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(54) Title: STATUS OF TUBERCULOSIS INFECTION IN AN INDIVIDUAL

(57) Abrégé/Abstract:

In a first aspect, the present invention relates to a method for diagnosing or determining the status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection. In a further aspect, a method for the stratification of the therapeutic regimen of an individual with tuberculosis infection is provided as well as a method for predicting a clinical outcome or determining treatment course in an individual afflicted with tuberculosis infection. Moreover, the present invention provides a method for monitoring the change from latent into active status of tuberculosis infection or vice versa in an individual. Furthermore, the present invention relates to a kit for use in diagnosing or detecting the status of tuberculosis infection as well as to *Mycobacterium tuberculosis* alanine dehydrogenase for use in specifically differentiating latent status from active diseases status of tuberculosis in an individual.

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(54) Title: STATUS OF TUBERCULOSIS INFECTION IN AN INDIVIDUAL

(57) Abstract: In a first aspect, the present invention relates to a method for diagnosing or determining the status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection. In a further aspect, a method for the stratification of the therapeutic regimen of an individual with tuberculosis infection is provided as well as a method for predicting a clinical outcome or determining treatment course in an individual afflicted with tuberculosis infection. Moreover, the present invention provides a method for monitoring the change from latent into active status of tuberculosis infection or vice versa in an individual. Furthermore, the present invention relates to a kit for use in diagnosing or detecting the status of tuberculosis infection as well as to *Mycobacterium tuberculosis* alanine dehydrogenase for use in specifically differentiating latent status from active disease status of tuberculosis in an individual.

Status of tuberculosis infection in an individual

Disclosed is a method for diagnosing or determining the status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection. In a further aspect, a method for the stratification of the therapeutic regimen of an individual with tuberculosis infection is provided as well as a method for predicting a clinical outcome or determining treatment course in an individual afflicted with tuberculosis infection.

Moreover, the present invention provides a method for monitoring the change from latent into active status of tuberculosis infection or vice versa in an individual. Furthermore, the present invention relates to a kit for use in diagnosing or detecting the status of tuberculosis infection as well as to *Mycobacterium tuberculosis* alanine dehydrogenase for use in specifically differentiating latent status from active diseases status of tuberculosis in an individual.

Prior art

Over the past decade substantial advances have been achieved in the in vitro immunological diagnosis of *Mycobacterium tuberculosis* (TB) infection (Machingaidze S. et al. , Pediatr Infect Dis J. 2011; 30:694-700). Three *Mycobacterium tuberculosis* specific interferon- γ release assays (IGRAs) are commercially available. They detect interferon- γ (INF- γ) release by mononuclear cells in the ELISpot (T-SPOT.TB - Oxford Immunotec, Abingdon, UK) or in whole blood in the ELISA (QuantiFERON-TB GOLD and QuantiFERON-TB GOLD in tube; QFT-G-IT; Cellestis, Carnegie, Australia) following *ex vivo* contact with specific *Mycobacterium tuberculosis* antigens. With respect of tuberculin skin test (TST), IGRAs have a number of

advantages: they are minimally influenced by previous Bacille Calmette-Guérin (BCG) vaccination or infection by non-tuberculosis mycobacteria, do not cause booster effect, do not necessitate a double access to health care facility , and interpretation of results is not operator-dependent (Chiappini E. et al., Clin 5 Ther. 2012 Apr 16). In adults IGRAs have been reported to be more specific and at least as sensitive as TST and are currently included in diagnostic algorithms in adult guidelines (Mazurek GH. et al., United States, 2010, MMWR Recomm Rep. 2010; 59:1-25.). However, reported IGRAs sensitivity and specificity largely vary among studies in paediatric populations and caution is 10 recommended regarding their use in children because of poor and contrasting data (Machingaidze S. et. al., Pediatr Infect Dis J. 2011;30:694-700; Sun L. et. al., FEMS Immunol. Med. Microbiol. 2011;63:165-73; Mandalakas AM, et. al., Int J Tuberc Lung Dis 2011;15:1018-32). Moreover, they do not allow to distinguish active from latent tuberculosis infection (LTBI) (Amanatidou V. et. 15 al., Eur J Clin Microbiol Infect Dis. 2012 Jan 4). This issue is fundamental for paediatricians since a definite TB diagnosis, confirmed by cultures, is rare in children. Paediatric TB disease is typically paucibacillary and most TB diagnoses in children are "probable "diseases, based on TST and IGRA results, clinical symptoms and signs, radiological findings, personal history, response to 20 TB therapy, and physicians' experience in this field.

A positive IGRA in a child with signs suggestive of active TB does not allow the definitive diagnosis especially if the child comes from a high TB prevalence area. The positive IGRA result may be due to TB disease as well as LTBI, which may be incidental to the disease causing the symptoms or signs 25 under investigation. In this situation, a test that accurately distinguishes active tuberculosis from latent TB infection would be valuable. At present, no success has been achieved in studies using ELISpot assays based on cytokines other than IFN- γ (i.e.:IL-10 or IL-2) (Amanatidou V. et. al., Eur J. Clin Microbiol Infect Dis. 2012 Jan 4) or exploring immune response to other mycobacterial antigens 30 besides ESAT-6, CFP-10 and TB7.7 which are currently included in the commercially available IGRAs.

Alternative methods for determining tuberculosis state include the determination of antibody profiles characteristic of the tuberculosis state. For

example in US 2007/0026473 A1 as well as in Davidow A., et al., 2005, Infection and Immunity, 73(10), 6846-6851, such antibody profiles are described. Therein, antigens (ESAT-6, 38kDa Ag, 16 kDa Ag, Rv 2626c, ferridoxine and alanine dehydrogenase were used to determine antibody profiles. It is described that antibodies to 38 kDa Ag, alanine dehydrogenase and Rv2626c are associated with active Tb, while antibodies to the 16 kDa AG, ferridoxine A, and ESAT-6 are associated with inactive TB. In contrast, the group identified later in Gennaro M.L., et al., 2007, In J. Tuberc. Lung Dis., 11(6), 624-631 that the antibody responses to antigens ESAT-6, 38 kDa Ag, 16 kDa Ag, malate synthase and MTS-10/CFP-10 are increased in the TB groups compared to the control group while other antigens, Rv 2626c, ferrodoxin A, alanine dehydrogenase, Ag85 and GluS show no difference on the antibody level. Hence, the data are controversial with respect to some antigens and the later published Gennaro et al., document identifies that inter alia alanine dehydrogenase but also Rv 2626c are not suitable for determining TB status.

