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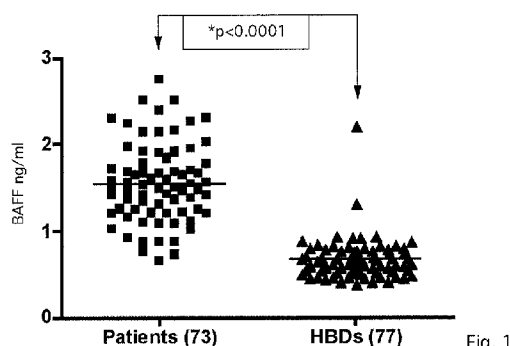
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(54) Title: USE OF THE SEROLOGICAL ASSAY OF THE CYTOKINE B-LYMPHOCYTE STIMULATOR (BLyS) FOR THE DIAGNOSIS, PROGNOSIS AND THE SCREENING OF THERAPEUTIC EFFICACY IN IMMUNE-RELATED DISEASES INCLUDING ORGAN-SPECIFIC AUTOIMMUNE DISEASES AND TRANSFUSION REACTIONS



(57) Abstract: Use of the assay in the serum of the cytokine B-Lymphocyte Stimulator (BLyS) for the diagnosis, prognosis and screening of therapeutic efficacy in the course of immune-mediated diseases including organ-specific autoimmune diseases (such as celiac disease and autoimmune thyroiditis) and / or transfusion reactions and / or IgA deficiency in a patient that includes an initial step of taking a sample of blood from the patient, a step of analyzing the blood sample to determine the concentration of cytokine BLyS, a step of comparing the BLyS levels determined in the previous step and one or more reference values of concentration of cytokine BLyS, a step of identifying a significant deviation between the determined concentration of cytokine BLyS and the reference values of concentration of cytokine BLyS indicated in the previous step and a step of assigning a diagnosis and / or prognosis and / or therapeutic effectiveness with respect to the immune-mediated diseases mentioned above, based on the previous steps.

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"USE OF THE SEROLOGICAL ASSAY OF THE CYTOKINE B-LYMPHOCYTE STIMULATOR (BLYS) FOR THE DIAGNOSIS, PROGNOSIS AND THE SCREENING OF THERAPEUTIC EFFICACY IN IMMUNE-RELATED DISEASES INCLUDING ORGAN-SPECIFIC
5 AUTOIMMUNE DISEASES AND TRANSFUSION REACTIONS"

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FIELD OF THE INVENTION

The present invention concerns the diagnostic and prognostic management of immune-related diseases, such as organ specific diseases and transfusion
10 reactions, based on the use of the serological assay of the cytokine B-Lymphocyte stimulator (BlyS) as a marker of predisposition, diagnostic confirmation, clinical course and therapeutic efficacy.

BACKGROUND OF THE INVENTION

The cytokine B-Lymphocyte stimulator (BlyS), also known as "B-cell
15 activating factor of the TNF family" (BAFF), was discovered and characterized in 1999 based on its homology with the members of the superfamily of the tumor necrosis factor (TNF) (Schneider P. et al. J Exp Med. 1999;189(11): 1747-56 and Nardelli B. et al. Blood. 2001; 97(1):198-204).

BLyS plays a very important role in immune response, since it is now counted
20 as one of the key factors in regulating B-cell development and differentiation (Mackay F., Browning JL. Nat Rev Immunol 2002;2:465-75 and Batten M et al. J Exp Med 2000; 192(10): 1453-66).

BLyS is synthesized, expressed as a membrane protein and released in soluble
25 form primarily by cells of the myeloid line such as monocytes, macrophages, neutrophils, dendritic cells (Huard B. et al. Int Immunol 2004;16:467-475 and Nardelli B. et al Blood. 2001;97(1):198-204).

Recently, the series of cell types able to secrete this cytokine has been further
enlarged, including also non-myeloid cells, such as the cells of the medullar
stroma (Gorelik L: et al. J Exp Med 2003;198:937-945), synoviocytes (Ohata J.
30 et al. J Immunol 2005;174(2):864-70), astrocytes (Markus Krumbholz et al. J Exp Med. 2005;201(2):195-200), the salivary gland epithelium (Ittah M, Miceli-Richard C., Eric Gottenburg J et al. Arthritis Res Ther. 2006;8(2):R51) and the intestinal epithelium (Xu W., He B., Chiu A. et al. Nature Immunol

2007;8(3):294-303).

BLyS exerts its function through interaction with three receptors, the most important of which, the BAFF receptor (BAFFR), is expressed in a peculiar manner by B lymphocytes (Ng LG et al. J Immunol. 2004;173(2):807-17). The link between BLyS and BAFFR induces an increase in expression of several anti-apoptotic factors (Bcl2, Bcl-xL, Mcl-1), thus promoting mature B cell survival and proliferation (Craxton A, et al. J Exp Med. 2005;202(10):1363-74).

BLyS has high homology with another member of the TNF superfamily called APRIL (A Proliferation Inducing Ligand) (Hahne M et al. J Exp Med 1998;188:1185-1190), which shares with BLyS two of its three receptors, TACI (transmembrane activator and calcium-modulating cyclophilin ligand) and BCMA (B-cell maturation antigen) (Thompson JS, Schneider P, Kalled SL et al. J Exp Med. 2002;192(1):129-35 and Seshasayee D, Valdez P, Yan M et al. Immunity 2003;18(2):279-88).

The importance of BLyS in B-cell homeostasis has been brought to light by studies on murine models.

In BLyS knock-out mice, where BLyS expression has been abrogated, a profound alteration is observed of the pool of mature B lymphocytes (Gross JA et al. Immunity 2001;15:289-302), whereas mice that hyperexpress BLyS (BLyS transgenic mice) develop many characteristics typical of autoimmune diseases.

These include spleno- and lymphoadeno-megaly, high serum levels of autoimmune-antibodies (rheumatoid factor, anti-DNA), B-cell infiltration of the parotid glands with subversion of the glandular architecture and loss of the secretory function, as is found in the course of Sjögren's syndrome (SS), renal alterations which greatly recall the glomerulonephritis typical of systemic lupus erythematosus (SLE) and finally they develop a B-cell neoplasia (Mackay F et al J Exp Med. 1999;190(11):1697-710 and Thien M et al. Immunity 2004;20(6):785-98).

The experimental evidence therefore shows that, at physiological levels, BLyS promotes B-cell survival and differentiation (Mackay F, Browning JL. Nat Rev Immunol 2002;2:465-75), but, at supraphysiological concentrations, it allows autoreactive B-lymphocytes to survive and proliferate, which would normally be suppressed by the immune system (Thien M et al. Immunity. 2004;20(6):785-98).

In accordance with this experimental evidence, recently high serum and tissue levels of BLYS have been described in several autoimmune diseases.

Among these autoimmune diseases, there are: Sjögren's syndrome (SS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), multiple sclerosis (MS), mixed cryoglobulinemia (MC) and Wegener's granulomatosis (Stohl W. B Curr Rheumatol Rep 2002;4(4):345-50, Seyler TM et al J Clin Invest. 2005;115(11):3083-92, Mariette X et al. Ann Rheum Dis. 2003;62(2):168-71, Matsushita T et al Arthritis Rheum. 2006;54(1):192-201, M. Thangaraj et al. J Neuroimmunol 2004;152:183-190, Fabris M et al, J Rheumatol 2007;46:37-43 and M. Krumbholz et al. J Autoimmun 2005;25:298-302).

In general, in these autoimmune diseases, the serum and tissue levels of BLYS are correlated with the levels of disease-specific autoantibodies and the presence and level of lymphocyte infiltration in the affected tissues (synovial membrane, salivary glands) and in particular the formation of ectopic germinal centers appeared correlated with the presence of BLYS and APRIL (Jonsson MV et al J Clin Immunol 2005;25:189-201, Szodoray P et al. Clin Immunol 2005;17:168-176).

Among the autoimmune diseases we can distinguish two main subgroups, the systemic and the organ-specific disorders. The former affect the entire human organism, whereas the latter affect a specific manner only one district of the body. BLYS has been thoroughly investigated in several systemic autoimmune diseases, but not in the organ-specific ones as in the present invention.

