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(54) **Title**: BIOMARKERS FOR CHRONIC FATIGUE SYNDROME

(57) **Abstract**: The present invention relates to biomarkers for diseases. In particular, the present invention relates to methods involving uses of a phenotype of a particular natural killer cell (NK) subset, that being CD56 ^{bright} expressing NK cells, and NK functionality as biomarkers for determining the incidence of and predisposition to chronic fatigue syndrome. The present invention also relates to kits for performing the methods of the invention.

Biomarkers for chronic fatigue syndrome

Technical Field

The present invention relates to biomarkers for diseases. In particular, the present invention relates to methods involving uses of natural killer cell phenotype and functionality as biomarkers for determining the incidence of and predisposition to chronic fatigue syndrome. The present invention also relates to kits for performing the methods of the invention.

Background of the Invention

Chronic fatigue syndrome (CFS), also known as myalgic encephalomyelitis (ME), is a heterogeneous multifactorial illness that severely obstructs normal function. It is characterised by flu-like symptoms, impairment in concentration and severe unexplained multi-joint pain. The aetiological factor(s) associated with CFS remain unknown. In some cases, the underlying causal factor may be due to the presence of a viral infection, while in other cases, the cause of fatigue is unclear. CFS patients are more likely than healthy controls to demonstrate abnormalities in leukocytes and erythrocytes. The leukocyte populations most studied in association with CFS are T lymphocytes, B lymphocytes and natural killer (NK) cells. The presence of unexplained fatigue in CFS may also be an indication of morphological abnormalities in erythrocytes.

CFS has been clinically defined as a new onset of unexplained fatigue persisting over a period of 6 months or more during which patients experience at least four of the following symptoms: (1) impairment in short term memory or concentration; (2) sore throat; (3) tender cervical or axillary lymph nodes; (4) multijoint pain without the presence of swelling; (5) severe headaches; (6) unrefreshing sleep; and (7) postexertional malaise enduring for more than 24 hours. These criteria therefore rule out psychiatric disorders such as melancholic depression, substance abuse, bipolar disorder, psychosis and eating disorders.

The worldwide prevalence rate of CFS is 0.4 - 1%, with the ratio of females to males being 6:1. The rate of recuperation has been estimated to be between 0 – 6%. The rate of unemployment among CFS patients in the United States is about 37%. CFS has no clearly defined pharmacological cure. However, drugs prescribed include analgesics, non-steroidal anti-inflammatory drugs and antidepressants.

Previous studies into CFS have disclosed significant increases in stomatocytes (erythrocytes with oval shaped cores) and it has thus been asserted that symptoms involved in CFS may be due to atypical erythrocyte shapes (Richards *et al.* 2007). It has also been investigated whether reduced red blood cell (RBC) aggregation is involved in CFS (Kennedy *et al.* 2006).

Other previous studies have identified genes related to CFS using various array protocols. For example, Powell *et al.* (2003) undertook a differential display analysis that suggested protein kinase R (PKR), tumour necrosis factor alpha (TNFα), cathepsin C and a molecule called MAIL were upregulated in CFS patients compared to controls. Further gene expression studies undertaken by Kaushik *et al.* (2005) and Kerr *et al.* (2008) also identified increases in the expression of various genes.

Due to the incidence of viral infections preceding or inducing some cases of CFS, previous studies have also attempted to investigate changes in number, function and subsets of NK cells in CFS patients. However, none of these studies measured NK cell number and function in conjunction with specific NK cell phenotypes, for example, such as measuring $CD56^{dim}$ versus $CD56^{bright}$ as distinct subsets of $CD56^+$ expression. For example, Klimas *et al.* (1990) reported normal levels of NK cells in CFS but with a 50% increase in NK cell $CD56^+$ expression and a 65% reduction in NK cell cytotoxicity compared to controls. Ojo-Amaise *et al* (1994) then evaluated reducible levels of antibody-dependent cellular cytotoxicity (ADCC) activity in NK cells in CFS patients. In this study, patients were classified according to the severity of their illness with A (8.0 ± 5.3 Lytic Units (LU)) representing most severe, B (18.3 ± 7.3 LU) intermediate and C (61.0 ± 21.7 LU) the least severe group. All groups recorded levels of NK activity less than 100 LU. However, no studies were undertaken into different NK phenotypes, nor were NK cell numbers examined for each of the various groups. Subsequently, using the same Lytic Unit classification system, See *et al.* (1997) undertook a comparison of NK cell biology among patients with CFS and AIDS versus healthy controls. This comparison disclosed decreased NK cell function in CFS (41.4±18.1 LU) and AIDS (14.7±7.2 LU) patients in contrast to controls (112.7±19.9 LU). Again, however, no studies were undertaken into different NK cell phenotypes such as $CD56^{dim}$ versus $CD56^{bright}$ NK phenotypes as distinct subsets of $CD56^+$ expression, nor were NK cell numbers examined for each of the various patient groups. Analysis by Barker *et al.* (1994) of peripheral blood mononuclear (PBM) cells in 23 CFS patients revealed comparable numbers of NK cells but with severely reduced lytic activity (38.8±26.65 LU in CFS patients versus 119.75±115.408 LU in healthy controls). In contrast, a duplicated study observed that the total number and function of NK cells was comparable in both CFS patients and a healthy control group (Mawle *et al.* 1997). Nevertheless, Maher *et al.* (2005) showed diminished NK cell numbers and function in CFS patients relative to healthy controls. Based on the present invention, the conflicting results in these studies may be related to the lack of consideration for distinct phenotype subsets of NK cells such as populations of $CD56^{bright}$ expressing NK cells and $CD56^{dim}$ expressing NK cells, respectively.

Some other previous studies have suggested changes in NK cell subtypes in CFS patients. Tirelli *et al.* (1994) immunophenotyped peripheral lymphocytes using flow cytometry and demonstrated both an increase in absolute numbers of $CD56^{+}/CD16^{-}$ NK cells and a reduction in $CD56^{-}/CD16^{+}$ NK cells in CFS patients compared to controls. In addition, Robertson *et al.* (2005) suggested that there was no significant change in the overall number of NK cells and specifically in relation to $CD16^{-}/CD56^{bright}$ NK cells in patients diagnosed with CFS, depression or multiple sclerosis compared with healthy controls. However, this study did show that the number of $CD16^{+}/CD3^{-}$ NK cells increased in CFS patients in comparison to MS patients, depression patients and healthy controls. The Robertson study showed no alteration in NK phenotypes in CFS patients and provided no indication as to NK activity.

A further study by Masuda *et al.* (1994) evaluated NK cell function among CFS patients, fatigued-non-CFS patients and healthy controls. Decreases in NK cytotoxic activity were observed in the CFS and fatigue-non-CFS groups in comparison to the control group. Additionally, compared to the controls, CFS and fatigued-non-CFS patients had reduced levels of $CD16^{+}$, $CD56^{+}$ and $CD57^{+}$ cells.

It is well known that CD56 and CD16 are expressed as surface antigens on many leukocytes including NK cells. Neutrophils, macrophages, dendritic cells and B cells are some of the cells that express both CD56 and CD16. The Masuda study did not specifically measure CD56 and CD16 expression on NK cells since the methodology in the study did not include an enrichment step for NK cells. Accordingly, measurements of CD56 and CD16 were taken using flow cytometric analysis with gating on total lymphocyte populations. Based on this methodology, the study also did not measure the density of the CD56 molecule on the surface of NK cells, that being $CD56^{dim}/CD56^{bright}$ NK phenotypes.

