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(54) Titre : METHODE POUR LE DIAGNOSTIC DE LA FIBROSE KYSTIQUE
(54) Title: METHOD FOR THE DIAGNOSIS OF CYSTIC FIBROSIS

(57) **Abrégé/Abstract:**

The present invention is related to a method for diagnosing cystic fibrosis in a subject comprising a step a), wherein the step a) comprises detecting a biomarker in a sample from the subject, wherein the sample is a sample selected from the group comprising blood, a blood product, urine, saliva, cerebrospinal liquid, stool, tissue and lymph liquid.

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(54) Title: METHOD FOR THE DIAGNOSIS OF CYSTIC FIBROSIS

(57) Abstract: The present invention is related to a method for diagnosing cystic fibrosis in a subject comprising a step a), wherein the step a) comprises detecting a biomarker in a sample from the subject, wherein the sample is a sample selected from the group comprising blood, a blood product, urine, saliva, cerebrospinal liquid, stool, tissue and lymph liquid.

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METHOD FOR THE DIAGNOSIS OF CYSTIC FIBROSIS

The present invention is related to a method for diagnosing cystic fibrosis in a subject, a method for determining the course of cystic fibrosis in a subject, a method for determining the effectiveness of at least one treatment applied to a subject being positively tested for suffering from or being at risk of suffering from cystic fibrosis, a method for determining the effectiveness of a compound for the treatment of cystic fibrosis, a method for determining the course of cystic fibrosis in a subject, use of mass spectrometric analysis for the detection of a biomarker, use of a biomarker for the diagnosis of cystic fibrosis, use of an internal standard to quantify a biomarker present in a sample from the subject, and a kit for determining the presence of a biomarker in a sample from a subject.

Background

Cystic fibrosis (abbr. CF), also known as mucoviscidosis, is a genetic disorder that affects mostly the lungs but also the pancreas, liver, kidneys, and intestine. Symptoms include difficulty in breathing and coughing up mucus as a result of frequent lung infections, sinus infections, poor growth, fatty stool, clubbing of the finger and toes, and infertility in males. Both mild and severe cases were described.

CF is inherited in an autosomal recessive manner. It is caused by the presence of mutations in both copies of the gene for the cystic fibrosis transmembrane conductance regulator (abbr. CFTR) protein. Ceramide accumulates in the lungs of CF patients and in individuals with other chronic lung diseases such as, e.g., emphysema.

The mechanism(s) underlying ceramide accumulation in CF are complex, but are in part due to alterations in lung pH that favor high activity of the ceramide producing enzyme, acid sphingomyelinase (abbr. ASM), and low activity of the ceramide clearing enzyme, acid ceramidase (abbr. AC). Previous studies suggested that a defect in the cystic fibrosis transmembrane regulator protein (abbr. CFTR) may itself be responsible for high ceramide levels in CF patients - PDZ interacting domain of the CFTR protein localizes the CFTR protein to lipid rafts in the cell membrane, and this localization suppresses cellular ceramide levels in

healthy individuals, but not in patients with CF, due potentially to a defect in the PDZ domain. High lung ceramide levels amplify the inflammatory pathology in CF patients and directly drive susceptibility to pathogens. Healthy individuals maintain low levels of ceramide in their lung epithelial cells through the homeostatic-like regulation of the enzymes ASM and AC and their activities. In CF lungs ceramide levels become high due, in part, to alterations of these enzymatic activities, typically high ASM and low AC. In addition, mutations in CFTR may alter delivery of the protein to the membrane rafts, which also contributes to ceramide elevation.

While there are no cures for cystic fibrosis, there are several treatment methods. The cornerstones of cystic fibrosis management are proactive treatment of airway infection, and encouragement of good nutrition and an active lifestyle. Pulmonary rehabilitation as a management of cystic fibrosis continues throughout a person's life, and is aimed at maximizing organ function, and therefore quality of life. At best, current treatments delay the decline in organ function taking into consideration the wide variation in disease symptoms. Targets for therapy are the lungs, gastrointestinal tract including pancreatic enzyme supplements, the reproductive organs including assisted reproductive technology (abbr. ART) and psychological support. Still, the most consistent aspect of therapy in cystic fibrosis is limiting and treating the lung damage caused by thick mucus and infection, with the goal of maintaining quality of life. Intravenous, inhaled, and oral antibiotics are used to treat chronic and acute infections. Mechanical devices and inhalation medications are used to alter and clear the thickened mucus.

Cystic fibrosis may be diagnosed by many different methods including newborn screening, sweat testing, and genetic testing. The newborn screen initially measures for raised blood concentration of immunoreactive trypsinogen. Infants with an abnormal newborn screen typically undergo a sweat test to confirm CF diagnosis. Trypsinogen levels can be increased in individuals who have a single mutated copy of the CFTR gene. Such individuals are also referred to as carriers. Most individuals are diagnosed after symptoms such as, e.g., sinopulmonary disease and gastrointestinal (abbr. GI) manifestations which prompt an evaluation for cystic fibrosis. The most commonly used form of testing is the sweat test. Sweat-testing involves application of a medication that stimulates sweating such as pilocarpine. People with CF have increased amounts of sodium and chloride in their sweat. In contrast, people with

CF have less thiocyanate and hypothiocyanite in their saliva and mucus. CF can also be diagnosed by identification of mutations in the CFTR gene. Several thousand mutations of the CFTR gene are known, whereas in commercial testing only the most frequent ones such as $\Delta F508$ are detected.

The above mentioned laboratory tests, i.e. immunoreactive trypsinogen and sweat test, are nonspecific, time consuming and demand high levels of resources such as biological sample, time and laboratory materials.

In light thereof, the problem underlying the present invention is the provision of a means for the diagnosis of cystic fibrosis in a high throughput manner which is suitable for mass screenings with low biological sample consumption. A further problem underlying the present invention is the provision of a means for the diagnosis of cystic fibrosis making use of a biomarker, preferably making use of a biomarker contained in blood or a blood product from the subject to be diagnosed.

These and other problems are solved by the subject matter of the attached independent claims. Preferred embodiments may be taken from the attached dependent claims.

These and other problems are equally solved by the subject matter of the following embodiments 1 to 153 and further embodiments disclosed herein. It is to be taken into consideration that the subject matter of embodiments 1, 34, 57, 87, 110, 139, 143, 146, 150 and 153 is each also referred to as aspect of the present invention.

Embodiment 1: A method for diagnosing cystic fibrosis in a subject comprising

a step a), wherein the step a) comprises detecting a biomarker in a sample from the subject.

Embodiment 2: The method according to embodiment 1, wherein the method comprises

a step b) wherein the step b) comprises determining a level of the biomarker present in the sample.

Embodiment 3: The method according to any one of embodiments 1 or 2, wherein the level of the biomarker is indicative whether or not the subject is suffering from cystic fibrosis or whether or not the subject is at risk of suffering from cystic fibrosis.

Embodiment 4: The method according to any one of embodiments 1 to 3, wherein the sample from the subject is a sample from a subject who has previously been treated for cystic fibrosis or a sample from a subject who has previously been diagnosed for cystic fibrosis.

Embodiment 5: The method according to any one of embodiments 1 to 3, wherein the sample from the subject is a sample from a subject who has not previously been treated for cystic fibrosis or a sample from a subject who has not been previously diagnosed for cystic fibrosis.

Embodiment 6: The method according to any one of embodiments 1 to 5, wherein the method comprises a step c), wherein the step c) comprises applying, maintaining, reducing, elevating or not applying a therapy based on whether the subject is suffering from cystic fibrosis or is at risk of suffering from cystic fibrosis.

Embodiment 7: The method according to embodiment 6, wherein the method comprises a step d), wherein the step d) comprises detecting the biomarker in a sample from the subject after a therapy has been applied, maintained, reduced, elevated or not applied in step c).

Embodiment 8: The method according to any one of embodiments 6 to 7, wherein the method comprises a step e), wherein the step e) comprises determining a level of the biomarker in the sample from the subject after a therapy has been applied, maintained, reduced, elevated or not applied in step c).

Embodiment 9: The method according to embodiment 8, wherein the method comprises a step f), wherein the step f) comprises determining whether the level of the biomarker determined in step b) is lower than the level of the biomarker determined in step e).

Embodiment 10: The method according to embodiment 9, wherein the method comprises a step g), wherein the step g) comprises applying, maintaining, reducing, elevating or not applying a therapy based on step f).

Embodiment 11: The method according to any one of embodiments 1 to 10, wherein the biomarker is selected from the group consisting of an isoform 1 compound and an isoform 2 compounds, wherein

the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;

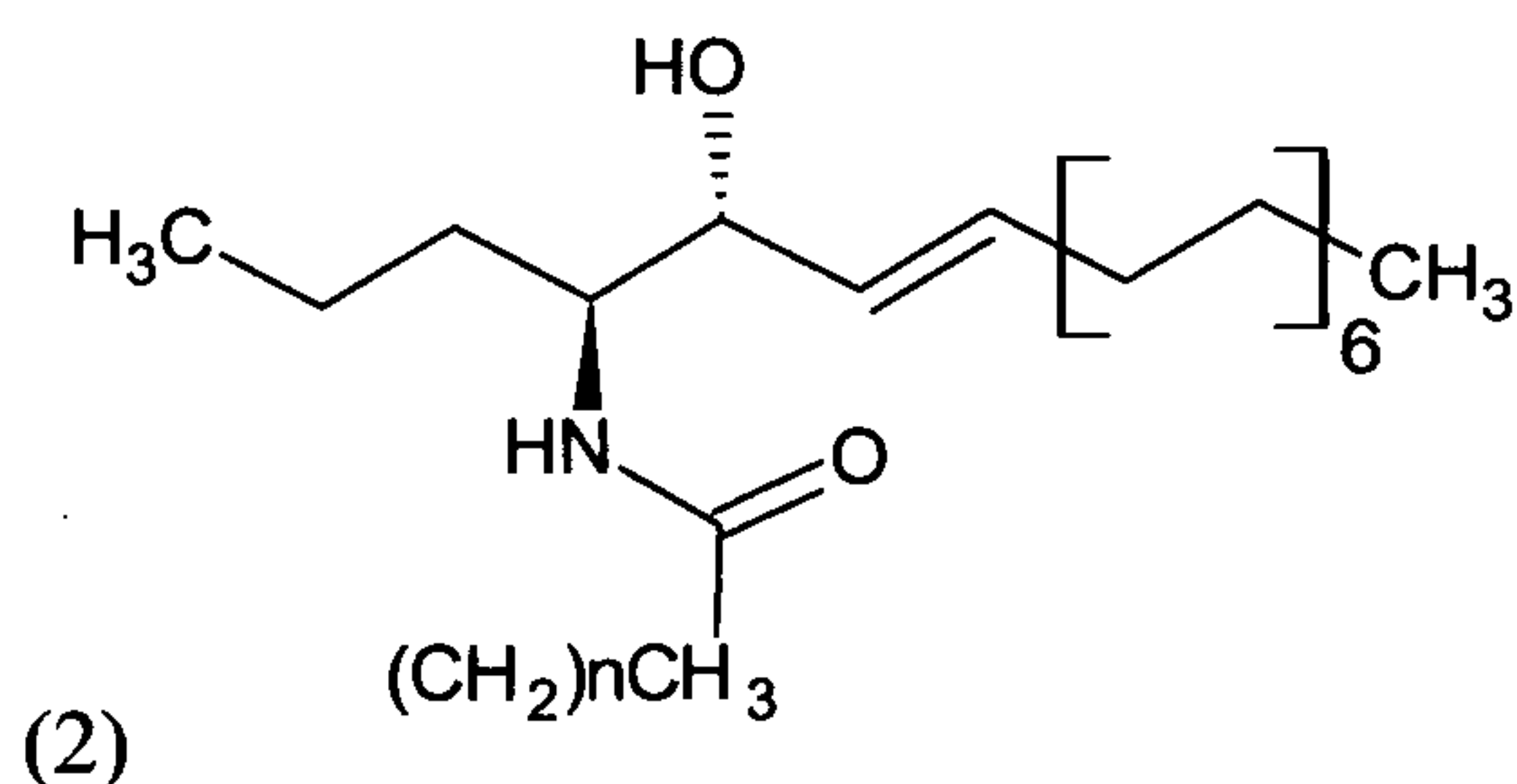
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 302.79 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 12: The method according to embodiment 11, wherein the biomarker is the total of isoform 1 compound and isoform 2 compound, preferably the sum of the amount of isoform 1 compound in a sample and the amount of isoform 2 compound in a sample, more preferably the sum of the amount of isoform 1 compound in a sample and the amount of isoform 2 compound in the sample, whereby preferably the sample is the sample in which the amount of isoform 1 compound is determined.

Embodiment 13: The method according to embodiment 11, wherein the biomarker is isoform 1 compound.

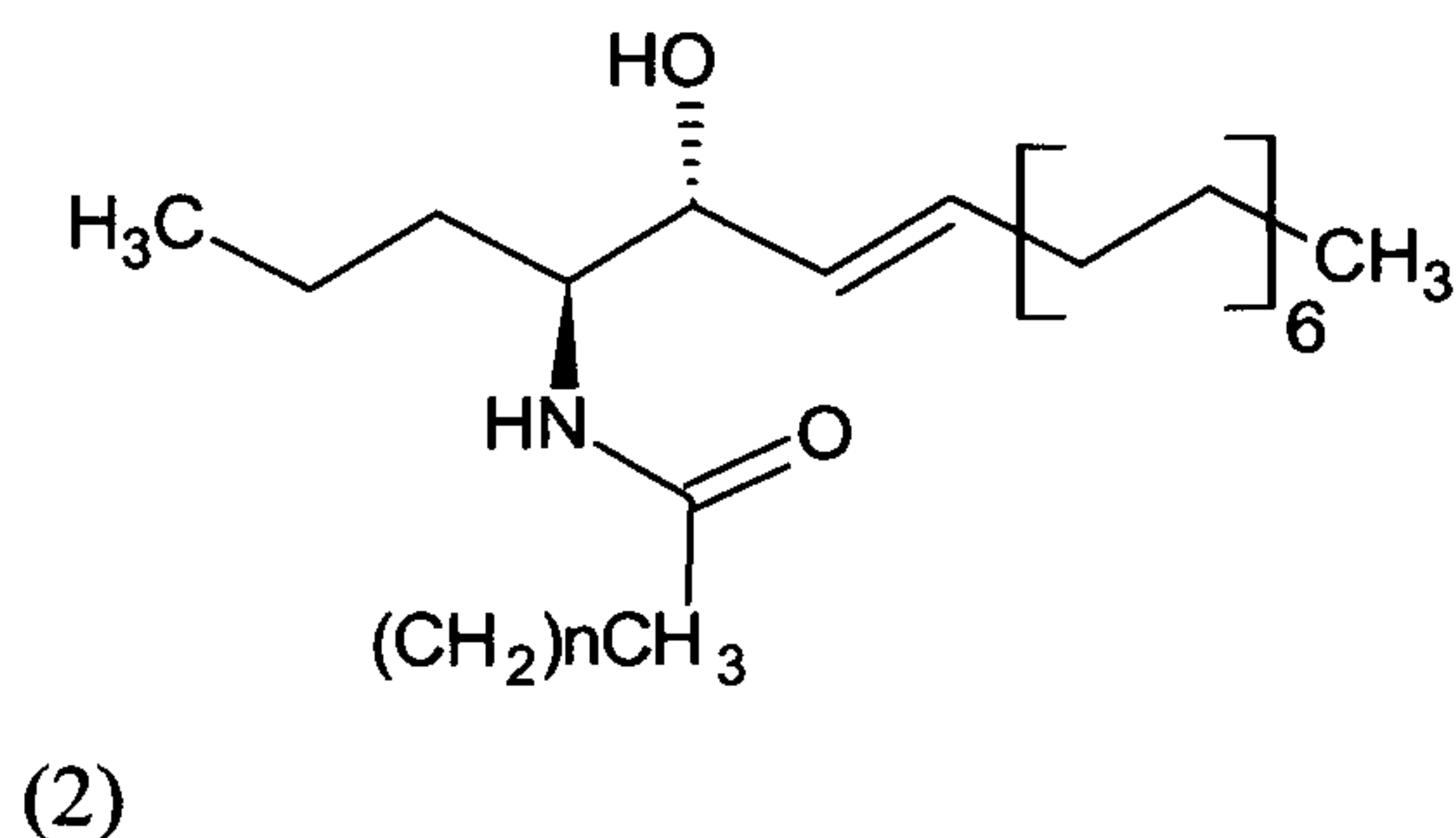
Embodiment 14: The method according to any one of embodiments 11 to 13, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of isoform 1 compound or the level of the total of isoform 1 compound and isoform 2 compound, wherein, preferably as used herein for each and any aspect of the invention, the level of the total of isoform 1 compound and isoform 2 compound is the sum of the level of the isoform 1 compound and the isoform 2 compound.

Embodiment 15: The method according to any one of embodiments 1 to 10, wherein the biomarker is one selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



wherein n is any integer from 18, 20, 22, 24, 26 and 28.

Embodiment 16: The method according to embodiment 15, wherein the biomarker is C26 ceramide.

Embodiment 17: The method according to any one of embodiments 15 to 16, wherein the biomarker is cis-C26 ceramide.

Embodiment 18: The method according to any one of embodiments 15 to 16, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide plus trans-C26 ceramide.

Embodiment 19: The method according to any one of embodiments 15 to 18, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the

level of cis-C26 ceramide or the level of total C26 ceramide, wherein, preferably as used herein for each and any aspect of the invention, the level of total C26 ceramide is the sum of the level of cis-C26 ceramide and the level of trans-C26 ceramide.

Embodiment 20: The method according to any one of embodiments 1 to 19, wherein the biomarker is detected by means of immunoassay, mass spectrometric analysis, affinity chromatography, affinity mass spectrometry, biochip array, functional nucleic acids and/or a derivative of the biomarker, wherein preferably the derivative of the biomarker is a labelled derivative of the biomarker, wherein more preferably the labeled derivative of the biomarker is selected from the group comprising a fluorescent derivative of the biomarker, a biologically labelled derivative of the biomarker, a physically labelled derivative of the biomarker and a chemically labelled derivative of the biomarker.

Embodiment 21: The method according to embodiment 20, wherein the biomarker is detected by means of mass spectrometric analysis.

Embodiment 22: The method according to embodiment 18, wherein mass spectrometric analysis is selected from the group comprising SELDI MS, MALDI MS, ESI MS, DESI MS and ion mobility MS.

Embodiment 23: The method according to any one of embodiments 20 to 22, wherein mass spectrometric analysis uses an analyzer selected from the group comprising ToF, QToF, ion trap, Triple Quad, orbitrap, ion mobility and any combination thereof.

Embodiment 24: The method according to any one of embodiments 20 to 23, wherein the mass spectrometric analysis comprises or uses MS/MS, MRM, SRM or any combination thereof, preferably MS/MS, MS/MRM or MS/SRM.

Embodiment 25: The method according to any one of embodiments 1 to 24, wherein the method comprises protein precipitation from the sample and/or extraction of the biomarker from the sample.

Embodiment 26: The method according to any one of embodiments 1 to 25, wherein the method comprises protein precipitation from the sample.

Embodiment 27: The method according to any one of embodiments 25 to 26, wherein the method comprises HPLC.

Embodiment 28: The method according to embodiment 37, wherein the method comprises

- a) protein precipitation from the sample and HPLC;
- b) extraction of the biomarker from the sample and HPLC; and/or
- c) protein precipitation from the sample, extraction of the biomarker from the sample and HPLC.

Embodiment 29: The method according to any one of embodiments 25 to 26, wherein the method further comprises at least one from the group consisting of HPLC, MS/MS, MRM and MS/MS-MRM.

Embodiment 30: The method according to embodiment 29, wherein the method comprises

- a) protein precipitation from the sample, HPLC and MS/MS or MRM;
- b) protein precipitation from the sample, HPLC and MS/MS.MRM;
- c) extraction of the biomarker from the sample, HPLC and MS/MS or MRM;
- d) extraction of the biomarker from the sample, HPLC and MS/MS.MRM;
- e) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS or MRM; and/or
- f) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS.MRM.

Embodiment 31: The method according to any one of embodiments 1 to 30, wherein the subject is a human being.

Embodiment 32: The method according to embodiment 31, wherein the subject is a human being subject suffering from or being at risk of suffering from cystic fibrosis.

Embodiment 33: The method according to any one of embodiments 7 to 32, wherein step d) comprising detecting the biomarker in a sample comprises subjecting the sample to a protein precipitation and/or biomarker extraction step, precipitating protein and/or extraction biomarker from the sample, providing a supernatant of the sample, subjecting the supernatant of the sample to HPLC and MS/MS and determining the level of the biomarker present in the supernatant and/or extract of the sample.

Embodiment 34: A method for diagnosing cystic fibrosis in a subject, wherein the method comprises the following steps:

- i) adding an internal standard to a sample from the subject, wherein preferably the sample from the subject is selected from the group comprising plasma, serum, blood and a blood product, whereby the sample is more preferably liquid blood or a dried blood spot;
- ii) optionally mixing the sample with the internal standard;
- iii) subjecting the sample to a protein precipitation and/or a biomarker extraction step, whereby protein from the sample is precipitated and/or the biomarker is extracted and a first supernatant of the sample is provided;
- iv) optionally subjecting the first supernatant of the sample or at least a part thereof to a first separation step which provides a second supernatant, whereby preferably the first separation step is a step of centrifugation;
- v) subjecting the first supernatant and/or the second supernatant, or at least a part thereof, to a second separation step, wherein the second separation step comprises injecting at least a part of the first supernatant and/or at least a part of the second supernatant into an HPLC-MS/MS system and using an HPLC column with a gradient from acidic water to acetonitrile/acetone;

wherein the HPLC column is preferably an HPLC column selected from the group comprising a C8 RP-HPLC column and a C18 RP-HPLC column, and wherein the second separation step provides a separated sample;

vi) subjecting the separated sample to MS/MS, wherein MS/MS comprises in source-fragmentation, collision induced dissociation, electron-capture dissociation, electron-transfer dissociation, negative electron dissociation, electron-detachment dissociation, charge transfer dissociation and/or photo dissociation,

and wherein the method comprises

a step a), wherein the step a) comprises detecting a biomarker in a sample from the subject,

and optionally

a step b), wherein the step b) comprises determining a level of the biomarker present in the sample, and

wherein the method is otherwise preferably a method according to any one of embodiments 1 to 33.

Embodiment 35: The method according to embodiment 34, wherein the internal standard is selected from the group comprising N-lauroyl sphingosine, lyso-Gb2, a C17 ceramide, a C19 ceramide, a C21 ceramide, a C23 ceramide and a C25 ceramide.

Embodiment 36: The method according to any one of embodiments 1 to 35, wherein step b), step c) and/or step e) comprises comparing the level of the biomarker in the sample with a cut-off value.

Embodiment 37: The method according to embodiment 36, wherein if the level of the biomarker in the sample from the subject is higher than the cut-off value this is indicative that the subject is suffering from cystic fibrosis or is at risk of suffering from cystic fibrosis.

Embodiment 38: The method according to embodiment 36, wherein if the level of the biomarker in the sample from the subject is lower than the cut-off value this is indicative that the subject is not suffering from or is not at risk of suffering from cystic fibrosis.

Embodiment 39: The method according to any one of embodiments 36 to 38, wherein the cut-off value is selected such that a or the sensitivity for diagnosing cystic fibrosis in a subject is from about 95% to 100%, preferably from about 98.5% to 100% more preferably 99.5% to 100%.

Embodiment 40: The method according to any one of embodiments 1 to 39, wherein step b) and/or step c) and/or step e) comprises that a level of the biomarker in said subject is compared to a level of the biomarker detected in a sample from a control sample.

Embodiment 41: The method according to embodiment 40, wherein the control sample is a sample from a subject not having cystic fibrosis.

Embodiment 42: The method according to any one of embodiments 40 to 41, wherein if the level of the biomarker in the sample from the subject is higher than the level of the biomarker in the control sample, this is indicative that the subject is suffering from and/or is at risk of suffering from cystic fibrosis.

Embodiment 43: The method according to any one of embodiments 1 to 42, wherein the sample from the subject is selected from the group comprising blood, a blood product, urine, saliva, cerebrospinal fluid, stool, tissue sample and lymph.

Embodiment 44: The method according to embodiment 43, wherein the sample from the sample from the subject is selected from the group comprising blood and a blood product.

Embodiment 45: The method according to any one of embodiments 43 to 44, wherein the blood product is dried blood spot (DBS).

Embodiment 46: The method according to any one of embodiments 1 to 45, preferably 45, wherein the method has a limit of detection for C26 ceramide of 0.1 nmol/L blood, wherein, preferably, the biomarker is detected by means of mass spectrometry.

Embodiment 47: The method according to any one of embodiments 36 to 46, wherein the method is for the diagnosis of cystic fibrosis and wherein the cut-off value is 69 nmol/L, preferably 69.1 nmol/L, in case the biomarker is the total of isoform 1 compound and isoform 2 compound, and wherein the cut-off value is 27.7 nmol/L in case the biomarker is isoform 1 compound. Embodiment 48: The method according to any one of embodiments 36 to 46, wherein the method is for the diagnosis of cystic fibrosis and wherein the cut-off value is 69 nmol/L, preferably 69.1 nmol/L, in case the biomarker is total C26 ceramide, and wherein the cut-off value is 27.7 nmol/L in case the biomarker is cis-C26 ceramide.

Embodiment 49: The method of any one of embodiments 47 and 48, wherein the sample from the subject is dried blood spot (DBS).

Embodiment 50: The method according to any one of embodiments 1 to 49, wherein the subject has been previously treated for cystic fibrosis and/or wherein the subject has been previously diagnosed for cystic fibrosis.

Embodiment 51: The method according to any one of embodiments 1 to 50, wherein the subject has not been previously treated for cystic fibrosis and/or wherein the subject has not been previously diagnosed for cystic fibrosis.

Embodiment 52: The method according to any one of embodiments 34 to 51, wherein the method comprises

a step c), wherein the step c) comprises applying, maintaining, reducing, elevating or not applying a therapy based on whether the subject is suffering from cystic fibrosis or is at risk of suffering from cystic fibrosis.

Embodiment 53: The method according to any one of embodiments 34 to 51, wherein the method comprises a step d), wherein the step d) comprises detecting the biomarker in a sample from the subject after a therapy has been applied, maintained, reduced, elevated or not applied in step c).

Embodiment 54: The method according to any one of embodiments 34 to 53, wherein the method comprises a step e), wherein the step e) comprises determining a level of the biomarker in the sample from the subject after a therapy has been applied, maintained, reduced, elevated or not applied in step c).

Embodiment 55: The method according to any one of embodiments 34 to 54, wherein the method comprises a step f), wherein the step f) comprises determining whether the level of the biomarker determined in step b) is lower than the level of the biomarker determined in step f).

