Subsequently, the expression level of a pro-inflammatory cytokine is determined in said sample at the end of step (b). First a nucleotide sequence encoding said pro-inflammatory cytokine and/or said pro-inflammatory cytokine are extracted and optionally purified using known methods to the skilled person. In a preferred
30 embodiment, at the end of step (b), the supernatant is isolated by centrifugation. Centrifugation may be carried out at 1200 rpm and at 4°C. Alternatively, one may add a detergent to the sample at the end of step b). Several detergents could be used such as Triton X 0.1 %. Adding a detergent is attractive since it is expected that no

centrifugation step is needed. One may determine the expression level of a pro-inflammatory cytokine in the sample comprising said detergent, which is also called a cell lysate.

In a more preferred diagnostic method, Lyme disease is diagnosed when the expression
5 of a pro-inflammatory cytokine is detectable or detected and optionally when the comparison leads to the finding of a detectable expression of said pro-inflammatory cytokine and/or an increase of the expression level of said pro-inflammatory cytokine. In control subjects and in control samples as defined before, pro-inflammatory cytokine expression is generally not detectable.

10 Detection of the expression of a pro-inflammatory cytokine or an increase of the expression level of said pro-inflammatory cytokine and/or an increase or a detection of the expression level of a nucleotide sequence encoding said pro-inflammatory cytokine (or steady state level of said pro-inflammatory cytokine) is preferably defined as being
15 a detectable expression level or a detectable change of the expression level of said pro-inflammatory cytokine and/or of a nucleotide sequence encoding said pro-inflammatory cytokine (or steady state level of the encoded pro-inflammatory cytokine or any detectable activity of said pro-inflammatory cytokine or detectable change in a biological activity of said pro-inflammatory cytokine) using a method as defined earlier on as compared to the expression level of said pro-inflammatory cytokine and/or of a
20 corresponding nucleotide sequence (or steady state level of the corresponding encoded pro-inflammatory cytokine) in a control subject or in a control. According to a preferred embodiment, detection or an increase of a pro-inflammatory cytokine activity is quantified using a specific mRNA assay for said pro-inflammatory cytokine gene as earlier defined herein. Preferably, an increase of the expression level of a nucleotide
25 sequence encoding a pro-inflammatory cytokine means an increase of at least 5% of the expression level of said nucleotide sequence using PCR. Preferred primers used for the PCR are identified as SEQ ID NO:9 and SEQ ID NO:10 when a pro-inflammatory cytokine is IL-1 β : forward CAGCTACGAATCTCCGACCAC and reverse GGCAGGGAACCAGCATCTTC. More preferably, an increase of the expression level
30 of a nucleotide sequence means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150%, or more.

Preferably, an increase of the expression level of a pro-inflammatory cytokine means an increase of at least 5% of the expression level of said pro-inflammatory cytokine using western blotting and/or using ELISA or a suitable assay. More preferably, an increase of the expression level of a polypeptide means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150%, or more.

Preferably, an increase of a pro-inflammatory cytokine activity means an increase of at least 5% of the polypeptide activity using a suitable assay. More preferably, an increase of the polypeptide activity means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150% or more. In a most preferred diagnostic method, Lyme disease is diagnosed when the detection or comparison leads to the finding of a detectable level or an increase of the level of expression of a pro-inflammatory cytokine or an increase or a detection of the expression level of a nucleotide sequence encoding said pro-inflammatory cytokine, said detection or increase being detected at the level of the amino acid sequence of said pro-inflammatory cytokine, more preferably an increase of at least 5% of the expression level of said pro-inflammatory cytokine using ELISA as defined herein.

The method of the invention is attractive since the diagnosis is reached early enough in order to treat a diagnosed subject to prevent damage. Furthermore, this method is non-invasive, simple, reproducible, sensitive, specific, and time and cost efficient.