Moreover, there are some immunological deficiencies generally predisposing to the development of autoimmune disorders, such as the immunoglobulin A (IgA) deficit.

Among the immune-related diseases not properly associated with autoimmunity, but which share with them the main mechanisms with which the organic damage originates and is produced, there are the immune-related transfusion reactions. The immune-related transfusion reactions comprise all the possible complications following a blood transfusion, due to plasmatic or erythrocytic incompatibility, such as thrill-hyperthermic syndrome, allergic reactions, but most of all post-transfusion haemolytic reactions. Recently, it has

been observed that autoimmune disease-affected patients present an increased production of irregular alloantibodies after blood transfusion compared to the general population. Irregular alloantibodies are antibodies produced against non-self erythrocytic antigens after blood transfusions, pregnancy, active immunizations or passively acquired after immunoglobulin or plasma infusions or organ or bone marrow transplantations. The frequency of the presence of alloantibodies varies between 0.3 and 38% of the general population and is continuously growing, through the increased sensibility of the new methods used to detect alloantibodies in the blood. These alloantibodies are responsible for most of the haemolytic transfusion reactions with clinical relevance.

Elevated BLyS levels have also been found in the course of organ rejection: in this context the BLyS/BAFFR interaction on T-lymphocytes promotes T cell activation and proliferation against the transplanted organ (Ye Q et al. Eur J Immunol 2004 ;34 :2750-59). In a murine model of cardiac transplantation rejection due to MHC-mismatch, the blockade of BLyS-BAFFR can significantly extend the survival of the transplanted organs.

In organ-specific autoimmune diseases, such as autoimmune thyroiditis (AITD) and celiac disease (CD), as in immune-related transfusion reactions (IRTR), the role of B lymphocytes has not yet been clarified, while that of T lymphocytes is better defined. In fact, AITD and CD are characterized by a prevalent T lymphocytes infiltrate in the involved tissue and the activation of T cell clones with autoantigenic specificity has been always considered at the basis of the pathological process. In contrast, autoantibodies secretion by B lymphocytes, cells rarely described in the infiltrate, is believed to be very important at the diagnostic level, but secondary towards the main trigger of the pathological process. In fact, CD and AITD can be diagnosed also when there are no circulating disease-specific autoantibodies. However, we can also find similar diagnostic options in many other systemic autoimmune diseases, such as RA, SS, SLE, SSc etc. But, in these latter conditions, the role of B lymphocytes is much more defined. In fact, beside the discoveries obtained through in vitro experiments or animal models, the recent introduction in vivo, in humans, of the B-cell depletion therapy has brought to light the fundamental role of B lymphocytes in sustaining the chronic pathological process, not only as producers

of pathogenic autoantibodies, but also as antigen presenting cells activating the T cell population. It is widely demonstrated that the proliferation of autoantibodies producing B cells is sustained specifically by the increased B_{LyS} levels.

The autoimmune thyroid diseases (AITD) comprise essentially two distinct pathologic entities: Hashimoto thyroiditis (HT) and Graves-Basedow disease (GBD). HT, also called chronic lymphocytic thyroiditis, is one of the most common autoimmune disease in humans. It affects females more than males (F:M=18:1) and it comprises several clinicopathological types: the goitrous (the most common) form, the atrophic form (also known as idiopathic myxedema), post-partum thyroiditis and the hyperthyroid "Hashitoxicosis". It is always characterized by a marked lymphocytic T CD4⁺ infiltration of the thyroid gland. The lymphocytes organize into true lymphoid follicles and come in close contact with the thyrocytes, the target of their destructive function. In the goitrous variant, in post-partum thyroiditis and in Hashitoxicosis the thyroid is enlarged, in the atrophic variant the thyroid is markedly reduced. The most common clinical manifestations are linked to hypothyroidism (pale skin; dry mucosa; bradycardia, muscle cramps; adynamia; widespread edema; weight gain; dyspnea; oligomenorrhea; anaemia; etc) due to thyroid dysfunction because of the presence of autoantibodies directed against fundamental components of the gland, such as thyroglobulin (Tg) and thyroperoxidase (TPO). Some patients over time develop antibodies that block the TSH receptor (TRBab).

The diagnosis relies both on clinical signs and symptoms and on the assay of thyroid hormones (FT4 and FT3), which may even be normal in the first phase of the disease, and of TSH, which will appear high even with normal levels of FT4, which condition is defined as subclinic hypothyroidism. AntiTPO antibodies are usually found in 90-95% of patients, while serum antithyroglobulin (antiTg) antibodies are positive in 60-80% of patients, but they are not considered a good clinical marker and can also be found in Graves-Basedow disease, thyroid cancer, and even in healthy people. The therapy depends on the stage: if full-blown hypothyroidism has been reached, the therapy is based on the daily administration of synthetic T4 (levo-Thyroxine, LT4). Treatment with L-T4 of goiter with euthyroidism is debated, it may prevent goiter growth and protect patients from the possible evolution to hypothyroidism but there is no universal

consensus on this issue. Atrophic thyroiditis is the form that most often leads to full-blown hypothyroidism and myxedema, it has very high levels of TSH, often (20-50% of cases) it has TRBab antibodies and has always been considered a disease sustained by a T cell proliferation with a Th2 disease. At the moment
5 there are no definite diagnostic criteria for this form of thyroiditis: LT4 replacement therapy is indicated in patients with asymptomatic AT, in order to slow down the progress of the thyroid degeneration.

Graves-Basedow disease (GBD) is an autoimmune thyroid disease clinically characterized by hyperthyroidism caused by circulating antibodies directed
10 against TSH-receptor with stimulating activity (TRAab). Although the etiology is unknown, it is believed to be due to the interaction between environmental factors that trigger it (low iodine diet, viral or bacterial infections) and genetic susceptibility. Diagnosis is based on the presence of hyperthyroidism (high levels of FT3 and FT4, reduced TSH, clinical manifestations linked to hyperthyroidism:
15 tachycardia, tremors, weight loss, diaphoresis, etc.) and pathognomonic antibodies (TRAab), positive in 95% of patients. Goiter is often present and in about 50% of cases there is the typical ophthalmopathy, and in 1-2% a characteristic dermopathy of the lower limbs. Current treatment is based on the severity of the symptoms and includes medical therapy with antithyroid activity
20 drugs (thionamides), radiotherapy with I¹³¹ and finally, surgical removal of the gland. Many patients obtain a relative advantage from medical therapy and often radiotherapy leads to hypothyroidism with the need for life-long replacement therapy. All current therapies treat the symptoms but not the disease. Recently rituximab has been used successfully, which is an antiCD20 monoclonal antibody
25 able to deplete the peripheral B cells.

It has been suggested recently that HT, AT and GBD are different expression of a basically similar inflammatory autoimmune process and that the clinical appearance reflects the spectrum of the immune response in the particular patient.

In celiac disease, the a-tTG antibodies are a fundamental feature, both from
30 the pathogenetic, and especially the diagnostic point of view (Dieterich W et al. Nat Med 1997;3:797-801 and Tonutti E et al. J Clin Pathol 2003;56:389-93).

Classically, active celiac disease is characterized by intestinal and/or extra-intestinal symptoms and by a strong positivity of the a-tTG antibodies.

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Histologically, there is a marked lymphocytic infiltrate T of the duodeno-jejunal mucosa, associated with the typical morphological alterations: hyperplasia of the vaults and atrophy of the villi.

5 The duodenal- jejunal biopsy is considered the gold standard for diagnosis, but in practice there are some problems, both of an analytical nature and also pre-analytical.

Celiac disease is frequently associated with other autoimmune diseases, in particular type I diabetes mellitus and Hashimoto's thyroiditis.

Lymphatic neoplasias are one of the worst complications of celiac disease.

10 Among these, the intestinal T lymphoma is certainly the most frequent and fearsome, but type B lymphomas are also described (Celier C et al. Lancet 2000;356:203-8).