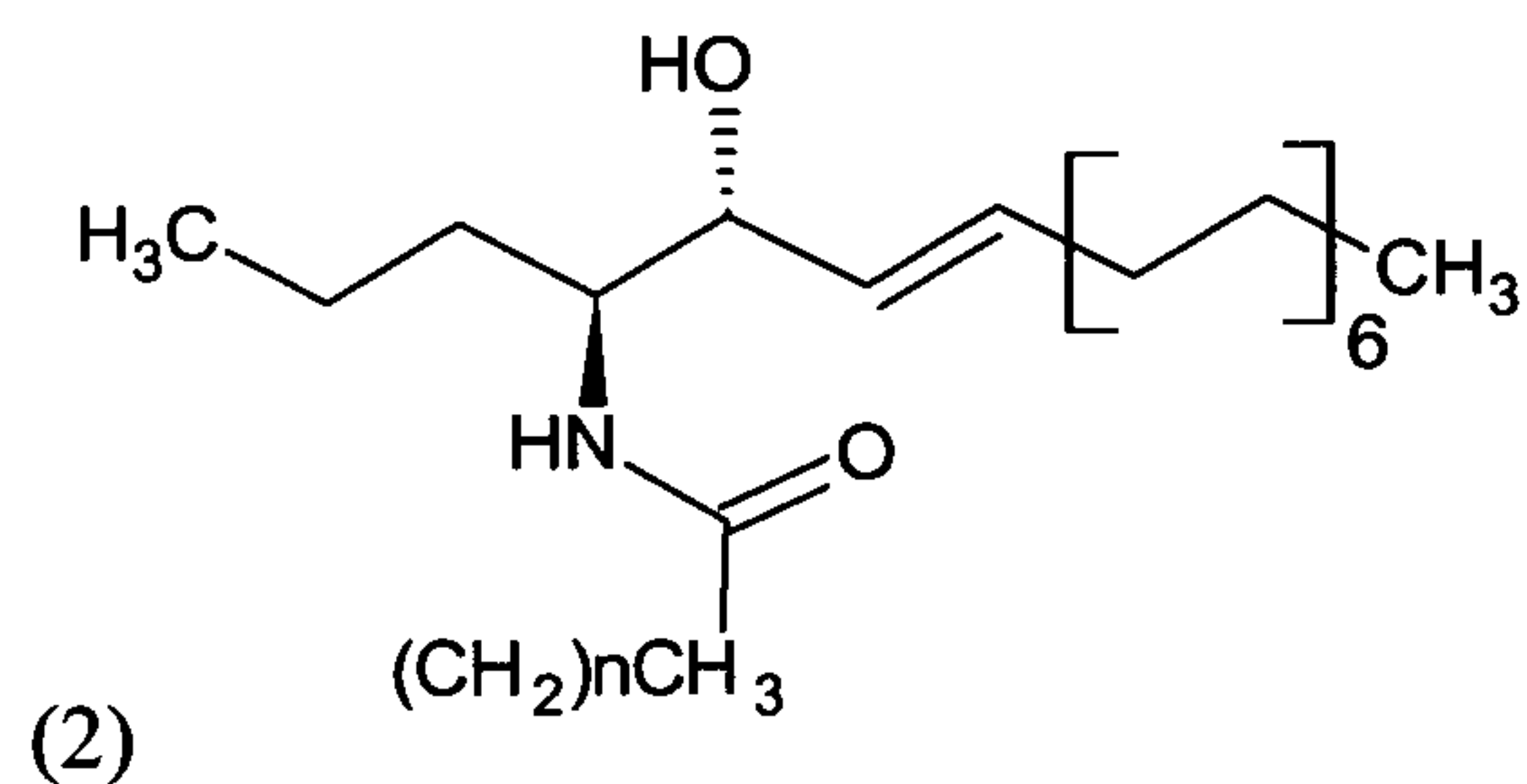
Embodiment 56: The method according to any embodiment 55, wherein the method comprises

a step g), wherein the step g) comprises applying, maintaining, reducing, elevating or not applying a therapy

Embodiment 57: A method for determining the effectiveness of at least one treatment applied to a subject being positively tested for suffering from or being at risk of suffering from cystic fibrosis comprising

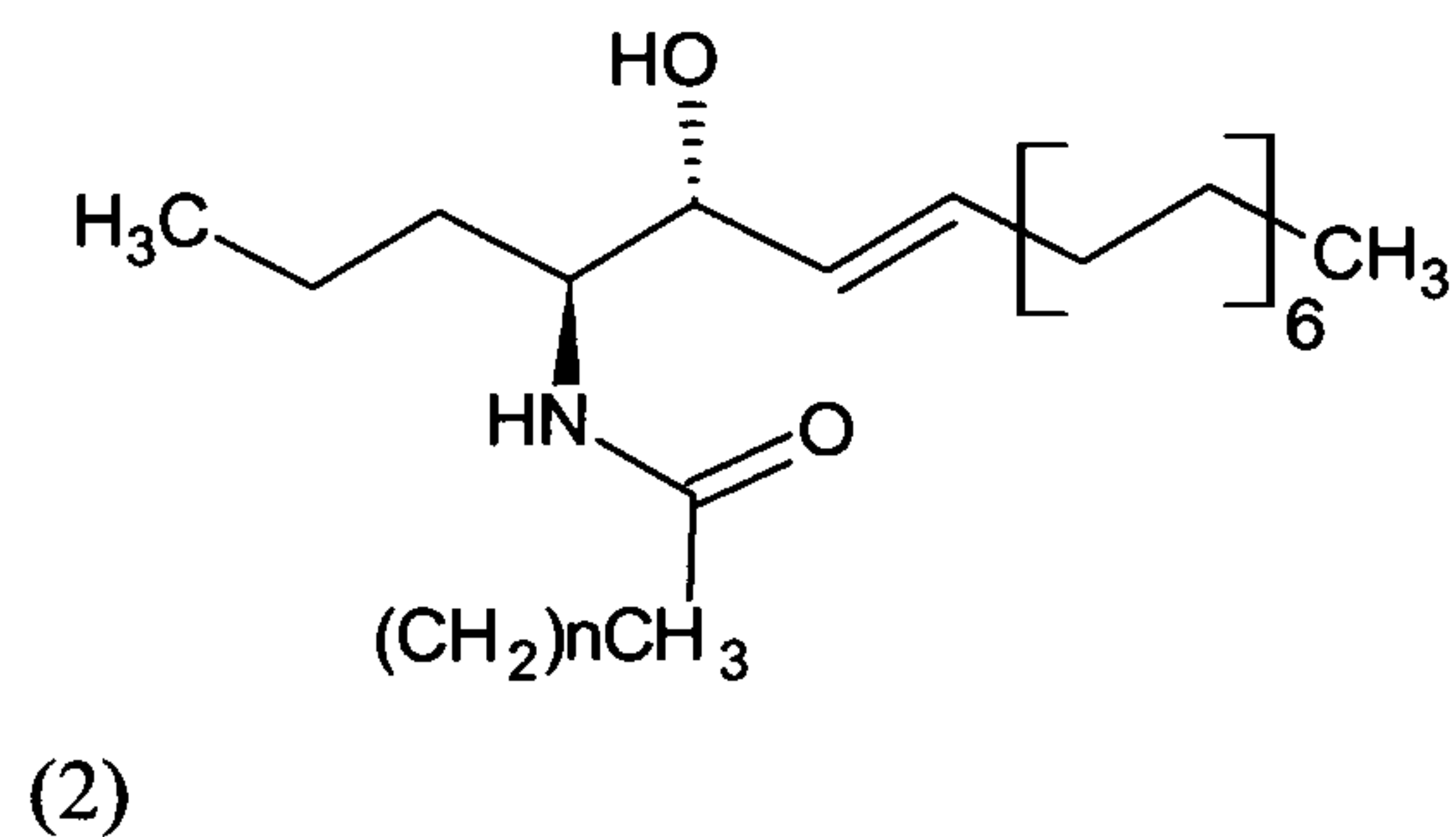
a step a), wherein the step a) comprises determining at several points in time a level of a biomarker in a sample from the subject, wherein the biomarker is preferably one selected from

the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



wherein n is any integer from 18, 20, 22, 24, 26 and 28 or wherein the biomarker is preferably one selected from the group comprising isoform compound 1 and isoform compound 2, wherein

the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z: 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 288.67 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $302.79 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 58: The method according to embodiment 57, wherein the biomarker is C26 ceramide.

Embodiment 59: The method according to any one of embodiments 57 to 58, wherein the biomarker is cis-C26 ceramide.

Embodiment 60: The method according to any one of embodiments 57 to 58, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide and trans-C26 ceramide.

Embodiment 61: The method according to any one of embodiments 57 to 60, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of cis-C26 ceramide or the level of total C26 ceramide, wherein, preferably as used herein for each and any aspect of the invention, the level of total C26 ceramide is the sum of the level of cis-C26 ceramide and the level of trans-C26 ceramide.

Embodiment 62: The method according to any one of embodiment 57, wherein the biomarker is isoform 1 compound, wherein isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 63: The method according to embodiment 57, wherein the biomarker is the total of isoform 1 compound and isoform 2 compound, wherein

the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;

- collision-cross-section: 302.79 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 64: The method according to any one of embodiments 57, 62 and 63, wherein the level of the biomarker in a or the sample, preferably the level of isoform 1 compound or the level of the total of isoform 1 compound and isoform 2 compound, wherein, preferably as used herein for each and any aspect of the invention, the level of total of the isoform 1 compound and isoform 2 compound is the sum of the level of isoform 1 compound e and the level of the isoform 2 compound.

Embodiment 65: The method according to any one of embodiments 57 to 64, wherein the subject has been previously treated for cystic fibrosis or diagnosed for cystic fibrosis.

Embodiment 66: The method according to any one of embodiments 57 to 64, wherein the subject has not been previously treated for cystic fibrosis or wherein the subject has not been previously diagnosed for cystic fibrosis.

Embodiment 67: The method according to any one of embodiments 57 to 66, wherein the method comprises a step b), wherein the step b) comprises applying, maintaining, reducing, elevating or not applying at least one treatment applied to the subject based on the decrease in the level of the biomarker as determined in step a)

Embodiment 68: The method according to any one of embodiments 57 to 67, wherein the method comprises a step c), wherein the step c) comprises detecting the biomarker in the sample from the subject, wherein the sample has been taken prior to the beginning of the treatment after applying, maintaining, reducing, elevating or not applying at least one treatment in step b) and, optionally determining a level of a biomarker present in a sample from the subject,

Embodiment 69: The method according to any one of embodiments 1 to 68, wherein the treatment and/or therapy is selected from the group comprising an inhibitor of acid sphingomyelinase (ASM) and enzyme replacement therapy, whereby preferably the inhibitor is selected from the group comprising Amitriptyline (Elavil), Clomipramine (Anafranil),

Desipramine (Norpramin), Doxepine (Sinequan), Imipramine (Tofranil), Lofepramine (Gamanil), Nortriptyline (Aventyl), Protriptyline (Vivactil) and Trimipramine (Surmontil) and whereby preferably the enzyme of the enzyme replacement therapy is acidic deramindase.

Embodiment 70: The method according to any one of embodiments 57 to 69, wherein the method comprises a step d), wherein the step d) comprises determining whether the level of the biomarker determined in step a) is lower than the level of the biomarker determined in step c);

Embodiment 71: The method according to embodiment 70, wherein the method comprises a step e), wherein step e) comprises applying, maintaining, reducing, elevating or not applying at least one treatment applied to the subject based on step d).

Embodiment 72: The method according to any one of embodiments 34 to 71, wherein the biomarker is detected by means of immunoassay, mass spectrometric analysis, affinity chromatography, affinity – mass spectrometry, biochip array, functional nucleic acids and/or a derivative of the biomarker, wherein preferably the derivative of the biomarker is a labelled derivative of the biomarker, wherein more preferably the labeled derivative of the biomarker is selected from the group comprising a fluorescent derivative of the biomarker, a biologically labelled derivative of the biomarker, a physically labelled derivative of the biomarker and a chemically labelled derivative of the biomarker.

Embodiment 73: The method according to embodiment 72, wherein the biomarker is detected by means of mass spectrometric analysis.

Embodiment 74: The method according to embodiment 73, wherein the biomarker is detected by means of mass spectrometric analysis.

Embodiment 75: The method according to any one of embodiments 73 and 74, wherein mass spectrometric analysis is selected from the group comprising SELDI MS, MALDI MS, ESI MS, DESI MS and ion mobility MS.

Embodiment 76: The method according to any one of embodiments 73 to 75, wherein mass spectrometric analysis uses an analyzer selected from the group comprising ToF, QToF, ion trap, Triple Quad, orbitrap, ion mobility and any combination thereof.

Embodiment 77: The method according to any one of embodiments 73 to 76, wherein the mass spectrometric analysis comprises or uses MS/MS, MRM, SRM or any combination thereof, preferably MS/MS, MS/MRM or MS/SRM.

Embodiment 78: The method according to any one of embodiments 57 to 77, wherein the method comprises protein precipitation from the sample and/or extraction of the biomarker from the sample.

Embodiment 79: The method according to any one of embodiments 57 to 78, wherein the method comprises protein precipitation from the sample.

Embodiment 80: The method according to any one of embodiments 78 to 79, wherein the method comprises HPLC.

Embodiment 81: The method according to embodiment 80, wherein the method comprises

- a) protein precipitation from the sample and HPLC;
- b) extraction of the biomarker from the sample and HPLC; and/or
- c) protein precipitation from the sample, extraction of the biomarker from the sample and HPLC.

Embodiment 82: The method according to any one of embodiments 78 to 79, wherein the method further comprises at least one from the group consisting of HPLC, MS/MS, MRM and MS/MS-MRM.

Embodiment 83: The method according to embodiment 82, wherein the method comprises

- a) protein precipitation from the sample, HPLC and MS/MS or MRM;

- b) protein precipitation from the sample, HPLC and MS/MS.MRM;
- c) extraction of the biomarker from the sample, HPLC and MS/MS or MRM;
- d) extraction of the biomarker from the sample, HPLC and MS/MS.MRM;
- e) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS or MRM; and/or
- f) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS.MRM.

Embodiment 84: The method according to any one of embodiments 57 to 83, wherein the subject is a human being.

Embodiment 85: The method according to embodiment 84, wherein the subject is a subject suffering from or being at risk of suffering from cystic fibrosis.

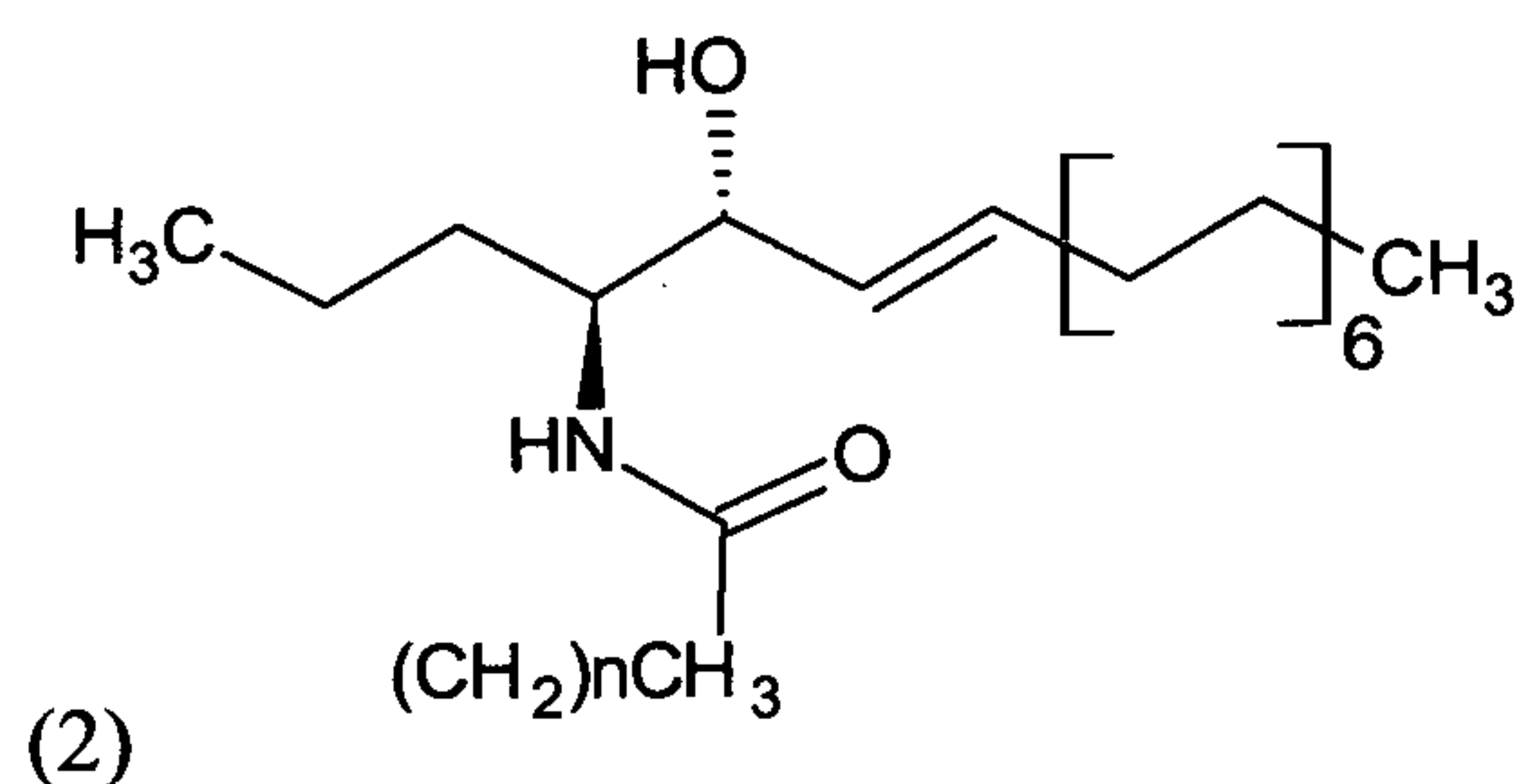
Embodiment 86: The method according to any one of embodiments 57 to 85, wherein the step of detecting the biomarker in the sample from the subject comprises precipitating protein and/or biomarker extraction from the sample from the subject, wherein precipitating protein and/or extracting biomarker from the sample provides a supernatant of the sample; subjecting a volume of the supernatant to HPLC and MS/MS and determining the level of the biomarker that is present in the sample from the subject.

Embodiment 87: A method of determining the effectiveness of a compound for the treatment of cystic fibrosis, wherein the method comprises the following steps:

- a) determining a level of a biomarker in a sample from a subject having cystic fibrosis;
- b) administering to said subject said compound;
- c) determining the level of the biomarker in a sample from the subject after the compound has been administered to the subject; and
- d) determining whether the level of the biomarker determined in step c) is lower than the level of the biomarker determined in step a);

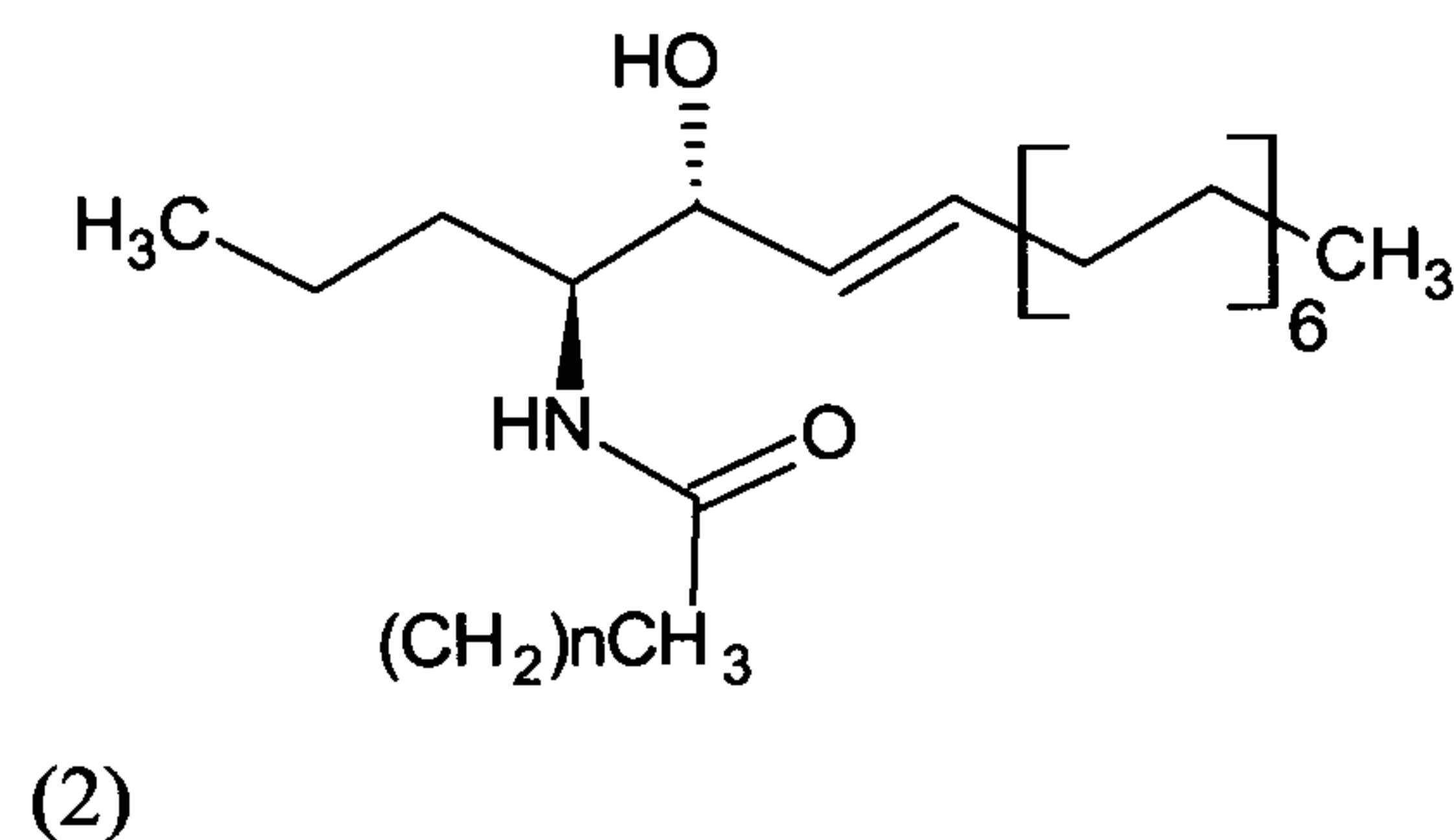
wherein if a level of the biomarker determined in step c) is lower than the level of the biomarker determined in step a) this indicates the effectiveness of said compound.

Embodiment 88: The method of embodiment 87, wherein the biomarker is one selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



wherein n is any integer from 18, 20, 22, 24, 26 and 28.

Embodiment 89: The method according to embodiment 88, wherein the biomarker is C26 ceramide.

Embodiment 90: The method according to any one of embodiments 88 to 89, wherein the biomarker is cis-C26 ceramide.

Embodiment 91: The method according to any one of embodiments 88 to 89, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide and trans-C26 ceramide.

Embodiment 92: The method according to any one of embodiments 88 to 91, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of cis-C26 ceramide or the level of total C26 ceramide, wherein, preferably as used herein for each and any aspect of the invention, the level of total C26 ceramide is the sum of the level of cis-C26 ceramide and the level of trans-C26 ceramide.

Embodiment 93: The method according to embodiment 87, wherein the biomarker is isoform 1 compound, wherein isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 94: The method according to embodiment 87, wherein the biomarker is the total of isoform 1 compound and isoform 2 compound, wherein

the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or

- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $302.79 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 95: The method according to any one of embodiments 93 to 94, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of isoform 1 compound or the level of the total of isoform 1 compound and isoform 2 compound, wherein, preferably as used herein for each and any aspect of the invention, the level of the total of isoform 1 compound and isoform 2 compound is the sum of the level of isoform 1 compound and the level of isoform 2 compound.

Embodiment 96: The method according to any one of embodiments 92 and 95, wherein the method comprises determining a level of the biomarker in a control sample.

Embodiment 97: The method according to any one of embodiments 87 to 96, wherein the biomarker is detected by means of immunoassay, mass spectrometric analysis, biochip array, functional nucleic acids and/or a fluorescent derivative of the biomarker.

Embodiment 98: The method according to embodiment 97, wherein the biomarker is detected by means of mass spectrometric analysis.

Embodiment 99: The method according to embodiment 98, wherein mass spectrometric analysis is selected from the group comprising SELDI MS, MALDI MS, ESI MS, DESI MS and ion mobility MS.

Embodiment 100: The method according to any one of embodiments 97 to 99, wherein mass spectrometric analysis uses an analyzer selected from the group comprising ToF, QToF, ion trap, Triple Quad, orbitrap, ion mobility and any combination thereof.

Embodiment 101; The method according to any one of embodiments 97 to 100, wherein the mass spectrometric analysis comprises or uses MS/MS, MRM, SRM or any combination thereof, preferably MS/MS, MS/MRM or MS/SRM.

Embodiment 102: The method according to any one of embodiments 87 to 101, wherein the method comprises protein precipitation from the sample and/or extraction of the biomarker from the sample.

Embodiment 103: The method according to any one of embodiments 87 to 102, wherein the method comprises protein precipitation from the sample.

Embodiment 104: The method according to any one of embodiments 102 to 103, wherein the method comprises HPLC.

Embodiment 105: The method according to embodiment 104, wherein the method comprises

- a) protein precipitation from the sample and HPLC;
- b) extraction of the biomarker from the sample and HPLC; and/or
- c) protein precipitation from the sample, extraction of the biomarker from the sample and HPLC.

Embodiment 106: The method according to any one of embodiments 102 to 103, wherein the method further comprises at least one from the group consisting of HPLC, MS/MS, MRM and MS/MS-MRM.

Embodiment 107: The method according to embodiment 106, wherein the method comprises

- a) protein precipitation from the sample, HPLC and MS/MS or MRM;
- b) protein precipitation from the sample, HPLC and MS/MS.MRM;
- c) extraction of the biomarker from the sample, HPLC and MS/MS or MRM;
- d) extraction of the biomarker from the sample, HPLC and MS/MS.MRM;
- e) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS or MRM; and/or
- f) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS.MRM.

Embodiment 108: The method according to any one of embodiments 87 to 107, wherein the subject is a human being.

Embodiment 109: The method according to embodiment 108, wherein the subject is a subject suffering from or being at risk of suffering from cystic fibrosis.

Embodiment 110: A method for determining the course of cystic fibrosis in a subject comprising the step of

- a) determining at several points in time a level of a biomarker present in a sample from the subject.

Embodiment 111: The method according to embodiment 110, wherein the subject has been previously treated or diagnosed for cystic fibrosis.

Embodiment 112: The method according to embodiment 110, wherein the subject has not been previously treated or wherein the subject has not been previously diagnosed for cystic fibrosis.

Embodiment 113: The method according to any one of embodiments 110 to 112, further comprising a step of

- b) applying, maintaining, reducing, elevating or not applying a therapy based on the diagnosis of whether the subject is suffering from or for being at risk for developing cystic fibrosis.

Embodiment 114: The method according to any one of embodiments 110 to 113, further comprising a step of

- c) detecting the biomarker in a sample from the subject after applying, maintaining, reducing, elevating or not applying a therapy in a step of b).

Embodiment 115: The method according to any one of embodiments 110 to 114, further comprising a step of

- d) determining a level of the biomarker in the sample from the subject after applying, maintaining, reducing, elevating or not applying a therapy in a step of b).