Assay device

In a second aspect, an assay device is provided for diagnosing Lyme disease in a subject, wherein said device comprises a molecule which specifically binds a pro-inflammatory cytokine. This device may be used in a diagnosis method of the invention. Any subject or physician could use this device at office/home, repeat the use of such device as often as necessary. Preferably, a pro-inflammatory cytokine is IL-1 β .

The type of molecules that are known to specifically bind a pro-inflammatory cytokine have already been earlier described herein. In a preferred embodiment, a molecule which specifically binds a pro-inflammatory cytokine and which is present in the device is an antibody.

In a preferred embodiment, an assay device is a lateral flow test strip also known as dipstick, preferably, though not necessarily, encased in a housing, designed to be read by the subject, and the assay is a sandwich immunoassay. Such devices are impregnated with reagents that specifically indicate the presence of a given molecule, here a cytokine by changing colour upon contact with a sample. Preferred subject's samples have already been defined herein. An antibody is preferably labelled by conjugation to a physically detectable label, and upon contacting with a sample containing a pro-inflammatory cytokine forms a complex. Said antibody-pro-inflammatory cytokine complex is then contacted with a second antibody, which recognizes said first antibody and which is immobilized on a solid support within the device. A second antibody captures said antibody-pro-inflammatory cytokine complex to form an antibody-pro-inflammatory cytokine-antibody sandwich complex, and the resulting complex, which is immobilized on the solid support, is detectable by virtue of the label. A test strip may then be inserted into a reader, where a signal from said label in the complex is measured. Alternatively, a test strip could be inserted into the reader prior to addition of the sample. Alternatively and according to a preferred embodiment, the presence of a pro-inflammatory cytokine is visualised by a subject as a change of colour of at least part of a device. Dipsticks are usually made of paper or cardboard. Usually additional molecules are present in a device as a positive or negative control. A typical positive control could be an antibody recognizing a molecule which is known to be present in a sample to be tested. A typical negative control could be an antibody recognizing a molecule which is known to be absent in a sample to be tested.

Kit of parts

In a further aspect, there is provided a kit of parts for diagnosing Lyme disease in a subject comprising a) a source of *Borrelia* antigens and b) reagents for detecting the expression level of a pro-inflammatory cytokine.

Each feature of this kit has already been defined herein.

In a preferred kit, the molecule which specifically binds a pro-inflammatory cytokine is an antibody.

Peptidomimetic or peptide mimetic

A peptide-like molecule (referred to as peptidomimetic) or non-peptide molecule that specifically binds to a cytokine as defined herein and that may be applied in a method of the invention as defined herein may be identified using a method known in the art per se, as e.g. described in detail in US 6,180,084 which incorporated herein by
5 reference. Such a methods includes e.g. screening libraries of peptidomimetics, peptides, DNA or cDNA expression libraries, combinatorial chemistry and, particularly useful, phage display libraries. These libraries may be screened for an agonists and/or an antagonist of said cytokine by contacting the libraries with a substantially purified polypeptide of the invention, fragments thereof or structural analogues thereof.

10

Sequence identity

"Sequence identity" is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (polynucleotide) sequences, as determined by comparing the sequences. The identity
15 between two amino acid or two nucleic acid sequences is preferably defined by assessing their identity within a whole SEQ ID NO as identified herein or part thereof. Part thereof may mean at least 50% of the length of the SEQ ID NO, or at least 60%, or at least 70%, or at least 80%, or at least 90%.

In the art, "identity" also means the degree of sequence relatedness between
20 amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. "Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to
25 those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and
30 Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program
5 package (Devereux, J., et al., Nucleic Acids Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990). The well-known Smith Waterman
10 algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program
15 useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison
20 matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be
25 clear to the skilled person. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine
30 and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution

groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence disclosed herein are those in which at least one residue in the disclosed sequences has been removed and a different residue inserted in its place. Preferably, the amino acid change is conservative. Preferred conservative substitutions for each of the naturally occurring amino acids are as follows: Ala to Ser; Arg to Lys; Asn to Gln or His; Asp to Glu; Cys to Ser or Ala; Gln to Asn; Glu to Asp; Gly to Pro; His to Asn or Gln; Ile to Leu or Val; Leu to Ile or Val; Lys to Arg, Gln or Glu; Met to Leu or Ile; Phe to Met, Leu or Tyr; Ser to Thr; Thr to Ser; Trp to Tyr; Tyr to Trp or Phe; and Val to Ile or Leu.