The lymphoma develops in stages starting from a reactive intra-epithelium lymphocyte infiltrate through a low-grade indolent proliferation, to the
15 transformation into a high-grade lymphoma which causes a persistently poor absorption even after the introduction of a gluten-free diet and immunosuppressive therapies (Cerf-Bensussan N et al. Gut 2002;51:304-5).

Celiac disease together with mixed cryoglobulinemic syndrome (Rodrigo L. World J Gastroenterol 2006;12(41):6585-6593; Ferri C et al. J Clin World Pathos
20 2002;55:4-13) are two very important models for studying the mechanisms which lead to the development of autoimmunity, since in celiac disease it has been widely proved that the altered response to gluten is associated with a precise genetic setting, HLA DQ2 and 8, whereas in mixed cryoglobulinemic syndrome it is well known that hepatitis C virus (HCV) infection has a very frequent role in
25 triggering the rheumatoid factor positive antibody response responsible for the formation of cryoglobulins and vasculitis.

The most recent evidence suggests, however, that a viral infection, precisely from Rotovirus (Troncone R, Aurecchio S. J Pediatr Gastroenterol Nutr. 2007;44(5):527-8), could also play an important role in the pathogenesis of celiac
30 disease.

Furthermore, the autocrine production of BLyS has recently been proved by neoplastic B lymphocytes in the course of some of the most frequent lympho-proliferative diseases (Hodgkin's and non-Hodgkin's lymphoma, chronic

lymphocytic leukemia, multiple myeloma, Waldenstrom's macroglobulinemia) (Chiu A et al. Blood 2007;109(2):729-39, J. Novak et al. Blood 2004;104:2247-2253, Kern C et al. Blood 2004;103(2):679-88, Mackay , Tangye SG. Curr Opin Pharmacol. 2004;4(4):347-54, Moreaux J et al. Blood 2004;103(8):3148-57).

- 5 Compared to the general population, autoimmune diseases generally have a higher risk of developing B cell clonality, more rarely T cells, which could then evolve into a frank lymphatic cancer.

At the basis of this increased risk there are several factors, both genetic and acquired (treatments) (Quartuccio L, et al. Haematologica 2006, 91 (5) :691-4).

- 10 Currently there are no definite biological or genetic markers that can predict the development of lymphocyte clonality in patients suffering from various autoimmune diseases, systemic or organ-specific. The IgA deficiency also has this increased risk, especially if it evolves into an extended immunoglobulin deficit.

- 15 At the present moment, in autoimmune diseases, as in the general population, the risk of neoplastic evolution is clinically monitored by means of a physical examination of the patient in order to identify the main signs and symptoms of lymphatic neoplasia (lympho-adenomegaly, splenomegaly, fever, itchiness, weight loss, asthenia, persistent parotid tumefaction in the case of Sjögren's syndrome, etc), or by means of radiological examinations (ultrasound, CT)
20 assisted or not by a biopsy and histological and molecular (amplification of the hypervariable regions of the immunoglobulin genes or T cell receptors) examinations.

- In the course of celiac disease the suspicion of a lymphomatous evolution
25 arises in cases of persistent high levels of anti-transglutaminase antibodies even when a gluten-free diet is being followed, however without associated lymphoma symptoms. In these cases persistently high levels of BLyS can suggest a pre-lymphoma condition and indicate a diagnostic and therapeutic course able to prevent a full neoplastic manifestation. In our current state of knowledge,
30 diagnostic confirmation is often late due to the difficulty of obtaining a reliable biopsy result. Therefore the possibility of preventing and hence treating the lymphoma evolution in these patients is very limited both due to the late diagnosis and also because they are very aggressive forms, which do not respond

very well to the currently available therapies.

In the current state of the art, except for celiac disease, in which the role of gluten is widely recognized, the triggers (viral, bacterial, environmental in general) responsible for setting off the pathological process of the diseases
5 described in the present invention are still largely unknown, and there is extensive research to find the factors that promote the perpetuation thereof, until the evident disease becomes chronic.

Furthermore, there is a lack of markers that are useful for monitoring the development of B/T cell clonality, which precedes lymphatic cancer in organ-specific autoimmune diseases and in immunological deficits.
10

It is known, from document WO-A-2004/074511, to use BLYS assay to determine predisposition to develop cancer in people being treated or candidates to be treated with anti-TNF antibodies. These drugs are commonly used in systemic autoimmune diseases, but there is no rationale for their use in organ-specific autoimmune diseases, in transfusion reactions or immune deficiencies as
15 in the present invention.

With such a background, one purpose of the present invention is to use the serological assay of cytokine B-Lymphocyte stimulator BlyS to confirm the diagnosis of immune-mediated diseases, including organ-specific autoimmune
20 diseases, (celiac disease, autoimmune thyroiditis), and immune-mediated transfusion reactions (post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions), which will overcome the limits in the approach currently in use in the event of suggestive situations and/or of doubt and/or predisposition (microbial infections, immunological deficiency, genetic
25 predisposition based on HLA genes).

Another purpose of the present invention is to use the assay of cytokine BLYS as a marker for risk of developing post-partum thyroiditis, which would discriminate in the differential diagnosis with post-partum depression.

Another purpose of the present invention is to use the assay of cytokine BLYS
30 as a prognostic marker in the course of acute thyroiditis and atrophic thyroiditis.

Another purpose of the present invention is to use the assay of cytokine BLYS as a marker of risk for a transfusion reaction in patients who are candidates to undergo a transfusion, especially with regard to patients with active immune-

mediated diseases.

Another purpose of the present invention is to use the assay of cytokine BLYS as a method for choosing a personalized therapy, monitoring the adhesion to treatment (e.g. gluten-free diet in celiac patients), the screening of effective
5 therapies not only in symptomatic terms but at the biological level of the disease (high levels of BLYS return to normal range after therapy) in diseases including organ-specific autoimmune diseases (celiac disease, autoimmune thyroiditis), the IgA deficiency and transfusion reactions, which will overcome the limits in the approach currently in use.

10 Still another purpose of the present invention is to use the serological assay of BLYS for the diagnosis and prognosis of B and T cell clonality in immune-mediated diseases, including organ-specific autoimmune diseases (celiac disease, autoimmune thyroiditis), and immunoglobulin deficiency, which will overcome the limits set out the approach currently in use.

15 Still another purpose of the present invention is to use BLYS as a marker for an innovative method of therapy to prevent and treat B and T cell clonality in the course of organ-specific autoimmune diseases and immunoglobulin deficiency.

To overcome the drawbacks of the state of the art and to obtain these and other purposes and advantages, the Applicant has studied, tested and embodied the
20 present invention.

SUMMARY OF THE INVENTION

The present invention is set forth and characterized in the independent claims, while the dependent claims describe other characteristics of the invention or variants to the main inventive idea.

25 The evidence regarding B cell population and the presence of BLYS was totally lacking in organ-specific autoimmune diseases and in immune-related transfusion reactions here described. Thus, it did not seem a likely explanation that BLYS could have an important pathogenetic role also in these disorders. The formulation of this hypothesis has been possible putting together the conspicuous
30 scientific background coming from the knowledge and the experience of systemic autoimmune diseases, with the investigation of the peculiar features of organ-specific autoimmune diseases, of the immunological deficit and of transfusion reactions, which are often associated with them. This process came

from the systematic integration among the different expertise of the co-inventors.

The results obtained by the inventors from preliminary studies regarding organ-specific autoimmune diseases (celiac disease and autoimmune thyroiditis), IgA deficit and transfusion reactions, have led the co-inventors to discover BLyS
5 as a new useful marker for the diagnostic and prognostic management of these immune-related diseases. In particular, BLyS appeared as a marker of risk and/or predisposition to the disease, as an indicator of disease severity and the presence of a B/T cell clonality, as a method to verify adherence to the therapy and screening of new therapeutic strategies, effective not only on the symptoms but
10 on the biology of the disease.