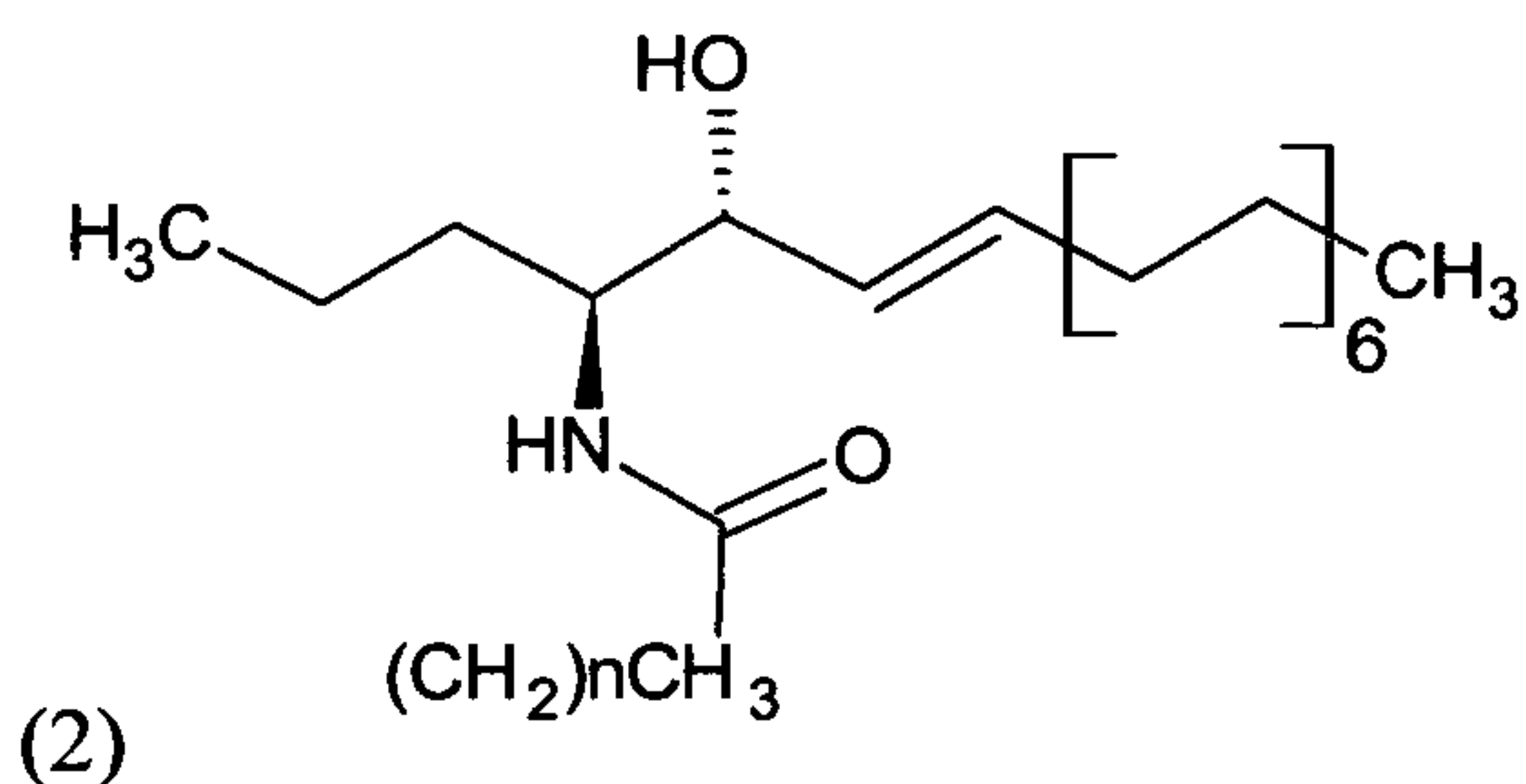
Embodiment 116: The method according to any one of embodiments 110 to 115, further comprising the steps of

- e) determining whether the level of the biomarker determined in step a) is lower than the level of the biomarker determined in step d);

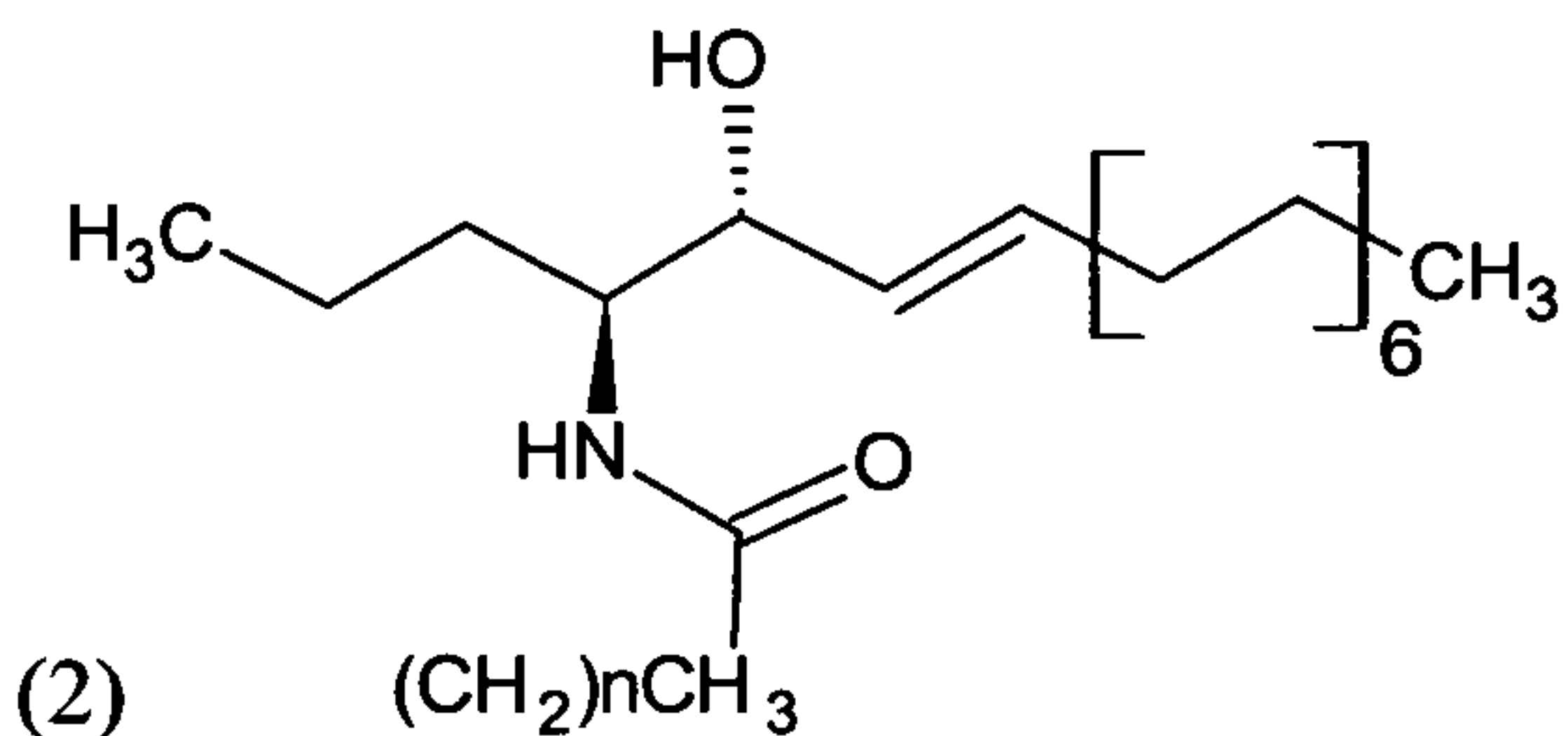
Embodiment 117: The method according to any embodiment 116, further comprising the step of

- f) applying, maintaining, reducing, elevating or not applying a therapy based on the step of e).

Embodiment 118: The method according to any one of embodiments 110 to 117, wherein the biomarker is one selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and a long ceramide is a ceramide of formula (2)



wherein n is any integer from 18, 20, 22, 24, 26 and 28.

Embodiment 119: The method according to embodiment 118, wherein the biomarker is C26 ceramide.

Embodiment 120: The method according to any one of embodiments 118 to 119, wherein the biomarker is cis-C26 ceramide.

Embodiment 121: The method according to any one of embodiments 118 to 119, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide and trans-C26 ceramide.

Embodiment 122: The method according to any one of embodiments 118 to 121, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of cis-C26 ceramide or the level of total C26 ceramide, wherein, preferably as used herein for each and any aspect of the invention, the level of total C26 ceramide is the sum of the level of cis-C26 ceramide and the level of trans-C26 ceramide.

Embodiment 123: The method according to any one of embodiments 110 to 117, wherein the biomarker is isoform 1 compound and wherein isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z: 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 124: The method according any one of embodiments 110 to 117, wherein the biomarker is the total of isoform 1 compound and isoform 2 compound, wherein

the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z: 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or

- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristic as measured with a high resolution ion mobility mass spectrometer s:

- molecular mass m/z: 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 302.79 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 125: The method according to any one of embodiments 123 to 124, wherein the method comprises determining the level of the biomarker in a or the sample, preferably the level of isoform 1 compound or the level of the total of isoform 1 compound and isoform 2 compound, wherein, preferably as used herein for each and any aspect of the invention, the level of the total of isoform 1 compound and isoform 2 compound is the sum of the level of isoform 1 compound and the level of isoform 2 compound.

Embodiment 126: The method according to any one of embodiments 110 to 125, wherein the biomarker is detected by means of immunoassay, mass spectrometric analysis, biochip array, functional nucleic acids and/or a fluorescent derivative of the biomarker.

Embodiment 127: The method according to embodiment 126, wherein the biomarker is detected by means of mass spectrometric analysis.

Embodiment 128: The method according to embodiment 127, wherein mass spectrometric analysis is selected from the group comprising SELDI MS, MALDI MS, ESI MS, DESI MS and ion mobility MS.

Embodiment 129: The method according to any one of embodiments 126 to 128, wherein mass spectrometric analysis uses an analyzer selected from the group comprising ToF, QToF, ion trap, Triple Quad, orbitrap, ion mobility and any combination thereof.

Embodiment 130: The method according to any one of embodiments 126 to 129, wherein the mass spectrometric analysis comprises or uses MS/MS, MRM, SRM or any combination thereof, preferably MS/MS, MS/MRM or MS/SRM.

Embodiment 131: The method according to any one of embodiments 110 to 130, wherein the method comprises protein precipitation from the sample and/or extraction of the biomarker from the sample.

Embodiment 132: The method according to any one of embodiments 110 to 131, wherein the method comprises protein precipitation from the sample.

Embodiment 133: The method according to any one of embodiments 131 to 132, wherein the method comprises HPLC.

Embodiment 134: The method according to embodiment 133, wherein the method comprises

- a) protein precipitation from the sample and HPLC;
- b) extraction of the biomarker from the sample and HPLC; and/or
- c) protein precipitation from the sample, extraction of the biomarker from the sample and HPLC.

Embodiment 135: The method according to any one of embodiments 131 to 132, wherein the method further comprises at least one from the group consisting of HPLC, MS/MS, MRM and MS/MS-MRM.

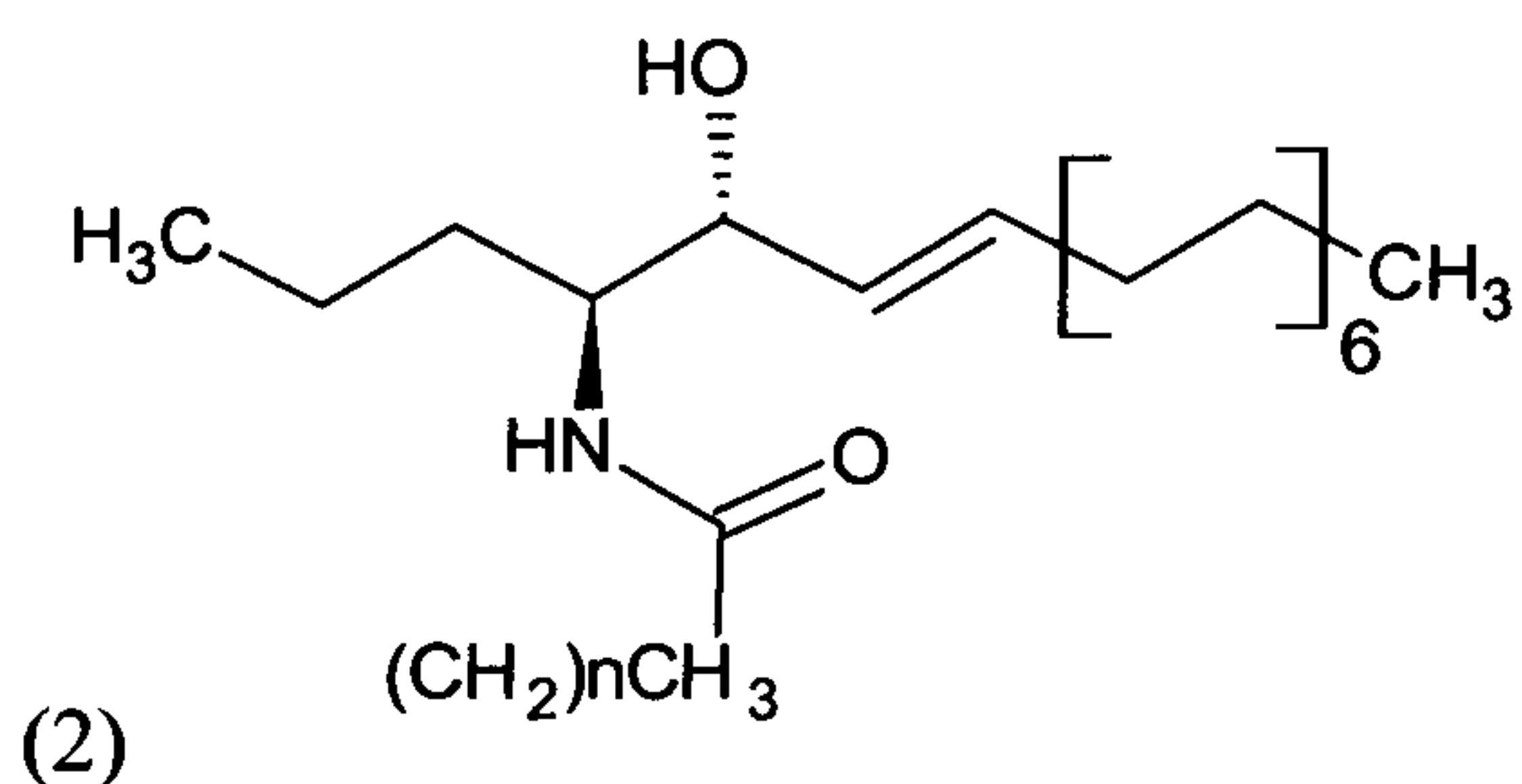
Embodiment 136: The method according to embodiment 135, wherein the method comprises

- a) protein precipitation from the sample, HPLC and MS/MS or MRM;
- b) protein precipitation from the sample, HPLC and MS/MS.MRM;
- c) extraction of the biomarker from the sample, HPLC and MS/MS or MRM;
- d) extraction of the biomarker from the sample, HPLC and MS/MS.MRM;
- e) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS or MRM; and/or
- f) protein precipitation and extraction of the biomarker from the sample, HPLC and MS/MS.MRM.

Embodiment 137: The method according to any one of embodiments 110 to 136, wherein the subject is a human being.

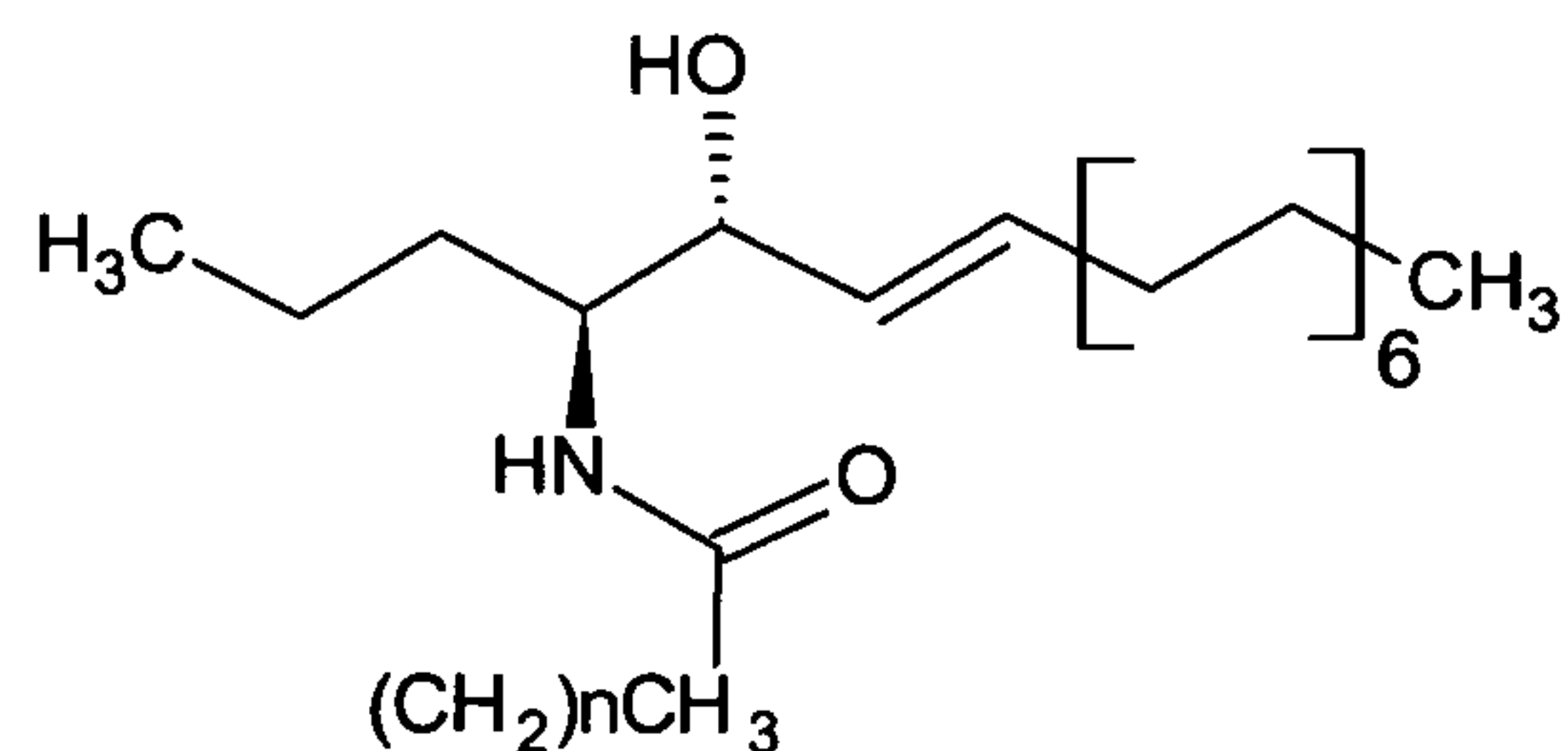
Embodiment 138: The method according to embodiment 137, wherein the subject is a subject suffering from or being at risk of suffering from cystic fibrosis.

Embodiment 139: Use of mass spectrometric analysis for the detection of a biomarker, wherein the biomarker is selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



(2)

wherein n is any integer from 18, 20, 22, 24, 26 and 28.

Embodiment 140: Use according to embodiment 139, wherein the biomarker is C26 ceramide.

Embodiment 141: Use according to any one of embodiments 139 to 140, wherein the biomarker is cis-C26 ceramide.

Embodiment 142: Use according to any one of embodiments 139 to 141, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide and trans-C26 ceramide.

Embodiment 143: Use of mass spectrometric analysis for the detection of a biomarker, wherein the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, the isoform 1 compound comprises the following characteristics:

- molecular mass m/z: 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 288.67 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

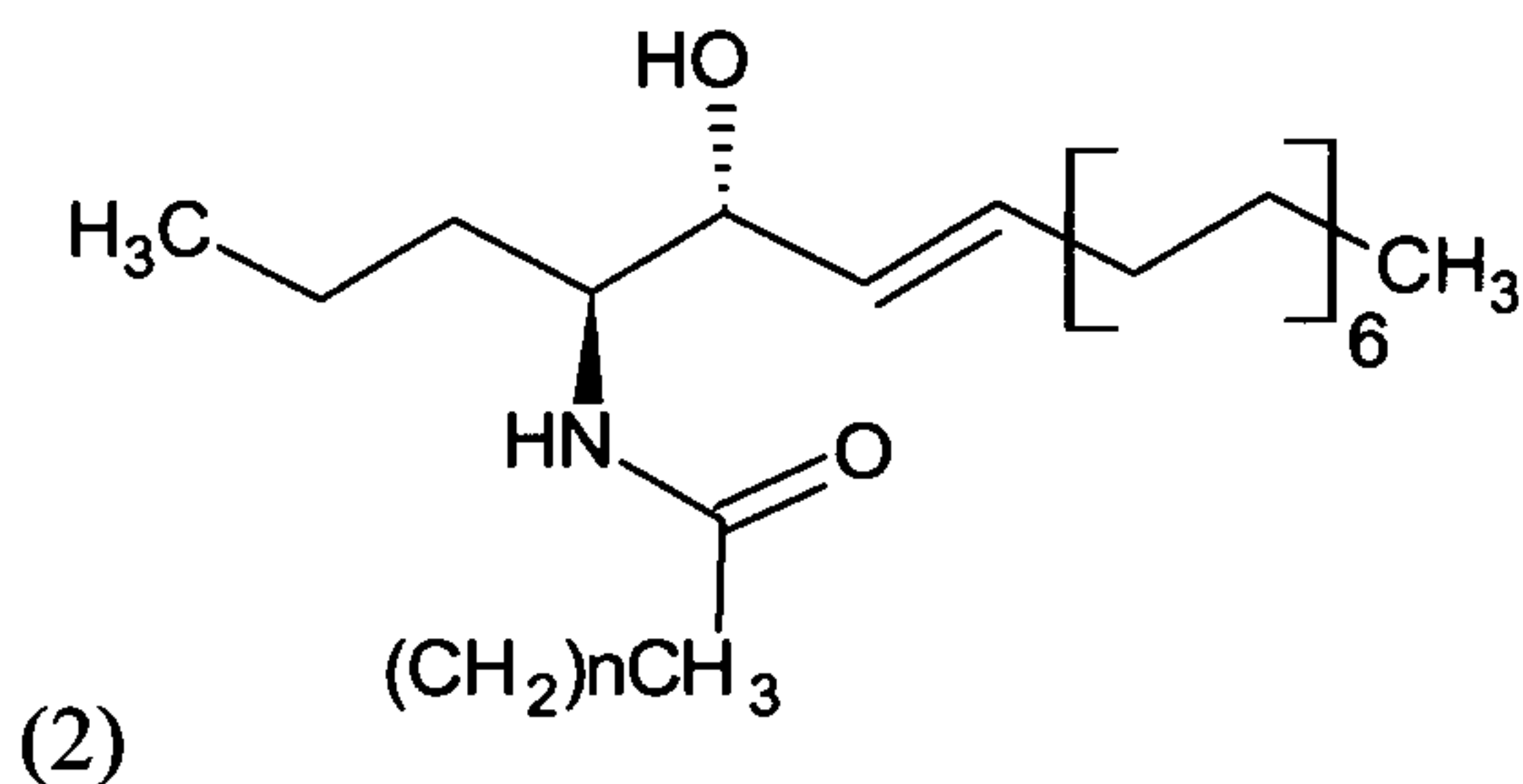
the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $302.79 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 144: Use according to any one of embodiments 139 to 143, wherein the detection comprises the use of HPLC.

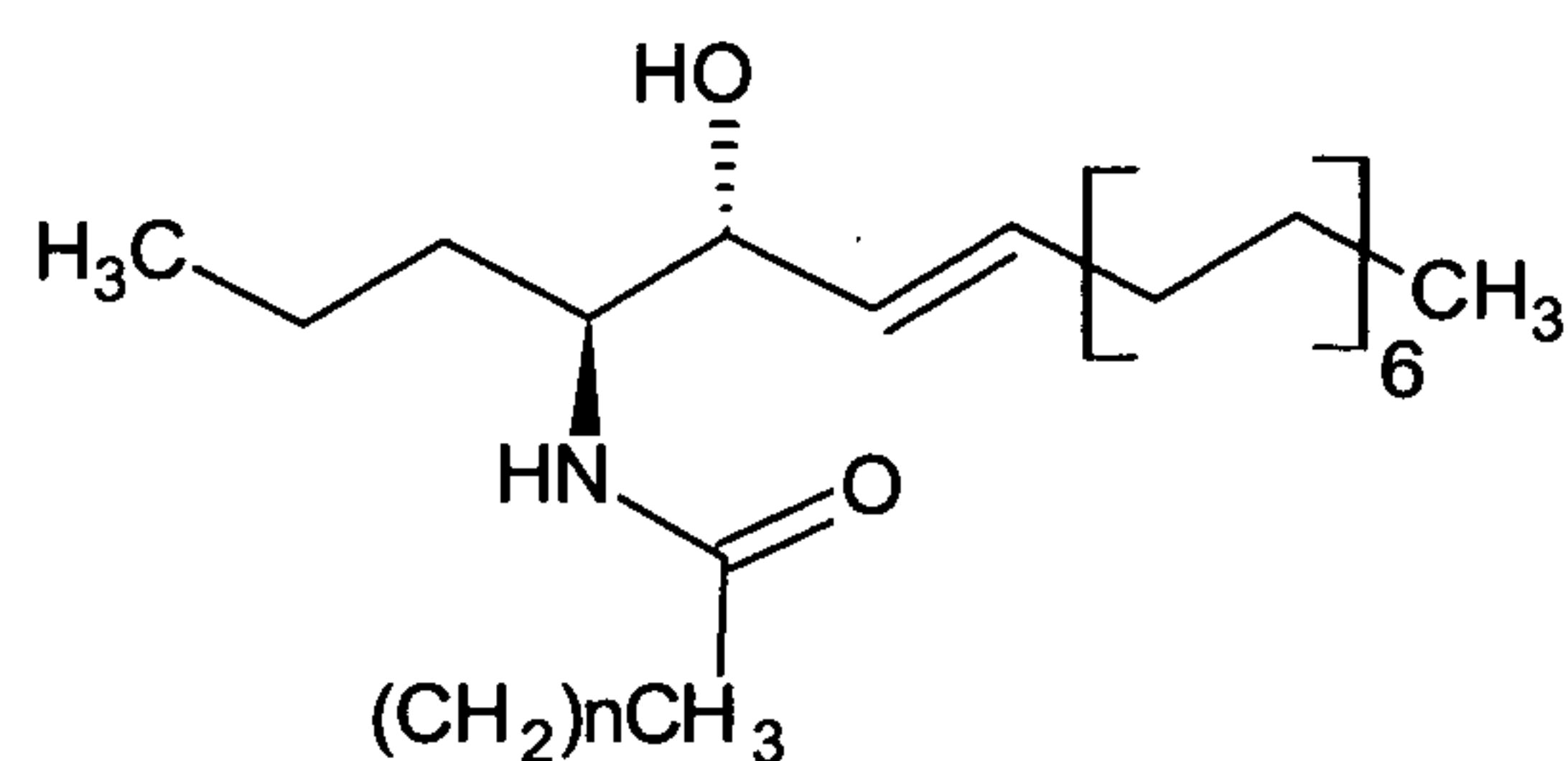
Embodiment 145: Use according to any one of embodiments 139 to 144, wherein the mass spectrometric analysis comprises or uses MS/MS.