$CD56^{+}$ NK cells can be divided into two phenotypes, the $CD56^{dim}$ NK phenotype and the $CD56^{bright}$ NK phenotype respectively. Along with CD16, these two phenotypes make up two major populations of human NK cells, one being a population of $CD16^{bright}/CD56^{dim}$ cells, which comprises about 90% of the total NK cell population, and the other population of $CD16^{dim}/CD56^{bright}$ cells, which constitutes about 10% of the total NK cell population. Besides being phenotypically distinct, these populations are also functionally distinct. The $CD16^{bright}/CD56^{dim}$ NK cells have been found to be highly cytotoxic, but lacking cytokine activity. The $CD16^{dim}/CD56^{bright}$ NK cells generate relatively large amounts of cytokines such as tumour necrosis factor (TNF)-α and interferon (IFN)-γ upon activation. However, these cells have relatively low natural cytotoxic activity. $CD16^{dim}/CD56^{bright}$ NK cells have also been found to express chemokine receptors and homing molecules such as CD62L and CCR7. These receptors and molecules assist the cells to hone to

secondary lymphoid organs via high endothelial venules. Therefore, it is apparent that NK cell populations of $CD56^{bright}$, $CD56^{dim}$ and $CD56^{+}$ are all distinct from each other. Thus, it was impossible based on the methodology in Masuda to accurately quantify the number of $CD56^{+}$ NK cells in a sample, let alone $CD56^{dim}/CD56^{bright}$ expression in NK cells. Hence the reported results are unlikely to reflect a true representation of NK cell phenotype for CFS patients.

Despite extensive research on the aetiology and pathophysiology of CFS, there remain no definitive known factors responsible for this disease. Based on the previous studies on NK cells, there is also no consensus on the relevance of NK cell function and activity in CFS patients. As CFS is known to impinge on the proper functioning of neurological, endocrine, immunological and cardiovascular systems, current methods for diagnosis of CFS therefore involve a difficult, time-consuming and expensive clinical process. Accordingly, there is a clear need to provide reliable, definitive diagnostic methods and kits for determining the incidence of and predisposition to chronic fatigue syndrome.

The present invention is based on the surprising and unexpected finding that a combination of a phenotype of a particular NK cell subset, that being $CD56^{bright}$ expressing NK cells, and NK cell activity is closely associated with chronic fatigue syndrome in human subjects.

Summary of the Invention

According to a first aspect of the present invention, there is provided a method for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

According to a second aspect of the present invention, there is provided a method for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer (NK) cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The level of natural killer cell activity may be determined by determining a level of natural killer cell cytotoxicity. The level of natural killer cell cytotoxicity may be determined by determining a level of natural killer cell lytic activity.

NK cells may be enriched by using any one or more isolation techniques known in the art. For example, an isolation technique may be a negative isolation technique. The negative isolation technique may include antibody binding to cells that are not of interest such as human hematopoietic cells that express the following surface antigens: CD3 as expressed on thymocytes and T cells; CD4 as expressed on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 as expressed on B cells; CD36 as expressed on platelets and monocytes; CD66b as expressed on granulocytes which includes neutrophils, eosinophils and basophils; and glycophorin A which is expressed on RBCs.

The number of natural killer cells expressing $CD56^{bright}$ may be determined by an immunoassay. The immunoassay may be selected from the group comprising flow cytometry, an enzyme-linked immunoassay, a radioimmunoassay, or an immunoassay comprising a biosensor.

The step of analyzing may further comprise determining a level or number of one or more additional biomarkers. The one or more additional biomarkers may include a level of cytotoxic T lymphocyte activity or a number of cytotoxic T lymphocytes, wherein a low level of cytotoxic T lymphocyte activity and/or a low number of cytotoxic T lymphocytes in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The level of cytotoxic T lymphocyte activity may be determined by determining a level of cytotoxic T lymphocyte cytotoxicity. The level of cytotoxic T lymphocyte cytotoxicity may be determined by determining a level of cytotoxic T lymphocyte lytic activity.

The number of cytotoxic T lymphocytes may be determined by an immunoassay. The immunoassay may be selected from the group comprising flow cytometry, an enzyme-linked immunoassay, a radioimmunoassay, or an immunoassay comprising a biosensor.

The biological test sample may be selected from the group comprising a bodily fluid or tissue. The bodily fluid or tissue may comprise blood, serum, plasma, lymph, thymus, lymph node,

spleen, bone marrow or tonsil. The biological test sample may comprise at least one isolated lymphocyte. The at least one isolated lymphocyte may comprise a natural killer cell.

The control sample may comprise a biological sample from one or more subjects known not to have chronic fatigue syndrome or a predisposition thereto.

The method may be used in conjunction with assessment of clinical symptoms.

The method may be used for diagnosing chronic fatigue syndrome or a predisposition thereto, for monitoring the progression or regression of chronic fatigue syndrome, or for predicting responses to therapy for chronic fatigue syndrome.

According to a third aspect of the present invention, there is provided a method for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

According to a fourth aspect of the present invention, there is provided a method for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

According to a fifth aspect of the present invention, there is provided a kit when used for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the kit comprises means for analyzing a biological test sample obtained from the subject to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

According to a sixth aspect of the present invention, there is provided a kit when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the kit comprises means for analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

According to a seventh aspect of the present invention, there is provided a kit when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the kit comprises means for analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The kit may include means for enriching NK cells from the biological sample. NK cells may be enriched by using any one or more isolation techniques known in the art. For example, an isolation technique may be a negative isolation technique. The negative isolation technique may include antibody binding to cells that are not of interest such as human hematopoietic cells that express the following surface antigens: CD3 as expressed on thymocytes and T cells; CD4 as

expressed on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 as expressed on B cells; CD36 as expressed on platelets and monocytes; CD66b as expressed on granulocytes which includes neutrophils, eosinophils and basophils; and glycophorin A which is expressed on RBCs.

For the purposes of the above aspects and embodiments, the subject may be a human or any other animal. In particular embodiments the subject is selected from the group consisting of human, non-human primate, equine, bovine, ovine, caprine, leporine, avian, feline or canine.

Definitions

The term "expression" as used herein refers interchangeably to expression of a gene or gene product, including the encoded polypeptide or protein. Expression of a gene product may be determined, for example, by immunoassay using an antibody(ies) that bind with the polypeptide. Alternatively, expression of a gene may be determined by, for example, measurement of mRNA (messenger RNA) levels.

As used herein the term "polypeptide" means a polymer made up of amino acids linked together by peptide bonds. Accordingly, the term "polypeptide" includes within its scope a full length protein and fragments thereof.

As used herein the term "polynucleotide" means a nucleic acid made up of nucleotide residues linked together by a phosphodiester backbone. Accordingly, a polynucleotide includes within its scope DNA, RNA and in particular messenger RNA (mRNA).

As used herein the term "oligonucleotide" means a single-stranded nucleic acid capable of acting as a point of initiation of template-directed nucleic acid synthesis. An oligonucleotide is a single-stranded nucleic acid typically ranging in length from 2 to about 500 bases. The precise length of an oligonucleotide will vary according to the particular application, but typically ranges from 15 to 30 nucleotides. An oligonucleotide need not reflect the exact sequence of the template but must be sufficiently complimentary to hybridize to the template, thereby facilitating preferential amplification of a target sequence. Thus, a reference to an oligonucleotide as being "specific" for a particular gene or gene product, such as mRNA, includes within its scope an oligonucleotide that comprises a complementarity of sequence sufficient to preferentially hybridize to the template, without necessarily reflecting the exact sequence of the target polynucleotide.