In accordance with the above purposes, one feature of the present invention concerns the use of the serological assay of cytokine B-Lymphocyte stimulator (BLyS) as a marker to confirm the diagnosis of immune-mediated diseases, including:

- 15 - organ-specific autoimmune diseases such as celiac disease, autoimmune thyroid disease;
- transfusion-related immune-mediated diseases, such as post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions;
- in patients with suggestive clinical symptoms and / or physical or biochemical
20 examination or doubtful situations or in patients with significant predispositions (chronic microbial infections, IgA deficiency, genetic predisposition, familiarity), in a method which comprises the following steps:
 - a first step of taking a blood sample from the patient from which the serum can be obtained by centrifugation;
 - 25 - a second step of examining the sample of serum in order to determine the concentration of cytokine BLyS, typically by using commercial kits;
 - a third step of comparing the concentration of cytokine BlyS determined in the second phase and the reference values of concentration of cytokine BlyS previously obtained on a healthy population;
 - 30 - a fourth step of identifying a significant deviation, deriving from the comparison in the third step, between the concentration of cytokine BlyS determined in the second phase and the reference values of concentration of cytokine BlyS previously obtained on a healthy population control, so as to select

the patient affected by one of said diseases;

- a fifth step of comparing the concentration of cytokine BLYS determined in the second step for the patient selected as affected by one of said diseases and one or more reference values of concentration of cytokine BLYS; these values are
5 in a range of benchmarks to BLYS previously obtained on a series of patients with a specific immune-mediated disease and / or established diagnosis of active disease or its particular course;

- a sixth step of assigning a diagnosis of one or more determinate diseases from among the immune-mediated diseases as above, according to the
10 comparison made in the fifth step.

Another innovative feature of the present invention is the use of cytokine B-lymphocyte Stimulator (BLYS) as a diagnostic marker, in particular the use of the assay of BLYS, in the method described above, in diseases where the role of this cytokine in the pathogenesis was not easily conceivable, since the T cell, not the
15 B cell, is the predominant component in the pathological infiltrate and in the pathogenetic mechanisms so far identified.

The present invention advantageously uses in the second step, for the analysis of the concentration of BLYS in the patients' serum, an automated apparatus able to perform immune-enzymatic assays (Enzyme-Linked Immunosorbent Assay:
20 ELISA), of the type usually present in the largest hospital analyses labs, without needing substantive modifications to the plants or the organizational structures of the wards concerned.

The present invention also concerns the use of BLYS in a method to monitor over time the onset of B/T cell clonality in the course of immune-mediated
25 diseases in a patient, comprising the following diseases:

- organ-specific autoimmune diseases (autoimmune thyroiditis, celiac disease);
- immunological deficit such as: IgA deficiency, common variable immunoglobulin deficiency;

which comprises the following steps:

- 30
- a first step of taking a sample of blood from the patient to obtain serum;
 - a second step of examining the serum sample to determine the concentration of cytokine BLYS;
 - a third step of comparing the concentration of cytokine BLYS determined in

the second step and one or more BlyS values previously detected in the patient;

- a fourth step of identifying a significant deviation, deriving from the comparison in the third step, that is, a significant increase between the concentration of cytokine BlyS determined in the second step and one or more

5 BlyS values previously detected in the patient;

- a fifth step of attributing a potential risk to develop a B or T cell clonality, or an evolution toward a B or T cell clonality in the course of immune-mediated diseases set forth above, according to the presence of BlyS, or to the significant deviation, detected in the fourth step;

10 - a sixth step of deciding to repeat over time, with predetermined time intervals, from the first to the fifth steps, based on the outcome of the assignment carried out in the fifth stage.

In this case we can evaluate, over time, the effectiveness of the diet in celiac patients, the markers of activation of the immune system, the markers of organ
15 functionality (FT4, FT3, TSH, specific autoantibodies in autoimmune thyroiditis), the clonal proliferation of B or T lymphocyte in peripheral blood or bone marrow of patients (by using molecular biology techniques), physical examinations, X-ray or biopsy on the patient, or other.

Another feature of the present invention provides for the use of cytokine BlyS
20 assay as a prognostic marker in a prognosis method, that is to say, risk of onset, course, severity, good response to therapies, etc., of immune-mediated diseases comprising:

- organ-specific autoimmune diseases such as:

celiac disease, autoimmune thyroiditis,

25 - immune-mediated diseases related to blood transfusions such as:

post-transfusion immunization, mother-fetal incompatibility, transfusion reactions;

comprising the following steps:

30 - a preliminary step to select patients characterized by one or more of the following situations:

i) presence of chronic microbial infection (hepatitis virus, herpes virus, chlamydia, etc)

ii) presence of a particular HLA genotypes / or genes encoding important

factors in the pathogenic process of the disease or target of the commonly used therapies;

- iii) subclinical hypothyroidism with or without goiter;
- iv) depression and / or hypothyroidism post-partum
- 5 v) presence of one or more active immune-mediated diseases
- vi) need for blood transfusion;
- vii) beginning of a new therapy;
- a first step of taking a sample of blood from a selected patient;
- a second step of examining the blood sample to determine the concentration
- 10 of cytokine BLYS;
- a third step of comparing the concentration of cytokine BLYS determined in the second step and one or more reference values of the concentration of cytokine BLYS;
- a fourth step of identifying a significant deviation, deriving from the third
- 15 step, between the concentration of cytokine BLYS determined in the second step and the reference values considered in the third step;
- a fifth phase of assigning a prognosis to the deviation identified in the fourth stage with regard to this immune-mediated disease.

Another feature of the present invention provides to use the assay of cytokine

20 BLYS as a method for screening new therapies effective in immune-mediated diseases such as:

- organ-specific autoimmune diseases such as: celiac disease, autoimmune thyroiditis;
- immune-mediated diseases related to blood transfusions such as: post-
- 25 transfusion immunization, mother-fetal incompatibility, transfusion reactions;
- immunological deficit such as: IgA deficiency, common variable immunoglobulin deficiency.

To this purpose it is possible to advantageously use the monitoring of the serological concentration of BLYS during therapy in order to verify the

30 effectiveness on the biology of the disease witnessed by the actual reduction in serum levels of BLYS to the levels included in the normal range, or to use the assay of BLYS as a marker in a method to control the effectiveness of a therapeutic treatment, of patients with immune-mediated diseases, that comprises

the following steps:

- a first step of taking a sample of blood from the patient to obtain serum before the new therapy;
- a second step of examining the serum sample to determine the concentration of cytokine BlyS;
- a third step of taking a sample of blood from a patient at fixed times after the start of the new therapy (for example: 1, 3, 6, 12 months);
- a fourth step of examining the sample/samples of blood taken in the third step, to determine the concentration of cytokine BlyS;
- a fifth step of comparing the concentration of cytokine BlyS determined in the second step and those determined in the fourth step;
- a sixth step of identifying a significant deviation, deriving from the comparison in the fifth step, between the concentration of cytokine BlyS determined in the second step and the values of the concentration of cytokine BlyS obtained in the fourth step;
- a seventh step of attributing efficacy to the new therapy to the deviation identified in the sixth step.

The present invention allows to improve the diagnostic / prognostic approach and the therapeutic monitoring of patients with immune-mediated diseases, both organ-specific autoimmune diseases, and also blood transfusion reactions.

The present invention is particularly effective for use in the diagnosis and prognosis of B and T cell clonality in the course of immune-mediated diseases including autoimmune thyroiditis, celiac disease and immunological deficiency, such as IgA deficiency.