However, antibody based assays have drawbacks regarding the sensitivity and specificity. Till today, no serologic test has been developed which could diagnose active TB with high sensitivity and specificity (Sarman 20 Singh and V.M. Katoch, Indian J Med Res 2011, 134(5): 583-587: doi : 10.4103/0971-5916.90980).

Hence, there is a need for further methods allowing to differentiate between latent and active status of tuberculosis infection. Accordingly, there is a need in the art for approaches that are for detection and determination of 25 the therapeutic regimen or, predicting the clinical outcome or determining the treatment course as well as determining the status of tuberculosis infection in an individual suffering therefrom.

The present invention aims for providing new methods and assays particularly useful in the issues described above.

Summary

Certain exemplary embodiments provide a method for diagnosing or determining a status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection, comprising:

- 5 a) determining a level or amount of cytokines released or produced by mononuclear cells after stimulation, whereby said mononuclear cells are from said individual; and b) determining or diagnosing a status of said individual based on the level or amount of cytokines released or produced from the mononuclear cells, wherein the mononuclear cells are stimulated with
- 10 mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.

Other exemplary embodiments provide a method for stratification of a therapeutic regimen of an individual with tuberculosis infection comprising:

- a) determining a level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are from said individual; and b) determining a status of tuberculosis infection based on the level or amount of cytokines released or produced from said mononuclear cells after stimulation allowing differentiation of a latent and active status of tuberculosis infection in said individual, wherein the mononuclear cells are
- 15 stimulated in the presence of mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.
- 20

- 25 Yet other exemplary embodiments provide a method for predicting a clinical outcome or determining a treatment course in an individual afflicted with tuberculosis infection comprising: a) determining a level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and b) predicting the clinical outcome or determining the treatment course based on the level or amount of cytokines released or produced from said mononuclear

cells after stimulation, wherein the stimulation includes cultivation of said mononuclear cells with mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.

5 Still yet other exemplary embodiments provide mycobacterial Alanine Dehydrogenase, for use as a stimulant to differentiate latent status and active status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection.

Still yet other exemplary embodiments provide composition comprising
10 mycobacterial Alanine Dehydrogenase (AlaDH) together with at least one excipient, carrier or diluent, for use to determine tuberculosis infection in an individual, wherein the composition is a stimulant.

Still yet other exemplary embodiments provide use of a composition comprising mycobacterial Alanine Dehydrogenase (AlaDH) together with at
15 least one excipient, carrier or diluent, to determine tuberculosis infection in an individual, wherein the composition is a stimulant.

In a first aspect, the present invention relates to a method for diagnosing or determining the status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection, comprising:

- a) determining the level or amount of cytokines released or produced by mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and
- b) determining or diagnosing the status of said individual based on the level or amount of cytokines released or produced from the mononuclear cells, characterized in that the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH).

5 In a further aspect, the present invention relates to a method for the stratification of the therapeutic regimen of an individual with tuberculosis
10 infection comprising:

- a) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and
- b) determining the status of tuberculosis infection based on the level or
15 amount of cytokines released or produced from said mononuclear cells after stimulation allowing differentiation of a latent and active status of tuberculosis infection in said individual, characterized in that the mononuclear cells are stimulated in the presence of mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.

20 In another aspect, the present invention relates to a method for predicting a clinical outcome or determining the treatment course in an individual afflicted with tuberculosis infection comprising:

- a) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and
- b) predicting the clinical outcome or determining the treatment course based
25 on the level or amount of cytokines released or produced from said mononuclear cells after stimulation, characterized in that the stimulation includes cultivation of said mononuclear cells with mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.

30 Another embodiment of the present invention relates to a method for monitoring the change from latent to active status of tuberculosis infection or vice versa in an individual comprising:

- a) determining the level or amount of cytokines released or produced from mononuclear cells from said individual after stimulation at a first point in time;
- b) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual at a second point in time; and
- c) comparing the level or amount of cytokines determined in step a) to the level or amount determined in step b) or to a reference value whereby an increase in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from latent to active status and a decrease in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from active to latent status,
characterized in that the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.
That is, the present inventors recognised that the mycobacterial, in particular, the *Mycobacterium tuberculosis* Alanine Dehydrogenase (AlaDH) represents a suitable stimulant for mononuclear cells obtained from the individuals afflicted with or suspected to be afflicted with tuberculosis infection and, subsequently, determining the amount or level of cytokines released or produced by said mononuclear cells after stimulation, thus, allowing to differentiate between latent and active status of tuberculosis infection in said individual, accordingly. In particular, contrary to other known mycobacterial components, e.g. as described in Amanatidou V. et. al., Eur J Clin Microbiol Infect Dis. 2012 Jan 4, it has been shown surprisingly that AlaDH results in cytokine release or production in active TB state only while during latent TB state cytokine release or production is significantly lower. This is particularly true for the cytokine IL-2.

The methods described herein are typically in vitro methods. The mononuclear cells are stemming from the individual to be tested. Said cells are obtained before from said individual whereby the step of taking a sample including the cells from the individual, e.g. taking a blood sample, is typically not part of the method according to the present invention. The methods as

described herein include embodiments wherein whole blood samples are used for stimulation or where the mononuclear cells are isolated in advance from the whole blood sample. Unless otherwise indicated herein, the term “mononuclear cells” include the embodiment of whole blood samples. In a preferred 5 embodiment, the mononuclear cells are isolated mononuclear cells isolated e.g. from whole blood.