Antibodies

Some aspects of the invention concern the use of an antibody or antibody-fragment that specifically binds a pro-inflammatory cytokine. Methods for generating antibodies or antibody-fragments that specifically bind to a polypeptide are described in e.g. Harlow and Lane (1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and WO 91/19818; WO 91/18989; WO 92/01047; WO 92/06204; WO 92/18619; and US 6,420,113 and references cited therein. The term "specific binding," as used herein, includes both low and high affinity specific binding. Specific binding can be exhibited, e.g., by a low affinity antibody or antibody-fragment having a K_d of at least about 10^{-4} M. Specific binding also can be exhibited by a high affinity antibody or antibody-fragment, for example, an antibody or antibody-fragment having a K_d of at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, at least about 10^{-10} M, or can have a K_d of at least about 10^{-11} M or 10^{-12} M or greater.

General

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a method or an assay device as defined herein may comprise additional step(s), respectively component(s) than the ones specifically identified, said additional step(s), respectively component(s) not altering the unique characteristic of the invention. In addition, reference to an element

by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". The word "about" when used in association with an integer (about 10) preferably means that the value may be the given value of 10 more or less 1 of the value: about 10 preferably means from 9 to 11. The word "about" when used in association with a numerical value (about 10.6) preferably means that the value may be the given value of 10.6 more or less 1% of the value 10.6.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety. The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Examples

Example 1 (figures 1, 2)

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers (C) and patients with Lyme disease (P). $1 \cdot 10^6$ cells/ml were stimulated in RPMI 1640 for 24h with the following strains of *Borrelia*: *B. burgdorferi* (ATCC 35210), *B. garinii* (ATCC 51383) and *B. afzelii* (ATCC 51567) that were first heat-inactivated by heating at 52°C for 30 minutes ($1 \cdot 10^5$ /ml). Thereafter IL-1 β was determined by ELISA using antibody from R&D systems (IL-1F2). IL-1 β was strongly detected in Lyme patients (see figures 1, 2).

Blood (lithiumheparine) was taken from a subject suspected to have the Lyme disease. Blood was subsequently diluted in culture medium (1:5) and stimulated for 4 hours with 3 species of *Borrelia* common in Europe (see above). Subsequently, the supernatant was isolated via centrifugation and the presence of IL-1b was assessed by ELISA as described above.

30

Example 3 (figure 3)

Venous blood was drawn from the cubital vein of healthy volunteers and Lyme patients into 10 mL ethylenediaminetetraacetic acid (EDTA) tubes (Monoject). Peripheral blood

mononuclear cells (PBMCs) were isolated according to standard protocols, with minor modifications. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in PBS over Ficoll-Paque (Pharmacia Biotech). Cells were washed three times in PBS and resuspended in RPMI 1640 (Dutch modified) supplemented with 50 mg/L gentamycin, 2 mM L-glutamin, and 1 mM pyruvate. Cells were counted in a Coulter Counter Z[®] (Beckman Coulter), and adjusted to 5×10^6 cells /mL.

Mononuclear cells (5×10^5) in a 100 μ L volume were added to round-bottom 96-wells plates (Greiner) and incubated with either 100 μ L of medium (negative control) or *B.burgdorferi* (ATCC 35210), at a dose of 1×10^6 spirochetes per mL. The *B.*

burgdorferi were first heat-inactivated by heating at 52°C for 30 minutes.