A variant of the present invention provides that the use of the BlyS assay according to the present invention can be integrated with one or more of the following analyses:

- physical examination of the patient, able to identify the main signs and symptoms of the specific disease;
- one or more X-rays of the patient;
- histological examination of a biptic specimen taken from the patient;
- molecular biology examination on a biptic sample or peripheral blood or bone marrow of the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other characteristics of the present invention will become apparent from the following description of a preferential form of embodiment, given as a non-restrictive example with reference to the attached drawings wherein:

- 5 - fig. 1 is a graph comparing serum B-Lymphocyte Stimulator (BLyS) levels in celiac patients (CD) with respect to healthy blood donors (HBDs) [range of normality: <1.145 ng/ml, mean $+2SD$];
- fig. 2 is a graph showing the significant correlation between the concentration of cytokine B-Lymphocyte stimulator (BLyS) and the concentration of antibodies a-tTG in celiac patients;
- 10 - fig. 3 is a graph showing the significant reduction of B-Lymphocyte stimulator (BLyS) concentration following the gluten-free diet (GFD) in celiac patients (from 1.619 ± 0.410 ng/ml to 1.283 ± 0.310 ng/ml; * $p=0.0122$, Wilcoxon signed rank test);
- 15 - fig. 4 is a graph comparing serum B-Lymphocyte Stimulator (BLyS) levels in IgAD patients, globally (IgAD tot) and when distinguished in 2 subgroups: IgAD with celiac disease (IgAD+CD) and without CD (IgAD) with respect to healthy blood donors (HBDs) [range of normality: <1.145 ng/ml, mean $+2SD$];
- 20 - fig. 5 is a graph comparing serum BLyS levels in patients with autoimmune thyroiditis (AITD) globally and when distinguished between Hashimoto's thyroiditis (HT) and Graves/Basedow's disease (GBD) with respect to healthy blood donors (HBDs) [range of normality: <1.145 ng/ml, mean $+2SD$];
- 25 - fig. 6 is a graph comparing serum BLyS levels in HT patients with normal or reduced (hypo) FT4 levels.

DETAILED DESCRIPTION OF A PREFERENTIAL FORM OF EMBODIMENT

The present invention takes as its base what is known in the state of the art
30 regarding cytokine B-Lymphocyte stimulator (BLyS) to perfect an innovative use of the serological assay this cytokine for the diagnosis and prognosis of immune-related diseases, including organ-specific autoimmune diseases (celiac disease, autoimmune thyroiditis), immunological deficiencies and immune-mediated

transfusion reactions.

In particular, the experimental results which Applicant has obtained concern the expression and role of BLyS in several immune-related pathologies:

- celiac disease;
- 5 - IgA deficiency;
- autoimmune thyroiditis;
- immune-mediated transfusion reactions.

characterized by an immune-mediated response responsible for specific organic symptoms, by autoantibody secretion, by a strong association among
10 them and with other autoimmune diseases and by an increased risk of developing B or T cell clonality, have led to identify and propose BLyS as a new diagnostic, prognostic and therapeutic marker in these pathologies.

Furthermore, based on the results obtained in these pathologies, the present invention can be extended to all the other immune-mediated disorders where
15 BLyS may be identified in future.

The previous results of the study of BLyS in mixed cryoglobulinemic syndrome have allowed Applicant, for the first time since this cytokine was discovered, to identify the probable key role in the up-regulation of the BLyS expression by Hepatitis C virus (HCV) infection and to hypothesize that other
20 viruses, with similar mechanisms, can produce the same effect. In organ-specific autoimmune diseases, object of the present invention, an important role was assumed by microbial infection, probably viral, but still unknown, to trigger the pathological process. The Applicant had shown that HCV infection per se is able to induce an increased expression of BLyS and contribute, in a subset of
25 predisposed subjects, to sustain the autoreactive B cell proliferation, until the appearance of the cryoglobulinemic syndrome. The development of the syndrome, however, coincided with a further increase in the expression of BLyS, given that the values of serum BLyS in patients with cryoglobulinemic syndrome were significantly higher than in subjects with only chronic HCV infection
30 (Fabris M et al, J Rheumatol 2007; 46:37-43).

With these premises, the Applicant has hypothesized that there could be an increased level of BLyS also in celiac disease and autoimmune thyroiditis.

This effect would seem mainly mediated by the endogen antiviral response,

that is, interferon, which various studies have shown to be in vitro a powerful inducer of BLyS expression.

The role of B lymphocytes in the pathogenesis of celiac disease has so far appeared marginal compared to the fundamental role played by HLA and T lymphocyte activation (LM Sollid, Thorsby E. Gastroenterology 1993; 105: 910-22, Spurkland A, et al. Tissue antigens 1997 ; 49:29-34 and Molberg Ø, et al. Gastroenterology 2003; 125:337-44). However, Applicant has shown that BLyS is found at high serum levels in a high percentage (>80%) of patients affected by celiac disease (fig. 1).

In particular, fig. 1 shows the serum levels of BLyS in celiac patients versus healthy controls (HBDs, consisting of healthy subjects, blood donors, comparable in age and sex to the patients in the study). The serum levels of BLyS are significantly higher in celiac patients compared with the healthy control population (Mann Whitney t-test, * $p < 0.0001$). [range of normality: <1.145 ng/ml, mean +2SD].

This result, never shown before, proposes a new key in the interpretation of the pathogenesis of this widespread (about 1.1% of the population) enteropathy. Of particular importance is that, compared with all the autoimmune disorders where BLyS has been studied until now, in celiac disease the up-regulation of BLyS is the widest ever recorded, since it concerns 4 patients out of 5. Furthermore, the serum levels of BLyS measured and analyzed by Applicant show a significant correlation with those of the specific-disease antibodies, the anti-transglutaminase (a-tTG) (fig. 2, which shows the correlation between serum levels of BLyS and levels of a-tTG IgA, Spearman rank test: $r = 0.399$, 95% CI: 0.1724-0.5856, $p = 0.0007$), and both BLyS and a-tTG diminish in concurrence with the introduction of the gluten-free diet and clinical remission of the disease (fig. 3, which shows the modulation of the serum levels of BLyS after a gluten-free diet). A general significant reduction can be seen in the BLyS levels after the diet (from 1.619 ± 0.410 ng/ml to 1.283 ± 0.310 ng/ml; * $p = 0.0122$, Wilcoxon signed rank test), even though 8/12 (75%) of the patients still have post-diet BlyS levels above the range of normality (>1.145 ng/ml, assay with ELISA kit R&D Systems Quantkine ELISA kit, Minneapolis, 55413 USA).

However, even in those cases which become a-tTG negative and which reach

clinical remission, the BLyS levels, although substantially reduced, do not reach the range of normality, suggesting the persistence of a sub-clinical state of disease or a higher base level of BLyS predisposing to the disease and widely shared, equal to the HLA genotype.

5 Recent evidence of a possible infective co-participation (Rotavirus) in the pathogenesis of celiac disease provides another hypothesis of the raising of the BLyS serum levels, similarly to what was observed in the cryoglobulinemic syndrome.

10 The BLyS assay could therefore represent an additional diagnostic tool in cases of doubt, with an atypical presentation or with negative serum levels of a-tTG, or where the intestinal biopsy is precluded or not ethically indicated or again as screening in classes of individuals at greater risk of developing the disease.

15 Furthermore, the persistence at a systemic level of high serum levels of BLyS could contribute to the development of other autoimmune diseases in genetically predisposed individuals, just as, thanks to its powerful anti-apoptotic effect, BLyS would promote further genetic mutations in the expanded B cells until escape from the initial trigger and generation of a clonal population.

20 In the 66 patients with mixed cryoglobulinemic syndrome previously analyzed by the Applicant, the only clinical feature that showed a significant association with higher levels of BLyS was the presence of a clonal B cell proliferation: patients with a B cell clonality had a percentage of subjects with very high levels of BLyS significantly higher compared to patients without B cell clonality (33.3% versus 9.8%; OR = 4.6, CI = 1.12-18.96, p = 0.04).

25 In these cases one might think that, as shown in some B cell neoplasms (Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, chronic lymphocytic leukemia) and more recently in B lymphocytes infiltrating the salivary glands of patients with primary SS (C Daridon et al. Arthritis Rheum 2007; 56:1134-44), B cell clones may also secrete BLyS and contribute to the
30 disease, by a mechanism of autocrine stimulation.

 BLyS, as previously shown in the course of cryoglobulinemia and SS and in several neoplastic disorders, could play an important role in the multi-step process which leads to the development of the lymphoma in celiac disease too, in

fact it can stimulate both B and T cells (Mackay F, Leung H. *Semin Immunol.* 2006;18(5):284-9). Applicant's finding of a very high BLyS level (8.5 ng/ml) in a celiac patient with a diffuse large B cell intestinal lymphoma is in accordance with this hypothesis.