As used herein, the term "dim" refers to a level of expression of a particular molecule or polynucleotide, such as CD56, by a particular cell or population of cells within a sample that is lower than the level of expression of that molecule or polynucleotide by another population, such as a larger subset population e.g. a CD56 cell population that comprises $CD56^{dim}$ and $CD56^{bright}$

expressing cells, or a population of cells comprising the whole of the sample being analyzed. For example, the term "$CD56^{dim}$" refers to a level of expression of CD56 by a particular cell or population of cells within the sample that is lower than the level of expression within a population comprising $CD56^{+}$ cells. More particularly, the term "dim" may refer to a distinct population of cells that express a particular molecule at a level that is lower than that expressed by one or more other distinct populations within a sample. Similarly, the terms "high" or "bright" are used interchangeably and may have corresponding meanings.

As used herein, the term "+" when used in relation to levels of expression of a particular molecule or polynucleotide, refers to the presence of a particular molecule or polynucleotide, such as CD56, by a particular cell or population of cells within a sample as opposed to the absence of that molecule or polynucleotide by the population of cells comprising the whole of the sample being analyzed.

As used herein, the term "-" when used in relation to levels of expression of a particular molecule or polynucleotide, refers to the absence of a particular molecule or polynucleotide, such as CD56, by a particular cell or population of cells within a sample as opposed to the presence of that molecule or polynucleotide by the population of cells comprising the whole of the sample being analysed.

As used herein, the term "dim" may refer to a quantity of particular cells in a biological sample such as $CD56^{dim}$ expressing NK cells that is lower than the quantity of $CD56^{dim}$ expressing NK cells in a control sample. Additionally or alternatively, the term "dim" may refer to a quantity of $CD56^{dim}$ expressing NK cells, the proportion of which in relation to the larger subset of CD56 expressing cells within the sample being analyzed or the whole of the analyzed sample, is lower compared to a control sample. The analyzed sample may comprise an enriched population of CD56 expressing NK cells. Typically a control sample may be a sample from a subject with no autoimmune, immunoinflammatory or allergic diseases, or predisposition thereto. The terms "high" or "bright" may have corresponding meanings.

As used herein, the term "low" or "lower" refers to a decrease in either total cell numbers or expression of a particular molecule or polynucleotide on cells within a particular population or in comparison to another population. The decrease in expression may be due to a decline in total cell numbers thus impacting on overall expression of a molecule on the surface of cells within a population. Alternatively, the decrease in expression may be due to a reduction in the density of the molecule on the surface of the cells.

All definitions included herein have an ordinary meaning as known to a person skilled in the art unless specifically stated otherwise.

Throughout this specification and the claims, unless the context requires otherwise, the word "comprise" and its variations, such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as an acknowledgement or any form of suggestion that prior art forms part of the common general knowledge in Australia.

Brief Description of the Drawings

Embodiments of the present invention will now be described, by way of example only, with reference to the accompanying drawings:

Figure 1. Natural killer cell cytotoxic activity: the patient group represents CFS patients while control refers to the healthy participants. Each bar represents percentage lysis of K562 cells by NK cells.

Figure 2. Variation in natural killer cell phenotype population in CFS and healthy subjects: this figure outlines the levels of natural killer cell phenotypes in terms of $CD56^{bright}$ natural killer cells and $CD56^{dim}$ natural killer cells. Light bars represent results from CFS patients while dark bars represent the results from healthy participants. Phenotype results are expressed as total numbers of cells expressing either $CD56^{bright}$ or $CD56^{dim}$.

Best Mode of Performing the Invention

The present invention is based on the surprising and unexpected finding that a combination of a particular phenotype of $CD56^+$ NK cells, that being $CD56^{bright}$ and NK cell activity is associated with chronic fatigue syndrome in human subjects.

In particular, the present studies demonstrate that the number of natural killer cells expressing $CD56^{bright}$ is lower in subjects with CFS compared with healthy control subjects. That is, the ratio of $CD56^{bright}$ expressing NK cells to $CD56^{dim}$ expressing NK cells is lower in subjects with CFS as compared to the same in healthy control subjects. The enrichment of NK cells and specific gating on $CD56^+$ cells by flow cytometry were necessary to accurately quantify total numbers of $CD56^{bright}$ expressing NK cells and $CD56^{dim}$ expressing NK cells which are two phenotypes that make up the $CD56^+$ NK cell population.

In addition, the present studies demonstrate that natural killer cell activity, namely, cytotoxicity, is lower in subjects with CFS compared with healthy control subjects. This was

determined by assessing the ability of NK cells to lyse and induce apoptosis in a leukemic cell line known as K562.

This combination of natural killer cell activity and natural killer cell phenotype provides the first definitive biomarker for diagnosing chronic fatigue syndrome. Prior to the present studies, CFS was a disease with no known biomarkers.

Natural killer (NK) cells are a subset of lymphocytes involved in innate immunity. The primary function of NK cells is to eliminate foreign pathogens, tumours and preferentially to provide an adequate supply of cytokines to the immune system. The different types of cytokines produced by NK cells include interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and granulocyte macrophage colony-stimulating factor (GM-CSF). NK cells also initiate antibody dependent cellular cytotoxicity (ADCC) through the FcγRIII receptor or CD16. The occurrence of ADCC in the immune system is essential in the lysis of viral pathogens. There are two main types of NK cells, being $CD56^{dim}CD16^{+}$ (representing approximately 90% of NK cells) and $CD56^{bright}CD16^{-}$ (representing approximately 10% of NK cells). $CD56^{dim}CD16^{+}$NK cells induce cytotoxicity and promote ADCC while $CD56^{bright}CD16^{-}$ NK cells facilitate the production of cytokines. Consequently, there are high levels of lytic granules such as perforin in $CD56^{dim}CD16^{+}$ NK cells compared to $CD56^{bright}CD16^{-}$ NK cells.

Methods for diagnosing chronic fatigue syndrome and methods for determining natural killer cell activity and natural killer cell surface expression

The present invention provides methods for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the methods comprise obtaining a biological test sample from the subject and analyzing the biological test sample to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$, wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The present invention also provides methods for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the methods comprise obtaining a biological test sample from the subject and analyzing the biological test sample to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$, wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing

$CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The present invention also provides methods for diagnosing chronic fatigue syndrome, or a predisposition thereto in a subject wherein the methods comprise obtaining a biological test sample from the subject and analyzing the biological test sample to determine a level of natural killer (NK) cell activity and a number of natural killer cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ when compared to a control sample is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The present invention also provides methods for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the methods comprise obtaining a biological test sample from the subject and analyzing the biological test sample to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ when compared to a control sample is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The technique for enriching NK cells is any technique or combination of techniques that achieves the enrichment of NK cells to carry out the invention. A person skilled in the art would understand that any technique that is useful in enriching NK cells is contemplated herein and forms part of the description of the invention.

Without limiting the scope of the invention or narrowing the description thereof, NK cells may be enriched by using any one or more isolation techniques known in the art. NK cells may be enriched by negative isolation or positive isolation. Negative isolation may include antibody binding to cells that are not of interest such as human hematopoietic cells that express the following surface antigens: CD3 as expressed on thymocytes and T cells; CD4 as expressed on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 as expressed on B cells; CD36 as expressed on platelets and monocytes; CD66b as expressed on granulocytes which includes neutrophils, eosinophils and basophils; and glycophorin A which is expressed on RBCs. The negative isolation technique may consist of or comprise the methodology as per the technical specification of the Rosettesep® NK enrichment cocktail by StemCell Technologies.