Moreover, the present invention relates to the use of a kit for diagnosing or determining the status of tuberculosis infection, or for predicting a clinical outcome or determining the treatment course in an individual afflicted with or 10 suspected to be afflicted with tuberculosis infection, or for the stratification of the therapeutic regimen of an individual with tuberculosis infection or for monitoring the progression of tuberculosis infection, or for determining the transition from latent to active status of tuberculosis infection in an individual, said kit comprises means for determining the level or amount of cytokines 15 released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual, a stimulating agent which is mycobacterial, e.g. of *Mycobacterium tuberculosis*, AlaDH and instructions on how to use said test kit for a method according to the present invention.

Further, the present invention relates to the use of mycobacterial 20 Alanine Dehydrogenase, in particular, of *Mycobacterium tuberculosis*, as a stimulant allowing to differentiate between latent and active status of tuberculosis infection in a individual afflicted with or suspected to be afflicted with tuberculosis infection.

Finally, a composition comprising mycobacterial AlaDH, in particular, 25 of *Mycobacterium tuberculosis*, in combination ESAT-6 and CFP-10 is provided for use in determining tuberculosis infection in an individual, in particular for use as stimulants, e.g. in whole blood or in cell-based assays.

Brief description of the drawings

30 Figure 1: Figure 2 shows ROC illustrating sensitivity and specificity of AlaDH IFN-gamma and IL-2 ELISpot results in discriminating children with latent (n = 21) and active (n = 25) tuberculosis.

Detailed description of the present invention

The present invention relates in a first aspect to a method for diagnosing or determining the status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with tuberculosis infection, comprising:

- 5 a) determining the level or amount of cytokines released or produced by mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and
- 10 b) determining or diagnosing the status of said individual based on the level or amount of cytokines released or produced from the mononuclear cells,
- 15 characterized in that the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*. That is, the present inventors recognised that depending on the level or amount of cytokines released or produced by mononuclear cells after stimulation whereby said mononuclear cells are obtained from the individual afflicted with
- 20 or suspected to be effected with tuberculosis infection, it is possible to diagnose or determine the status of tuberculosis infection in said individual when nuclear cells are stimulated with mycobacterial Alanine Dehydrogenase, in particular, of *Mycobacterium tuberculosis*, for cytokine release or production.

In contrast to assays commercially available, the assays and methods described herein allows to differentiate between the latent and active status of tuberculosis infection. The differentiation between the two different types of status is important for determining the treatment course and the risk of the course of tuberculosis infection as well as for predicting the clinical outcome. Moreover, it is important to monitor the development of the tuberculosis infection in said individual. It has been shown that when using AlaDH as stimulant, the cytokine pattern released or produced from the mononuclear cells stimulated with the AlaDH molecule is different between individuals having latent TB infection and individuals having active TB infection. In contrast, the molecules of commercially available IGRAS allow to differentiate between non-infected and infected individuals only without differentiating between the two status.

As used herein, the term "individual" in the context of the present invention is typically a mammal, in particular, the mammal is a human, a non-

human primate or other mammals susceptible for mycobacterial infection. The individual can be male or female. The individual can be one who has been previously diagnosed with or identified as suffering from tuberculosis infection and, optionally, but need not to have already undergone treatment for

5 tuberculosis infection. An individual can also be one who has been diagnosed with or identified as suffering from tuberculosis infection but who has shown improvement in the disease as a result of receiving one or more treatments for the infection, accordingly. Moreover, the individual may also be one who has not been previously diagnosed or identified as having tuberculosis infection.

10 The term "determining" or "detecting" as used herein refers to assessing the presence, absence, quantity, level or amount of either a given substance within a clinical or subject derived sample, including quality and/or quantitative concentration levels of substances otherwise evaluating values or categorisation of a subject's clinical parameter.

15 The term "comprises" or "comprising" or "contains" or "containing" includes the embodiments of "consist" or "consisting".

As used herein, the term "AlaDH" comprises antigenic fragments or the whole polypeptide of AlaDH. In particular, the AlaDH is an antigenic oligopeptide or polypeptide derived from the peptide of Seq ID No. 1. That is,

20 the mycobacterial AlaDH is preferably the ALaDH of TB with the NCBI-reference No. NP_217296.1 or the TubercuList database No. AlaDH-Rv2780. The skilled person is well aware of suitable fragments, e.g. as described in EP 0966544. In addition, the term comprises homologs thereof of Seq. ID. No. 1 having the same stimulatory activity on mononuclear cells as AlaDH of Seq. ID. No. 1.

25 As used herein, the term "latent" includes the latent status of TB infection (LTBI) as well as the cured status after successful treatment unless otherwise indicated.

As used herein, the term "mononuclear cells obtained from...an individual" refers to cells obtained before from said individual. The cells are

30 stemming from the individual but the method according to the present invention typically does not include the sampling step. Rather, the cells are provided as a

sample. The sample may be whole blood or a sample containing the cells after isolation steps from the sample from said individual.

Moreover, the present invention relates to method for the stratification of the therapeutic regimen of an individual with tuberculosis

5 infection comprising:

- a) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and
- b) determining the status of tuberculosis infection based on the level or 10 amount of cytokines released or produced from said mononuclear cells after stimulation allowing differentiation of a latent and active status of tuberculosis infection in said individual, characterized in that the mononuclear cells are stimulated in the presence of mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.

15

That is, it is possible to stratify the therapeutic regimen of an individual suffering from tuberculosis infection based on determining the status of infection, namely to differentiate between latent and active status of tuberculosis infection.

20

Further, the present inventors recognised that AlaDH represents a valuable tool, namely, a valuable stimulant for whole blood samples, in particular, mononuclear cells obtained from an individual afflicted with or suspected to be afflicted with tuberculosis infection for diagnosing or determining the status of said tuberculosis infection in said individual.

25

Hence, a method is provided for monitoring the change from latent to active status of tuberculosis infection or vice versa in an individual comprising:

- a) determining the level or amount of cytokines released or produced from mononuclear cells from said individual after stimulation at a first point in time;
- b) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual at a second point in time; and 30

c) comparing the level or amount of cytokines determined in step a) to the level or amount determined in step b) or to a reference value whereby an increase in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from 5 latent to active status and an decrease in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from active to latent status characterized in that the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.