5 This result therefore suggests a possible use of the BLyS assay as a diagnostic support in cases of a celiac patient where a B/T cell clonality is suspected (persistence of high a-tTG levels despite the strict adherence to the gluten-free diet).

10 Selective primary IgA deficiency (IgAD) is the most common form of immunodeficiency, with an estimated incidence at 1:600 in Caucasians. Individuals with isolated IgAD have normal IgA genes, but have a defect of terminal lymphocyte differentiation, which leads to underproduction of serum and mucosal IgA (Cunningham-Rundles C. *J Clin Immunol* 2001;21(5):303-9). There have been many diseases reported in association with IgAD, such as
15 allergies, gastrointestinal tract and recurrent upper respiratory tract diseases and, in particular, autoimmune diseases (Liblau RS et al. *Int Arch Allergy Immunol* 1992;99(1):16-27). The most common association is with celiac disease (CD), which has special significance since CD is usually diagnosed by detection of specific IgA antibodies, which are obviously lacking in IgAD patients. As
20 illustrated in Figure 4, Applicant discovered that BLyS serum levels are significantly more elevated in IgAD patients (1.57 ± 0.51 ng/ml) than in controls (0.66 ± 0.24 ng/ml; $p < 0.0001$). In particular, 77.8% (35/45) of IgAD patients have BLyS levels over the range of normality (> 1.14 ng/ml). Among the IgAD patients analyzed, 26 were affected by celiac disease CD but they did not differ
25 significantly from the 19 patients with IgAD and without celiac disease CD (1.49 ± 0.46 ng/ml versus 1.67 ± 0.57 ng/ml, $p = \text{ns}$). No difference was found between BLyS levels in IgAD patients and the previously described series of patients with CD and normal IgA, (1.54 ± 0.46 ng/ml). Thus, BLyS is upregulated in patients with IgAD, and could be one of the factors in the strong association
30 between IgAD and autoimmune diseases, but also in the increased risk of developing B cell clonality. The present invention makes innovative use of the BLyS assay as a prognostic marker of the development of B cell clonality in subjects affected by IgAD.

Autoimmune thyroid diseases (AITD) are common autoimmune diseases, affecting up to 5% of the general population, with females affected more than males. Thyroid-directed autoimmunity is manifested in two classical autoimmune conditions: Hashimoto's Thyroiditis (HT) resulting in hypothyroidism (anti-TPO and anti-Thyroglobulin) and Graves – Basedow's disease (GBD) resulting in hyperthyroidism (TSH-Receptor agonist autoantibodies). AITD are frequently associated with other autoimmune diseases (celiac disease, type 1 diabetes mellitus, systemic connectivitis). Probably they share a common autoimmune-prone phenotype. Like other autoimmune diseases, AITDs present an increased risk of developing B-cell clonal diseases (especially HT patients).

As illustrated in fig. 5, Applicant has studied a series of 77 Caucasian patients with AITD, 10M/67F, mean age 48.2 ± 16.1 , 52 with HT and 25 with GBD, and analyzed BLYS serum levels compared to 77 age/sex matched healthy controls. AITD patients showed a significant increase of BLYS levels ($p < 0.0001$), GBD patients tended to have higher BLYS than HT ($p = 0.06$). No significant correlation was found between BLYS levels and autoantibodies, both in HT and in GD. In contrast, a positive correlation was found between BLYS and FT4 ($r = 0.31$; $p = 0.012$), while an inverse correlation was found with TSH ($r = -0.45$; $p = 0.0002$).

In fact, in HT patients BLYS is significantly more elevated in patients with normal FT4 levels than in patients with hypothyroidism ($*p = 0.0396$)(fig. 6).

In the present invention, for the first time, Applicant hypothesized and found elevated BLYS levels in AITD patients, suggesting an important pathogenetic role of BLYS also in these autoimmune disorders. In an innovative manner than previously demonstrated in systemic autoimmune diseases (RA, SS, LES), levels of BLYS correlate with thyroid functionality, but not with autoantibodies secretion. BLYS is therefore higher in the first euthyroideal phase of HT, and correlates with the level of hyperthyroidism in GBD, as a marker of gland activation, but not of plasma cells autoantibody secretion; it decreases when the gland loses its function, clinically manifested by hypothyroidism. Moreover, BLYS overexpression may represent one of the possible mechanisms explaining the increased percentage of AITD patients developing other autoimmune or lymphoproliferative diseases.

Thus, the present invention suggests using BLYS serological assay for the

diagnosis, prognosis and screening of treatment efficacy in AITD patients.

It has been observed recently that patients with autoimmune diseases have a greater tendency to produce irregular alloantibodies after blood transfusion than the general population. At present, there are no known markers that can predict the development of transfusion reactions. The association with autoimmune diseases and the immune-mediated mechanism led the Applicant to consider a possible role of cytokine BLyS also in transfusion reactions. To this aim, a pilot study was conducted on 5 patients from the Blood Products Distribution Laboratory of Udine University Hospital: 2 patients (pts Type A) that despite repeated transfusions had never demonstrated the development of allo/autoantibodies; and 3 patients (pts Type B) who had developed allo/autoantibodies after transfusion of multiple units of concentrated red blood cells. In patients with allo/autoantibody reactions (Type B) the levels of BLyS/BAFF tended to be higher than in patients without reactions (Type A), (average 2.48 ng / ml versus 1.29 ng / ml). In addition, all type B patients showed BLyS levels above the threshold of normality (> 1.14 ng / ml).

The present invention therefore provides, in an innovative manner, the assay of serum BLyS in the following situations:

i) diagnostic / prognostic marker (confirmation, severity, course, B / T cell clonality) and screening of therapeutic efficacy in the course of immune-mediated diseases including:

- organ-specific autoimmune diseases such as:
 - celiac disease, Hashimoto's thyroiditis, atrophic thyroiditis, post-partum thyroiditis, Graves-Basedow disease,
- immune-mediated diseases related to blood transfusion such as:
 - post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions;
 - immunological deficiency such as: IgA deficiency, common-variable immunodeficiency.

The present invention applied to the diagnosis and/or prognosis and / or screening of effective therapies in immune-mediated disease in a patient therefore comprises the following steps:

- a step of taking a sample of blood from which to obtain the patient's serum;

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- a step of examining the serum sample to determine the concentration, or assay, of cytokine BLYS, using the ELISA technique;

- a step of comparing the concentration of cytokine BLYS determined in the previous step and one or more reference values of concentration of cytokine BLYS, which values may be those determined on a healthy population (healthy blood donors: HBDs) or a population of patients with a certain diagnosis of immune-mediated disease and / or the presence of a particular clinical manifestation (e.g. B or T cell clonality) or on a sample of serum from the same patient analyzed before the initiation of therapy;

- a step of identifying a significant deviation, deriving from the previous step, between the determined concentration of cytokine BLYS and the reference values of concentration of cytokine BLYS indicated in the previous step;

- a step, of the decisional - deductive type, so as to assign a diagnosis and / or prognosis regarding a particular clinical manifestation, (e.g. B or T cell clonality), or to a level of therapeutic effectiveness in the course of immune-mediated diseases mentioned above, according to the previous steps.

In the particular case of the diagnostic method, the comparison step is carried out between the concentration of cytokine BLYS determined in the patient and one or more reference values of concentration of cytokine BLYS determined on a healthy population. According to this, from the step of identifying a significant deviation we select the patient as affected by one of said above-mentioned diseases. Moreover, between the step of identifying a significant deviation and the last step of the decisional – deductive type, we have a further comparison step, between the concentration of cytokine BLYS determined in the patient and the values of concentration of cytokine BLYS of a population of patients with a certain diagnosis of immune-mediated disease and/or the presence of a particular clinical manifestation, so as to assign, in the last step, a diagnosis of a determined immune-mediated disease from among all the above-mentioned immune-mediated diseases.