Non-limiting examples of other isolation techniques which may be negative or positive isolation or a combination thereof would include magnetic-based techniques and/or affinity chromatography. Affinity chromatography may be in the form of column chromatography. In a non-limiting example, affinity chromatography may consist of or comprise immunoaffinity, immobilized metal ion affinity chromatography, recombinant protein technology and lectin-based techniques. One non-limiting example of a magnetic-based technique is a magnetic bead technique. The beads may be coated with one or more entities that allow for specific binding to the NK cells. The surface of the beads may be coated with antibodies that are directed to CD56 and CD16. Alternatively, the surface of the beads may be coated with antibodies that have a specific affinity to cell surface antigens on human hematopoietic cells that are not present on NK cells, for example, CD3 on thymocytes and T cells; CD4 on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 on B cells; CD36 on platelets and monocytes; and CD66b on granulocytes which includes neutrophils, eosinophils and basophils. The beads may also comprise glycophorin A which is directed to red blood cells.

As contemplated herein, tag(s) may be used in any one or more of the above described techniques and/or any technique(s) that achieves NK enrichment. As a non-limiting example, a tag may be a peptide-based tag, immuno-based tag, and/or fluorescent tag.

The level of natural killer cell activity may be determined by determining a level of natural killer cell cytotoxicity. The level of natural killer cell cytotoxicity may be determined by determining a level of natural killer cell lytic activity. In some embodiments, the level of natural killer cell activity may be determined by assessing the ability of NK cells to lyse and induce apoptosis in a cell line.

In some embodiments, the level of natural killer cell activity may therefore be determined by assessing the level of lysis by NK cells of another population of cells, such as a cell line. As would be apparent to the person skilled in the art, the level of NK cell-induced lysis of such cells is most accurately indicative of chronic fatigue syndrome when it is compared to the level of NK cell-induced lysis of cells in a control sample. Hence, the NK-induced lysis of cells in a biological test sample as a percentage of the NK-induced lysis of cells in a control sample is a preferred calculation when practicing the methods of the present invention. The percentage of NK-induced lysis of cells in a biological test sample may be indicative of chronic fatigue syndrome in a subject where the percentage is less than about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51%, 50%, 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%,

22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the NK-induced lysis of cells in a control sample.

In other embodiments, the level of natural killer cell activity may be determined by determining the reducible levels of antibody-dependent cellular cytotoxicity (ADCC) activity in NK cells, expressed as lytic units (LU), as disclosed in for example Ojo-Amaise *et al* (1994), See *et al.* (1997), Barker *et al.* (1994) and Mawle *et al.* (1997), the entire contents of which is incorporated herein by reference. The reducible levels of antibody-dependent cellular cytotoxicity (ADCC) activity in NK cells may be indicative of chronic fatigue syndrome in a subject where the ADCC activity is a range of from 0 to 100 LU, although levels above 100 LU may not necessarily be indicative that the subject does not have chronic fatigue syndrome.

The number of natural killer cells expressing $CD56^{bright}$ and $CD56^{dim}$ may be determined by any useful technique that allows for enumeration of the cells. Any technique that a person skilled in the art would use to quantify both $CD56^{bright}$ expressing NK cells and $CD56^{dim}$ expressing NK cells to carry out the invention is contemplated herein. In a non-limiting example, an immunoassay may be such a technique. The immunoassay typically involves determination of CD56 NK cell surface expression using an anti-CD56 antibody. The immunoassay may also involve determination of CD16 NK cell surface expression using an anti-CD16 antibody. The level of expression of other molecules expressed on the surface of NK cells may also be undertaken.

Antibody binding may be detected by virtue of a detectable label on the primary anti-CD56 antibody or primary anti-CD16 antibody. Alternatively, the antibodies may be detected by virtue of binding with a secondary antibody or reagent that is appropriately labeled to enable detection. A variety of methods is known in the art for detecting binding in an immunoassay and is within the scope of the present invention. For example, determinations of NK cell surface CD56 and/or CD16 expression can be accomplished by any one of a number of techniques known in the art including, for example, enzyme-linked immunosorbent assays (ELISA); sandwich immunoassays, competitive immunoassays, immunoradiometric assays (IRMA), radioimmunoassays (RIA), immunoelectrophoresis assays, *in situ* immunoassays, immunodiffusion assays, immunofluorescence assays, Western blots, ligand-binding assays, biosensors and flow cytometry.

Flow cytometry may include one or more single wavelengths (lasers) that are detected by one or more detectors whereby the one or more detectors may include one or more fluorescent detectors. Flow cytometry may occur subsequent to the enrichment of NK cells. Analysis of the NK cells may include sorting of the cells via various parameters. One parameter may be the expression of CD56 and/or CD16 on the surface of NK cells. Flow cytometry comprises a flow cytometer and may comprise a flow cell - liquid stream (sheath fluid), which carries and aligns the

cells so that they pass single file through the light beam for sensing; an optical system - commonly used are lamps (mercury, xenon); high-power water-cooled lasers (argon, krypton, dye laser); low-power air-cooled lasers (argon (488 nm), red-HeNe (633 nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals; a detector and Analogue-to-Digital Conversion (ADC) system which generates FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer; an amplification system either displayed in linear or logarithmic form and a computer for analysis of the signals.

The process of collecting total numbers of NK cells from samples may be via a computer physically connected to the flow cytometer and software which handles the digital interface with the cytometer. The software is capable of adjusting parameters such as voltage and compensation for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to insure that parameters are set correctly. As contemplated herein, the number of lasers and detectors may vary depending on the need for multiple labeling such as antibody labeling. Digital imaging of individual cells is also contemplated herein.

As contemplated herein, the data generated by a flow cytometer can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity by use of gating for example. Any gating approach that achieves accurate enumeration of $CD56^{bright}$ expressing NK cells and $CD56^{dim}$ expressing NK cells is contemplated herein.

Data accumulated using the flow cytometer can be analyzed using software, e.g., WinMDI(depricated), Flowjo, or CellQuest Pro. Any one or more computational methods can be used as an alternative to or to complement any one or more gating approaches contemplated herein. Examples of such methods include but are not limited to FLOCK in ImmPort, FLAME in GenePattern and flowClust in Bioconductor.

Fluorescence labels are contemplated herein. The labels may be fluorochromes and/or cytochromes. Selection of one or more labels for use will depend on the lasers used to excite the fluorochromes and/or cytochromes and the available detectors. An example of a label may include but not limited to Blue (488 nm); Green (usually labelled FL1): FITC, Alexa Fluor 488, GFP, CFSE, CFDA-SE, DyLight 488; Orange (usually FL2): PE, PI; Red channel (usually FL3): PerCP, PerCP-Cy5.5, PE-Alexa Fluor 700, PE-Cy5 (TRI-COLOR), PE-Cy5.5.; Infra-red (usually FL4; not provided by all FACS machines as standard): PE-Alexa Fluor 750, PE-Cy7; Red diode laser (635 nm); APC; APC-Cy7, APC-eFluor 780; Alexa Fluor 700; Cy5; Draq-5; Violet laser (405 nm); Pacific Orange; Amine Aqua; Pacific Blue; DAPI; Alexa Fluor 405; eFluor 450; eFluor 605 Nanocrystals; eFluor 625 Nanocrystals and eFluor 650 Nanocrystals.