Concentrations of human IL-1 β or IFN- γ were determined using either specific or commercial ELISA kits (R&D Systems, Minneapolis or Pelikine Sanquin, Amsterdam, The Netherlands), in accordance with the manufacturers' instructions. Detection limits were 40 pg/mL for IL-1 β and for IFN- γ ELISA (12 pg/mL).

Example 4

Blood samples were taken from patients and healthy individuals in EDTA vacutainer tubes (Becton and Dickinson, Leiden, The Netherlands). From these samples 200 μ l were diluted 1:5 in culture medium (RPMI 1640) and incubated in 24 wells tissue culture plates (Costar, Badhoevedorp, The Netherlands). As a stimulus, 100 ng/ml of formaldehyde-inactivated (i.e. formalin fixated) *B. burgdorferi* (ATCC 35210), *B. garinii* (ATCC 51383) or *B. afzelii* (ATCC 51567) that were first heat-inactivated by heating at 52°C for 30 minutes was added to these cultures. Formaldehyde-inactivated cells were prepared by transferring or incubating them with 4% formaldehyde for one hour. Subsequently cells were washed several times with PBS. No stimulus was added to the control cultures. The cultures were incubated at 37 °C and 5% CO₂ for 48 hours. After this incubation period, the supernatants were harvested and centrifuged at 15000g for 5 minutes, and thereafter stored at -20 °C until measurement of interferon γ (IFN γ) and/or IL- β .

IFN γ was measured using a specific ELISA (Pelikine Sanquin, Amsterdam, The Netherlands). IL-1 β was measured as described in example 1.

Claims

1. A method for diagnosing Lyme disease in a subject, the method comprising the steps of:
 - 5 (a) obtaining a sample from said subject,
 - (b) contacting said sample with a source of *Borrelia* antigens and
 - (c) determining the expression level of a pro-inflammatory cytokine in said sample at the end of step (b).
- 10 2. A method according to claim 1, wherein the pro-inflammatory cytokine is selected from the group consisting of: IL-1 β , IFN γ , IL-6 and IL-17.
3. A method according to claim 1 or 2, wherein Lyme disease is diagnosed when step (c) leads to the finding of a detectable expression level or an increase of the expression
15 level of a pro-inflammatory cytokine.
4. A method according to any one of claims 1 to 3, wherein the expression level of a pro-inflammatory cytokine is determined by directly quantifying the amount of said pro-inflammatory cytokine and/or indirectly by quantifying the amount of a nucleotide
20 sequence encoding said pro-inflammatory cytokine.
5. A method according to any one of claims 1 to 4, wherein said source of *Borrelia* antigen is a whole *Borrelia* cell, preferably a heat-inactivated or formalin-fixed *Borrelia* cell is being used .
25
6. A method according to any one of claims 1 to 5, wherein the sample is a fluid obtained from the subject.
7. A method according to claim 6, wherein the fluid is selected from: blood, spinal cord
30 fluid, or synovial fluid

8. A method according to any one of claims 1 to 7, wherein in step (b) the *Borrelia* antigen is from a species of *Borrelia* selected from: *B. burgdorferi*, *B. garinii* and *B. afzelii*.
- 5 9. A method according to any one of claims 1 to 8, wherein in step (b) if several species of *Borrelia* are used as a source of *Borrelia* antigens, the sample of step (a) is divided into several sub-samples, each sub-sample being contacted with a source of antigens from one species of *Borrelia*.
- 10 10. An assay device for diagnosing Lyme disease in a subject, wherein the device comprises a molecule which specifically binds a pro-inflammatory cytokine.
11. A device according to claim 10, wherein the molecule which specifically binds a pro-inflammatory cytokine is an antibody.
- 15 12. A device according to claim 10 or 11, wherein the device is a lateral flow test strip.
13. A kit of parts for diagnosing Lyme disease in a subject comprising a) a source of *Borrelia* antigens and b) reagents for detecting the expression level of a pro-inflammatory cytokine.
- 20 14. A kit according to claim 13, wherein the molecule which specifically binds a pro-inflammatory cytokine is an antibody.