10 In the context of the present invention, the term "reference value" refers to an index value, a value derived from one or more computer indices, a value derived from a subject with known active or latent tuberculosis infection, in particular, with a cohort of the same. In particular, the reference values obtained from individuals afflicted with latent and active status tuberculosis 15 infection and, in addition, the reference value represents a range or index obtained from at least two samples collected from individual of said disease state.

20 In a preferred embodiment, the mononuclear cells are obtained from a sample of said individual. Said sample may be blood, a blood derived sample or other samples containing mononuclear cells suitable for allowing cytokine release assays or assays determining the amount of cytokines both on protein and nucleic acid level. In particular, the sample is a blood sample or is derived from other body fluids including Bronchoalveolar lavage and urine and tissues.

25 That is, the mononuclear cells may be obtained from blood or other body fluids and, optionally, are isolated by common techniques.

A person skilled in the art will appreciate a number of different methods are useful for obtaining said mononuclear cells from the individual.

30 That is, the present inventors recognized that AlaDH represents a suitable stimulant in a whole blood assay and in a cell-based assay for a method according to the present invention.

It is preferred that the cytokine level or amount is determined by immuno based assays. A person skilled in the art will appreciate that a number of methods are useful for determining the amount or level of the cytokine in a

sample, including immunoassays such as western blot, ELISA and ELIspot. It is particularly desired that the cytokines are determined at protein level with ELIspot or ELISA technique. However, the cytokine levels can also be measured by determining the mRNA levels corresponding to the said cytokines, 5 accordingly. The skilled person is well aware of suitable methods for determining the same on nucleic acid level including PCR techniques.

It is preferred that the cytokine determined is at least one selected from IL-2 or interferon- γ . It is particularly desired that the IL-2 amount or level is determined released or produced by said mononuclear cells after stimulation 10 with mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*.

As demonstrated herein, IL-2 ELIspot and/or IFN- γ ELIspot based ELIspot assays allow to differentiate between latent and active status of tuberculosis infections in either children or adults.

15 According to a preferred embodiment, the reference value for the latent status of tuberculosis infection is below 25, preferably below 10, while the reference value for the active status of tuberculosis infection is above 50, preferably above 100, for IL-2 ELIspot.

When monitoring the change from latent to active status of tuberculosis 20 infection or vice versa in an individual, the first sample from the individual is preferably obtained at a first time point while the second sample obtained after a, preferably predetermined, time point, e.g. after undergoing treatment of tuberculosis infection or undergoing treatment for autoimmune diseases. For example in case of the treatment of autoimmune diseases or other diseases 25 whereby the immune system is modulated, it is helpful to determine the status of TB infection to avoid any onset of the TB infection. It is known that when treatment RA patients, latent status TB infection may proceed to active status. That is, the present invention allows to determine the treatment course in said individual or predicting the clinical outcome by identifying the actual status of 30 tuberculosis infection in said individual.

In particular, the method according to the present invention allows for identifying or monitoring or supervising individuals afflicted with tuberculosis infection to determine a change from latent to active status of tuberculosis or

vice versa. When the relative or absolute level or amount of cytokines increases, it is indicative for a transition from the latent to active status while a decrease in the relative or absolute level or amount is indicative for a transition from active to latent status.

5 In clear contrast to mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*, other stimulants used e.g. in ELIspot or ELISA assays as stimulants allow to differentiate between TB infection and non TB infection only, e.g. the well known mycobacterial molecules ESAT-6, CFP-10 or other molecules.

10 However, it has been determined herein that a composition comprising mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*, and ESAT-6 as well as CFP-10 allows to increase the sensitivity and specificity of tests allowing to differentiate between uninfected and infected individuals, in particular, tests wherein these components are used as 15 stimulants. That is, the components of said composition may be used in separate assays or may be used in combination.

20 Important clinical implications arise from the finding described herein. Due to the possibility to differentiate between latent and active status it is possible to stratify the therapeutic regimen or to determine the treatment course in the individual accordingly.

In a further aspect, the present invention relates to the use of a kit for diagnosing or determining the status of tuberculosis infection, or for predicting a clinical outcome or determining the treatment course in an individual afflicted with or suspected to be afflicted with tuberculosis infection, or for the 25 stratification of the therapeutic regimen of an individual with tuberculosis infection or for monitoring the progression of tuberculosis infection, or for determining the transition from latent to active status of tuberculosis infection in an individual, said kit comprises means for determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, 30 whereby said mononuclear cells are obtained from said individual, a stimulating agent which is mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*, and instructions on how to use said test kit for a method according to the present invention.

In particular, in a preferred embodiment said kit is an ELIspot or ELISA allowing for determining the level or amount of the cytokine. It is particular preferred that the ELIspot or ELISA is an assay allowing determination of the amount or level of IL-2 or interferon- γ , in particular, IL-2. Alternatively, the kit is 5 a PCR kit with means suitable for analysing cytokine expression on nucleic acid level.

Moreover, the present invention relates to the use of mycobacterial Alanine Dehydrogenase (AlaDH), in particular, of *Mycobacterium tuberculosis*, in differentiating latent status and active status of tuberculosis infection in an 10 individual afflict with or suspected to be afflicted with tuberculosis infection. The mycobacteria Alanine Dehydrogenase is particularly useful for differentiating the same in human, in particular, children.

Finally, the present invention provides assays useful for performing or conducting the method according to the present invention. Said assay 15 comprises means for culturing mononuclear cells and AlaDH as stimulant in a form suitable to stimulate said mononuclear cells when added to a culture thereof. Furthermore, the assay may contain means for determining the amount or level of the cytokine to be detected. Typically, said assay is based on an immunoassay, in particular, said assay is an EliSpot or ELISA. Detection is 20 effected by antibodies detecting specifically the desired cytokine. Alternatively, the assay is a PCR-based assay.