Particular embodiments of the invention therefore provide for the use of one or more antibodies raised against CD56 and CD16, either free or in association with other molecules, for the detection of NK cell surface expression of CD56 and CD16. The antibodies may be polyclonal or monoclonal and may be raised by the use of CD56 or CD16 or an antigenic fragment or portion thereof as an antigen. Persons skilled in the art will readily understand and appreciate that several methods of generation may be used to produce antibodies, either monoclonal or polyclonal, suitable for performance of the invention. Suitable antibodies include, but are not limited to polyclonal, monoclonal, chimeric, humanised, single chain, Fab fragments, Fv fragments and Fab expression libraries.

Suitable antibodies may be prepared from discrete regions or fragments of the CD56 and/or CD16 polypeptide. An antigenic CD56 and/or CD16 polypeptide contains at least about 5, and typically at least about 10, amino acids. Methods for the generation of suitable antibodies will be readily appreciated by those skilled in the art. For example, an anti-CD56 monoclonal antibody, typically containing Fab portions, may be prepared using the hybridoma technology described in Antibodies - A Laboratory Manual, Harlow and Lane, eds., Cold Spring Harbour Laboratory, N.Y. (1988). In essence, in the preparation of monoclonal antibodies directed toward CD56 and/or CD16, a fragment or analogue thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include the hybridoma technique originally developed by Kohler et al., Nature, 256:495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, Immunology Today, 4:72 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy, pp. 77-96, Alan R. Liss, Inc., (1985)]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier *et al.*, "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies and T-cell Hybridomas" (1981); Kennett *et al.*, "Monoclonal Antibodies" (1980).

A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Similarly, there are various procedures known in the art which may be used for the production of polyclonal antibodies to CD56 and/or CD16, or fragments or analogues thereof. For

the production of polyclonal antibody, various host animals can be immunized by injection with the CD56 and/or CD16 polypeptide, or a fragment or analogue thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. Further, the CD56 and/or CD16 polypeptide or fragment or analogue thereof can be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Also, various adjuvants may be used to increase the immunological response, including but not limited to Freund's (complete and incomplete), nitrocellulose, cellulose acetate, mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Screening for the desired antibody can also be accomplished by a variety of techniques known in the art. Assays for immunospecific binding of antibodies may include, but are not limited to, radioimmunoassays, ELISAs (enzyme-linked immunosorbent assay), sandwich immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays, Western and dot blots, precipitation reactions, agglutination assays, complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, biosensors and the like (see, for example, Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York).

The methods may further comprise determining a level or number of one or more additional biomarkers. The one or more additional biomarkers may include a level of cytotoxic T lymphocyte activity or a number of cytotoxic T lymphocytes, wherein a low level of cytotoxic T lymphocyte activity and/or a low number of cytotoxic T lymphocytes in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

Cytotoxic T lymphocytes (CTLs) are CD8+ T cells that monitor all the cells of the body. They have both suppressive and cytotoxic functions. CTLs readily remove cells that have the potential to cause harm to the body's homeostasis and alter the integrity of immune function. They are responsible for killing virally infected cells and thus preventing the upregulation of viral pathogens in the body which can potentially reduce function. Additionally, CTLs potentially protect against spontaneous malignant tumors, as they are able to identify antigenic differences in malformed or mutated cells. CD8 receptor molecules on CTLs are responsible for recognizing a ligand and initiating signal transduction. Circulating CD8+ T cells may also be classified into naive (CD27+CD28+CD45RA+CD8+), effector (CD27-CD28-CD45RA+CD8+), effector/memory (CD27+CD28–CD45RA–CD8+/CD27-CD28–CD45RA–CD8+), and memory

(CD27+CD28+CD45RA-CD8+) cells. Differentiation of naive CD8+ T cells to memory T cells occurs once the cells are exposed to antigens. These cells later develop into effector T cells with cytotoxic effects and high perforin, granzyme and IFN-γ production. CTLs can kill target cells via direct cell-cell contact and via cytokines such as IFN-γ and TNF-α. Cytokines such as TNF-α initiate the caspase cascade, resulting in target-cell apoptosis, while IFN-γ activates MHC class I antigen presentation and Fas ligands in affected cells, thus promoting effective target-cell lysis. In direct cell-cell contact, cytotoxic activity causes target cells to undergo apoptosis either through Fas ligand expression on the surface of the CTLs or the release of perforin and granzyme in to the target cells, both cases cause cytotoxicity.

The level of cytotoxic T lymphocyte activity may therefore be determined by determining a level of cytotoxic T lymphocyte cytotoxicity. The level of cytotoxic T lymphocyte cytotoxicity may be determined by determining a level of cytotoxic T lymphocyte lytic activity. In some embodiments, the level of cytotoxic T lymphocyte activity may be determined by assessing the ability of CTLs to lyse and induce apoptosis in a cell line.

In some embodiments, the level of CTL activity may therefore be determined by assessing the level of lysis by CTLs of another population of cells, such as a cell line. As would be apparent to the person skilled in the art, the level of CTL-induced lysis of such cells is most accurately indicative of chronic fatigue syndrome when it is compared to the level of CTL-induced lysis of cells in a control sample. Hence, the CTL-induced lysis of cells in a biological test sample as a percentage of the CTL-induced lysis of cells in a control sample is a preferred calculation when practicing the methods of the present invention. The percentage of CTL-induced lysis of cells in a biological test sample may be indicative of chronic fatigue syndrome in a subject where the percentage is less than about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51%, 50%, 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the CTL-induced lysis of cells in a control sample.

In other embodiments, the level of CTL activity may be determined by determining the reducible levels of antibody-dependent cellular cytotoxicity (ADCC) activity in CTLs, expressed as lytic units (LU), as disclosed in for example Ojo-Amaise *et al* (1994), See *et al.* (1997), Barker *et al.* (1994) and Mawle *et al.* (1997), the entire contents of which is incorporated herein by reference. The reducible levels of antibody-dependent cellular cytotoxicity (ADCC) activity in CTLs may be

indicative of chronic fatigue syndrome in a subject where the ADCC activity is a range of from 0 to 100 LU, although levels above 100 LU may not necessarily be indicative that the subject does not have chronic fatigue syndrome.

The number of cytotoxic T lymphocytes may be determined by an immunoassay. The immunoassay may be selected from the group comprising flow cytometry, an enzyme-linked immunoassay, a radioimmunoassay, or an immunoassay comprising a biosensor as described elsewhere herein.

The biological test sample may be selected from the group comprising a bodily fluid or tissue. The bodily fluid or tissue may comprise blood, serum, plasma, lymph, thymus, lymph node, spleen, bone marrow or tonsil. The biological test sample may comprise at least one isolated lymphocyte. The at least one isolated lymphocyte may comprise a natural killer cell.

The control sample may comprise a biological sample from one or more subjects known not to have chronic fatigue syndrome or a predisposition thereto. In some embodiments, the control may be analysed at the same time the biological test sample is analysed and/or when the NK cells are enriched from the biological sample. In other embodiments, samples from healthy subjects, known not to have chronic fatigue syndrome or a predisposition thereto, may be tested in order to establish a normal range of natural killer cell activity and a normal number of natural killer cells expressing $CD56^{bright}$ against which biological test samples may be compared.

The methods may also be used in conjunction with assessment of clinical symptoms. Clinical symptoms may be clinically defined as a new onset of unexplained fatigue persisting over a period of 6 months or more during which patients experience at least four of the following symptoms: (1) impairment in short term memory or concentration; (2) sore throat; (3) tender cervical or axillary lymph nodes; (4) multijoint pain without the presence of swelling; (5) severe headaches; (6) unrefreshing sleep; and (7) postexertional malaise enduring for more than 24 hours.