1/2

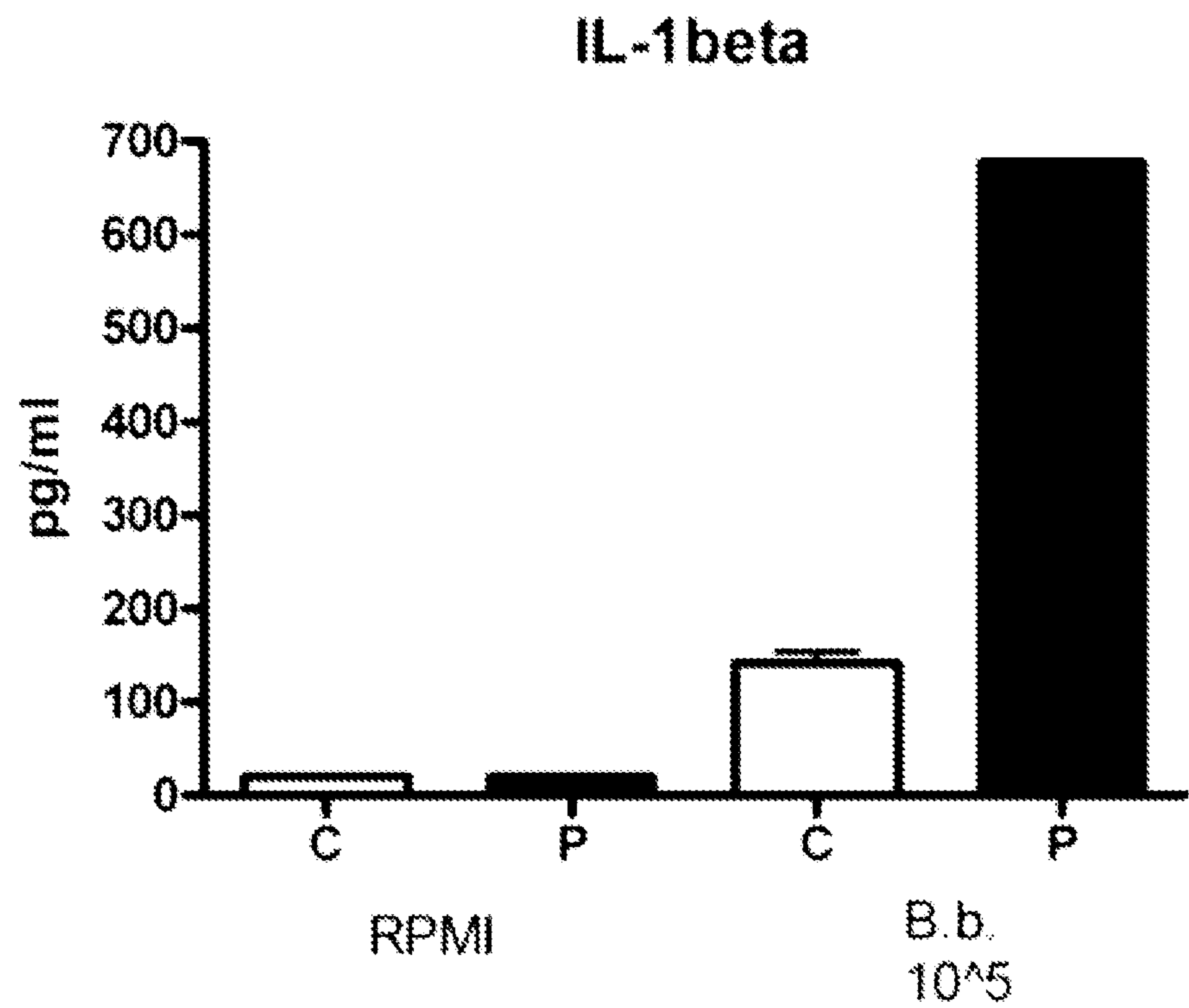