The above disclosure generally describes the present invention. More complete understanding can be obtained by reference to the following specific examples of third embodiments of the invention without being limited thereto.

25

Examples

Materials and Methods

Study subjects

Consecutive children at risk for TB infection referred to the Infectious 30 Disease Unit, Department of Sciences for Woman and Child's Health University of Florence, Italy, were prospectively enrolled between 2009 and 2010. Study children were those with clinical suspicion of TB disease and/or in close contact with recently diagnosed cases of contagious TB disease

and/or internationally adopted or recently immigrated children coming from countries with a high-prevalence of TB. A maximum of two years was considered as period of recent immigration. Children with congenital or acquired immunodeficiency disorders (based on their medical history, clinical 5 examination and/or laboratory tests) were excluded from the study.

Tuberculin skin test

TST was administered according to the Mantoux method by injecting intradermally 5 tuberculin units (in 0.1 mL) of purified protein derivative (Biocine Test-PPD, Chiron, Siena, Italy) into the volar surface of the forearm. 10 The transversed skin induration was recorded (in mm) after 48-72 hours. Following the American Academy of Pediatrics guidelines (American Academy of Pediatrics. [Tuberculosis]. In: Pickering LK, Baker CJ, Kimberlin DW, Long SS, eds. Red Book: 2009 Report of the Committee on Infectious Diseases. 28th ed. Elk Grove Village, IL: American Academy of Pediatrics; 2009:[680- 15 701]. Amanatidou V. et. al, see above) a positive TST was defined as an induration size \geq 5 mm for children in close contact with known or suspected contagious case of TB disease or for children suspected to have TB disease (based on clinical evidence and/or chest radiograph) and \geq 10 mm for children born in countries with a high prevalence of TB and recently immigrated.

20 QFT-G-IT

The QFT-IT test was performed according to the manufacturer's instructions as described previously (Millington KA, et. al., Proc Natl Acad Sci USA.2011;108:5730-5. Vilaplana C, et. al., Scand J Immunol 2008;67:610-7). After subtracting the value from the negative control, the result was positive if, 25 the antigen-dependent response was \geq 0.35 IU, negative if the mitogen-induced response was \geq 0.5 IU/mL and the antigen-dependent response was <0.35 IU/mL, and indeterminate if both mitogen-induced and antigen-dependent responses were below cut-off or mitogen-induced response >8 IU/mL.

ELISpot Assay

30 In-house ELIsop for IFN-gamma and IL-2 were performed using Lionex M. tuberculosis. antigens. Briefly, T cell blasts (10⁵ cells) of each patients line were stimulated with M. tuberculosis antigen in the presence of irradiated autologous APCs (5 \times 10⁴ cells) and seeded in triplicate in 96-well plates

coated with anti-IFN- γ or anti-IL-2 antibody. Cells stimulated with medium alone served as negative controls. Cells stimulated with PHA served as positive controls. IFN- γ and IL-2 ELISPOT microplates were then incubated at 37 °C in 5% CO₂ for 24 hours. At the end of the culture period, plates were washed and 5 incubated for 3 hours with the appropriate biotinylated anti-IFN- γ or anti-IL-2 mAb. Streptavidin-HRP complex was then added for 2 hours, followed by the substrate solution. SFCs were counted using an automated ELISPOT reader (Autoimmune Diagnostika GmbH).

Study design

10 Information regarding socio-demographic data, prior TB exposure, and past medical history was obtained from each child's parents or tutors or from medical documentation and recorded into the study database. Children were considered vaccinated with BCG whether a clear documentation was available and/or a scar was present. All children underwent clinical evaluation, TST and 15 venipuncture for IGRAs (QFT-G-IT and in-house ELISpot assay). Blood was taken during the first examination after the parent's or tutor's informed consent had been obtained and before starting any anti-tubercular treatment. Chest radiography was performed in all symptomatic children, in those with a positive TST, and in all contacts aged less than 5 years. Children with suspected 20 pulmonary TB had three sputum or early morning gastric aspirates samples collected for *Mycobacterium tuberculosis* detection (by means of microscopy, polymerase chain reaction and culture). Chest computed tomography (CT) scan was performed in selected cases at the paediatrician's discretion. No suspected or ascertained extra-pulmonary TB cases presented at the Infectious 25 Disease Unit during the study period. The study received local Ethical Committee approval and informed consent for the study was obtained by parents or tutors.

Definition of study groups

Study children were classified as not-infected, LTBI cases, or active lung 30 TB disease cases, following the American Academy Guidelines definition (American Academy of Pediatrics. [Tuberculosis]. In: Pickering LK, Baker CJ, Kimberlin DW, Long SS. eds. Red Book: 2009 Report of the Committee on Infectious Diseases. 28th ed. Elk Grove Village, IL: American Academy of

Pediatrics: 2009:[680-701]). In the event of discordant TST/QFT-G-IT results, children were assigned to the corresponding group on the bases on the TST result, according to the most recent U.S. and European Guidelines (American Academy of Pediatrics. [Tuberculosis]. In: Pickering LK, Baker CJ, Kimberlin 5 DW, Long SS. eds. Red Book: 2009 Report of the Committee on Infectious Diseases. 28th ed. Elk Grove Village, IL: American Academy of Pediatrics: 2009:[680-701]. Amanatidou V. et. al., see above). In particular, asymptomatic children with negative TST were defined as uninfected. LTBI diagnosis was assigned to any child with a positive TST and no clinical nor radiographic 10 evidence of active TB. Cases of active TB were defined according to two categories: definite TB, children with *Mycobacterium tuberculosis* cultured or detected by microscopy or molecular methods from sputum or gastric aspirate culture, and probable TB: absence of microbiological confirmation but presence of all of the following criteria: (A) clinical symptoms and signs of active TB, (B) 15 abnormal radiography and/or CT scan consistent with lung TB, (C) response to TB therapy plus, (D) either a history of TB contact or travel to a TB-endemic country within the last 24 months (Amanatidou V. et. al, see above).