The methods may be used for diagnosing chronic fatigue syndrome or a predisposition thereto, for monitoring the progression or regression of chronic fatigue syndrome, or for predicting responses to therapy for chronic fatigue syndrome.

For the purposes of the above aspects and embodiments, the subject may be a human or any other animal. In particular embodiments the subject is selected from the group consisting of human, non-human primate, equine, bovine, ovine, caprine, leporine, avian, feline or canine.

Kits for diagnosing chronic fatigue syndrome and kits for determining natural killer cell activity and natural killer cell surface expression

The present invention provides kits when used for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the kits comprise means for analyzing a biological test sample obtained from the subject to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$, wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

The present invention also provides kits when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the kit comprises means for analyzing the biological test sample to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$, wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

Yet further, the present invention provides kits when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$ when compared to a control sample wherein the kit comprises means for analyzing the biological test sample to determine a level of natural killer cell activity and a number of natural killer cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ when compared to a control sample is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

In one embodiment, the kits may include means for enriching NK cells from the biological sample. NK cells may be enriched by using any one or more isolation techniques known in the art. For example, an isolation technique may be a negative isolation technique. The negative isolation technique may include antibody binding to cells that are not of interest such as human hematopoietic cells that express the following surface antigens: CD3 as expressed on thymocytes and T cells; CD4 as expressed on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 as expressed on B cells; CD36 as expressed on platelets and monocytes; CD66b as expressed on granulocytes which includes neutrophils, eosinophils and basophils; and

glycophorin A which is expressed on RBCs. One example of a negative isolation technique is the Rosettesep® human NK cell enrichment cocktail (StemCell Technologies).

Typically, kits for carrying out a method of the invention contain all the necessary reagents to carry out the method. For example, in one embodiment the kit may comprise a first container containing a capture antibody raised against CD56 and/or CD16, and a second container containing a detection antibody raised against CD56 and/or CD16, as well as means for determining the level of natural killer cell activity as described herein. The anti-CD56 and/or anti-CD16 capture antibody may be immobilized onto a solid surface, such as the well of a microtitre plate or a bead. The anti-CD56 and/or anti-CD16 detection antibody may be conjugated to a marker such as biotin.

Typically, the kits described above will also comprise one or more other containers, containing for example, wash reagents, and/or other reagents capable of quantitatively detecting the presence of bound antibodies. For example, a signal generator such as a streptavidin peroxidase, may be provided for binding to the detection antibody, and a substrate such as 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or tetramethylbenzidine (TMB) may be provided for binding to the signal generator.

Additionally or alternatively, kits of the invention may comprise a competitive ELISA, wherein CD56 and/or CD16 may be immobilized onto a solid surface. The immobilized CD56 and/or CD16 may then compete with endogenous CD56 and/or CD16 present in the biological test sample for binding with an anti-CD56 and/or anti-CD16 antibody. Additionally or alternatively, the anti-CD56 and/or anti-CD16 antibody may comprise a marker, for example, biotin, suitable for binding with a signal generator such as a streptavidin peroxidase.

Additionally or alternatively, kits of the invention may comprise reagents including for example, antibodies that recognize and bind at least one other NK cell surface expressed molecule.

In the context of the present invention, a compartmentalized kit includes any kit in which reagents are contained in separate containers, and may include small glass containers, plastic containers or strips of plastic or paper. Such containers may allow the efficient transfer of reagents from one compartment to another compartment whilst avoiding cross-contamination of the samples and reagents, and the addition of agents or solutions of each container from one compartment to another in a quantitative fashion. Such kits may also include a container which will accept the test sample, a container which contains the antibody(s) used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, and like), and containers which contain the detection reagent.

Typically, a kit of the present invention will also include instructions for using the kit components to conduct the appropriate methods.

Kits and methods of the invention may be used in conjunction with automated analysis equipment and systems, such as diagnostic systems enabling the analysis of multiple samples and/or multiple biomarkers, for example, the automated bead-based multiplexing BioRad BioPlex 2200 analyser. For example, an automated analyser may be used to determine the expression level of CD56 and/or CD16 in conjunction with determining the level of at least one other NK cell surface expressed molecule.

Methods and kits of the present invention are equally applicable to any animal, including humans, and also including but not limited to non-human primate, equine, bovine, ovine, caprine, leporine, avian, feline and canine species. Accordingly, for application to different species, a single kit of the invention may be applicable, or alternatively different kits, for example containing reagents specific for each individual species, may be required. Methods and kits of the present invention find application in any circumstance in which it is desirable to determine S100A12 levels or to obtain an indication of cardiovascular disease.

The present invention will now be further described in greater detail by reference to the following specific examples, which should not be construed as in any way limiting the scope of the invention.

Examples

Example 1. Methods and materials

1.1. Natural killer cell activity assay

1.1.1 Cell culture

Cell culture was performed using complete culture media containing RPMI-1640 solution without phenol red (Invitrogen), but with fetal bovine serum (FBS), penicillin, streptomycin, HEPES and sodium pyruvate. Typically, 500ml RPMI-1640, 10ml HEPES, 5ml sodium pyruvate, 50ml FBS and 2ml penicillin/streptomycin were combined to form the culture media used for culturing K562 cells.

Prior to culture, K562 cells stored in liquid nitrogen were quickly thawed in a 37°C water bath with constant agitation for approximately 12 minutes. Cells were then combined with 50% complete culture media and 50% FBS, gently shaken and centrifuged at a relative centrifugation force (rcf) of 125 for 5 minutes. After discarding the supernatant, pelleted cells were placed in a sterile T25 flask

containing 10ml of complete culture media. The T25 flask was then placed in a humidified incubator at 37°C and 5% CO_2. Media was changed every 48 hours. Prior to use in experimental assays, an aliquot of cultured cells was used to determine total cell concentration. For natural killer cell cytotoxic assays, cells were routinely resuspended in 500µl of culture medium at a concentration of $1x10^5$ cells / ml.

1.1.2 Lymphocyte separation and staining

Heparinised whole blood (6ml) from all samples was collected and gently mixed with 300µl Rosettesep® NK enrichment cocktail (StemCell Technologies) for 20 minutes. Samples were then layered over 6ml Ficoll-hypaque density gradient solution for separation of white blood cells from red blood cells (RBCs). Centrifugation was applied for 20 minutes at 1200rcf. Cells were washed in RPMI twice at 400 rcf for 10 minutes and then at 400 rcf for 5 minutes. To promote viability, cells were resuspended in 1mL diluent C solution. 1ml of solution containing PKH-26 dye (4ul) was then added to isolated NK cells in 1mL of diluent C in order to stain and distinguish NK cells from K562 cells. Staining was performed for 15 minutes in the dark at room temperature. Staining was stopped by adding 2ml of 1:5 diluted serum supreme for 1 minute at room temperature. Cells were then centrifuged at 400rcf for 5 minutes and washed twice as previously described using RPMI. Isolated NK cells were resuspended and the concentration adjusted to 5×10^6 cells / ml in a volume of 1mL.

1.1.3 Natural killer cell lysis of K562 cells

NK cell lysis was performed at effector to target ratios (E:T) of 25:1 and 0:1, where the effector cells were natural killer cells and the target cells were K562 cells. 50µl aliquots of K562 cells at 1×10^5 cells / ml were placed into 5ml sample tubes, as well as a control tube (the 0:1 tube). 25µl aliquots of NK cells at 5×10^6 cells / ml were then added to each of the 5ml sample tubes except the control tube. Samples were centrifuged at 50g for 5 minutes and then incubated at 37°C in 5% CO_2 for 4 hours. Incubated samples were centrifuged at 400rcf for 5 mins. 5µl of Annexin V-FITC (BD Bioscience) and 7-Amino Actinomycin D (7-AAD) (BD bioscience) were placed in each tube and incubated in the dark for 15 minutes at room temperature. 500µl of 1:10 diluted Annexin V binding buffer was subsequently added prior to flow cytometry.