Advantageously, the assay can be selected from among the methods able to identify and quantify BLYS on different biological matrixes (blood, urine, cerebrospinal fluid, cavitary effusions, histological sections, cell cultures).

Moreover, the adoption of this assay does not entail substantive modifications

to the plants or organizational structures of the wards involved in using this new marker, since the assay is effected with the ELISA technique using an automated apparatus commonly present in the major hospitals.

The present invention therefore provides, in a innovative manner, the use of
5 BLYS assay also in the following situations:

ii) diagnostic and / or prognostic marker in conditions predisposing the occurrence of organ-specific autoimmune diseases and blood transfusion reactions, such as immunological deficiency (IgA deficiency), presence of other systemic or organ-specific autoimmune diseases, HLA genotypes, chronic viral
10 infections;

iii) monitoring organ-specific autoimmune diseases specified in section i) (compliance to diet in celiac patients, marker of activation of the immune system, effectiveness of therapy in general, reactivation of the disease, residual function of the affected tissue, atrophic evolution, etc.);

15 in this case, based on the present invention, the method of monitoring comprises the same steps as the above described diagnostic method, applied to a patient with an autoimmune disease during his clinical follow-up with or without treatment, in which, in the third phase, the comparison may also be made with one or more values of cytokine BLYS concentration previously detected in the
20 patient and where on the basis of the fifth step of diagnosis, in a subsequent sixth step it may be decided to repeat, at predetermined time intervals, the preceding five steps, in order to assess over time the evolution of the immune-mediated disease in the patient.

v) additional "decision-maker" in the diagnostic process before more invasive
25 approaches (e.g. in celiac patients or in autoimmune thyroiditis).

It is clear that modifications and/or additions of parts and/or steps may be made to the use of the serological assay of the cytokine B-Lymphocyte stimulator in a diagnostic and prognostic method in the course of the immune-mediated diseases as described heretofore, without departing from the field and scope of
30 the present invention. It is also clear that, although the present invention has been described with reference to specific examples, a skilled in the art shall be able to achieve other equivalent forms of the use of the serological assay of the cytokine B-Lymphocyte stimulator in diagnostic and prognostic method, having the

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characteristics as set forth in the claims and hence all coming within the field of protection defined thereby.

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CLAIMS

1. Use of the assay of cytokine B-lymphocyte Stimulator (BLyS) as a diagnostic marker for confirmation of immune-mediated diseases, included in a group comprising:
 - 5 - organ-specific autoimmune diseases such as:
celiac disease, autoimmune thyroiditis, and
 - immune-mediated diseases related to blood transfusion such as:
post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions,
 - 10 in patients who have symptoms or signs suggestive thereof and / or doubtful situations and / or situations predisposing thereto;
in a method that includes the following steps:
 - a first step of taking a sample of blood from the patient;
 - a second step of examining the blood sample to determine the concentration of
 - 15 cytokine BLyS;
 - a third step of comparing the concentration of cytokine BLyS determined in the second step and one or more reference values of concentration of cytokine BlyS previously obtained on a healthy control group;
 - a fourth step of identifying a significant deviation, deriving from the
 - 20 comparison in the third step, between the concentration of cytokine BlyS determined in the second step and the reference values of concentration of cytokine BlyS previously obtained on the healthy control group, so as to select the patient as affected by one of said diseases;
 - a fifth step of comparing the concentration of cytokine BLyS determined in the
 - 25 second step for the patient selected as affected by one of said diseases and one or more reference values of concentration of cytokine BlyS; these values are in a range of BLyS reference values previously obtained on a series of patients with said immune-mediated disease and / or established diagnosis of active disease or its particular course;
 - 30 - a sixth step of attributing a diagnosis of one or more determinate diseases among the immune-mediated diseases as above, based on the comparison made in the fifth step.
2. Use as in claim 1, characterized in that the second step of the method is carried

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out using an automated apparatus based on the ELISA technique.

3. Use of the assay of cytokine BLYS as a marker in a method for monitoring over time a patient in the appearance of a clonal B or T cell in the course of immune-mediated diseases included in the following group:

- 5 - organ-specific autoimmune diseases such as:
 celiac disease, autoimmune thyroiditis, and
 - immune-mediated diseases related to blood transfusion such as:
 post-transfusion immunization, maternal-fetal incompatibility, transfusion
 reactions, and
- 10 - immunological deficiency such IgA deficiency, common variable
 immunodeficiency;
 which includes the following steps:
 - a first step of taking a sample of blood from the patient with an immune-
 mediated disease as above
- 15 - a second step of examining the blood sample to determine the concentration of
 cytokine BLYS;
 - a third step of comparing the concentration of cytokine BLYS determined in the
 second step and one or more reference values of concentration of cytokine BLYS
 and / or one or more values of cytokine BLYS previously detected in the patient;
- 20 - a fourth step of identifying a significant deviation, deriving from the
 comparison in the third step, between the concentration of cytokine BLYS
 determined in the second step and the reference values of concentration of
 cytokine BLYS indicated in the third step;
 - a fifth step of attributing a risk of evolving into a B / T cell clonality in the
- 25 course of immune-mediated diseases as above to the deviation identified in the
 fourth step;
 - a sixth step of deciding to repeat over time, at predetermined time intervals, the
 steps from the first to the fifth, on the base of the outcome of the fifth step.
4. Use of the cytokine B-Lymphocyte Stimulator (BLYS) as a prognostic marker
- 30 in a method for prognosis of immune-mediated diseases included in a group
 including:
 - organ-specific autoimmune diseases such as:
 celiac disease, autoimmune thyroiditis,

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- immune-mediated diseases attributable to transfusion such as:
post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions,
which includes the following steps:
- 5 - a preliminary step of selecting patients characterized by one or more of the following situations:
 - i) presence of chronic microbial infection (hepatitis virus, herpes virus, chlamydia, etc),
 - ii) presence of a particular HLA genotypes / or genes encoding important
 - 10 factors in the pathogenic disease process or target of the commonly used therapies;
 - iii) subclinical hypothyroidism with or without goiter;
 - iv) depression and / or hypothyroidism post-partum
 - v) presence of one or more active immune-mediated diseases
 - 15 vi) need for blood transfusion
 - vii) beginning of a new therapy;
- a first step of taking a sample of blood from a selected patient;
- a second step of examining the blood sample to determine the concentration of cytokine BLYS;
- 20 - a third step of comparing the concentration of cytokine BLYS determined in the second step and one or more reference values of the concentration of cytokine BLYS;
- a fourth step of identifying a significant deviation, deriving from the comparison in the third step, between the concentration of cytokine BLYS
- 25 determined in the second step and the reference values considered in the third step;
- a fifth step of assigning a prognosis to the deviation identified in the fourth step with regard to said immune-mediated disease.
- 5. Use of the assay of cytokine BLYS as a marker in a method for monitoring the
- 30 treatment of patients with immune-mediated diseases included in a group comprising:
 - organ-specific autoimmune diseases such as:
celiac disease, autoimmune thyroiditis,

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- immune-mediated diseases attributable to transfusion such as:

post-transfusion immunization, maternal-fetal incompatibility, transfusion reactions

which includes a therapeutic step of treating the above mentioned disease and the following steps:

- a first step of taking a sample of blood from the patient before therapy;

- a second step of examining the blood sample to determine the concentration of cytokine BLyS;

10 - a third step of taking one or more samples of blood from the patient at predefined time intervals from the initiation of the therapy;

- a fourth step of examining said one or more blood samples of the third step to determine the concentration of cytokine BLyS;

15 - a fifth step of comparing the concentration of cytokine BLyS determined in the second step and the values of the concentration of cytokine BLyS detected in the patient in the fourth step;

- a sixth step of identifying a significant deviation, deriving from the comparison of the fifth step, between the concentration of cytokine BLyS determined in the second step and the values of BLyS detected in the fourth step;

20 - a seventh step of attributing effectiveness to the therapeutic treatment on the basis of the deviation identified in the sixth step.