Statistical analysis

Categorical data were compared using the Chi-squared test (or Fisher's 20 exact test, when expected cell sizes were smaller than five). The Wilcoxon-Mann-Whitney test was used for continuous measurements to test relationships in unpaired analysis, when assumed that the dependent variable is a not normally distributed interval variable. Test concordance was assessed by Cohen's κ -statistics with agreement considered 'slight' for $\kappa \leq 0.2$, 'fair' for 25 $0.2 < \kappa \leq 0.4$, 'moderate' for $0.4 < \kappa \leq 0.6$, 'substantial' for $0.6 < \kappa \leq 0.8$ and 'optimal' for $0.8 < \kappa \leq 1.0$. Receiver operating characteristic (ROC) curve analysis was conducted to determine the best IL-2 and IFN- γ ELISpot result thresholds in discriminating between children with active or latent TB, relatively to specific *M. tuberculosis* antigens and correspondent sensitivity and specificity were 30 reported. The area under the ROC curve (AUC) and 95% confidence interval (CI) were also calculated. Statistical analysis was performed using the statistical software SPSS for Windows, version 14.0. $P < 0.05$ was considered statistically significant.

Results

Seventy five children (males); median age: 75 months; interquartile range [IQR] 39-116) were included in the study. Previous BCG vaccination was documented in 21 (28.0%) children.

5 The diagnosis of definite TB disease was made in 5 children. All these children presented with symptoms compatible with TB (e.g. persistent cough, fever, night sweats or weight loss) and/or pulmonary disease documented by chest X-ray and confirmed by CT scan results and *Mycobacterium tuberculosis* was detected from the cultures and/or microscopy and/or polymerase chain

10 reaction. Probable TB was diagnosed in 20 children. All these children underwent at least three gastric aspirate or sputum examination for *Mycobacterium tuberculosis* detection which yielded negative results. Table 1 shows the BCG vaccination status, the TST size and the QFT-G-IT result according to final diagnosis.

15 Table 1: BCG vaccination status, TST size and QFT- G-IT result according to final diagnosis of the study children

	uninfected n = 29 n (%)	latent TB n = 21 n (%)	Probable TB disease n = 20 n (%)	Definite TB disease n = 5 n (%)
Age, months (median and IQR)	77 (28-101)	116 (61-151)	52(27-82)	47 (8-97)
BCG vaccinated	5	12	1	0
TST, induration diameter (mm)				
< 5	28	0	0	1
≥ 5 and < 10	1	1	2	0
≥ 10 and < 15	0	5	1	1
≥ 15	0	15	17	3
QFT-G-IT result				
negative	29	5	3	5
positive	0	15	17	0
indeterminate	0	1	0	0

BCG = Bacille Calmette-Guérin, TST = tuberculin skin test, IGRA = interferon- γ

release assay

Discordant TST/QFT-G-IT result was obtained in four children (one child with definite TB, positive QFT-G-IT and negative TST and three children with probable TB, negative QFT-G-IT and positive TST). The overall agreement 5 between the IGRA and the TST (without considering the indeterminate result) was substantial with a k value of 0.679.

IFN- γ and IL-2 ELISpot results

ELISpot results are summarized in Table 2.

Table 2. ELISpot results

	uninfected n = 29	latent TB n = 21	Probable TB disease n = 20	Definite TB disease n = 5	P Uninfected vs. infected children	P Latent vs. overt disease
IFN-γ						
ELISpot						
- ESAT-6	35 (1-102)	320 (157-520)	292 (170-631)	330 (190-1575)	<0.0001	0.724
- CFP-10	40 (1-120)	305 (130-625)	280 (76-986)	875 (200-3532)	<0.0001	0.691
- H1-hybrid	60 (12-192)	300 (127-437)	295 (120-825)	180 (115-1737)	<0.0001	0.651
- Ag85B	65 (12-152)	150 (47-322)	107 (46-192)	90 (27-1517)	0.063	0.724
- AlaDH	35 (12.5-110)	85 (17-180)	147 (71-303)	280 (115-1565)	<0.001	0.021
- PstS1	40 (1-122)	50 (1-197)	60 (11-241)	95 (18-982)	0.0512	0.504
IL-2						
ELISpot						
- ESAT-6	10 (1-35)	220 (35-410)	110 (60-402)	420 (155-1365)	<0.0001	0.791
- CFP-10	10 (1-20)	210 (85-710)	160 (100-637)	510 (355-1735)	<0.0001	0.574
- H1-hybrid	20 (1-50)	180 (35-535)	195 (95-595)	430 (265-1635)	<0.0001	0.247
- Ag85B	20 (10-75)	40 (10-140)	45 (17-195)	200 (135-1485)	0.063	0.188
- AlaDH	1 (1-20)	1 (1-10)	75 (36-130)	340 (110-1365)	=0.001	<0.0001
- PstS1	10 (1-50)	20 (1-75)	20 (10-95)	10 (5-155)	<0.0001	0.755

10

Comparing uninfected children vs. children with TB infection (LTBI plus probable and definite TB disease) significantly higher median values were evidenced in infected than in uninfected children considering IFN- γ ELISpot responses to ESAT-6 ($p<0.0001$), CFP-10 ($p<0.0001$), H1-hybrid ($p<0.0001$) or 15 AlaDH ($p=0.001$) while differences were not significant considering Ag85B ($p=0.063$) and PstS1 ($p=0.512$). Considering IL-2 ELISpot results significantly different results were evidenced with ESAT-6, CFP-10, H1hybrid, and AlaDH

(p=0.001); but not with Ag85 (p=0.063).

Comparing results between children with LTBI and those with TB disease (probable plus definite disease) differences were significant for IFN- γ ELISpot only considering response to AlaDH antigen (p=0.021) (Table 2). With regard to

5 IL-2 ELISpot significant differences were confirmed for AlaDH antigen

(p<0.0001) while no difference was evidenced considering other antigens.

Comparing results between uninfected children and children with LTBI

significant difference were observed in IFN- γ ELISpot responses to ESAT-6

(p>0.0001), CFP-10(p>0.0001), H1-hybrid (p=0.001), Mix (p=0.017) and in IL-2

10 ELISpot responses to ESAT-6 (p>0.0001), CFP-10 (p>0.0001), H1-hybrid

(p>0.0001), and mix (p>0.0001).