1.2. Natural killer cell phenotyping

6ml of blood from heparinized tubes was placed into 15ml tubes, with 250µL RosetteSep® Human NK cell Enrichment Cocktail then added to each of the blood samples. The cocktail contains a combination of mouse and rat monoclonal antibodies purified from mouse ascites fluid or

hybridoma culture supernatant, by affinity chromatography using Protein A or Protein G Sepharose. These antibodies have a specific affinity to cell surface antigens on human hematopoietic cells such as CD3 as expressed on thymocytes and T cells; CD4 as expressed on thymocytes, helper and inflammatory T cells, monocytes and macrophages; CD19 as expressed on B cells; CD36 as expressed on platelets and monocytes; CD66b as expressed on granulocytes which includes neutrophils, eosinophils and basophils; and glycophorin A which is expressed on RBCs. The mouse and rat antibodies are bispecific and tetrameric which allows them to bind to RBCs and cells that are not of interest as described above which also allows for subsequent separation from the NK cells through layering and centrifugation. Specifically, samples were incubated for 20 minutes at room temperature with gentle mixing and then gently layered on a Ficoll-hypaque density gradient solution. Samples were centrifuged for 20 minutes at 1200rcf at room temperature. The layer containing the NK cells which is the layer between the plasma top layer and ficoll-hypaque was removed and places in a new 15ml tube. The remaining layers which contain RBCs (including cells that are not of interest as described above), plasma and Ficoll-hypaque were discarded. Two washing steps were applied using PBS initially at 700rcf for 10 minutes and then at 400rcf for 5 minutes, discarding the supernatant each time. Cells were then resuspended in 100-300μl of PBS. Monoclonal antibodies for CD56-FITC and CD16-PE were added to the samples and vortexed briefly. Samples were incubated for a period of 30 minutes on ice in the dark. Fixation of samples was then achieved using 500μl formalin subsequent to analysis by flow cytometry.

Example 2. Results

The results shown in Figures 1 and 2 were obtained after application of the protocols described above. These results provide an indication of the ability of NK cells to effectively lyse K562 cells and therefore eliminate viral infections. In addition, the results indicate populations of distinct NK phenotypes.

2.1 Natural killer cell activity assay

Figure 1 illustrates the efficiency of NK cell function in both healthy controls and CFS patients. Percentage lysis of K562 cells is lower in CFS patients compared to healthy controls. In particular, results from CFS patients show a value of 10.3% percentage lysis while results from healthy controls are approximately 34%. This percentage was deduced after flow cytometry results for the 0:1 ratio control sample was deducted from the 25:1 ratio samples.

2.2 Natural killer cell phenotype analysis

Figure 2 shows total cell numbers for NK phenotypes, that being $CD56^{bright}$ and $CD56^{dim}$ expressing NK cell populations. Cell numbers were determined subsequent to enrichment of NK cells via the RosetteSep® Human NK cell Enrichment Cocktail and specific gating on $CD56^+$ cells via flow cytometry. The accurate determination of total cell numbers of the above-mentioned NK phenotypes could have only been made possible through the combination of NK cell population enrichment and specific gating on $CD56^+$ cells which is described elsewhere herein.

As shown in Figure 2, the total number of $CD56^{bright}$ expressing NK cells is characteristically lower than the total number of $CD56^{dim}$ expressing NK cells. In addition, the total number of $CD56^{bright}$ expressing NK cells in CFS patients (light bars) is significantly lower when compared to the total number of $CD56^{bright}$ expressing NK cells in healthy controls (dark bars). In other words, the ratio of $CD56^{bright}$ expressing NK cells to $CD56^{dim}$ expressing NK cells in CFS patients is lower than the ratio of same in healthy controls. Staining patterns for $CD56^{dim}$ expressing NK cell populations were not significantly different between the two groups.

The examples clearly demonstrate that a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in a subject. Thus the present invention shows that natural killer cell activity and numbers of natural killer cells expressing $CD56^{bright}$ can be assessed in tandem, and together, is an effective biomarker for diagnosing chronic fatigue syndrome.

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Claims

1. A method for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

2. A method for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

3. A method for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to

a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

4. A method for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the method comprises:

(a) obtaining a biological test sample from the subject; and

(b) analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

5. The method according to claim 2 or claim 4 wherein said NK cells are enriched by using an isolation technique.

6. The method according to any one of claims 1 to 5, wherein the biological test sample is selected from the group comprising a bodily fluid or tissue.

7. The method according to claim 6, wherein the bodily fluid or tissue comprises blood, serum, plasma, lymph, thymus, lymph node, spleen, bone marrow or tonsil.

8. The method according to claim 6, wherein biological test sample comprises at least one isolated lymphocyte.

9. The method according to claim 8, wherein at the least one isolated lymphocyte comprises a natural killer cell.

10. The method according to any one of claims 1 to 9, wherein the level of natural killer cell activity is determined by determining a level of natural killer cell cytotoxicity.

11. The method according to claim 10, wherein the level of natural killer cell cytotoxicity is determined by determining a level of natural killer cell lytic activity.

12. The method according to any one of claims 1 to 11, wherein the number of natural killer cells expressing $CD56^{bright}$ is determined by an immunoassay.

13. The method according to claim 12, wherein the immunoassay is selected from the group comprising flow cytometry, an enzyme-linked immunoassay, a radioimmunoassay, or an immunoassay comprising a biosensor.

14. The method according to any one of claims 1 to 13, wherein the control sample comprises a biological sample from one or more subjects known not to have chronic fatigue syndrome or a predisposition thereto.

15. The method according to any one of claims 1 to 14, wherein the method is used in conjunction with assessment of clinical symptoms.

16. The method according to any one of claims 1 to 15, wherein the step of analyzing further comprises determining a level or number of one or more additional biomarkers.

17. The method according to claim 16, wherein the one or more additional biomarkers comprises a level of cytotoxic T lymphocyte activity or a number of cytotoxic T lymphocytes, wherein a low level of cytotoxic T lymphocyte activity and/or a low number of cytotoxic T lymphocytes in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

18. The method according to any one of claims 1 to 17, wherein the method is used for diagnosing chronic fatigue syndrome or a predisposition thereto, for monitoring the progression or regression of chronic fatigue syndrome, or for predicting responses to therapy for chronic fatigue syndrome.

19. A kit when used for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the kit comprises means for analyzing a biological test sample obtained from the subject to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

20. A kit when used for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the kit comprises means for analyzing a biological test sample obtained from the subject to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

21. A kit when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the kit comprises means for analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

22. A kit when used for determining in a subject a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, wherein the kit comprises means for analyzing the biological test sample to determine:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity in the biological test sample and a lower number of natural killer cells expressing $CD56^{bright}$ in the biological test sample, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

23. The kit according to claim 20 or claim 22 wherein said NK cells are enriched by using an isolation technique.

24. The kit according to any one of claims 19 to 23, wherein the biological test sample is selected from the group comprising a bodily fluid or tissue.

25. The kit according to claim 24, wherein the bodily fluid or tissue comprises blood, serum, plasma, lymph, thymus, lymph node, spleen, bone marrow or tonsil.