Embodiment 146: Use of a biomarker for the diagnosis of cystic fibrosis, preferably in a method according to any one of embodiments 1 to 138, wherein the biomarker is selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



(2)

wherein n is any integer from 18, 20, 22, 24, 26 and 28.

Embodiment 147: Use according to embodiment 146, wherein the biomarker is C26 ceramide.

Embodiment 148: Use according to any one of embodiments 146 to 147, wherein the biomarker is cis-C26 ceramide.

Embodiment 149: Use according to any one of embodiments 146 to 148, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide and trans-C26 ceramide.

Embodiment 150: Use of a biomarker for the diagnosis of cystic fibrosis, preferably in a method according to any one of embodiments 1 to 138, wherein the biomarker is selected from the group comprising isoform 1 compound, isoform 2 compound and the total of isoform 1 compound and isoform 2 compound, the isoform 1 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z: 678.4847;
- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 288.67Å²; and/or

- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44;

and

the isoform 2 compound comprises the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: 302.79 Å²; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

Embodiment 151: A kit for determining the presence of a biomarker in a sample from a subject, wherein the kit comprises

- a) an interaction partner of the biomarker;
- b) optionally a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds the biomarker; and
- c) instructions for using the solid support to detect the biomarker, wherein the biomarker is C26 ceramide, preferably cis-C26 ceramide.

Embodiment 152: The kit according to embodiment 151, wherein the kit is for

- a) use in a method for diagnosing cystic fibrosis;
- b) use in a method for determining the course of cystic fibrosis in a subject; and/or
- c) use in a method for determining the effectiveness of at least one treatment applied to a subject,

wherein preferably the method of a), b) and/or c) is a method according to any one of embodiments 1 to 138.

Embodiment 153: Use of a compound selected from the group consisting of N-lauroyl sphingosine, lyso-Gb2, a C17 ceramide, a C19 ceramide, a C21 ceramide, a C23 ceramide and a C25 ceramide as an internal standard in

- a) use in a method for diagnosing cystic fibrosis;
- b) use in a method for determining the course of cystic fibrosis in a subject; and/or
- c) use in a method for determining the effectiveness of at least one treatment applied to a subject,

wherein preferably the method of a), b) and/or c) is a method according to any one of embodiments 1 to 138.

The present inventors have surprisingly found that compound C26 ceramide constitutes a biomarker which allows for a method for diagnosing cystic fibrosis in a subject, more specifically diagnosing cystic fibrosis in a subject with high specificity and sensitivity using said C26 ceramide as the biomarker. In a preferred embodiment of the present invention C26 ceramide is cis-C26 ceramide. In a further embodiment of the present invention C26 ceramide is total C26 ceramide. In light of these embodiments, it is within the present invention that whenever it is referred to C26 ceramide, such reference also includes any embodiment of C26 ceramide, including cis-C26 ceramide and total C26 ceramide.

The present inventors have also surprisingly found that isoform 1 compound, isoform 2 compound and the total of isoform compound 1 and isoform compound 2 each constitutes a biomarker which allows for a method for diagnosing cystic fibrosis in a subject, more specifically diagnosing cystic fibrosis in a subject with high specificity and sensitivity. In a preferred embodiment of the present invention the biomarker is isoform 1 compound. In a further embodiment of the present invention the biomarker ceramide is the total of isoform 1 compound and isoform 2 compound.

The present inventors have also surprisingly found that the level of total C26 ceramide and cis-C26 ceramide in particular, determined in the sample from a subject by the methods of the present invention allows for diagnosing cystic fibrosis with high sensitivity and high specificity.

The present inventors have also surprisingly found that the level of the total of isoform compound 1 and isoform compound 2, and the level of isoform 1 compound in particular, determined in the sample from a subject by the methods of the present invention allows for diagnosing cystic fibrosis with high sensitivity and high specificity.

Furthermore, the present inventors have also surprisingly found that total C26 ceramide and cis-C26 ceramide in particular, which can be detected by the methods of the present invention, is circulating in the blood of a subject. Moreover, the present inventors have surprisingly found that total C26 ceramide and cis-C26 ceramide in particular, which is present in the blood of a subject is useful in a method for diagnosing cystic fibrosis in a subject comprising a step of detecting a biomarker in a sample from the subject, wherein the biomarker is total C26 ceramide and cis-C26 ceramide in particular. The present inventors have also surprisingly found that the level of total C26 ceramide and cis-C26 ceramide in particular determined in the sample from a subject by the methods of the present invention allows for diagnosing cystic fibrosis with high sensitivity and high specificity.

Furthermore, the present inventors have also surprisingly found that total of the isoform 1 compound and isoform 2 compound, and isoform 1 compound in particular, which can be detected by the methods of the present invention, is circulating in the blood of a subject. Moreover, the present inventors have surprisingly found that the total of isoform 1 compound and isoform 2 compound, and isoform 1 compound in particular, which is present in the blood of a subject, is useful in a method for diagnosing cystic fibrosis in a subject comprising a step of detecting a biomarker in a sample from the subject, wherein the biomarker is the total of isoform 1 compound and isoform 2 compound, and isoform 1 compound in particular. The present inventors have also surprisingly found that the level of the total of isoform 1 compound and isoform 2 compound, and isoform 1 compound in particular determined in the sample from

a subject by the methods of the present invention allows for diagnosing cystic fibrosis with high sensitivity and high specificity.

The present inventors also have surprisingly found that total C26 ceramide and cis-C26 ceramide is a useful biomarker contained in blood and any blood product derived therefrom such as plasma, serum or dried blood spots, for use in any of the methods and uses disclosed herein.

The present inventors also have surprisingly found that the total of isoform 1 compound and isoform 2 compound, and isoform 1 compound in particular is a useful biomarker contained in blood and any blood product derived therefrom such as plasma, serum or dried blood spots, for use in any of the methods and uses disclosed herein.

Finally, the present inventors have surprisingly found that the total concentration of C26 ceramide in a sample from a subject, which is preferably the sum of cis-C26 ceramide and trans-C26 ceramide in said sample, allows distinguishing a subject being carrier of cystic fibrosis from a subject being a non-carrier of cystic fibrosis, preferably such non-carrier of cystic fibrosis is a healthy subject.

Finally, the present inventors have surprisingly found that the total of isoform 1 compound and isoform 2 compound in a sample from a subject, which is preferably the sum of isoform 1 compound and isoform 2 compound in said sample, allows distinguishing a subject being carrier of cystic fibrosis from a subject being a non-carrier of cystic fibrosis, preferably such non-carrier of cystic fibrosis is a healthy subject.

In connection with the instant invention it is referred to the concentration or level of C26 ceramide. Such concentration or level of C26 ceramide is preferably determined as follows. In the analytical set-up as described in the example part in more detail an internal standard is added to the sample to be analyzed. In the course of such analysis a chromatogram is obtained indicating as individual peaks the various compounds detected in the sample. The various compounds include, among others, C26 ceramide and the internal standard. In order to

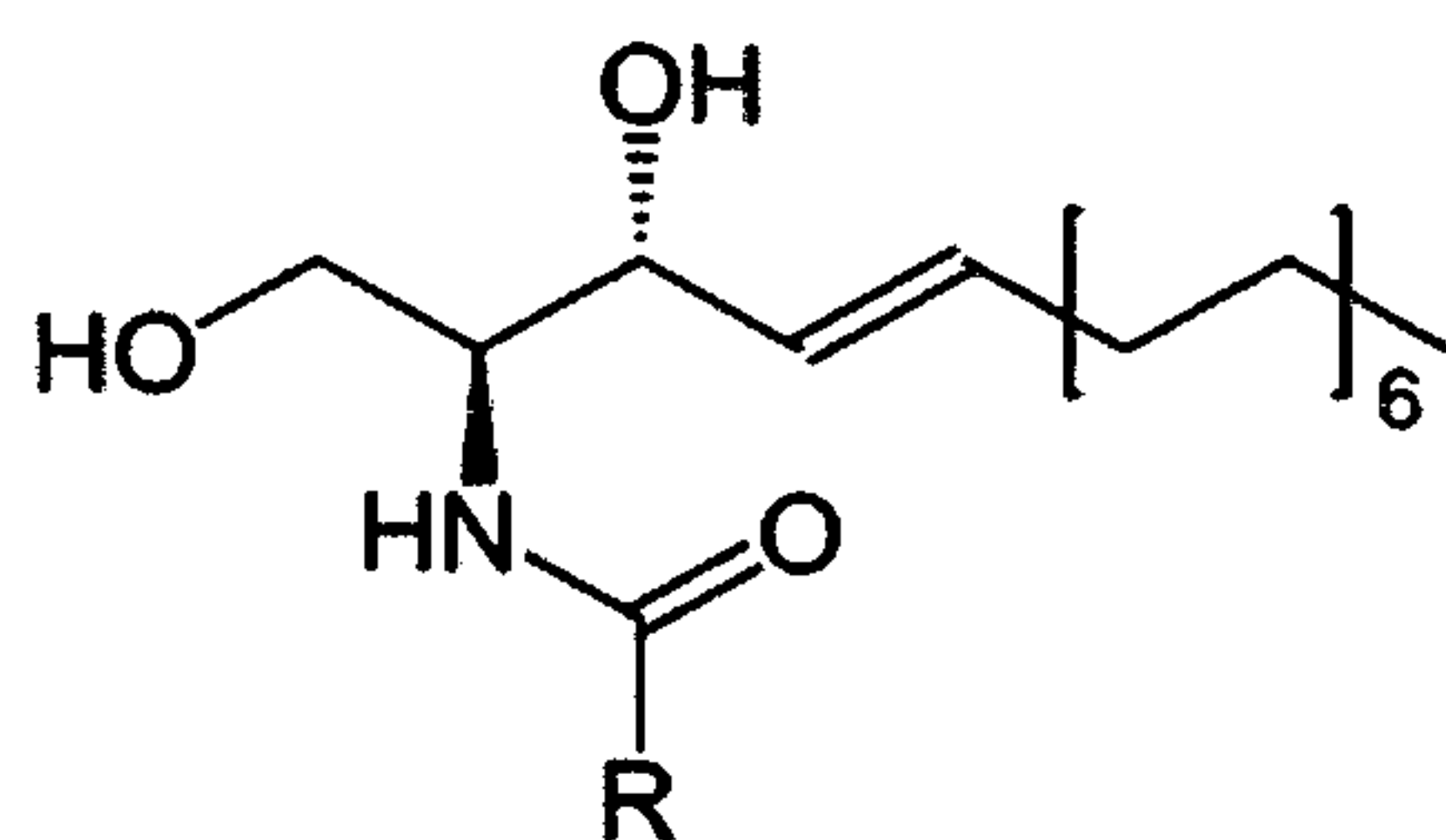
determine from such chromatogram and the peaks indicated therein the concentration or level of C26 ceramide the peak area of the peak corresponding to C26 ceramide and the peak area of the peak corresponding to the internal standard is determined. The concentration of C26 ceramide is obtained using a standard curve of the C26 ceramide at different concentrations in the presence of internal standard at known concentration. In those embodiments of the methods of the present invention where the concentration or level of C26 ceramide is used, the concentration of C26 ceramide is preferably the normalized concentration of C26 ceramide. The same equally applies to the concentration or level of isoform compound 1, isoform compound 2 and the total of isoform compound 1 and isoform compound 2.

Cystic fibrosis (abbr.) is a genetic disease inherited in an autosomal recessive manner. It is caused by the presence of mutations in both copies of the gene for the cystic fibrosis transmembrane conductance regulator (abbr. CFTR) protein. The mechanism(s) underlying ceramide accumulation in CF are complex, but in part are due to alterations in lung pH that favor high activity of the ceramide producing enzyme, acid sphingomyelinase (abbr. ASM), and low activity of the ceramide clearing enzyme, acid ceramidase (abbr. AC). Previous studies suggested that a defect in the cystic fibrosis transmembrane regulator protein (abbr. CFTR) may itself be responsible for high ceramide levels in cystic fibrosis patients. High lung ceramide levels amplify the inflammatory pathology in cystic fibrosis patients and directly drive susceptibility to pathogens. Healthy individuals maintain low levels of ceramide in their lung epithelial cells through the regulated activities of the enzymes ASM and AC. In cystic fibrosis lungs ceramide levels become high due, in part, to alterations of these enzymatic activities, typically high ASM and low AC. In addition, mutations in CFTR may alter delivery of the protein to the membrane rafts which also contributes to ceramide elevation.

Mutations of CFTR protein associated with cystic fibrosis and of CFTR mutations tested in the diagnosis of cystic fibrosis are known to the person skilled in the art and can be retrieved from scientific papers using routine measures.

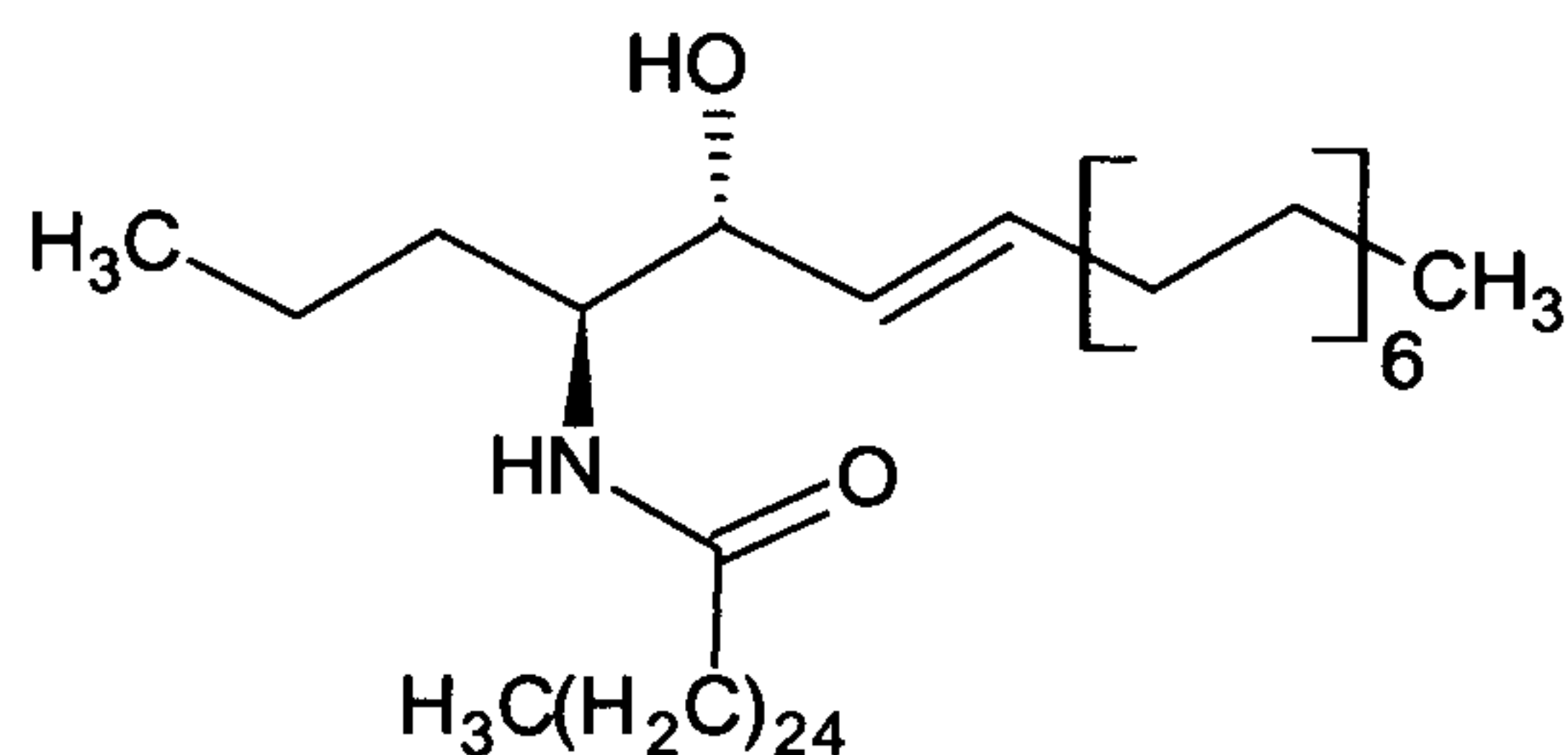
Ceramides are a family of lipid molecules. A ceramide is composed of sphingosine and a fatty acid. Ceramides are found in high concentrations within the cell membrane of cells. They are

one of the component lipids that make up sphingomyelin, one of the major lipids in the lipid bilayer membrane of the cells. Ceramides and other sphingolipids found in cell membrane are not purely structural elements, they can participate in a variety of cellular signaling: examples include regulating differentiation, proliferation, and programmed cell death of cells. A typical ceramide has the formula:



wherein R indicates the alkyl portion of the fatty acid.

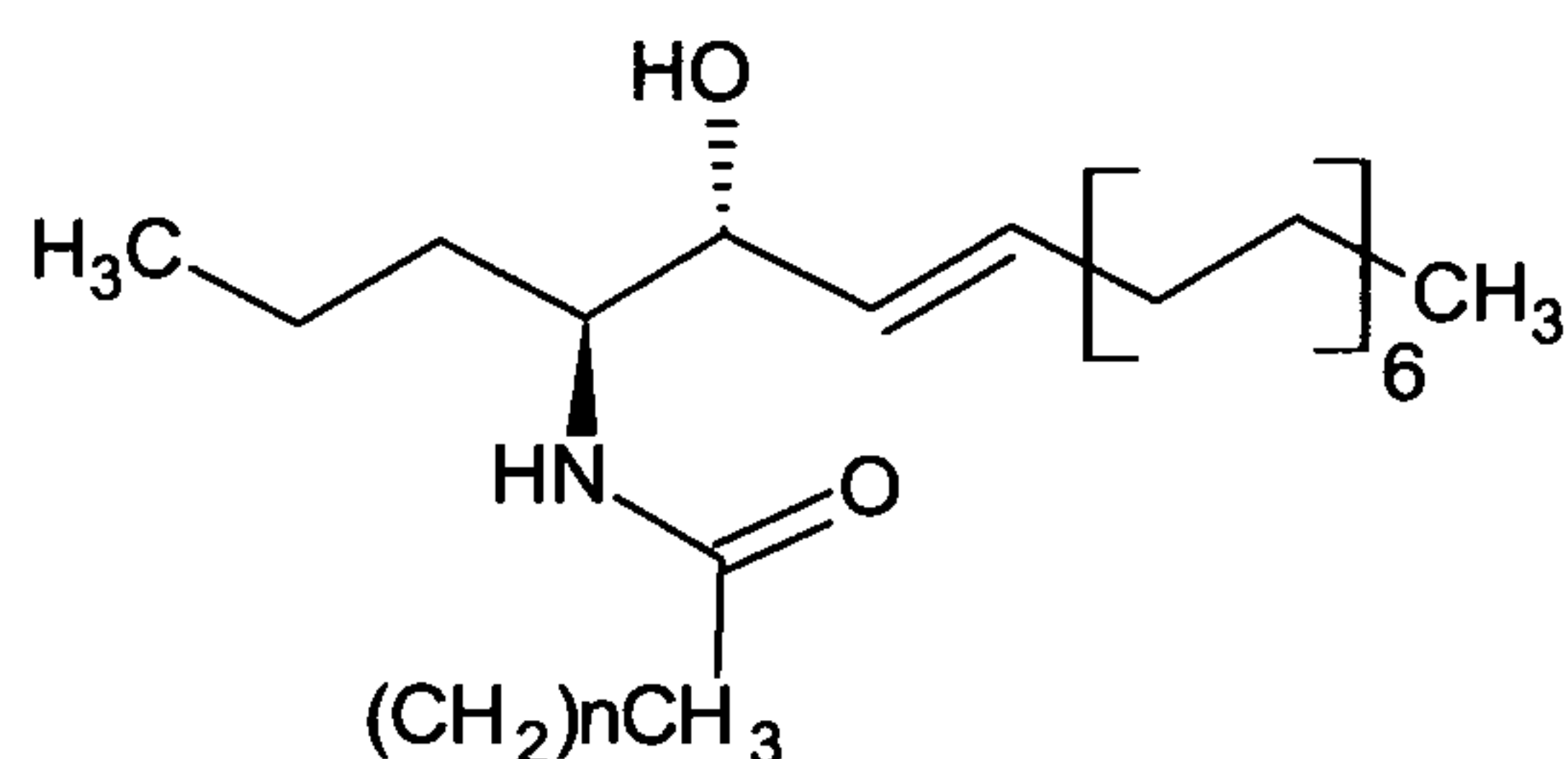
C26 ceramide, which is also referred to herein as ceramide C26, is a compound of formula (1):



(1)

Compound C26 ceramide, which is used as a biomarker according to the present invention, can be detected, among others, by the methods disclosed herein. C26 ceramide has a molecular weight of 678, detected as MRM transition in positive mode 679 m/z to 264.4 m/z.

Apart from C26 ceramide, medium ceramides as well as long ceramides may be used as a biomarker in the methods and uses of the invention. As preferably used herein, a medium ceramide is a ceramide of formula (2)



(2)

wherein n is any integer from: 8, 10, 12, 14 and 16.

As preferably used herein, a long ceramide is a ceramide of formula (2), wherein n is any integer from 18, 20, 22, 24, 26 and 28.

The term "sample" as used herein means preferably a limited quantity of a subject's material, wherein said subject's material is part of or has been taken from a subject and/or a subject's body. Preferably, said material is selected from the group comprising body fluids such as blood, a blood product, urine, saliva, cerebrospinal fluid and lymph, as well as stool or any kind of tissue and or cell material being part of a subject and/or a subject's body. It will be acknowledged by a person skilled in the art that the presence of and/or a level of a biomarker of the invention in said sample is intended to be similar to and represent the presence and/or the level of the biomarker in a larger amount of that subject's material. More precisely and as an illustrative, non-limiting example, a level of a biomarker of the invention determined in a sample of, e.g., some ml of blood from a subject also represents a level of said biomarker in the blood of the subject's body. Furthermore, in an embodiment of the method of the invention for diagnosing cystic fibrosis in a subject, a sample from the subject comprises said subject's material in a form, for example processed, fixed and/or preserved such that said sample is suitable for use in the method of the invention, whereby such processing, fixing and/or preserving preferably does neither generate C26 ceramide nor isoform compound 1 or isoform compound 2 which was not as such present in the blood of the patient. The subject's material in the sample may thus be diluted, for example with a solvent suitable for the method of the invention such as methanol and/or water, may be dried, for example on a filter card, may be resolved after having been dried such, for example with a solvent suitable for the method of the

invention such as methanol and/or water, or a substance may be added, wherein said substance prevents blood from coagulation such as for example EDTA or heparin. It will be further understood by a person skilled in the art that it is within the present invention and its various aspects and embodiments that said subject's material is separated into single components of said subject's material and/or single components of said subject's material are extracted from said subject's material, for example blood is separated into plasma or serum and cellular blood components or protein is precipitated from the sample. Accordingly, in an embodiment of any method according to the present invention wherein the method comprises protein precipitation and/or HPLC, precipitation of protein preferably results in a) a precipitation of cellular blood components and/or protein, more preferably forming a pellet after a step of centrifugation, and b) the biomarker being preferably not precipitated and being present in the supernatant after a step of centrifugation. A person skilled in the art will immediately understand that in an embodiment of any method according to the present invention wherein the method comprises HPLC a supernatant containing the biomarker(s) of the present invention or a part thereof is subjected to HPLC. In connection therewith it is important to understand that the supernatant or a part thereof which is subjected to HPLC comprises the biomarker to be detected as well as, preferably, an internal standard. In an embodiment of the method of the invention wherein an internal standard is added to the sample, the internal standard may be added to the sample before or after a precipitation step, i.e. the internal standard may be added into the sample immediately after the sample is taken from the subject, or may be added to the supernatant which is subjected to HPLC, as well as in between these points in time. A person skilled in the art will know, how and when an internal standard is preferably added to the sample in order to achieve an accurate detection and determination of a level of the biomarker.

It will be immediately understood that after such processing, fixing and/or preserving the sample is subjected to the methods of the invention for detecting and/or determining a or the level of a biomarker contained in said sample, whereby such processing, fixing and/or preserving preferably does not generate C26 ceramide which was not present in the sample from the patient as such.

In an embodiment of the methods of the present invention a sample as used in such methods is prepared from a primary such as whole blood. In an embodiment of the various aspects of the invention the primary sample is whole blood which is, in an embodiment, processed such that it is collected on a dry blood filter card; preferably approximately 3 μ l of full blood are collected on a spot of said dry blood filter card having a diameter of 3 mm. A person skilled in the art will acknowledge that the exact volume thus collected may vary depending on the hematocrit of the specific patient.

As particularly evident from the example part the present invention provides methods for the diagnosis of cystic fibrosis and biomarkers used in said methods which allow the diagnosis of cystic fibrosis with high sensitivity and high specificity suitable of clinical application.

The term "cystic fibrosis status" as used herein, preferably refers to the status of the disease in the subject. Examples of cystic fibrosis disease statuses include, but are not limited to, the subject's risk of suffering or developing cystic fibrosis, the stage of the disease in a subject and the effectiveness of treatment of the disease. Other statuses and degrees of each status are known in the art. In an embodiment of the present invention the cystic fibrosis status comprises a severe, mild, or healthy cystic fibrosis status.

The term "diagnosing" as used herein, preferably means determining the presence or the absence of a disease or disorder in a subject and/or determining whether a subject is at risk for developing a disease, a disorder or symptoms related to a disease or disorder as well as predicting a status of a disease. "Diagnosis" or "diagnosing" as used herein also preferably means that a cause of symptoms of a disease which are present or will be present is identified. In connection therewith it is important to note that a person skilled in the art, such as a skilled clinician consulted by a subject suffering from symptoms or suspected to be ill, applies the methods of the present invention or makes the methods of the present invention applied and thus determines whether a subject is at risk for developing a disease, particularly cystic fibrosis, whether a subject suffers from such disease or predicts the status of such disease, preferably based on the result obtained by the practicing of the methods of the present invention.

Based on said diagnosis the person skilled in the art will recommend to apply, maintain, reduce, elevate or not apply a therapy or to perform further diagnostic tests.

It is thus an embodiment of the method of the present invention for diagnosing cystic fibrosis that the method comprises giving a recommendation whether a therapy should be applied, maintained, reduced, elevated or not applied.

The term "differentially diagnosing" as used herein in connection with the method of the present invention preferably means that the method allows determining the presence or the absence of a disease or disorder in a subject and/or determining whether a subject is at risk for developing a disease, a disorder or symptoms related to a disease or disorder as well as predicting a status of a disease, wherein the disease is cystic fibrosis.