6. Use as in any claim hereinbefore, characterized in that the assay of cytokine BLyS can be integrated by a step of one examination or several examinations chosen from a group comprising:

25 - physical examination of the patient able to identify the main signs and symptoms of the disease;

- one or more X-rays of the patient;

- histological examination of a bioptic specimen taken from the patient;

- molecular analyses on the bioptic specimen or peripheral blood or bone marrow taken from the patient.

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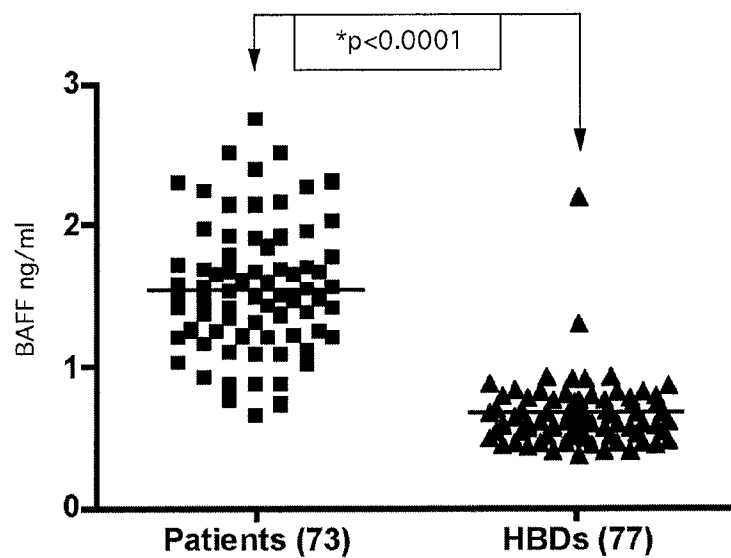


Fig. 1

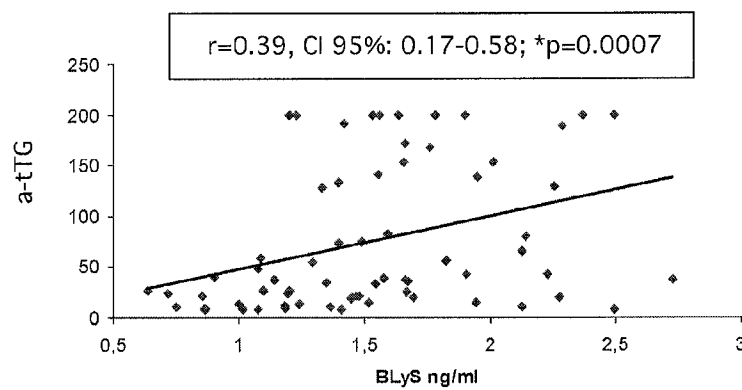


Fig. 2

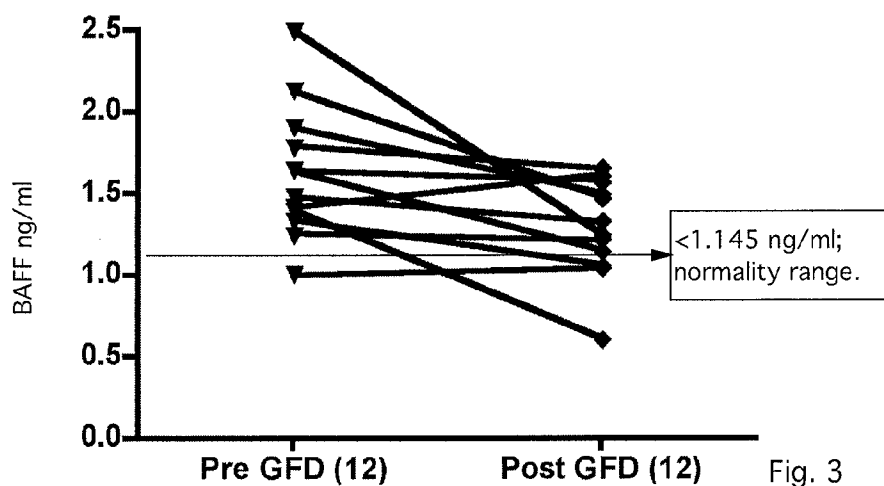


Fig. 3

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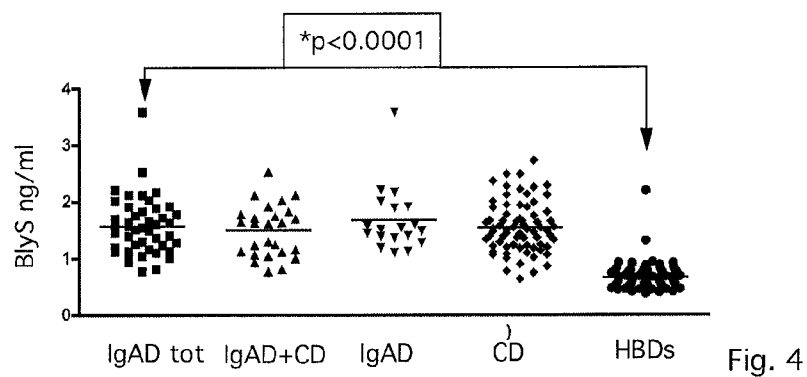


Fig. 4

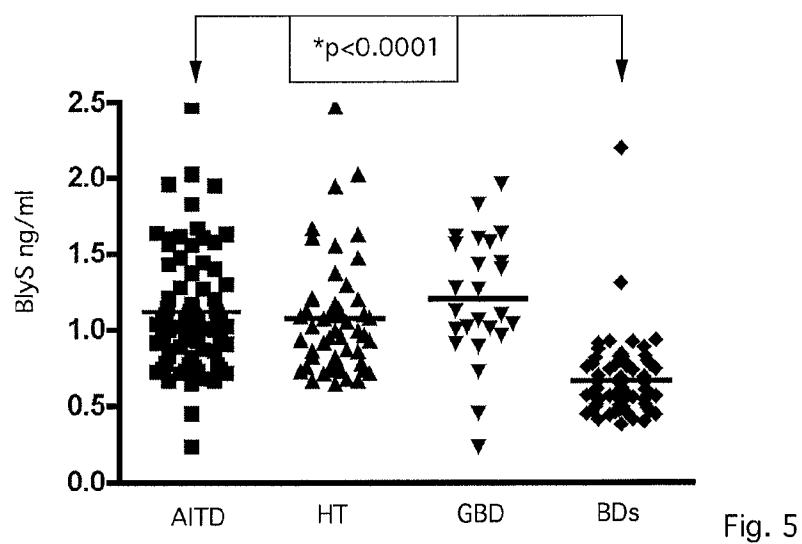


Fig. 5

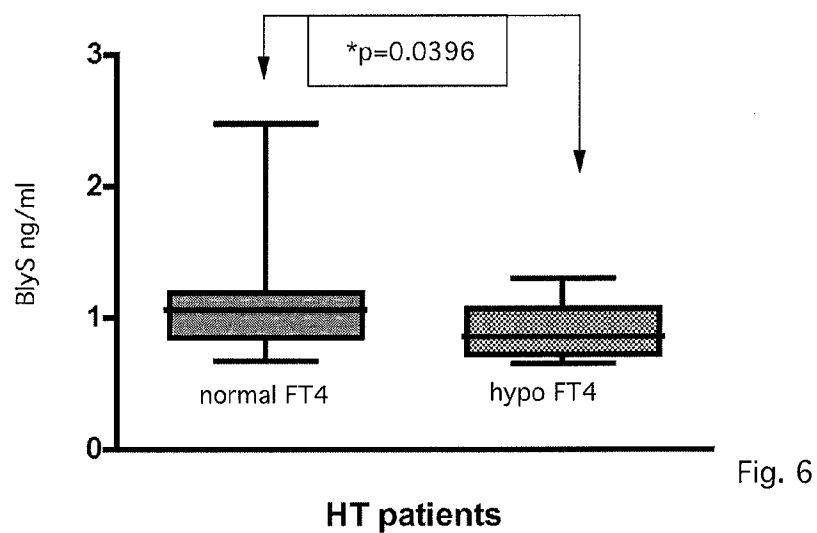


Fig. 6