ROC analyses demonstrated that, considering response to AlaDH, for IL-2 ELISpot value of 12.5 SCF per million PBMC (after the subtraction of *background spots*) sensitivity in discriminating between latent and active TB

15 was 100% and specificity was 81%. For IFN- γ ELISpot the performance was

much poorer: for the best threshold of 42.0 SCF per million PBMC sensitivity was 88% and specificity 56% (Figure 1).

Similar results were obtained in adult LTBI patients, see table 3.

Table 3. Adult ELISpot results

	uninfected n = 31	latent TB n = 39	Probable TB disease n = 12	Definite TB disease n = 18
IFN-γ ELISpot				
- ESAT-6	29	343	301	342
- CFP-10	34	298	276	722
- H1-hybrid	52	312	221	173
- Ag85B	71	179	114	102
- AlaDH	24	99	266	328
- PstS1	51	43	72	86
IL-2 ELISpot				
- ESAT-6	7	215	114	433
- CFP-10	5	227	148	471
- H1-hybrid	14	198	201	445
- Ag85B	25	52	63	217
- AlaDH	2	8	193	429

- PstS1

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As demonstrated by way of examples, the performances of interferon- γ and IL-2 based ELIspot assays in children and adult when using *Mycobacterium tuberculosis* AlaDH as stimulant (antigen) allows to differentiate between latent and acute status of tuberculosis infection.

5 Moreover, as expected, the antigens currently included in commercial available IGRAs like ESAT-6 and CFP-10 allows to differentiate between uninfected and infected individuals only.

Moreover a mixture or composition of mycobacterial *Mycobacterium tuberculosis* AlaDH and ESAT-6 as well as CFP-10 allows to determine 10 tuberculosis infection in an individual with higher sensitivity and specificity. As demonstrated by ROC-analysis, sensitivity of discriminating a latent and active status reached 100 % while specificity was 96 % by the use of IL-2 based ELIspot assay. The performance of the interferon- γ ELIspot assay using AlaDH antigen was not as good as the IL-2 based ELIspot assay but still allow to 15 differentiate between the two different types of status.

Moreover, as demonstrated, the methods and assay systems according to the present invention are particularly useful for discrimination of children with active from those with latent TB when using AlaDH antigen.

Claims:

1. A method for diagnosing or determining a status of tuberculosis infection in an individual afflicted with or suspected to be afflicted with
5 tuberculosis infection, comprising:
 - a) determining a level or amount of cytokines released or produced by mononuclear cells after stimulation, whereby said mononuclear cells are from said individual; and
 - 10 b) determining or diagnosing a status of said individual based on the level or amount of cytokines released or produced from the mononuclear cells,
- 15 wherein the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.
- 20 2. The method of claim 1, wherein the AlaDH is of *Mycobacterium tuberculosis*.
3. A method for stratification of a therapeutic regimen of an individual with tuberculosis infection comprising:
25
 - a) determining a level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are from said individual; and

5 b) determining a status of tuberculosis infection based on the level or amount of cytokines released or produced from said mononuclear cells after stimulation allowing differentiation of a latent and active status of tuberculosis infection in said individual, wherein the mononuclear cells are stimulated in the presence of mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.

10 4. The method of claim 3, wherein the AlaDH is of *Mycobacterium tuberculosis*.

15 5. A method for predicting a clinical outcome or determining a treatment course in an individual afflicted with tuberculosis infection comprising:

20 a) determining a level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual; and

25 b) predicting the clinical outcome or determining the treatment course based on the level or amount of cytokines released or produced from said mononuclear cells after stimulation, wherein the stimulation includes cultivation of said mononuclear cells with mycobacterial Alanine Dehydrogenase (AlaDH), and wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.

6. The method of claim 5, wherein the AlaDH is of *Mycobacterium tuberculosis*.

7. The method according to claim 1 for monitoring the change from latent to active status of tuberculosis infection or vice versa in an individual comprising:

5 a) determining the level or amount of cytokines released or produced from mononuclear cells from said individual after stimulation at a first point in time;

10 b) determining the level or amount of cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual at a second point in time; and

15 c) comparing the level or amount of cytokines determined in step a) to the level or amount determined in step b) or to a reference value, whereby an increase in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from latent to active status and a decrease in the level or the amount relative to a reference value or to the level or amount determined in step a) is indicative for a transition from active to latent status,

20 wherein the mononuclear cells are stimulated with mycobacterial Alanine Dehydrogenase (AlaDH).

25 8. The method of claim 7, wherein the AlaDH is of *Mycobacterium tuberculosis*.

9. The method according to any one of claims 1 to 8, wherein the mononuclear cells stem from a sample of the individual.

10. The method according to claim 9, wherein the sample is a blood sample or of a body fluid other than blood.
11. The method according to claim 10, wherein the body fluid is
5 Bronchoalveolar lavage, urine or tissue fluid.
12. The method according to any one of claims 1 to 11, wherein the level or amount of cytokines is determined at a protein level, or at a nucleic acid level.

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13. The method of claim 12, wherein the level or amount of cytokines is determined by an immunoassay or PCT technique.
14. The method according to claim 13, wherein the immunoassay is ELISpot
15 or ELISA.
15. The method according to any one of claims 1 to 14, wherein the cytokine to be determined is IL-2.

20 16. The method according to any one of claims 1 to 15, wherein the reference value in IL-2 Elispot is below 25.

17. The method according to any one of claims 1 to 16, wherein the individuals are children.

25

18. Use of a kit for diagnosing or determining the status of tuberculosis infection, or for predicting a clinical outcome or determining treatment course in an individual afflicted with or suspected to be afflicted with tuberculosis infection, or for the stratification of a therapeutic regimen

of an individual with tuberculosis infection or for monitoring a progression of tuberculosis infection, or for determining a transition from latent to active status of tuberculosis infection in an individual, said kit comprising means for determining the level or amount of

5 cytokines released or produced from mononuclear cells after stimulation, whereby said mononuclear cells are obtained from said individual, a stimulating agent which is mycobacterial Alanine Dehydrogenase (AlaDH), and instructions on how to use said test kit for a method according to any one of claims 1 to 17, wherein the cytokines

10 to be determined are selected from IL-2 and interferon Gamma.