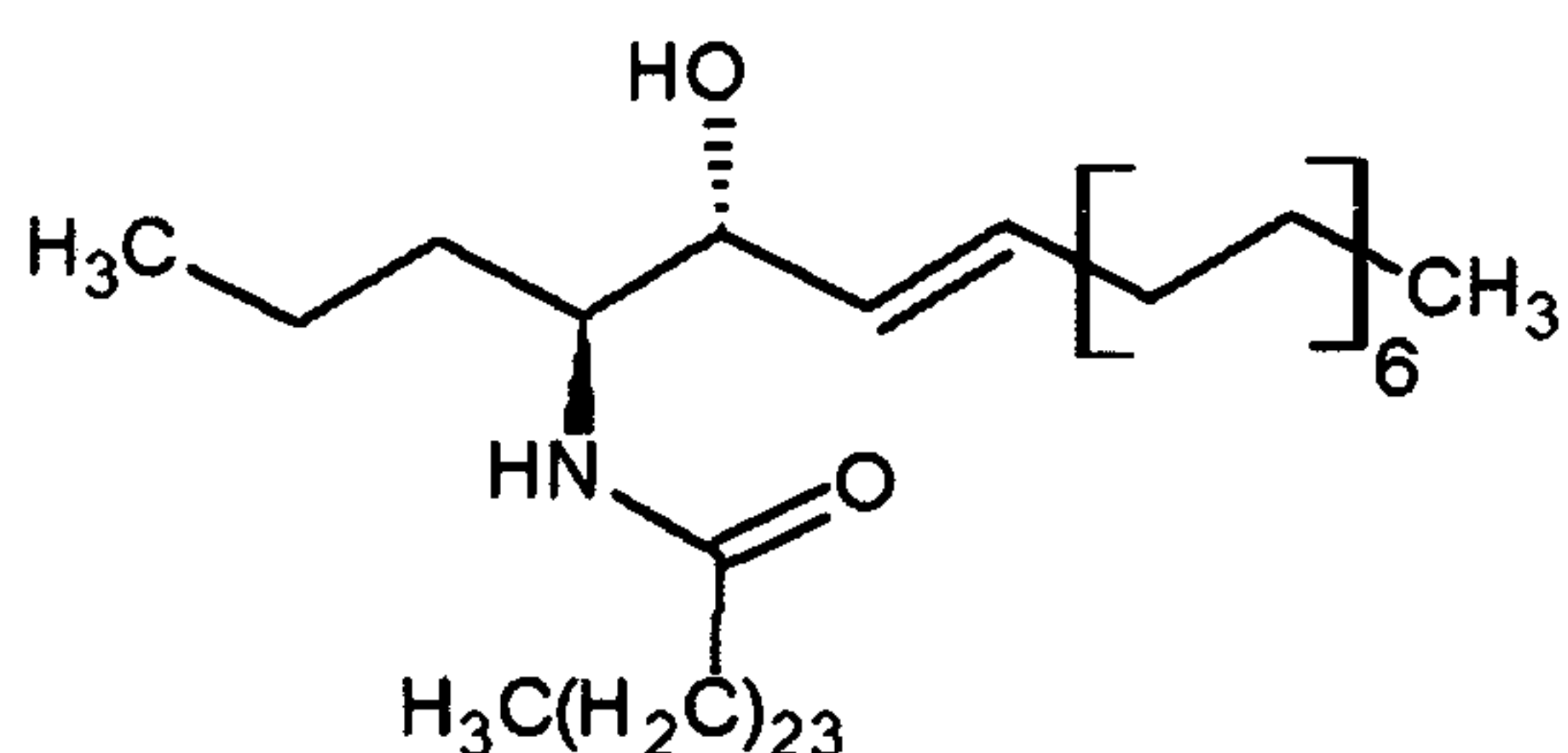
The term "detecting" in the context of the present invention means methods which include detecting the presence or absence of a substance in a sample and/or qualifying the type of said substance. Detecting can be accomplished by methods known in the art and those further described herein, including, but not limited to, the direct measurement of the affected protein(s) e.g. the sequencing of the CFTR gene. It will, however, be acknowledged by a person skilled in the art that cystic fibrosis is typically characterized by CFTR protein deficiency. Any suitable method can be used to detect one or more of the biomarkers described herein. These methods include, without limitation, mass spectrometry such as, e.g. HPLC-MS/MS, fluorescence such as, e.g., sandwich immunoassay, HPLC-fluorescence or HPLC-UV.

A biomarker as used herein, preferably is any biological compound, such as a protein and a fragment thereof, a peptide, a polypeptide, a proteoglycan, a glycoprotein, a lipoprotein, a carbohydrate, a lipid, a nucleic acid, an organic or inorganic chemical, a natural polymer, and a small molecule, which is differentially present in a sample from a subject of one phenotypic status (e.g. having a disease) as compared with another phenotypic status (e.g. not having the disease) and which may be isolated from, or measured in the sample from the subject. In an embodiment of the present invention the biomarker is different from an enzyme, preferably different from acid sphingomyelinase (abbr. ASM) and more preferably different from human

acid sphingomyelinase, or a part thereof, and/or different from acid ceramidase (abbr. AC) and more preferably human acid ceramidase, or a part thereof. In an alternative embodiment of the present invention the biomarker is an enzyme, preferably an acid sphingomyelinase, preferably a human acid sphingomyelinase, or a part thereof, and/or a ceramidase and more preferably a human acid ceramidase, or a part thereof. Furthermore, the biomarker can be the entire intact molecule, or it can be a portion thereof which is preferably detected by mass spectrometric analysis, an antibody, another protein specifically binding the biomarker, functional nucleic acids specifically binding the biomarker and/or a fluorescent label. A biomarker is furthermore considered to be informative if a measurable aspect of the biomarker is associated with a given status of the patient, such as a particular status of cystic fibrosis. Such a measurable aspect may include, for example, the presence, absence, or the level of the biomarker in the sample from the subject and/or its presence as part of a profile of biomarkers. A profile of biomarkers comprises at least two such measurable aspects, where the measurable aspects can correspond to the same or different classes of biomarkers such as, for example, a nucleic acid and a carbohydrate. A biomarker profile may also comprise at least three, four, five, 10, 20, 30 or more measurable aspects. In one embodiment, a biomarker profile comprises hundreds, or even thousands, of measurable aspects. In another embodiment, the biomarker profile comprises at least one measurable aspect of at least one biomarker and at least one measurable aspect of at least one internal standard.

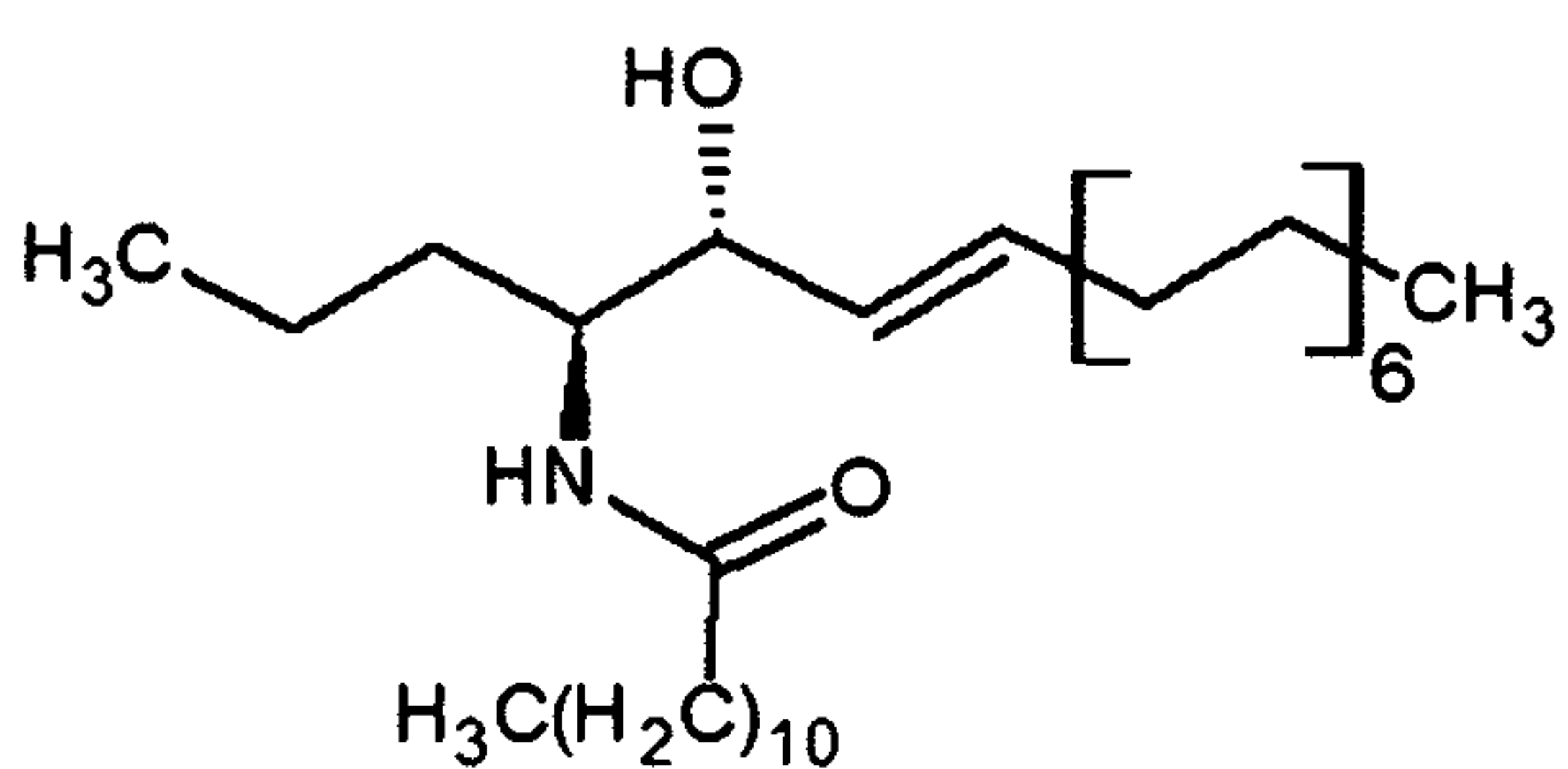
In an embodiment of any method according to the present invention an internal standard is added to a sample from a or the subject. It will be acknowledged that by said addition of internal standard, also referred to herein as IS, to the sample, i.e. spiking of the sample, to be subjected to such method according to the present invention, the concentration of IS in the sample is known and, e.g., by determining the area under the peak, i.e. the peak area, of the internal standard in, e.g., an HPLC-mass spectrometric chromatogram the relation between a peak area and a concentration of a substance, e.g. of IS, and/or the biomarker of the present invention, i.e. C26 ceramide, isoform 1 compound and/or isoform 2 compound, is established and thus a means provided for determining the level of the biomarker in the sample. A person skilled in the art will further acknowledge that various molecules may be used as an IS. Nevertheless an IS having a similar chemical structure compared to the molecule such as the biomarker, e.g. a

ceramide or ceramide derivative, is preferable. In a preferred embodiment the molecule being the IS can be distinguished from the biomarker of the present invention, e.g. C26 ceramide, in the methods of the present invention. In a further preferred embodiment the IS is selected such that a molecule which is ideally not present or rare in nature. In accordance therewith, the present inventors have in an embodiment chosen C25 ceramide as an internal standard which is not present as such in nature. C25 ceramide is of the following structure:

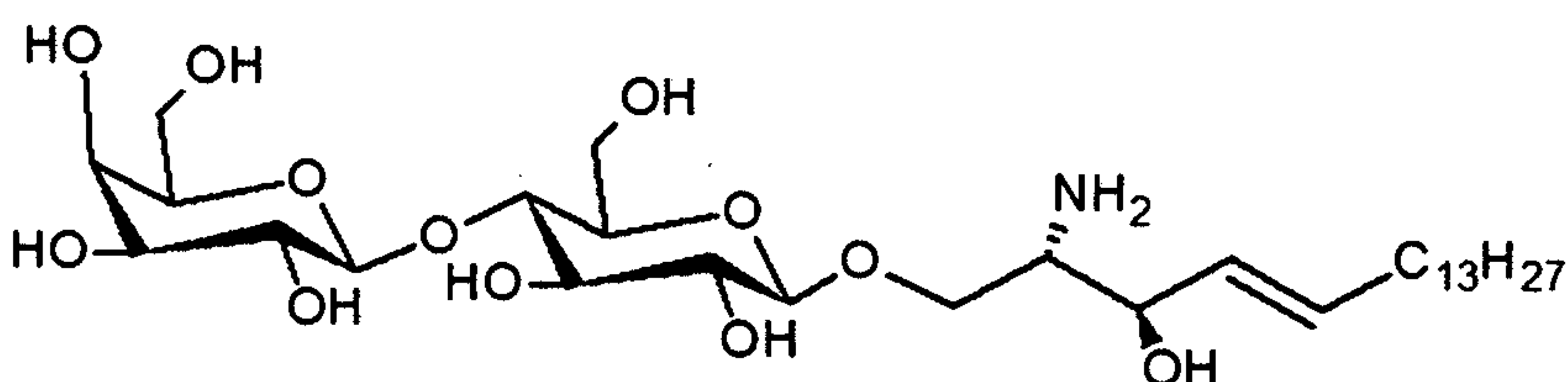


Other compounds which are useful as internal standard in the practicing of the methods of the invention include, but are not limited to,

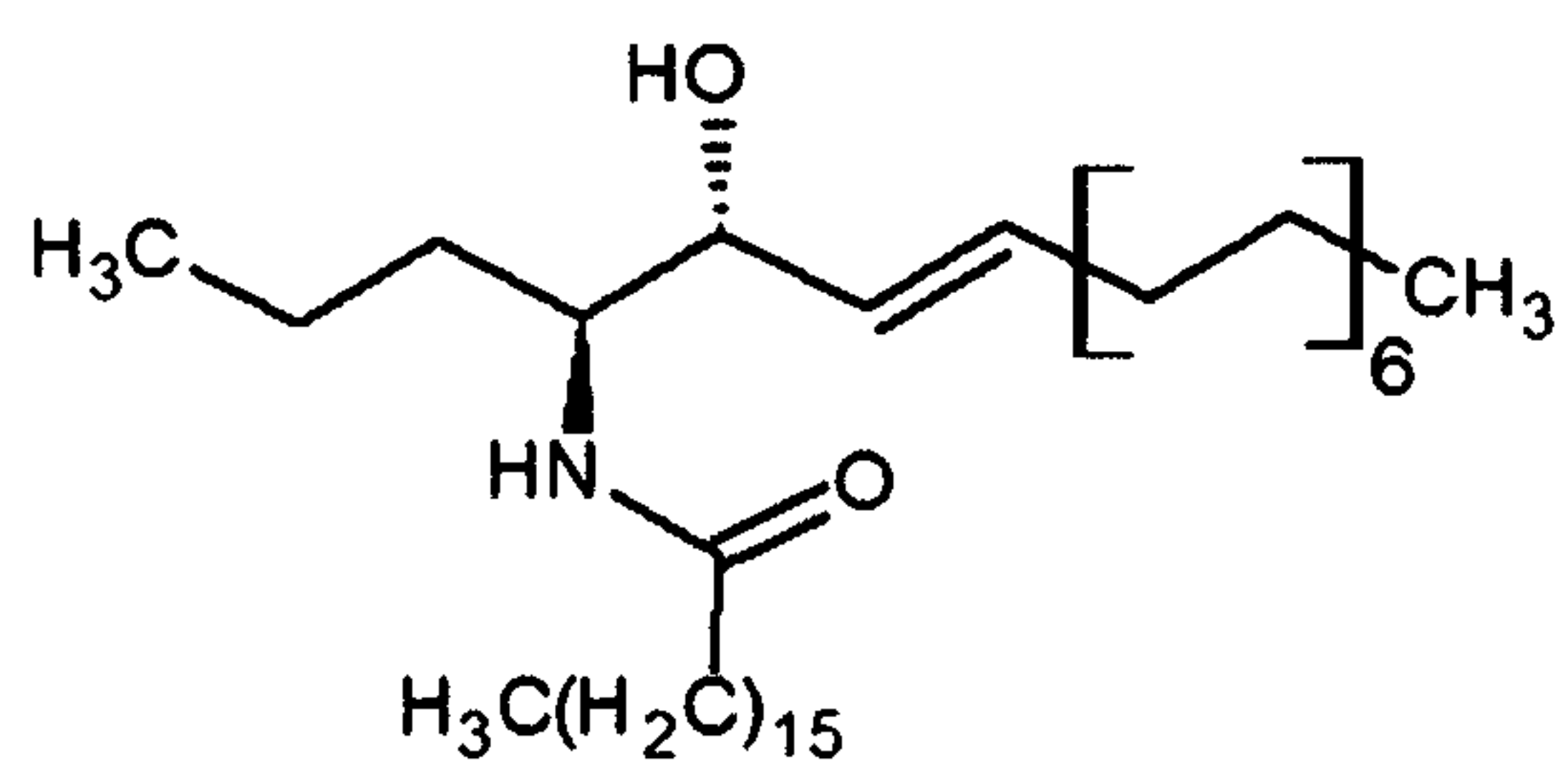
N-lauroyl sphingosine of the following formula