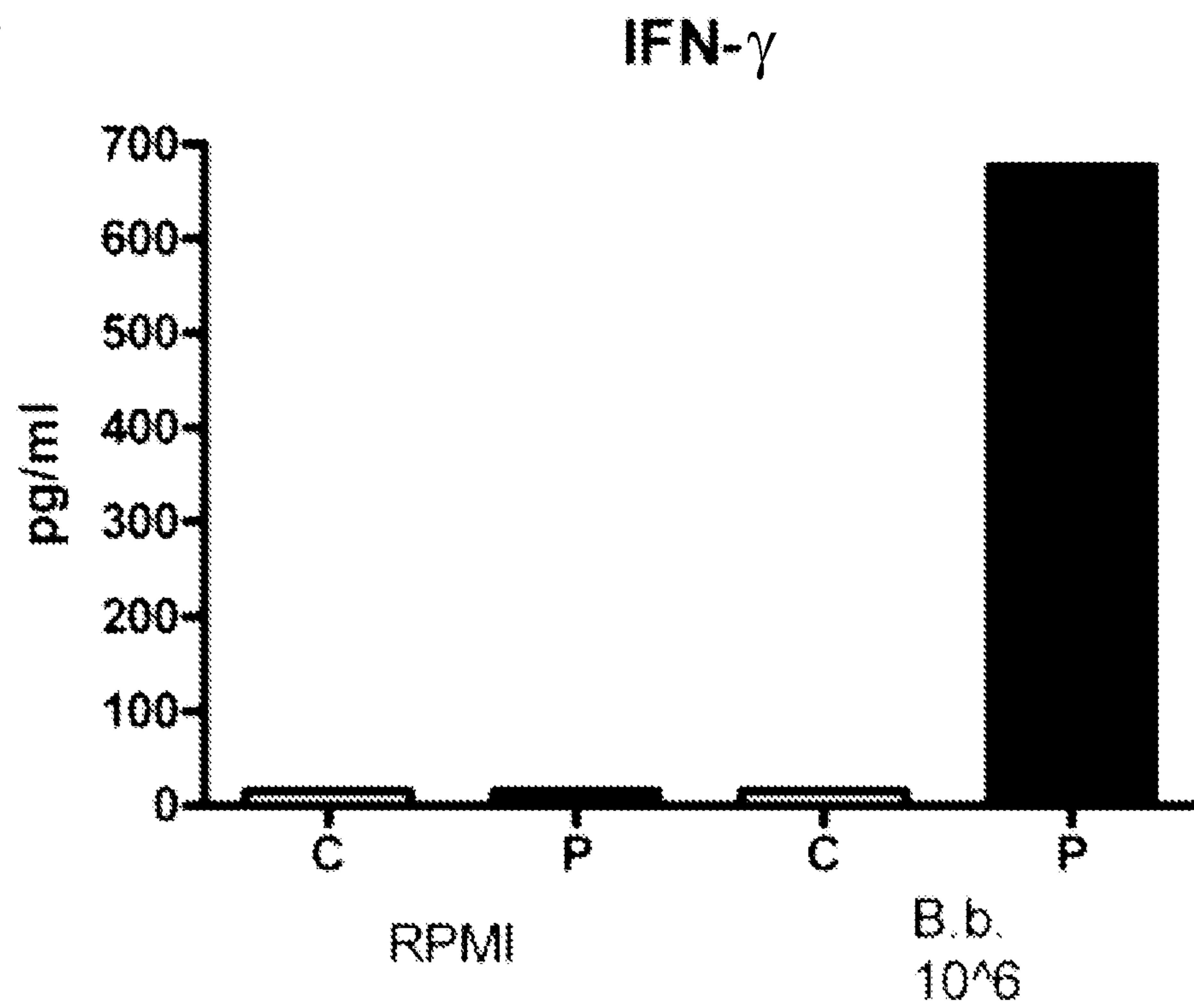
Fig. 1*Fig. 2*