19. The use of claim 18, wherein the AlaDH is of *Mycobacterium tuberculosis*.

15 20. The use of a kit according to claim 18 or 19 whereby said kit is an ELISpot or an ELISA or a PCR kit.

21. Mycobacterial Alanine Dehydrogenase, for use as a stimulant to differentiate latent status and active status of tuberculosis infection in

20 an individual afflicted with or suspected to be afflicted with tuberculosis infection.

22. The mycobacterial Alanine Dehydrogenase (AlaDH) of claim 21, of *Mycobacterium tuberculosis*.

25 23. The mycobacterial Alanine Dehydrogenase (AlaDH) of claim 21 or 22, wherein the individual is a child.

24. An assay for conducting the method according to any one of claims 1 to 17 comprising means for culturing mononuclear cells, means for determining the level or amount of cytokines and mycobacterial Alanine Dehydrogenase (AlaDH), wherein the cytokines to be determined are selected from IL-2 and interferon Gamma.

5

25. The assay of claim 24, wherein the AlaDH is of *Mycobacterium tuberculosis*.

10 26. Composition comprising mycobacterial Alanine Dehydrogenase (AlaDH) together with at least one excipient, carrier or diluent, for use to determine tuberculosis infection in an individual, wherein the composition is a stimulant.

15 27. The composition of claim 26, wherein the AlaDH is of *Mycobacterium tuberculosis*.

28. The composition of claim 27, wherein the composition is for use to determine tuberculosis infection in an individual.

20 29. Use of a composition comprising mycobacterial Alanine Dehydrogenase (AlaDH) together with at least one excipient, carrier or diluent, to determine tuberculosis infection in an individual, wherein the composition is a stimulant.

25

30. The use of claim 29, wherein the AlaDH is of *Mycobacterium tuberculosis*.

31. The use of claim 29, wherein the individual is a child.

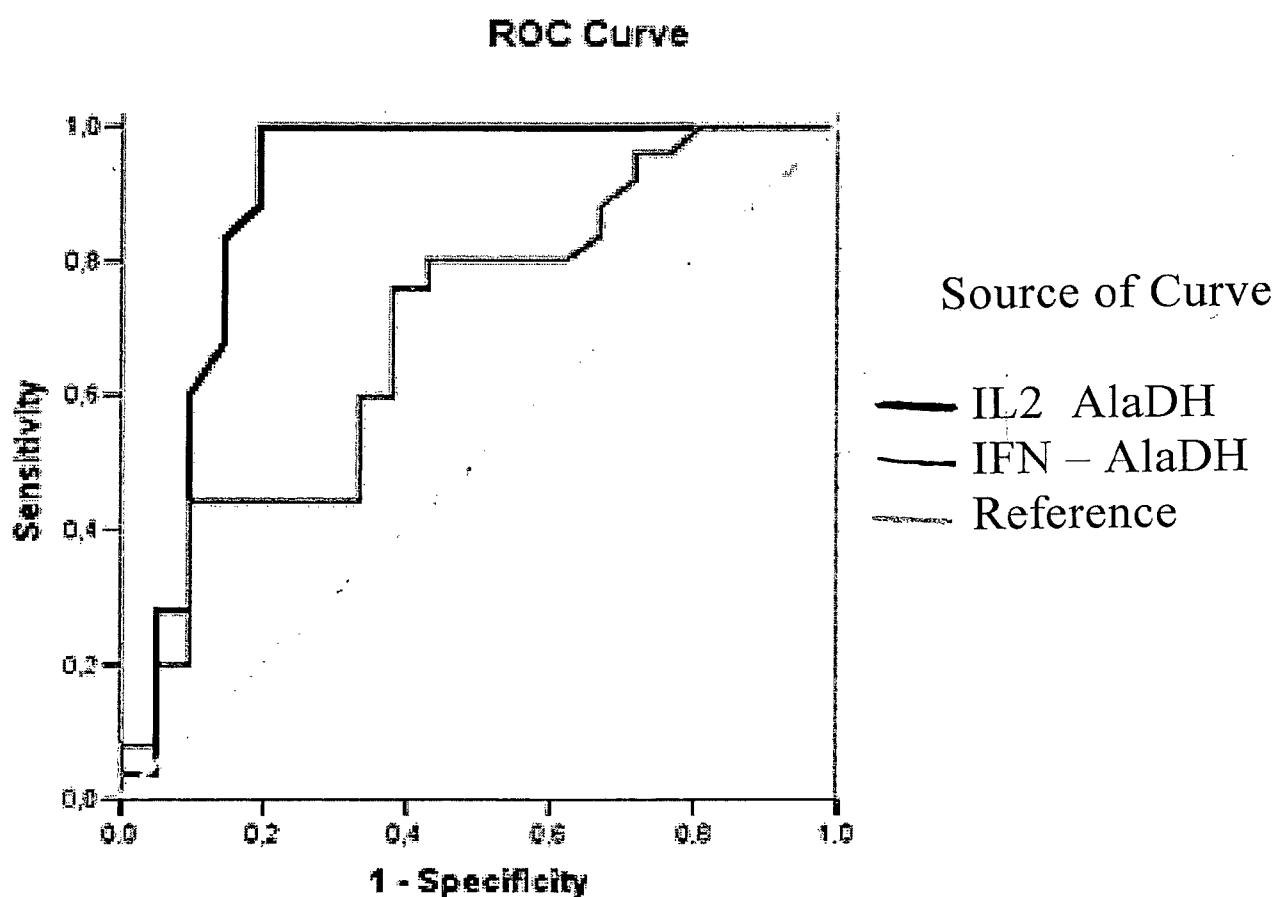


Figure 1