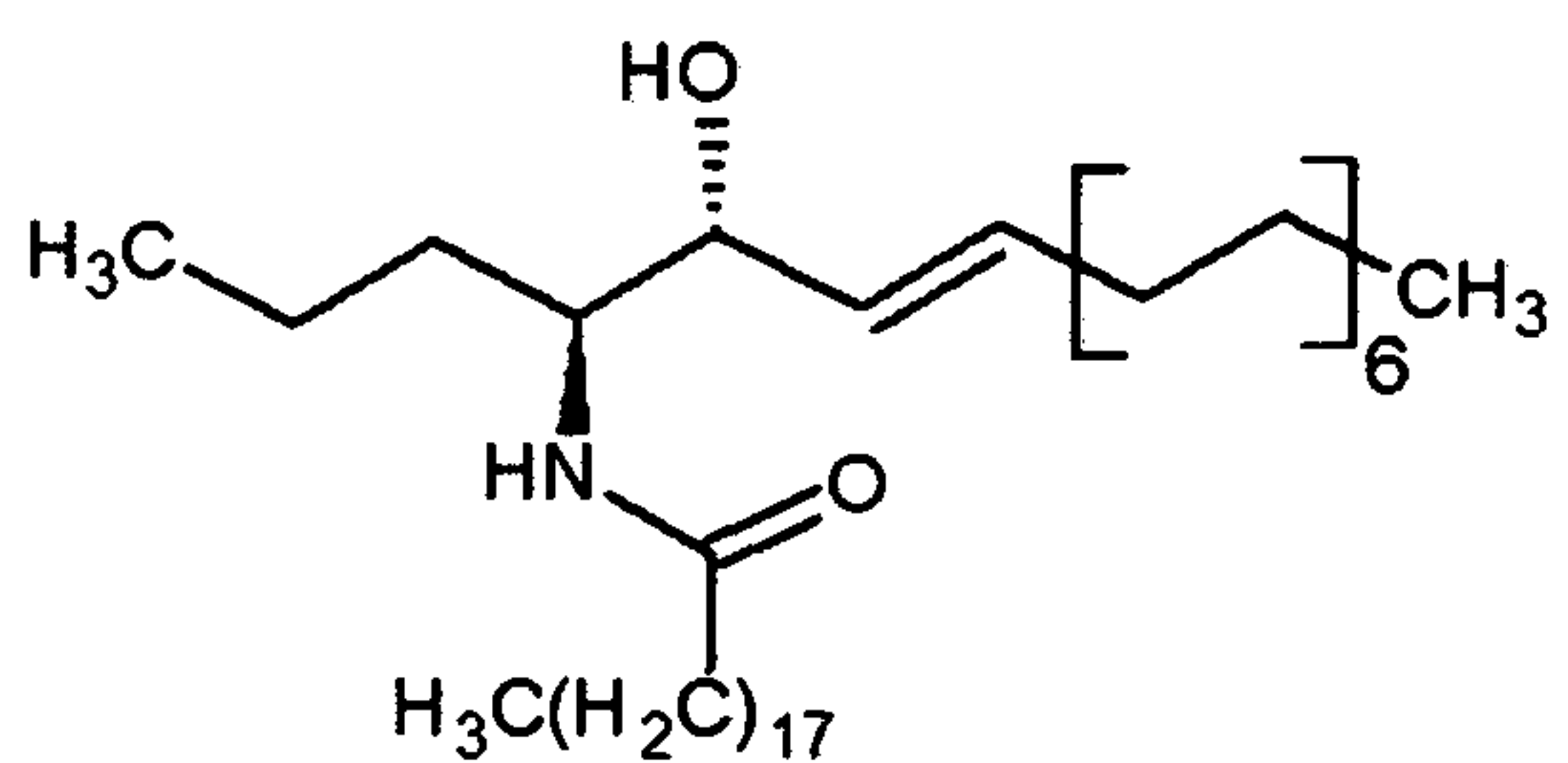
lyso-Gb2 of the following formula



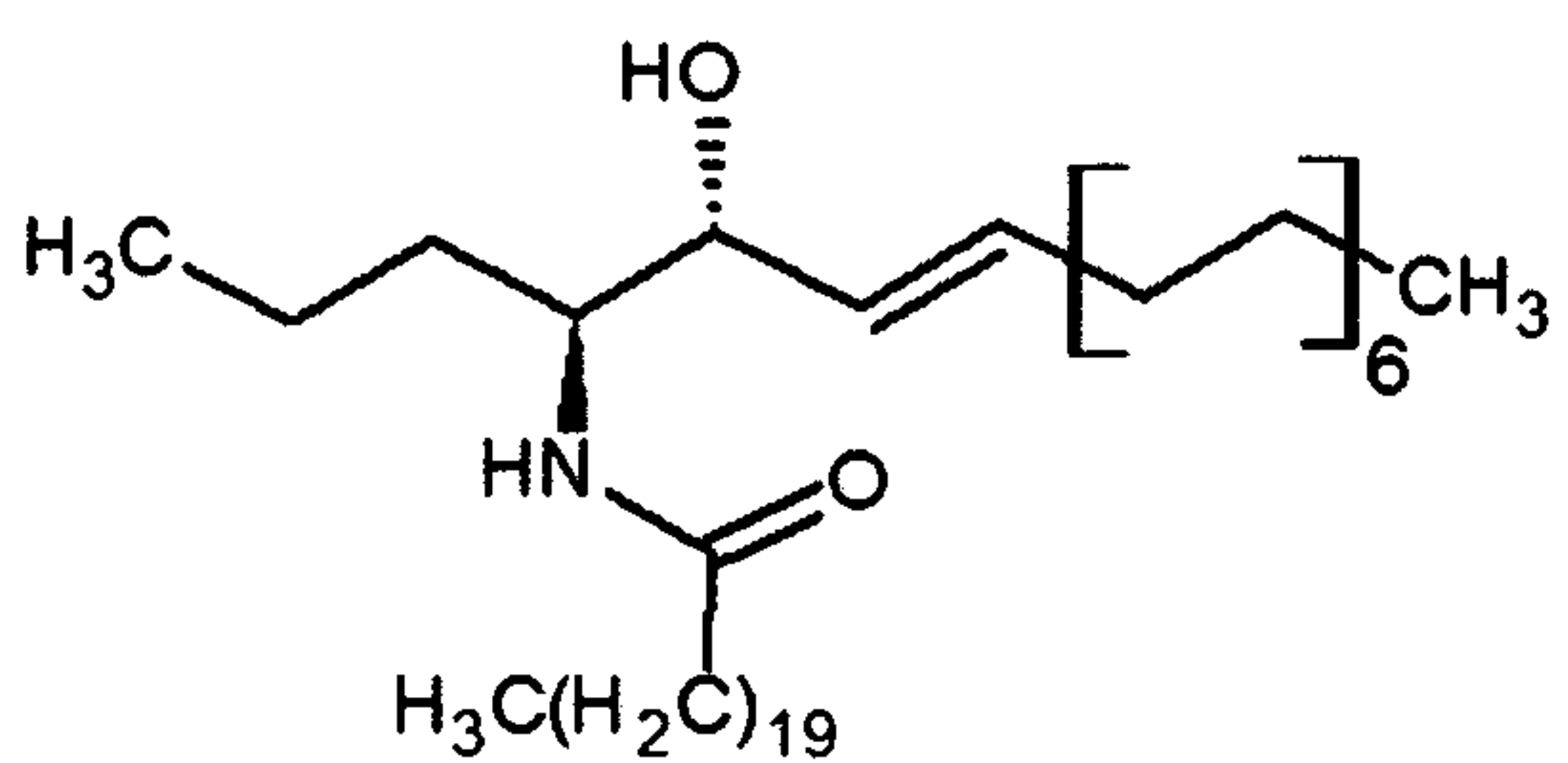
C17 ceramide of the following formula



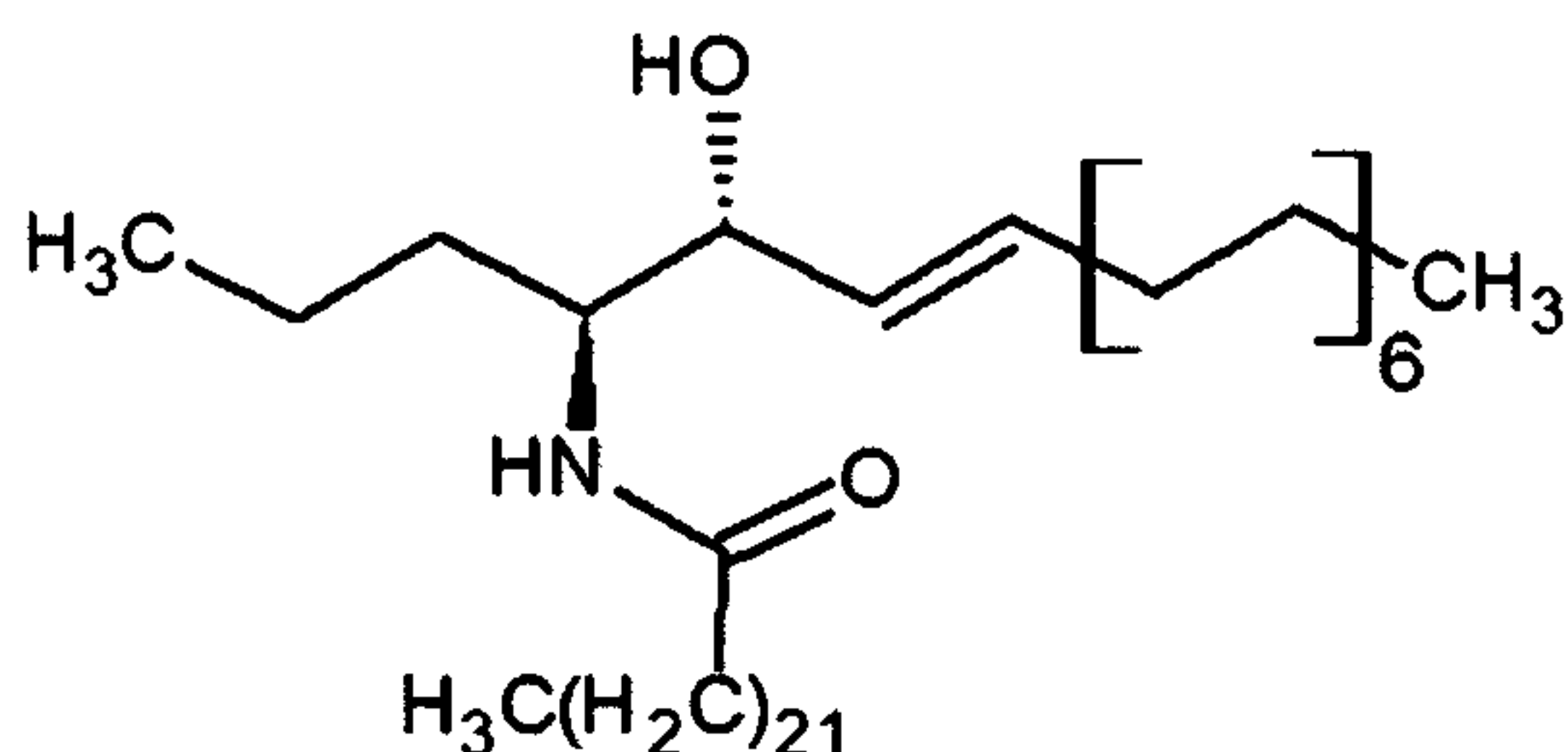
C19 ceramide of the following formula



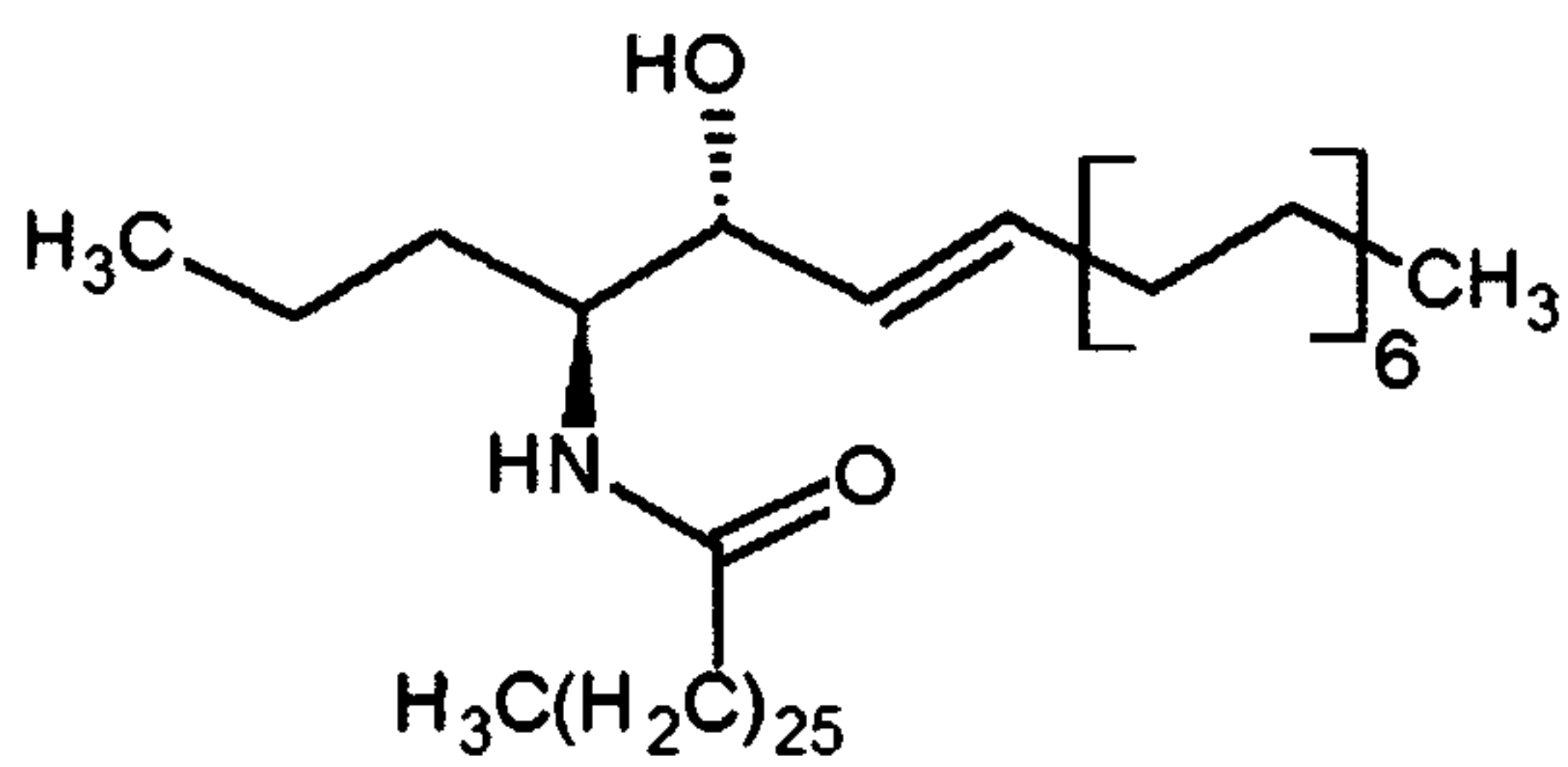
C21 ceramide of the following formula



C23 ceramide of the following formula



and C27 ceramide of the following formula



In an embodiment of the various aspects of the present invention where the internal standard is added to a sample from a subject, it is preferred that the IS is added such that it is dissolved in a solvent, e.g. *ethanol*, prior to said addition to the sample. In a further preferred embodiment that the solvent is selected such that said solvent is capable of causing protein precipitation, preferably is capable of causing the protein precipitation step as subject to the method of the present invention.

In some embodiments of the methods of the present invention a protein precipitation and/or protein precipitation step is part of the methods of the present invention. It will be understood that precipitation as used herein, preferably means the formation of a solid in a solution, i.e. for example the formation of a protein precipitate in a sample, e.g. serum, from a subject. When precipitation, e.g. protein precipitation, occurs in a sample, the solid formed is called the precipitate, or when compacted by a centrifuge, a pellet. The liquid remaining above the solid is in either case called the supernatant. The present invention contemplates different methods of precipitation and/or separating said supernatant and said precipitate or pellet, comprising, among others, settling or sedimentation and centrifugation. A person skilled in the art will know further methods for protein precipitation and/or for separating a supernatant and a protein

precipitate, nevertheless said skilled person will acknowledge that if a method, preferably a method of the invention, is applied where precipitated protein will disable a device such as a column or HPLC-column used in connection with the present invention the precipitated protein is preferably separated from the solvent and/or the sample.

In some embodiments of the methods of the present invention a level of a biomarker of the present invention is compared to a level of the same or another biomarker of the present invention determined in another sample, e.g. from the same patient, from another patient, from a control and/or from the same or different points in time, and/or a cut-off value, and/or a level of a control and/or a level of an IS. In connection therewith "comparing" or "compared to" as used herein, preferably means the mathematical comparison of the two or more values of the levels of the biomarker(s). It will thus be immediately evident whether one of said values is higher, lower or identical if at least two of such values are compared with each other.

The term "cut-off value" as used herein preferably refers to a level, concentration and/or a titer of a biomarker of the present invention. In some embodiments where a ratio of two levels, concentrations and/or titers of the biomarkers of the present invention is considered said cut-off value is referred to a value of a ratio to which the ratio of two levels, concentrations and/or titers of the biomarkers is compared, and wherein if said ratio of two levels, concentrations and/or titers of the biomarkers of the present invention determined in the practicing of the methods of the present invention is elevated, increased or higher compared to the cut-off value to which the ratio of two levels, concentrations and/or titers of the biomarkers is compared, this is indicative that the subject is suffering from or is at risk for developing cystic fibrosis. In one particular embodiment thereof using C26 ceramide as the biomarker allows for diagnosing cystic fibrosis in dried blood spots using a cut-off value for C26 ceramide of 69 nmol/L, preferably 69.1 nmol/L blood in case the biomarker is total C26 ceramide, i.e. both or the sum of cis-C26 ceramide and trans-C26 ceramide, or in case the biomarker is to total of isoform 1 compound and isoform 2 compound, and is 27.7 nmol/L in case the biomarker is cis-C26 ceramide or in case the biomarker is isoform 1 compound. It will be acknowledged by a person skilled in the art that the cut-off value and the above cut-off values in particularly depend on the method applied when determining the same. The method used in determining said cut-off

values is the one described in the Example part of the instant application and Example 1 thereof in particular the disclosure of which is incorporated into this general part of the description by reference. As preferably used herein, the cut-off value is set at the mean value of a cohort of healthy individuals plus 2x standard deviation.

In some embodiments of the methods of the present invention the level of the biomarker is also determined in a control. As used herein, a control is preferably a sample from a subject, wherein the cystic fibrosis status of said subject is known. In an embodiment a control is a sample of a healthy patient. In a further embodiment an amount of said biomarker is added to said sample of a healthy patient prior to determining the level of said biomarker in said sample of a healthy patient comprising said added biomarker, preferably in the practicing of a method of the present invention. In a further embodiment the control is a sample from at least one subject having a known cystic fibrosis status, e.g. a control patient, and in a still further preferred embodiment also comprises the genetic status with regard to mutations of the gene, affected in said disease, comprising CFTR protein, i.e. comprising the subject having homozygous and/or compound heterozygous mutations, the subject being a carrier of a mutation. In a further preferred embodiment the control is a sample from a subject not being treated for cystic fibrosis. In a still further preferred embodiment the control is a sample from a single subject or a pool of samples from different subjects and/or samples taken from the subject(s) at different points in time.

The term "level" or "level of a biomarker" as used herein, preferably means the concentration of a substance and/or titer of a substance, preferably of a biomarker of the invention within a sample of a subject. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound. It will be understood by a skilled person that in certain embodiments of the methods of the invention said sample is used as such in the practicing of said methods. It is, however, also within the present invention that said sample is subject to processing prior or during its use in the methods of the present invention. In an embodiment said sample may be subjected, e.g., to a step of protein precipitation, separation, e.g., centrifugation and/or HPLC

and subsequently subjected to a step of determining the level of the biomarker, e.g., using mass spectrometric analysis. It should be further noted that whenever the term "a" level of a biomarker is used in connection with a level of the biomarker of the invention which is to be determined according to or in the practicing of the present invention, "the" level of the biomarker of the present invention which is to be determined by the methods of to the present invention and which is contained in the sample subjected to the method(s) of the invention is meant.

The level of a biomarker is different between different statuses of cystic fibrosis, if the mean or median level of the biomarker in the different groups is calculated to be statistically significant. Biomarkers, alone or in combination, provide measures of relative risk that a subject belongs to one phenotypic status or another. Therefore, a biomarker of the present invention is useful in an embodiment of the present invention as markers for disease, or for therapeutic effectiveness of a drug or a treatment.

The term "determining the level" of a biomarker as used herein, preferably means methods which include quantifying an amount of at least one substance in a sample from a subject and/or quantifying an amount of said substance contained in a part of the body of the subject, such as saliva, blood, lymph, serum, plasma or liquor and/or quantifying an amount of said substance in the subject, preferably the substance being selected from the group comprising a biomarker.

It will be understood by a person skilled in the art that detecting and/or determining the level of C26 ceramide in a sample from the subject, not chemically converted, transformed or derivatized. In a further preferred embodiment a step of detecting and/or determining the level of a biomarker in a sample from the subject, wherein the biomarker is C26 ceramide, is performed subsequent to separation using HPLC by application of mass spectrometric analysis. The latter is also applicable in case the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound.

A subject is considered to be a healthy subject with regard to cystic fibrosis, if the subject does not suffer from symptoms associated with cystic fibrosis. In an embodiment of the methods of

the invention a subject will be considered to be healthy regarding cystic fibrosis, if it has no mutation of the functional parts of the CFTR gene resulting in a reduction of or deficiency of the respective protein or the activity thereof, resulting in symptoms associated with cystic fibrosis.

In connection with the present invention and more specifically therewith a “patient” is a person presenting one homozygous mutation or multiple heterozygous mutations of the CFRT gene resulting in reduction or deficiency of the respective protein and /or protein activity, resulting in symptoms associated with cystic fibrosis. Furthermore, in connection therewith a “carrier” is a person presenting one heterozygous mutation of the CFRT gene resulting or not resulting in reduction or deficiency of the respective protein and /or protein activity, usually or preferably not resulting in symptoms associated with cystic fibrosis. In an embodiment of each and any aspect of the present invention the sensitivity of the cis-C26 ceramide used as biomarker is 100 %, in other words all patient samples can be identified. The same is equally applicant in case the biomarker is isoform 1 compound. In an embodiment of each and any aspect of the present invention the specificity of the cis-C26 ceramide biomarker for cystic fibrosis patients is 99.5 % versus normal controls, preferably versus healthy individuals. In an embodiment of each and any aspect of the present invention the specificity of the cis-C26 ceramide biomarker for cystic fibrosis carriers is 83 % versus normal controls, preferably versus healthy individuals. The same is equally applicable in case the biomarker is the total of isoform 1 compound and isoform 2 compound.

In an embodiment of each and any aspect of the present invention the sensitivity of the total C26 ceramide used as biomarker is 100 %, in other words all patient samples can be identified. In an embodiment of each and any aspect of the present invention the specificity of the total C26 ceramide biomarker for cystic fibrosis patients is 98.9 % versus normal controls, preferably versus healthy individuals. The same is equally applicant in case the biomarker is isoform 1 compound. In an embodiment of each and any aspect of the present invention the specificity of the total C26 ceramide biomarker for cystic fibrosis carriers is 85 % versus normal controls, preferably versus healthy individuals. The same is equally applicable in case the biomarker is the total of isoform 1 compound and isoform 2 compound.

Said mutations of CFTR gene will be detected if a sample from the subject is subjected to a genetic testing for such mutations as described herein. In a further embodiment of the present invention a sample from a healthy subject is used as a control sample or as a blank matrix in the methods of the present invention. A blank matrix as used herein is preferably a sample from a healthy subject. Nevertheless, it will be understood that such a blank matrix may contain a native level of biomarker. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

In an embodiment of the present invention the level of a biomarker is indicative for the subject for suffering from or for being at risk for developing a disease or disorder, wherein the disease or disorder is cystic fibrosis. The level of the biomarker determined in accordance with the present invention is compared to a control level of the biomarker, wherein the result of said comparison allows for diagnosing cystic fibrosis.

More specifically, comparing the level of the biomarker in the sample from the subject to the control level of the biomarker comprises comparing the level of the biomarker in the sample from the subject to a cut-off value, wherein if a level of the biomarker in the sample from the subject is elevated, increased or higher compared to the cut-off value, this is indicative that the subject is suffering from or is at risk for developing cystic fibrosis or is a cystic fibrosis carrier; and/or wherein if a level of the biomarker in the sample from the subject is decreased or lower compared to the cut-off value this is indicative that the subject is not suffering from or is not at risk for developing cystic fibrosis.

The term "being at risk for developing a disease" as used herein preferably means that it is likely that a subject will suffer from said disease and/or will develop said disease or symptoms associated with said disease, particularly if no treatment is applied. In connection therewith it has to be acknowledged that cystic fibrosis is a genetic disorder and thus the occurrence of

relatives, particularly parents having said disease or having a mutation known to be the cause of said disease are indicative for a subject, e.g. the child of two cystic fibrosis patients or two cystic fibrosis carriers, to be at risk for developing said disease. It will furthermore be acknowledged that the progression of a disease is linked to the occurrence of symptoms as well as the severity of said symptoms. Accordingly, a person not suffering from symptoms at present, however, may be at risk for developing the disease, for example, because although genetically mutations of a gene, known to cause a disease are present, no symptoms or no severe symptoms occur. Nevertheless, it will be immediately understood that the methods and biomarkers of the present invention, particularly if the level of said biomarker according to the present invention is elevated, allow for diagnosing that such subject is at risk for developing the disease independent from the presence or absence of symptoms. Accordingly, the methods according to the present invention allow for determining whether a subject is at risk of suffering from cystic fibrosis. It is also within the present invention that a therapy is applied, maintained, reduced, elevated or not applied based on whether the subject is at risk of suffering from cystic fibrosis or not.

It is also within the present invention that comparing the level of the biomarker in the sample from the subject to a control level allows for determining the severity of cystic fibrosis, wherein if a level of the biomarker in the sample from the subject is elevated, increased or higher compared to the control level that is indicative that the subject is suffering from or is at risk for developing cystic fibrosis of a more severe status or progression; and wherein if a level of the biomarker in the sample from the subject is decreased or lower compared to the control level that is indicative that the subject is suffering from or is at risk for developing cystic fibrosis of a less severe status or progression. In a further embodiment of the present invention that comparing the level of the biomarker in the sample from the subject to the control level comprises comparing a level of the biomarker in said subject to a level of the biomarker detected in a sample from a control, wherein if a level of the biomarker in the sample from the subject is elevated, increased or higher compared to the control sample this is indicative that the subject is suffering from and/or is at risk for developing cystic fibrosis; and/or a level of the biomarker in the sample from the subject is elevated, increased or higher compared to the control sample this is indicative that the subject is suffering from or is at risk for developing cystic fibrosis of

a more severe status or progression. Said control preferably is selected from the group comprising healthy subjects, subjects suffering from cystic fibrosis or being at risk of suffering from cystic fibrosis symptoms, subjects being positively tested for a mutation or a combination of mutations of the gene CFTR; in more preferred embodiment the control is a subject suffering from cystic fibrosis, whereby the subject suffering from cystic fibrosis is a subject different from the subject undergoing diagnosis or the same subject as the subject undergoing diagnosis with the sample examined and the sample used as a reference or control having been taken from the same subject at different points in time. In a further embodiment of the present invention a control level is determined in a sample from a control, wherein optionally C26 ceramide is added to the sample from the control in a specific quantity prior to determining the level of C26 ceramide in the sample from the control.

It is the merit of the present inventors that a method for diagnosing cystic fibrosis in a subject could be established wherein the method comprises detecting a biomarker in a sample from a subject, wherein, preferably, the biomarker is C26 ceramide, preferably further comprising determining a level of the biomarker in the sample from the subject, and more preferably further comprising comparing the level of the biomarker in the sample from the subject to a cut-off value, which shows high sensitivity. In other words, the sensitivity, which means the proportion of actual positives which are correctly identified as such, is high, which means that the percentage of cystic fibrosis patients correctly identified as having the disease is as high as has been outlined above. In contrast, in a statistic test as described herein specificity means the proportion of negatives which are correctly identified as negatives, in other words the percentage of healthy patients correctly identified as not having cystic fibrosis. A person skilled in the art will acknowledge that thus an optimal prediction of a diagnostic test such as in some embodiments of the methods according to the present invention in general aims to achieve 100% sensitivity, i.e. predict all patients having a disease, such as cystic fibrosis or being at risk of suffering from said disease, as having the disease or being at risk from suffering from said disease, respectively.

In an embodiment of the methods according to the present invention a specificity of at least 95%, to 100% is preferred. In a further embodiment of the present invention of the methods

according to the present invention the methods allow for diagnosing cystic fibrosis in a subject independent from a progression status of cystic fibrosis in the subject. More specifically, the methods of the present invention allow for diagnosing cystic fibrosis in a subject having an early status of cystic fibrosis as well as in a subject having an advanced or progressed status of cystic fibrosis.

A person skilled in the art will acknowledge that although the specificity and/or the sensitivity of the methods according to the present invention are as high as described above and were determined as described in the examples hereinafter, individual cases may not be excluded where a patient having cystic fibrosis will be tested false negative or where a patient not having cystic fibrosis will be tested false positive with a method of the invention. A person skilled in the art will thus immediately acknowledge that according to the methods according to the present invention, wherein a level of a biomarker is compared to a cut-off value, such comparison to said cut-off value is for use to differentially diagnose a disease and/or from a level and/or a value in a healthy subject. Having said this, it is obvious for the person skilled in the art that also according to the methods of the present invention, wherein the method is for diagnosing cystic fibrosis patient and carrier, individual cases may not be excluded where a patient having cystic fibrosis will be tested false negative or where a patient not having cystic fibrosis will be tested false positive, or where the type and/or status is diagnosed incorrectly with a method of the invention.

Therefore, it is important to note that although the sensitivity and the specificity of the method of the present invention may vary if patient collectives other than the one reported in the Example part, e.g. varying in number of patients, will be subject to the methods of the present invention, it is the firm belief of the inventors that no method known in the prior art, especially using biomarkers, will achieve a higher specificity and a higher sensitivity compared to the methods according to the present invention. This is especially true since the limit of detection of the methods of the present invention allows for determining the level of C26 ceramide in healthy subjects.

A "limit of detection" of a substance such as C26 ceramide, as used herein, preferably is a level of the substance determined by a method for determining a level of the substance, wherein a level less than or lower than said limit of detection cannot be determined by said method. It is thus immediately clear that a "cut-off value" and a "limit of detection", as used herein, are preferably not necessarily identical, although both reflect a certain level of a substance, e.g. of a biomarker of the present invention, i.e. C26 ceramide. It will be immediately understood that a cut-off value will be selected preferably such that selectivity and sensitivity of the method are as high as possible. In contrast thereto a limit of detection represents an absolute level of the biomarker of the present invention which reflects the minimum level of biomarker which can be detected with a method for determining the level of said biomarker. It is thus immediately clear that a limit of detection depends on the method for determining a level of a substance and on the substance the level of which is to be determined by the method. A skilled person will immediately understand that a high limit of detection, e.g. higher than an ideal cut-off value would possibly result in a low sensitivity of the method since the percentage of true positives that are predicted by a test to be positive also depends on whether a level of the biomarker may be determined for said true positives. In other words, if the limit of detection is higher than an ideal cut-off value, true positives having a level of the biomarker slightly higher than the cut-off value may not be distinguished from true negatives having a level of the biomarker lower than the cut-off value since no level of the biomarker may be determined for both true positives having a level of the biomarker slightly higher than the cut-off value and negatives having a level of the biomarker lower than the cut-off value. It is thus immediately clear that a low limit of detection is of advantage. It is therefore also the merit of the inventors to show that a lower limit of detection allows for a method for diagnosing cystic fibrosis in a subject comprising a step of determining a level of a biomarker present in the sample with higher selectivity and sensitivity. An "ideal cut-off value" as used herein, preferably is the cut-off value as described herein, wherein the method using said ideal cut-off value has the highest selectivity and sensitivity.

In an embodiment of the methods according to the present invention the methods comprise a step of validating said method by diagnosing a disease or disorder, preferably cystic fibrosis in a subject by the method of the present invention; a step of diagnosing the disease or disorder,

preferably cystic fibrosis, in a subject by a genetic testing, comprising sequencing of a gene, preferably sequencing of a gene a mutation of which is known to the one skilled in the art to cause the disease or disorder, more preferably sequencing the CFTR gene; and comparing the results of said method and said genetic testing. A healthy subject as used herein, preferably is considered to be healthy with regard to a disease or disorder if said subject is not suffering from symptoms associated with said disease or disorder and/or if the result of a genetic testing reveals no mutations of a gene a mutation of which is known to the one skilled in the art to cause the disease or disorder. A healthy subject also is understood to be a subject being positively tested for not having cystic fibrosis. In a preferred embodiment a healthy subject is a subject not being a carrier of cystic fibrosis.

The term "qualifying cystic fibrosis status" in a subject as used herein, preferably means a classification of a subject's biomarker profile selected from the group comprising to identify or detect the presence or absence of cystic fibrosis in the subject, to predict the onset of or the risk for developing of cystic fibrosis in the subject, to determine the course of cystic fibrosis in a subject, to determine and/or predict the severity of cystic fibrosis in a subject, to determine whether a subject suffers from an early status of cystic fibrosis or an advanced or progressed status of cystic fibrosis or to determine whether a level of a biomarker in a subject has significantly changed over time.

The term "managing subject treatment" or "subject management" as used herein, preferably refers to the behavior of the clinician or physician subsequent to the determination of cystic fibrosis status. For example, if the result of the methods according to the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order new tests, such as testing for the function of the affected proteins and/or sequencing of the CFTR gene. Alternatively, if the status indicates that treating for cystic fibrosis is appropriate, the physician may schedule the subject for treating for cystic fibrosis. Likewise, if the status is negative or if the results show that treatment has been successful, no further management may be necessary. Nevertheless a person skilled in the art will immediately acknowledge that besides gene therapy any suitable and/or effective therapy may be applied. Furthermore it is an embodiment of the present invention that managing subject treatment comprises titrating of a

dose of a drug applied as a treatment for cystic fibrosis, e.g. amount of ASM inhibitor applied or administered to a patient and/or subject. In some embodiments of the methods of the present invention wherein a level of a biomarker present in a sample from a subject is determined at several points in time, or is compared to other levels of the biomarker, a cut-off value and/or a level of said biomarker in a control and/or another value of a ratio of the levels of two biomarkers, a skilled person will apply or not apply a therapy, or amend a therapy already applied in order to treat or not to treat, or to continue treating cystic fibrosis.

It is within the present invention that a skilled person will apply a dosage and/or maintain a dosage or amend a dosage, e.g. apply a dosage or a higher dosage, i.e. elevate a dosage, if such a comparison of the level of a biomarker e.g. that the level of said biomarker is higher than for example, a cut-off value, i.e. the patient is diagnosed to have cystic fibrosis; or that a level determined in the same patient earlier in time is lower or the same, i.e. a therapy applied is not sufficient, i.e. does not result in a decrease in the level of the biomarker. On the other hand, a skilled person will apply or not apply a dosage or maintain or reduce a dosage, e.g. apply no dosage or a lower dosage, i.e. decrease a dosage, if such a comparison of the level shows e.g. that the level of said biomarker is lower than for example, a cut-off value, i.e. the patient is diagnosed not to have cystic fibrosis; or that a level determined in the same patient earlier in time is higher, i.e. a therapy applied is sufficient, i.e. does result in a decrease in the level of the biomarker. Nevertheless, it will also be immediately understood that a skilled person will consider a patient's history, i.e. a skilled person managing treatment of a patient suffering from cystic fibrosis and being treated such that a level of biomarker is lower than a cut-off value, for example, will not decide to stop treatment rather than decrease a dosage and increase the time between further applications of the methods of the present invention.