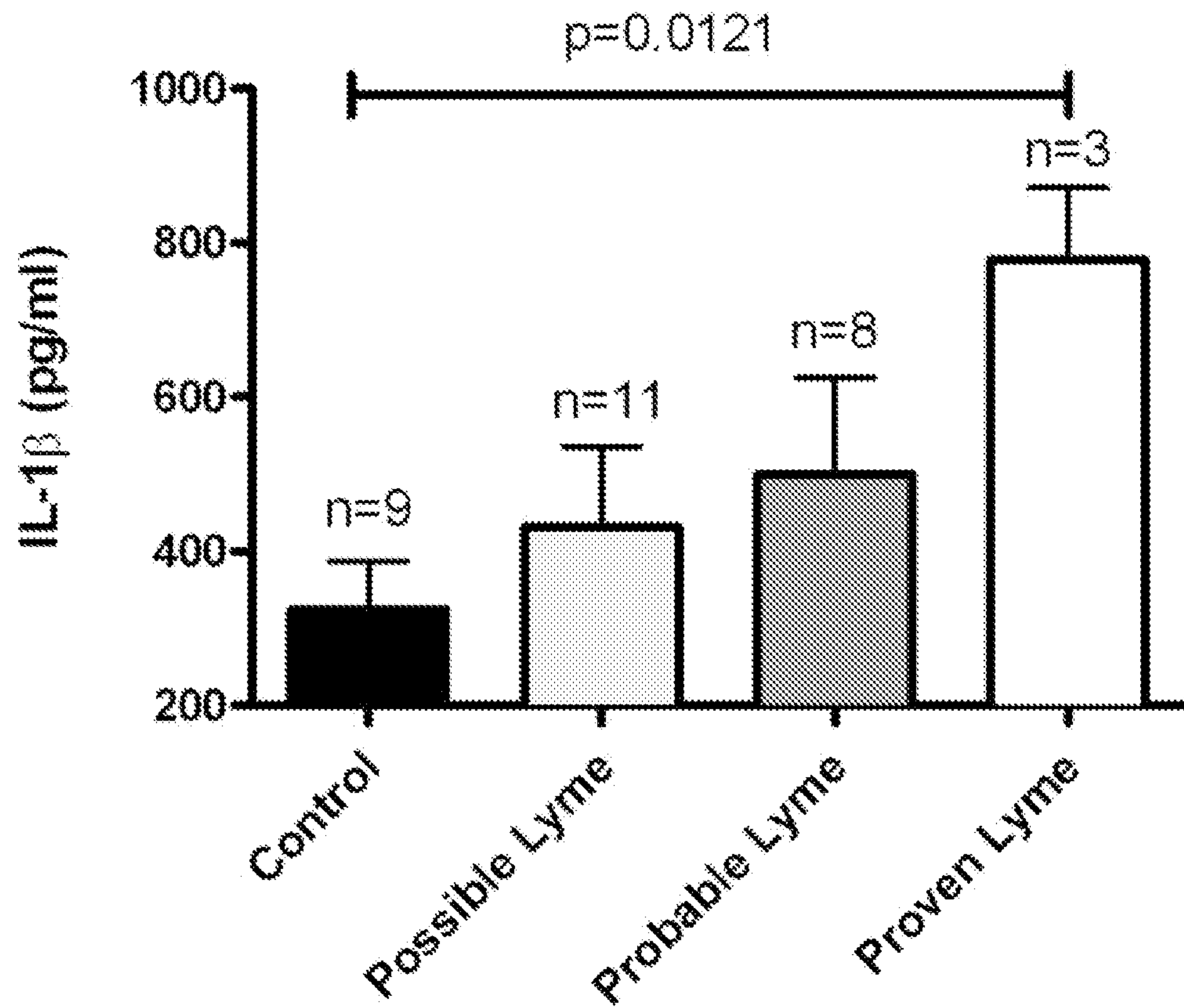