26. The kit according to claim 24, wherein biological test sample comprises at least one isolated lymphocyte.

27. The kit according to claim 26, wherein the at least one isolated lymphocyte comprises a natural killer cell.

28. The kit according to any one of claims 19 to 27, wherein the level of natural killer cell activity is determined by determining a level of natural killer cell cytotoxicity.

29. The kit according to claim 28, wherein the level of natural killer cell cytotoxicity is determined by determining a level of natural killer cell lytic activity.

30. The kit according to any one of claims 19 to 29, wherein the number of natural killer cells expressing $CD56^{bright}$ is determined by an immunoassay.

31. The kit according to claim 30, wherein the immunoassay is selected from the group comprising flow cytometry, an enzyme-linked immunoassay, a radioimmunoassay, or an immunoassay comprising a biosensor.

32. The kit according to any one of claims 19 to 31, wherein the control sample comprises a biological sample from one or more subjects known not to have chronic fatigue syndrome or a predisposition thereto.

33. The kit according to any one of claims 19 to 32, wherein the kit is used in conjunction with assessment of clinical symptoms.

34. Use of a biomarker for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the biomarker comprises:

(i) a level of natural killer cell activity; and

(ii) a number of natural killer cells expressing $CD56^{bright}$

wherein a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

35. Use of a biomarker for diagnosing chronic fatigue syndrome, or a predisposition thereto, in a subject, wherein the biomarker comprises:

(i) a level of natural killer cell activity; and

(ii) a number of NK cells expressing $CD56^{bright}$ and $CD56^{dim}$ identified within an enriched population of NK cells from the sample

wherein a lower level of natural killer cell activity and a lower number of natural killer cells expressing $CD56^{bright}$, when compared to a control sample, is indicative of chronic fatigue syndrome, or a predisposition thereto, in the subject.

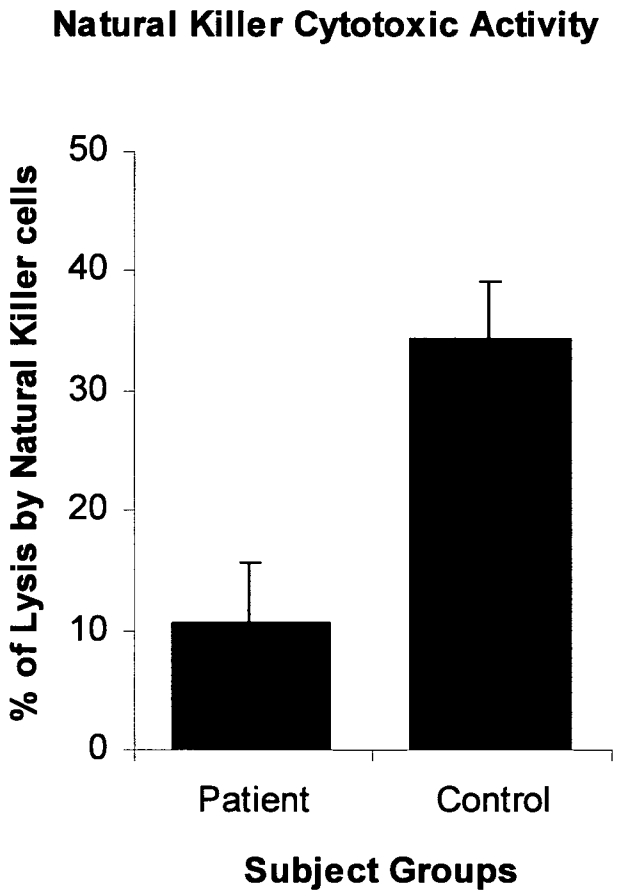
Natural Killer Cytotoxic Activity
% of Lysis by Natural Killer cells
50
40
30
20
10
0
Patient
Control
Subject Groups

Figure 1

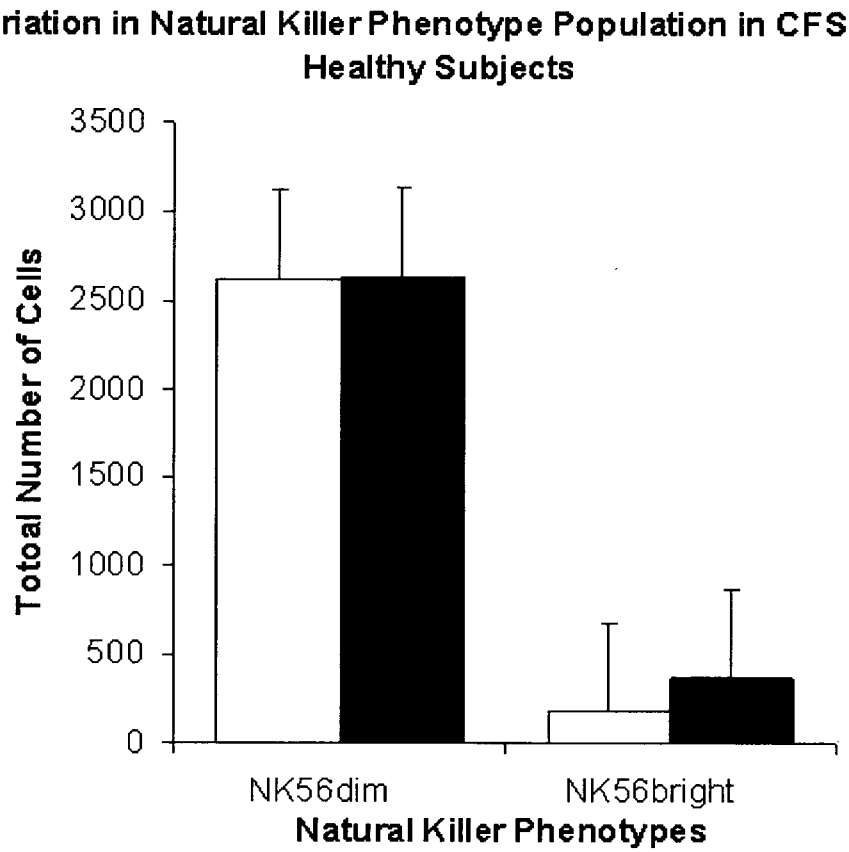
Variation in Natural Killer Phenotype Population in CFS and Healthy Subjects
3500
3000
2500
2000
1500
1000
500
0
Totoal Number of Cells
NK56dim
NK56bright
Natural Killer Phenotypes

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/000239

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/53 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC, WPI, MEDLINE, BIOSIS, HCAPLUS & keywords (Chronic fatigue Syndrome, Myalgic encephalomyelitis, CD56+ and similar terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MASUDA, A. et al., 'Psychobehavioral and Immunological Characteristics of Adult People with Chronic Fatigue and Patients with Chronic Fatigue Syndrome', Psychosomatic Medicine, 1994, Vol.56, pages 512-518. See Abstract, page 512	1-35
A	COOPER, M.A. et al., 'The biology of human natural killer-cell subsets', Trends in Immunology. 2001. Vol. 22, pages 633-640. Published online 30 October 2001 See Pages 638-639	1-35
P,X	BRENU, E.W. et al., 'Immune and hemorheological changes in Chronic Fatigue Syndrome', Journal of Translational Medicine', 2010, vol. 8, pages 1-10 Published online 11 January 2010 Abstract, Page 2, Right column, 4th paragraph, Page 4, Page 7, Right paragraph, lines 1-6 and Figure 3	1-16, 18-35

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2010	25 MAR 2010

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