The course of cystic fibrosis may be determined by the method according to the present invention by determining a level of the biomarker in the sample from the subject at different points in time in the course of the disease. It is important to note that a single application of a method for diagnosing cystic fibrosis according to the present invention allows for diagnosing cystic fibrosis and in certain embodiments comprises a step of managing subject treatment based on the diagnosis of whether the subject is suffering from or is at risk for developing cystic

fibrosis. If a subject a sample of whom is thus subjected to the method of the present invention, is tested positive for suffering from or being at risk for developing cystic fibrosis a skilled clinician will know how to decide concerning managing subject treatment. It will be immediately understood that independent of the decision of a skilled clinician on how to manage subject treatment the skilled clinician may decide for at least one additional application of the method according to the present invention at a later point in time. It is thus an embodiment of the present invention that the levels of the biomarker are determined at the different points in time, wherein different points in time means at least two points in time, may be compared. Without wishing to be bound by any theory the present inventors have found that the level of the biomarker of the present invention in samples from one particular patient may be correlated to the severity of the disease in said patient at the point in time the sample from the patient is taken. It will thus immediately be understood that an elevated level of the biomarker determined in the sample of a later point in time compared to the level of the biomarker determined in the sample of an earlier point in time is indicative for a more severe status of the subject at the later point in time compared to the status of the subject at the earlier point in time. A decreased level of the biomarker and/or ratio of the levels of two biomarkers determined in the sample of a later point in time compared to the level of the biomarker determined in the sample of an earlier point in time is indicative for a less severe status of the subject at the later point in time compared to the status of the subject at the earlier point in time. Accordingly, in one aspect the present invention provides a method for determining the course of cystic fibrosis in a subject comprising the step of determining at several points in time a level of a biomarker present in a sample from the subject. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

In a further aspect the invention concerns a method for determining the effectiveness of at least one treatment applied to a subject being positively tested for suffering from or being at risk for developing cystic fibrosis comprising the step of determining at several points in time a level of a biomarker present in a sample from the subject. Preferably the biomarker is C26 ceramide,

more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.. It will be immediately understood by a person skilled in the art that the methods of the present invention thus allow for selecting a therapy and/or adjusting the doses and/or dosage of a selected therapy based on the results of the method of the invention. If, for example, the subject is scheduled for treatment for cystic fibrosis the method for diagnosing cystic fibrosis in a subject according to the present invention may be applied every 3 months and levels of the biomarker thus determined will be compared in order to determine the effectiveness of the treatment(s) and/or therapy/therapies applied to the subject. If the subject reaches a status, wherein a stable level of the biomarker is maintained over time the frequency of application of the method for diagnosing cystic fibrosis in a subject according to the present invention may be reduced to every 6 month. If the dosage of the therapy is changed, e.g. the amount of ASM inhibitor applied to the subject are reduced or increased, the frequency of application of the method for diagnosing in a subject according to the present invention may be set back to every 3 month. By comparison of the determined levels of the biomarker in the samples from the subject the skilled physician will recognize whether the level of the biomarker increases, decreases or whether a stable level of the biomarker is maintained over time. Accordingly, the skilled physician may decide to reduce the dosage of the therapy, e.g. the amount of ASM inhibitor applied; to increase the dosage of the therapy; or to maintain the dosage of the therapy according to the comparison of the levels of the biomarker with the method according to the present invention.

A person skilled in the art thus will acknowledge that the progression, i.e. course of cystic fibrosis, as well as the effectiveness of a therapy in a single subject can be monitored by frequent determining of the level of the biomarker in samples from the subject. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

In a further aspect the invention concerns a method for determining the effectiveness of at least one treatment applied to a subject being positively tested for suffering from or being at risk for developing cystic fibrosis, wherein the method comprises the step of determining at several points in time a level of a biomarker present in a sample from the subject. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound. In connection with what has been outlined above in relation to managing subject treatment a person skilled in the art will immediately understand that the effectiveness of one treatment or the combination of at least two treatments may be compared by applying the methods of the present invention. Thus, it is possible to test and compare several new drugs, dosage forms, dosages or treatments for cystic fibrosis by the method of the present invention.

It is an embodiment of the present invention that the method for diagnosing cystic fibrosis according to the present invention is independent of whether the subject has or has not been previously treated for cystic fibrosis. In accordance therewith, the sample from the subject may be a sample from a subject who has been previously treated for cystic fibrosis as well as a sample from a subject who has not been previously treated for cystic fibrosis. It is thus a further embodiment of the present invention that the methods of the present invention comprise a step of managing subject treatment and/or determining a level of the biomarker in the sample from the subject after subject management. Said subject treatment can be based on the diagnosis of whether the subject is suffering from or is at risk for developing cystic fibrosis; on the detection of the biomarker in a sample from the subject after subject management; or on the determining of the level of the biomarker in the sample from the subject after subject management. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound. Nevertheless, a person skilled in the art will understand that a sample of some patients not

having cystic fibrosis or of some patients being successfully treated for cystic fibrosis will show a level of such biomarker lower than the limit of detection and/or the cut-off value.

Without wishing to be bound by any theory the data presented in the example part demonstrates that the level C26 ceramide present in a sample from a subject correlates with the severity of the disease in a subject suffering from cystic fibrosis, whereby preferably the severity of a given disease including cystic fibrosis is independent from the healthy control and the severity of said given disease including cystic fibrosis is determined by comparison between different patients suffering from said given disease including cystic fibrosis. In connection therewith the present inventors assume that although, in principle, the level of C26 ceramide is different in particular individuals, and more specifically may be different in particular individuals having the same mutation(s), that the higher is a level of C26 ceramide, the higher is the severity of a course of cystic fibrosis in terms of a statistical mean according to a clinical score. Thereby the level of C26 ceramide correlates with the severity of cystic fibrosis in that in patients being positively tested for distinct mutations of the CFTR gene, being known to generally cause a mild or a more severe course of cystic fibrosis, a level of C26 ceramides, determined in said patients statistically correlates with the severity generally related to such mutation. The same equally applies to the biomarker being isoform 1 compound, isoform 2 compound or the total of isoform 1 and isoform 2 compound.

Thus a further aspect of the present invention concerns a method for determining the severity of cystic fibrosis in a subject comprising

- a) a step of determining a level of the biomarker present in a sample from the subject and
- b) a step of determining the severity of cystic fibrosis, e.g. by comparing the level of the biomarker in a subject preferably determined by a method of the present invention to a clinical score. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

The level of the biomarker will be correlated with the severity of the disease and subsequently with the type of mutation in the CFTR gene. A "mutation usually linked to a more severe course of cystic fibrosis " as used herein preferably is known to cause a more severe course of cystic fibrosis - this is especially true in case the subject is homozygous as to said mutation. Corresponding to that in an embodiment a higher mean-level of the biomarker is determined in the homozygous compared to the homozygous mutation usually linked to a milder course of cystic fibrosis. A person skilled in the art will know clinical scores to categorize the severity of cystic fibrosis or symptoms or a group of symptoms thereof. It is thus an embodiment of the method of the present invention that the course of cystic fibrosis in a patient is predicted and more particularly the severity of cystic fibrosis is determined based on the level of the biomarker determined according to the method of the present invention. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

A person skilled in the art will acknowledge that a level of the biomarker of the present invention determined in a sample from a subject wherein said level of the biomarker is correlated with the severity of cystic fibrosis as described above, will be indicative for applying a certain therapy and/or dose or dosage of said therapy. For example, if the level of the biomarker determined according to the methods of the invention is correlated with „severe" cystic fibrosis status the subject is scheduled for treatment of cystic fibrosis and the method for diagnosing cystic fibrosis in a subject according to the present invention may be applied every 3 months and levels of the biomarker thus determined will be compared in order to determine the effectiveness of the treatment(s) and/or therapy/therapies applied to the subject. If the subject reaches a status where the level of the biomarker is correlated with a "mild" cystic fibrosis or wherein a stable level of the biomarker is maintained over time, the frequency of application of the method for diagnosing cystic fibrosis in a subject according to the present invention may be reduced to every 6 month. It will be acknowledged by a person skilled in the art that such repeated diagnosing of a subject using the method for diagnosing cystic fibrosis of the present invention, preferably in case such subject having previously been diagnosed as

suffering from cystic fibrosis, is actually a monitoring of such subject and insofar, preferably in case such subject having previously been diagnosed as suffering from cystic fibrosis, the method for diagnosing of the present invention can, particularly under such conditions, also be referred to as a method of monitoring cystic fibrosis.

In another aspect the present invention is related to a method of determining the effectiveness of a composition for the treatment of cystic fibrosis. Such method may comprise the steps of determining a level a biomarker in a subject having cystic fibrosis; administering to said subject said compound in an amount sufficient to determine the effectiveness of said compound; re-determining the level of the biomarker in said subject; comparing the level of the biomarker determined before and after administering said composition, wherein a lower level of the biomarker determined after administering said composition compared to the level of the biomarker determined prior to administering said composition indicates the effectiveness of said compound for treating cystic fibrosis. Preferably the biomarker is C26 ceramide, more preferably cis-C26 ceramide or total C26 ceramide. Alternatively but also preferably, the biomarker is isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound, more preferably the biomarker is isoform 1 compound or the total of isoform compound 1 and isoform 2 compound.

It is the merit of the present inventors having found that the biomarkers of the present invention and C26 ceramide isoform 1 compound, isoform 2 compound and the total of isoform 1 compound and isoform 2 compound in particular are/is useful for the diagnosis of cystic fibrosis in a subject independent from the age of the subject. It is thus an embodiment of the present invention that the method of the present invention allows for diagnosing cystic fibrosis in a subject independent from age.

As preferably used herein, the term isoform 1 compound refers to a compound comprising the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z : 678.4847;

- retention time: 1.68 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $288.67 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44

As preferably used herein, the term isoform 2 compound refers to a compound comprising the following characteristics as measured with a high resolution ion mobility mass spectrometer:

- molecular mass m/z: 678.4847;
- retention time: 2.17 min, preferably as determined in the experimental set-up described in example 1;
- collision-cross-section: $302.79 \text{ \AA}^2$; and/or
- fragmentation pattern: 184.07, 247.20, 264.27, 282.27, 467.21, 482.26, 620.44, 660.66, 678.48, 679.44.

As preferably used herein, the term total of isoform 1 compound and isoform 2 compound means that both isoform 1 compound and isoform 2 compound are taken into consideration together. In case said term refers to the level of the total of isoform 1 compound and isoform 2 compound, this preferably means that the level or concentration of isoform 1 compound and the level or concentration of isoform 2 compound are added so as to preferably providing a single level figure of single concentration figure.

It will be understood by a person skilled in the art that, preferably, in each and any embodiment of any aspect of the present invention, cystic fibrosis may be form of cystic fibrosis selected from the group comprising pneumothorax related to cystic fibrosis, nasal polyps related to cystic fibrosis, chronic sinusitis related to cystic fibrosis, meconium ileus related to cystic fibrosis, intussusception related to cystic fibrosis, undescended testis associated to cystic fibrosis, hydrocele associated with cystic fibrosis, , pancreatic insufficiency related cystic fibrosis, sinusitis related cystic fibrosis, bronchiectasis related cystic fibrosis, intussusception related to cystic fibrosis, undescended testis associated to cystic fibrosis, hydrocele associated

with cystic fibrosis. It will be acknowledged that the above forms are complications of the disease cystic fibrosis, the cause is always CFTR mutation of a kind.

All of the methods disclosed in connection with the various aspects of the invention as to the diagnosis of cystic fibrosis may be equally used for prognosis of cystic fibrosis.

The present invention is now further illustrated by the following figures and examples from which further features, embodiments and advantages may be taken.

More specifically,

Fig. 1a is a histogram indicating levels of cis-C26 ceramide in dried blood spot (abbr. DBS) of healthy controls, i.e. healthy subjects;

Fig. 1b is a histogram indicating levels of total C26 ceramide in dried blood spot (abbr. DBS) of healthy controls, i.e. healthy subjects, i.e. healthy subjects;

Fig. 2 is a diagram (whisker plot) indicating the level of cis- C26 ceramide in dried blood spot (abbr. DBS) of cystic fibrosis patients, cystic fibrosis carriers and healthy subjects; and

Fig. 3 is a diagram (whisker plot) indicating the level of total C26 ceramide in dried blood spot (abbr. DBS) of cystic fibrosis patients, cystic fibrosis carriers and healthy subjects.

Examples

In the Examples described in the following a dried blood spot (abbr. DBS) on a filter card was used as a sample from a subject. Nevertheless, a person skilled in the art will acknowledge that depending on the used type of sample from a subject, e.g. comprising saliva, liquor, plasma, serum, full blood, blood on a dry blood filter card or another blood product, the method of the present invention has to be adjusted to the type of sample and furthermore a cut-off value has to be determined for each type of sample according to the method described in the following examples.

Example 1: Method for the detection of C26 ceramide in DBS*Equipment*

For detecting C26 ceramide and/or a substance with molecular weight of 678, detected as MRM transition in positive mode 678.7 m/z to 264.3 m/z, in a sample of plasma from a subject the following equipment was used:

Apparatus / Piece of Equipment Type / Producer

UPLC Aquity Series (pumps, autosampler, column manager), Waters, UK

Mass selective detector TripleQuad 5500, AB SCIEX, Germany

Multi-tube vortexer DVX-2500 Henry Troemner LLC, USA

Vortex mixer Vortex Genie 2; Scientific Industries, USA

Centrifuge Megafuge 1.0; Heraeus, Germany

Multipette(s), pipette(s) Eppendorf, Germany

Water bath SW21-C, Julabo, Germany

Reagents

For detecting C26 ceramide in a sample from a subject the following reagents were used. To that extent that values depend on temperature (e.g. the pH value) such values were determined at a temperature of 25°C.

Acetonitrile (ACN) HPLC-grade or Gradient

Acetone 99.5 %

Dimethylsulfoxide (DMSO) HPLC grade

Ethanol (EtOH) p.a., 96 %

Formic acid (FA) p.a., 98 - 100 %

Methanol (MeOH) Gradient (LiChrosolv)

Trifluoroacetic acid (TFA) purum > 98 %

Water ASTM-I

The abbreviation "p.a." as used herein means "pro analysis".

The term "purum" as used herein, preferably means a commercial grade of a chemical compound having a purity of the above specified value.

ASTM-I as used herein refers to a water grade standard purity achieved by purification methods comprising Reverse Osmosis and Ultraviolet (UV) Oxidation.

Preparation of Calibration Standards

For calibration, as calibration standards five concentration levels between 2.00 and 200 ng/mL C26 ceramide (as provided by Carbosynth, UK) were used.

Preparation of Internal Standard

The Internal Standard (IS 1) stock solution was prepared dissolving 25 µg of C25 ceramide (as provided by Avanti Polar Lipids, US) in DMSO / EtOH (1/1; vol/vol) to a concentration of 50 ng/mL.

Storing of Samples and Solutions

Control samples or study samples either were immediately stored below -20°C. Standard solutions as well as Internal Standard working solutions were stored between 2°C and 8°C until use.

Sample Preparation for Analysis

3 punches Ø of 3.2 µm were cut from the filtercard with dried blood spots and subjected to extraction in presence of internal standard solution in DMSO: water: ethanol vol. 1:1:2 for 60 minutes at 37°C and sonicated 20 minutes at 40°C. The solution containing internal standard and blood extract was transferred to a PTFE (polytetrafluoroethylene) filter plate (Acroprep™, Pall, Germany) and then to a 96 well plate by centrifugation at 3.500 rpm.

Methods

A person skilled in the art will acknowledge that methods for detecting C26 ceramide in a sample from a subject using mass spectrometric analysis may also employ other transitions and

fragments which allow for specific detection of and/or quantification of C26 ceramide and its isoforms in said sample from a subject.

LC/MRM-MS analyses of the C26 ceramide for DBS extracts were performed using a Waters Acquity UPLC (Waters, UK) coupled with an ABSciex 5500 TripleQuad mass spectrometer (ABSciex, Germany). Chromatographic run was performed on a C8 column (ACE columns, Germany). The 10 μ L extract were injected on the column and the compounds were eluted using a linear gradient from 40 % A (50 mM formic acid in water) to 100 % B (50 mM formic acid in acetonitrile/acetone). The following MRM transitions were monitored: 664.3 $\rightarrow$ 264.3 for the internal standard (with DP of 34V, CE of 46 V and CXP of 13 V) and 678.7 $\rightarrow$ 264.3 for C26 ceramide isoforms (with DP of 30V, CE of 38.4 V and CXP of 11 V).

MRM-MS analyses were performed in positive ion mode using the following parameters: CUR gas 10 psi, IS voltage 5 kV, CAD 8 psi, cone temperature 200 °C, GS 1 45 psi, GS2 60 psi, EP 10 V. For all batches analyzed a standard curve was measured using 5 dilutions of C26 ceramide in ethanol .

Data analysis was performed using Analyst 1.6.2 and Microsoft Office 2010.

Software

Data acquisition, data processing, statistics and calculations were performed using Analyst software 1.6.2 or higher (AB SCIEX, USA/Canada).

Example 2: Determining C26 ceramide in DBS from healthy subjects and cystic fibrosis patients and cystic fibrosis carriers

Using the methods outlined in Example 1, the content of cis-C26 ceramide (referred to as “Isoform 1” in Table 1) and trans-C26 ceramide (referred to as “Isoform 2” in Table 1) was determined in DBS from a total of 192 healthy subjects. Similarly, the content of cis-C26 ceramide (referred to as “Isoform 1” in Table 1) and trans-C26 ceramide (referred to as

“Isoform 2” in Table 1) was determined in DBS from a total of 15 previously genetically diagnosed cystic fibrosis patients and in DBS from a total of 40 cystic fibrosis carriers (a priori genetically proven). From such data, the ratio (cis-C26 ceramide/trans-C26 ceramide), in referred to as “Ratio” in Table 1, and the total content of C26 ceramide (content of cis-C26 ceramide + content of trans-C26 ceramide), referred to as “Total” in Table 1, were determined. The cut-off value was set at the mean value of the cohort of healthy individuals plus 2x standard deviation.

The results thereof are shown in Tables 1a, 1b and 1c.

Table 1a: Summary of C26 ceramide levels in healthy controls, cystic fibrosis patients and cystic fibrosis carriers

Samples	Ceramide C26:0 (nmol/L)				
	N	Isoform1	Isoform2	Ratio	Total
<i>Cut-off</i>		27.7	-	-	69.1
Cystic Fibrosis patients	15	67.3 ± 26.0	39.3 ± 29.4	1.9 ± 1.0	106.5 ± 40.74
Cystic Fibrosis carriers	40	49.3 ± 52.8	44.9 ± 20.96	1.5 ± 1.1	93.9 ± 55.28
All Normal Controls	192	18.3 ± 4.7	31.6 ± 8.6	0.6 ± 0.2	49.9 ± 9.6

Table 1b: C26 ceramide levels in healthy controls

No	Healthy control description	C26Cer Isoform 1 nmol/L	C26Cer Isoform 2 nmol/L	ratio	Total C26Cer nmol/L
1	Newborn 1	13.1	51.5	0.3	64.6
2	Newborn 2	20.0	45.0	0.4	65.0
3	Newborn 3	15.3	46.4	0.3	61.7
4	Newborn 4	13.6	40.4	0.3	54.0
5	Newborn 5	14.2	22.0	0.6	36.2
6	Newborn 6	19.1	18.4	1.0	37.5
7	Newborn 7	18.8	45	0.4	63.8
8	Newborn 8	18.9	32.6	0.6	51.5
9	Newborn 9	16.9	20.1	0.8	37.0
10	Newborn 10	19.6	32.4	0.6	52.0
11	Newborn 11	27.0	19.7	1.4	46.7
12	Newborn 12	15.9	33.3	0.5	49.2
13	Newborn 13	16.9	25.1	0.7	42.0
14	Newborn 14	25.3	40.4	0.6	65.7
15	Newborn 15	19.3	32.7	0.6	52.0
16	Newborn 16	19.9	23.0	0.9	42.9
17	Newborn 17	19.0	42.8	0.4	61.8
18	Newborn 18	13.2	30.8	0.4	44.0
19	Newborn 19	20.9	45.0	0.4	65.9
20	Newborn 20	9.5	24.1	0.4	33.6
21	Newborn 21	6.9	60.7	0.1	67.6
22	Newborn 22	17.0	34.5	0.5	51.5
23	Newborn 23	12.6	40.0	0.3	52.6
24	Newborn 24	21.1	25.9	0.8	47.0
25	Newborn 25	27.0	17.4	1.6	44.4
26	Newborn 26	9.3	40.0	0.2	49.3
27	Newborn 27	26.0	27.2	1.0	53.2
28	Newborn 28	27.0	42.0	0.6	69.7
29	Newborn 29	25.8	33.5	0.8	59.3
30	Newborn 30	17.0	32.5	0.5	49.5

31	Newborn 31	13.0	42.0	0.3	55
32	Juvenile_0.5-4y_01	22.8	28.0	0.8	50.8
33	Juvenile_0.5-4y_02	14.0	16.9	0.8	30.9
34	Juvenile_0.5-4y_03	10.4	23.5	0.4	33.9
35	Juvenile_0.5-4y_04	22.1	34.2	0.6	56.3
36	Juvenile_0.5-4y_05	21.4	15.5	1.4	36.9
37	Juvenile_0.5-4y_06	24.6	13.5	0.9	38.1
38	Juvenile_0.5-4y_07	13.0	20.4	0.6	33.4
39	Juvenile_0.5-4y_08	24.9	27.8	0.9	52.7
40	Juvenile_0.5-4y_09	10.5	23.9	0.4	34.4
41	Juvenile_0.5-4y_10	18.4	36.0	0.5	54.4
42	Juvenile_0.5-4y_11	17.6	24.6	0.7	42.2
43	Juvenile_0.5-4y_12	17.5	27.3	0.6	44.8
44	Juvenile_0.5-4y_13	18.7	21.6	0.9	40.3
45	Juvenile_0.5-4y_14	10.9	20.7	0.5	31.6
46	Juvenile_0.5-4y_15	27.0	26.6	1.1	53.6
47	Juvenile_0.5-4y_16	17.7	43.6	0.4	61.3
48	Juvenile_0.5-4y_17	20.7	31.3	0.7	52
49	Juvenile_0.5-4y_18	19.2	23.6	0.8	42.8
50	Juvenile_0.5-4y_19	19.6	34.8	0.6	54.4
51	Juvenile_0.5-4y_20	17.4	24.7	0.7	42.1
52	Juvenile_0.5-4y_21	26.1	32.9	0.8	59
53	Juvenile_0.5-4y_22	22.6	29.2	0.8	51.8
54	Juvenile_0.5-4y_23	21.4	28.8	0.7	50.2
55	Juvenile_0.5-4y_24	23.8	19.1	1.2	42.9
56	Juvenile_0.5-4y_25	18.9	16.4	1.1	35.3
57	Juvenile_0.5-4y_26	23.0	31.2	0.7	54.2
58	Juvenile_0.5-4y_27	23.2	33.4	0.7	56.6
59	Juvenile_0.5-4y_28	27.0	40.4	0.8	67.4
60	Juvenile_0.5-4y_29	7.1	23.3	0.3	30.4
61	Juvenile_0.5-4y_30	14.4	46.8	0.3	61.2
62	Juvenile_0.5-4y_31	20.9	28.4	0.7	49.3
63	Juvenile_0.5-4y_32	8.7	29.5	0.3	38.2
64	Juvenile_0.5-4y_33	27.0	16.1	2.0	43.1

65	Juvenile_0.5-4y_34	27.0	31.4	0.9	58.4
66	Juvenile_0.5-4y_35	23.0	17.7	1.6	40.7
67	Juvenile_0.5-4y_36	26.3	38.5	0.7	64.8
68	Juvenile_4-17y_01	19.5	37.2	0.5	56.7
69	Juvenile_4-17y_02	13.3	36.2	0.4	49.5
70	Juvenile_4-17y_03	25.8	33.6	0.8	59.4
71	Juvenile_4-17y_04	22.0	45.0	0.5	67.0
72	Juvenile_4-17y_05	24.8	29.6	0.8	54.4
73	Juvenile_4-17y_06	23.6	36.4	0.6	60.0
74	Juvenile_4-17y_07	16.0	50.8	0.4	66.8
75	Juvenile_4-17y_08	23.8	31.6	0.8	55.4
76	Juvenile_4-17y_09	21.5	40.8	0.5	62.3
77	Juvenile_4-17y_10	18.4	43.0	0.3	61.4
78	Juvenile_4-17y_11	11.8	50.4	0.2	62.2
79	Juvenile_4-17y_12	19.0	31.3	0.6	50.3
80	Juvenile_4-17y_13	20.7	17.0	1.2	37.7
81	Juvenile_4-17y_14	22.9	35.3	0.6	58.2
82	Juvenile_4-17y_15	16.9	38.7	0.4	55.6
83	Juvenile_4-17y_16	16.0	38.5	0.4	54.5
84	Juvenile_4-17y_17	23.0	29.8	0.8	52.8
85	Juvenile_4-17y_18	24.8	38.0	0.7	62.8
86	Juvenile_4-17y_19	19.0	28.4	0.7	47.4
87	Juvenile_4-17y_20	13.4	22.5	0.6	35.9
88	Juvenile_4-17y_21	23.9	19.0	1.3	42.9
89	Juvenile_4-17y_22	20.9	39.3	0.5	60.2
90	Juvenile_4-17y_23	14.7	35.3	0.4	50.0
91	Juvenile_4-17y_24	10.3	30.0	0.3	40.3
92	Juvenile_4-17y_25	17.0	21.6	0.8	38.6
93	Juvenile_4-17y_26	18.4	35.6	0.5	54.0
94	Juvenile_4-17y_27	20.2	40.8	0.5	61.0
95	Juvenile_4-17y_28	26.6	38.9	0.7	65.5
96	Juvenile_4-17y_29	14.9	48.0	0.3	62.9
97	Juvenile_4-17y_30	18.2	31.6	0.6	49.8
98	Juvenile_4-17y_31	21.0	30.5	0.7	51.5

99	Juvenile_4-17y_32	25.9	28.0	0.9	53.9
100	Juvenile_4-17y_33	16.1	37.0	0.4	53.1
101	Juvenile_4-17y_34	24.4	27.9	0.9	52.3
102	Juvenile_4-17y_35	19.2	26.7	0.7	45.9
103	Juvenile_4-17y_36	24.7	33.2	0.7	57.9
104	Juvenile_4-17y_37	21.0	31.5	0.7	52.5
105	Juvenile_4-17y_38	19.1	38.4	0.5	57.5
106	Juvenile_4-17y_39	21.4	47.0	0.4	69.4
107	Juvenile_4-17y_40	14.8	22.3	0.7	37.1
108	Juvenile_4-17y_41	10.2	18.0	0.6	28.2
109	Juvenile_4-17y_42	17.2	17.6	1	34.8
110	Adult_01	19.3	46.0	0.4	65.3
111	Adult_02	13.5	33.2	0.4	46.7
112	Adult_03	11.3	26.4	0.4	37.7
113	Adult_04	15.1	38.6	0.4	53.7
114	Adult_05	12.3	32.2	0.4	44.5
115	Adult_06	21.3	44.0	0.5	65.3
116	Adult_07	14.5	48.4	0.3	62.9
117	Adult_08	14.4	31.0	0.5	45.4
118	Adult_09	10.9	29.5	0.4	40.4
119	Adult_10	22.0	45.0	0.6	67.0
120	Adult_11	15.3	37.3	0.4	52.6
121	Adult_12	14.6	44.4	0.3	59.0
122	Adult_13	22.3	35.9	0.6	58.2
123	Adult_14	18.4	24.3	0.8	42.7
124	Adult_15	14.9	21.1	0.7	36.0
125	Adult_16	15.9	28.0	0.6	43.9
126	Adult_17	15.3	25.3	0.6	40.6
127	Adult_18	25.1	35.1	0.7	60.2
128	Adult_19	14.7	42.0	0.3	56.7
129	Adult_20	17.6	28.7	0.6	46.3
130	Adult_21	15.4	24.3	0.6	39.7
131	Adult_22	24.9	39.6	0.6	64.5
132	Adult_23	18.1	26.7	0.7	44.8

133	Adult_24	17.4	34.7	0.5	52.1
134	Adult_25	20.6	31.8	0.6	52.4
135	Adult_26	15.7	28.4	0.6	44.1
136	Adult_27	16.5	29.7	0.6	46.2
137	Adult_28	13.1	34.2	0.4	47.3
138	Adult_29	18.5	27.2	0.7	45.7
139	Adult_30	22.9	36.5	0.6	59.4
140	Adult_31	14.8	28.7	0.5	43.5
141	Adult_32	11.0	34.6	0.3	45.6
142	Adult_33	18.8	30.4	0.6	49.2
143	Adult_34	17.8	36.8	0.5	54.6
144	Adult_35	15.9	40.4	0.4	56.3
145	Adult_36	16.5	43.2	0.4	59.7
146	Adult_37	25.2	36.3	0.7	61.5
147	Adult_38	15.1	24.1	0.6	39.2
148	Adult_39	27.9	36.5	0.8	64.1
149	Adult_40	17.6	32.1	0.5	49.7
150	Adult_41	18.3	21.9	0.8	40.2
151	Adult_42	16.9	25.9	0.7	42.8
152	Adult_43	15.4	19.7	0.8	35.1
153	Adult_44	19.7	27.0	0.7	46.7
154	Adult_45	16.1	28.4	0.6	44.5
155	Adult_46	14.0	28.7	0.5	42.7
156	Adult_47	19.4	25.6	0.8	45.0
157	Adult_48	16.6	32.1	0.5	48.7
158	Adult_49	22.6	34.3	0.7	56.9
159	Adult_50	19.4	37.0	0.5	56.4
160	Adult_51	15.0	32.5	0.5	47.5
161	Adult_52	13.7	31.8	0.4	45.5
162	Adult_53	19.2	23.4	0.8	42.6
163	Adult_54	15.4	26.5	0.6	41.9
164	Adult_55	16.1	18.8	0.9	34.9
165	Adult_56	26.1	27.7	0.9	53.8
166	Adult_57	25.7	31.4	0.8	57.1

167	Adult_58	14.2	25.8	0.5	40.0
168	Adult_59	17.7	32.0	0.6	49.7
169	Adult_60	19.2	32.6	0.6	51.8
170	Adult_61	17.8	28.8	0.6	46.6
171	Adult_62	15.4	32.7	0.5	48.1
172	Adult_63	19.5	32.6	0.6	52.1
173	Adult_64	13.3	28.3	0.5	41.6
174	Adult_65	13.7	31.0	0.4	44.7
175	Adult_66	21.1	32.9	0.6	54.0
176	Adult_67	18.9	33.1	0.6	52.0
177	Adult_68	14.5	32.7	0.4	47.2
178	Adult_69	14.2	21.8	0.7	36.0
179	Adult_70	22.2	22.9	1.0	45.1
180	Adult_71	17.7	31.4	0.6	49.1
181	Adult_72	17.5	30.0	0.6	47.5
182	Adult_73	16.9	33.6	0.5	50.5
183	Adult_74	27.0	40.0	0.7	67.0
184	Adult_75	11.7	24.6	0.5	36.3
185	Adult_76	12.6	28.9	0.4	41.5
186	Adult_77	20.4	39.3	0.5	59.7
187	Adult_78	19.9	28.3	0.7	48.2
188	Adult_79	12.6	40.0	0.3	52.6
189	Adult_80	15.2	24.2	0.6	39.4
190	Adult_81	11.1	27.6	0.4	38.7
191	Adult_82	16.0	24.9	0.6	40.9
192	Adult_83	15.6	25.2	0.6	40.8
minimum		6.9	3.5	0.1	28.1
maximum		27.9	60.7	2.0	69.7
mean		18.3	31.6	0.6	49.9
Standard Deviation		4.7	-	-	9.6
Cut-off		27.7	-	-	69.1

The normal distribution of cis-C26 ceramide and total C26 ceramide for healthy controls is shown in Fig. 1a and Fig 1b.