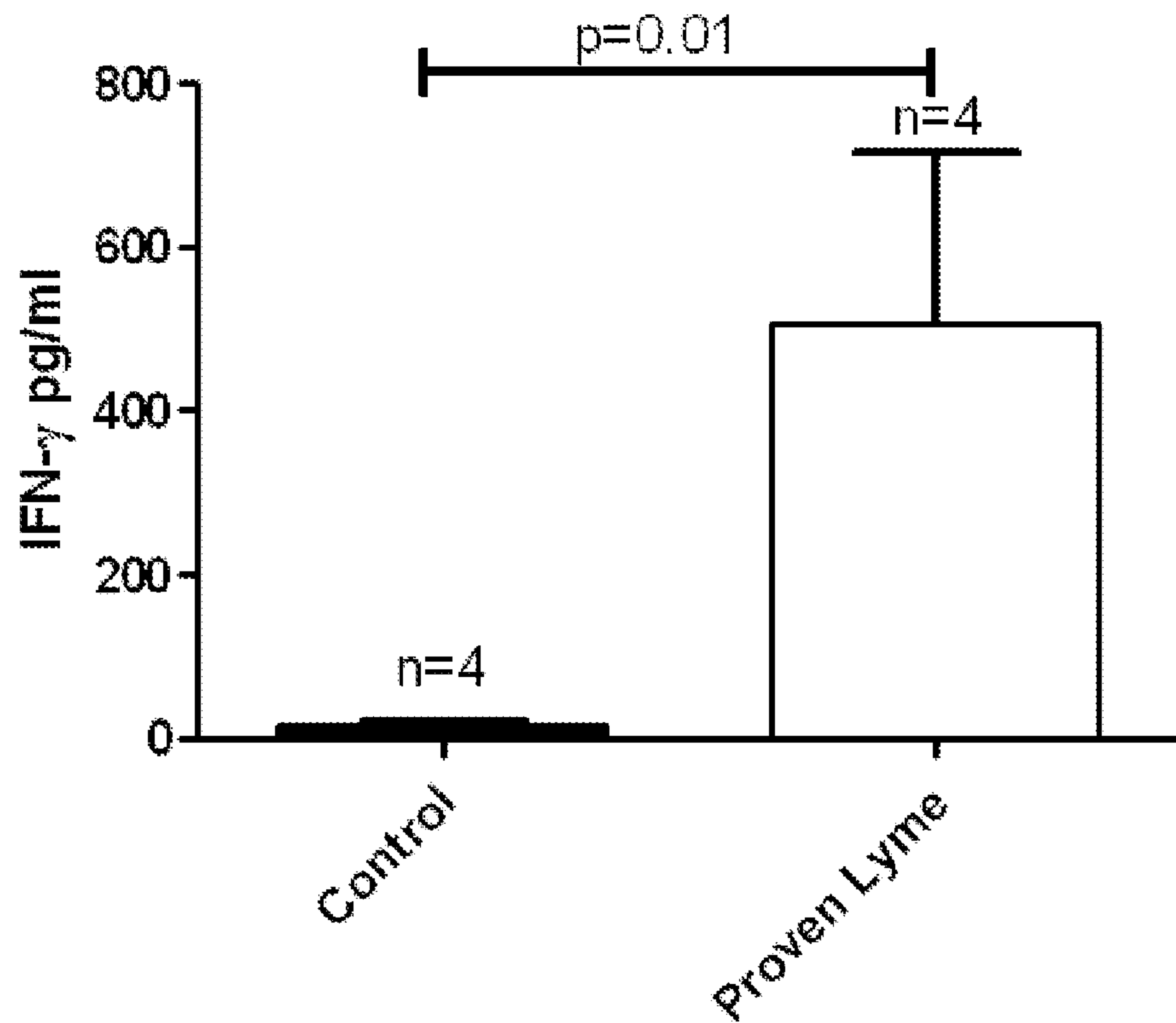
Fig. 3a*Fig. 3b*

Fig. 3a