Table 1c: C26 ceramide levels in cystic fibrosis patients and carriers

No	Sample description		C26Cer Isoform 1 nmol/L	C26Cer Isoform 2 nmol/L	ratio	Total C26Cer nmol/L
Cystic Fibrosis - Affected patients						
1	Cystic Fibrosis _01	patient	110.7	61.3	1.8	171.9
2	Cystic Fibrosis _02	patient	53.5	34.2	1.6	87.7
3	Cystic Fibrosis _03	patient	82.8	52.7	1.6	135.5
4	Cystic Fibrosis _04	patient	78.7	45.7	1.8	124.4
5	Cystic Fibrosis _05	patient	40.7	58.3	0.7	99.0
6	Cystic Fibrosis _06	patient	55.0	31.2	1.8	86.2
7	Cystic Fibrosis _07	patient	69.7	19.7	3.6	89.5
8	Cystic Fibrosis _08	patient	49.7	31.6	1.6	81.3
9	Cystic Fibrosis _09	patient	38.8	31.8	1.2	70.7
10	Cystic Fibrosis _10	patient	47.6	23.3	2.1	70.9
11	Cystic Fibrosis _11	patient	78.3	47.3	1.7	125.6
12	Cystic Fibrosis _12	patient	57.5	24.9	2.3	82.4
13	Cystic Fibrosis _13	patient	95.4	27.5	3.5	122.9
14	Cystic Fibrosis _14	patient	64.0	63.0	1.0	127.0
15	Cystic Fibrosis _15	patient	86.3	36.4	2.5	122.7
minimum			38.8	19.7	0.7	70.7
maximum			110.7	63.0	3.6	171.9
mean			67.3	39.3	1.9	106.5

Cystic Fibrosis - Carriers

1	CF Carrier_01	carrier	48.4	60.6	0.8	109.0
2	CF Carrier_02	carrier	97.6	22.2	4.5	119.7
3	CF Carrier_03	carrier	15.8	33.8	0.5	49.5
4	CF Carrier_04	carrier	10.9	55.1	0.2	66.1
5	CF Carrier_05	carrier	13.8	33.7	0.4	47.4
6	CF Carrier_06	carrier	28.7	67.9	0.4	96.6
7	CF Carrier_07	carrier	47.3	48.0	1.0	95.4
8	CF Carrier_08	carrier	69.2	49.1	1.8	107.9
9	CF Carrier_09	carrier	73.5	58.2	1.3	131.8

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10	CF Carrier_10	carrier	58.0	32.7	1.8	90.7
11	CF Carrier_11	carrier	12.6	51.1	0.3	63.7
12	CF Carrier_12	carrier	94.5	31.8	3.1	126.4
13	CF Carrier_13	carrier	33.9	31.2	1.1	65.1
14	CF Carrier_14	carrier	92.5	43.1	2.2	135.6
15	CF Carrier_15	carrier	10.8	37.4	0.3	48.2
16	CF Carrier_16	carrier	110.5	38.2	2.9	148.7
17	CF Carrier_17	carrier	10.7	37.4	0.3	48.1
18	CF Carrier_18	carrier	13.8	47.9	0.3	61.7
19	CF Carrier_19	carrier	14.6	52.0	0.3	66.6
20	CF Carrier_20	carrier	103.9	64.0	1.7	167.8
21	CF Carrier_21	carrier	12.9	31.3	0.4	44.1
22	CF Carrier_22	carrier	16.4	55.7	0.3	72.2
23	CF Carrier_23	carrier	28.9	35.5	0.8	64.3
24	CF Carrier_24	carrier	36.6	34.1	1.1	70.7
25	CF Carrier_25	carrier	14.6	27.5	0.5	42.0
26	CF Carrier_26	carrier	60.1	41.3	1.5	101.4
27	CF Carrier_27	carrier	66.3	40.0	1.7	106.2
28	CF Carrier_28	carrier	67.1	28.8	2.3	95.9
29	CF Carrier_29	carrier	50.5	5.1	9.8	55.6
30	CF Carrier_30	carrier	89.9	31.8	2.9	121.7
31	CF Carrier_31	carrier	43.1	56.8	0.8	99.8
32	CF Carrier_32	carrier	56.1	56.0	1.0	112.1
33	CF Carrier_33	carrier	12.5	105.1	0.1	117.5
34	CF Carrier_34	carrier	19.4	72.2	0.3	91.6
35	CF Carrier_35	carrier	94.7	37.6	2.5	132.4
36	CF Carrier_36	carrier	102.5	78.4	1.3	181.0
37	CF Carrier_37	carrier	45.2	41.3	1.1	86.5
38	CF Carrier_38	carrier	131.0	40.1	3.3	171.2
39	CF Carrier_39	carrier	51.8	33.6	1.6	85.4
40	CF Carrier_40	carrier	12.0	47.7	0.3	59.8
minimum			10.7	5.1	0.1	42.0
maximum			131.0	105.1	9.8	181.0
mean			49.3	44.9	1.5	93.9

Example 3: Determining the cut-off value for C26 ceramide for the diagnosis of cystic fibrosis

Based on the results presented in Examples 1 and 2, the cut-off values for the diagnosis of cystic fibrosis patients and cystic fibrosis carrier based on cis-C26 ceramide and total C26 ceramide were determined. Using dried blood spots, the cut-off value for the diagnosis of cystic fibrosis based on the level of cis-C26 ceramide is 27.7 nmol/L blood of the subject to be tested and, respectively, diagnosed, and the cut-off value for the diagnosis of cystic fibrosis based on the level of total C26 ceramide is 69.1 nmol/L blood of the subject to be tested and, respectively, diagnosed.

These results are also shown as a whisker plot in Figs. 2 and 3. From said Fig. 2 it is evident that using cis-C26 ceramide as a biomarker in blood of a subject with a cut-off value of 27.7 nmol/L, cystic fibrosis patients can be distinguished from normal, i.e. healthy control subjects. From said Fig. 3 it is evident that using total C26 ceramide as a biomarker in blood of a subject with a cut-off value of 69.1 nmol/L, cystic fibrosis carriers can be distinguished from normal, i.e. healthy control subjects.

Example 4: Positive predictive value and negative predictive value of C26 ceramide in the diagnosis of cystic fibrosis

All of the samples described in Example 1 were used for calculating the positive predictive value (PPV) and the negative predictive value (NPV) versus the healthy controls. The results are shown in Table 2.

Table 2: PPV and NPV for C26 ceramide for the diagnosis of cystic fibrosis

		Patients with Cystic fibrosis (as confirmed on molecular genetics)		
		Condition positive	Condition negative	
C26Cer Isoform 1 (cis-C26 ceramide)	Test outcome positive	True positive (TP) = 15	False positive (FP) = 1	Positive predictive value = TP / (TP + FP) = 0,937
	Test outcome negative	False negative (FN) = 0	True negative (TN) = 192	Negative predictive value = TN / (FN + TN) =1
		Sensitivity = TP / (TP + FN) = 1	Specificity = TN / (FP + TN) = 0.995	
		Patients with cystic fibrosis (as confirmed on molecular genetics)		
		Condition positive	Condition negative	
Total C26Cer	Test outcome positive	True positive (TP) = 15	False positive (FP) = 2	Positive predictive value = TP / (TP + FP) =
	Test outcome negative	False negative (FN) = 0	True negative (TN) = 192	Negative predictive value = TN / (FN + TN) = 1
		Sensitivity = TP / (TP + FN) = 1	Specificity = TN / (FP + TN) = 0.989	

The features of the present invention disclosed in the specification, the claims, the sequence listing and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

CLAIMS

1. A method for diagnosing cystic fibrosis in a subject comprising

a step a), wherein the step a) comprises detecting a biomarker in a sample from the subject, wherein the sample is a sample selected from the group comprising blood, a blood product, urine, saliva, cerebrospinal liquid, stool, tissue and lymph liquid.
2. The method according to claim 1, wherein the method comprises

a step b) wherein the step b) comprises determining a level of the biomarker present in the sample.
3. The method according to any one of claims 1 or 2, wherein the level of the biomarker is indicative whether or not the subject is suffering from cystic fibrosis or whether or not the subject is at risk of suffering from cystic fibrosis.
4. A method for diagnosing cystic fibrosis in a subject, wherein the method comprises the following steps:
 - i) adding an internal standard to a sample from the subject, wherein the sample from the subject is selected from the group comprising blood, a blood product, urine, saliva, cerebrospinal liquid, stool, tissue and lymph liquid;
 - ii) optionally mixing the sample with the internal standard;
 - iii) subjecting the sample to a protein precipitation and/or a biomarker extraction step, whereby protein from the sample is precipitated and/or the biomarker is extracted and a first supernatant of the sample is provided;
 - iv) optionally subjecting the first supernatant of the sample or at least a part thereof to a first separation step which provides a second supernatant, whereby preferably the first separation step is a step of centrifugation;

- v) subjecting the first supernatant and/or the second supernatant, or at least a part thereof, to a second separation step, wherein the second separation step comprises injecting at least a part of the first supernatant and/or at least a part of the second supernatant into an HPLC-MS/MS system and using an HPLC column with a gradient from acidic water to acetonitrile/acetone; wherein the HPLC column is preferably an HPLC column selected from the group comprising a C8 HPLC column and a C18 HPLC column, and wherein the second separation step provides a separated sample;
- vi) subjecting the separated sample to MS/MS, wherein MS/MS comprises collision induced dissociation, in source-fragmentation, electron capture and/or transfer dissociation;

and wherein the method comprises

a step a), wherein the step a) comprises detecting a biomarker in a or the sample from the subject,

and optionally

a step b), wherein the step b) comprises determining a level of the biomarker present in the sample, and

wherein the method is preferably a method according to any one of claims 1 to 3.

5. The method of claim 4, wherein the internal standard is selected from the group comprising N-lauroyl sphingosine, lyso-Gb2, a C17 ceramide, a C19 ceramide, a C21 ceramide, a C23 ceramide, a C25 ceramide and a C27 ceramide.

6. A method of determining the effectiveness of a compound for the treatment of cystic fibrosis, wherein the method comprises the following steps:

- a) determining a level of a biomarker in a sample from a subject having cystic fibrosis;

- b) administering to said subject said compound;
- c) determining the level of the biomarker in a sample from the subject after the compound has been administered to the subject; and
- d) determining whether the level of the biomarker determined in step c) is lower than the level of the biomarker determined in step a);

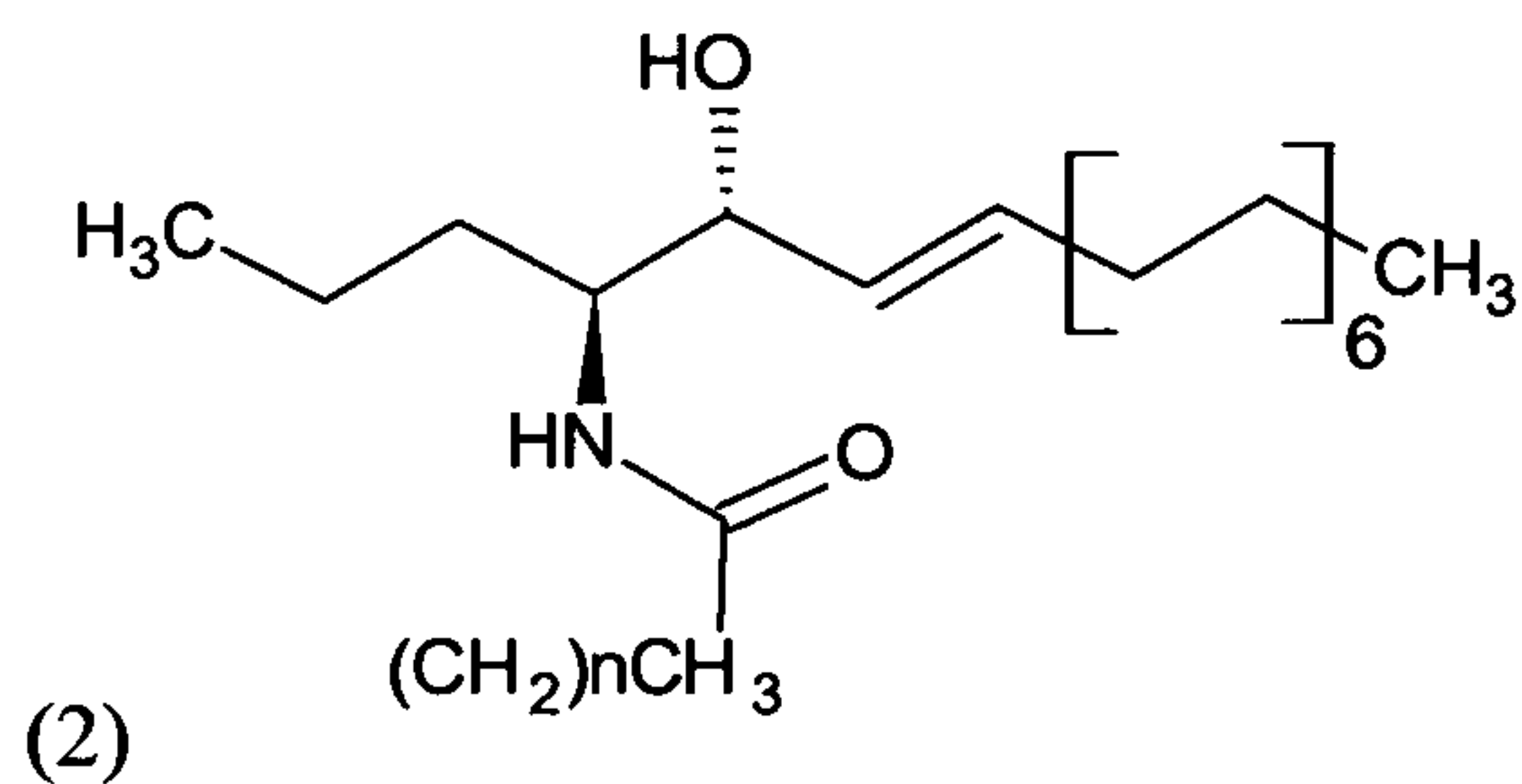
wherein if a level of the biomarker determined in step c) is lower than the level of the biomarker determined in step a) this indicates the effectiveness of said compound.

7. A method for determining the effectiveness of at least one treatment applied to a subject being positively tested for suffering from or being at risk of suffering from cystic fibrosis comprising

a step a), wherein the step a) comprises determining at several points in time a level of a biomarker in a sample from the subject.

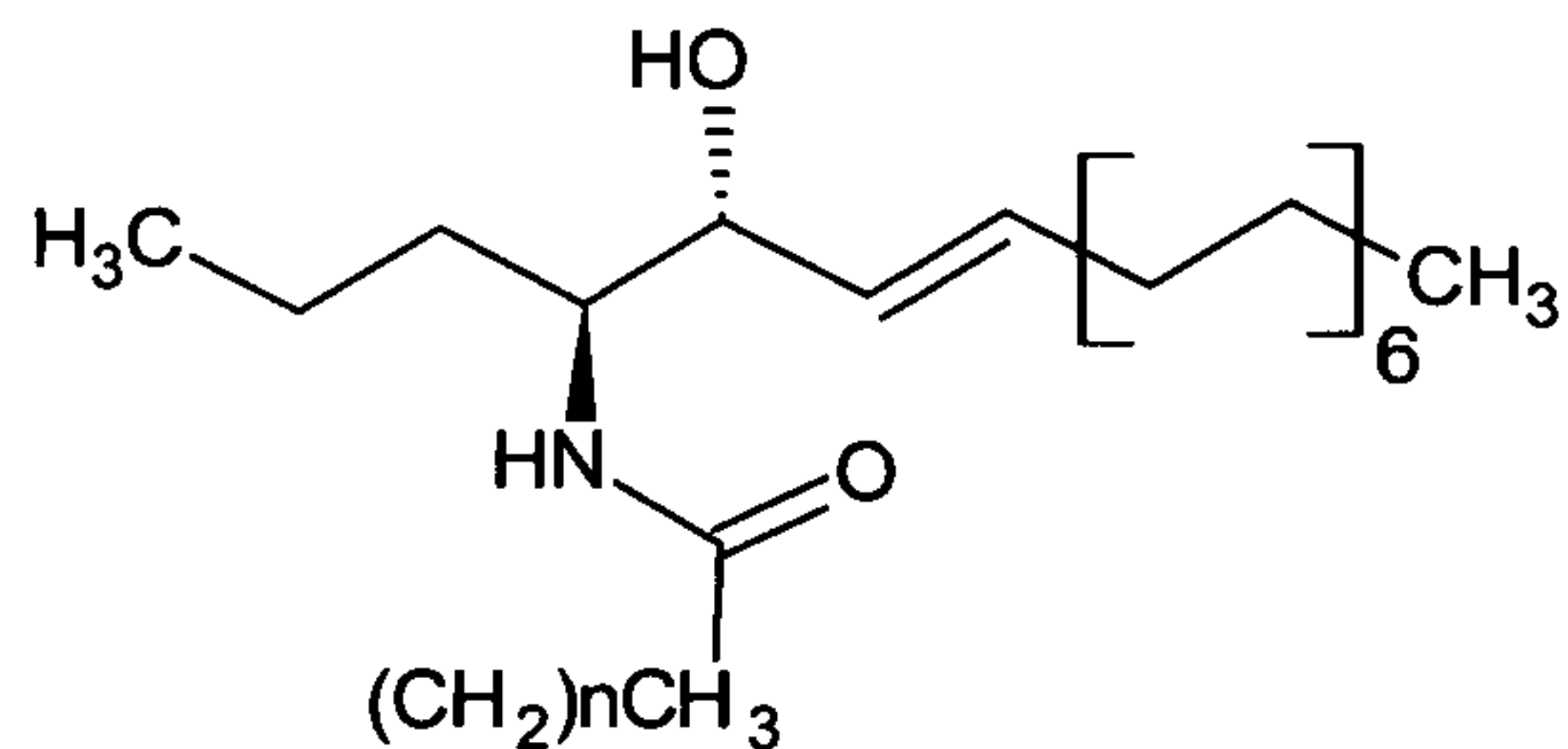
8. A method for determining the course of cystic fibrosis in a subject comprising the step of determining at several points in time a level of a biomarker present in a sample from the subject.

9. The method according to any one of claims 1 to 8, wherein the biomarker is one selected from the group comprising of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)



(2)

wherein n is any integer from 18, 20, 22, 24, 26, and 28.

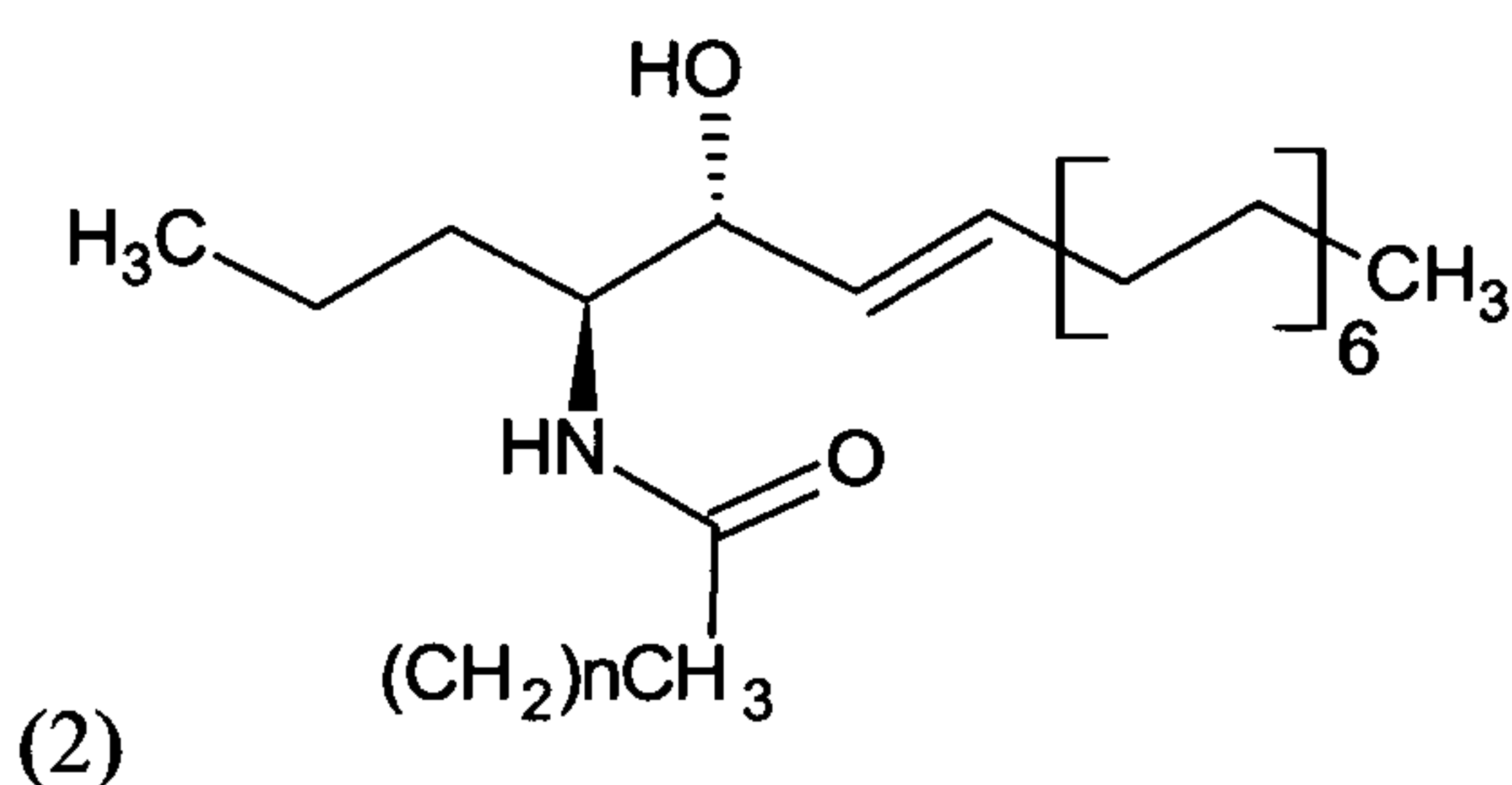
10. The method according to claim 9, wherein the biomarker is C26 ceramide.
11. The method according to any one of claims 9 to 10, wherein the biomarker is cis-C26 ceramide.
12. The method according to any one of claims 9 to 10, wherein the biomarker is total C26 ceramide, preferably total C26 ceramide is cis-C26 ceramide plus trans-C26 ceramide.
13. The method according to any one of claims 1 to 8, wherein the biomarker is selected from the group comprising isoform 1 compound, isoform 2 compound and the total of isoform 1 compound and isoform 2 compound.
14. The method according to any one of claims 1 to 13, wherein the biomarker is detected by means of mass spectrometric analysis.
15. The method according to any one of claims 1 to 14, wherein the subject is a human being.
16. The method according to any one of claims 1 to 15, wherein if the level of the biomarker in the sample from the subject is higher than a cut-off value this is indicative that the subject is

suffering from cystic fibrosis or is at risk of suffering from cystic fibrosis and/or wherein if the level of the biomarker in the sample from the subject is lower than a cut-off value this is indicative that the subject is not suffering from or is not at risk of suffering from cystic fibrosis, preferably the cut-off value is 69 nmol/L blood, more preferably 69.1 nmol /L blood in case the biomarker is total C26 ceramide and the cut-off value is 27.7 nmol/L blood in case the biomarker is cis-C26 ceramide.

17. The method according to any one of claims 6 to 16, wherein the sample is selected from the group consisting of blood, a blood product, urine, saliva, cerebrospinal liquid, stool, tissue and lymph liquid.

18. The method according to any one of claims 1 to 17, preferably claim 16, wherein the blood product is dried blood spots (DBS).

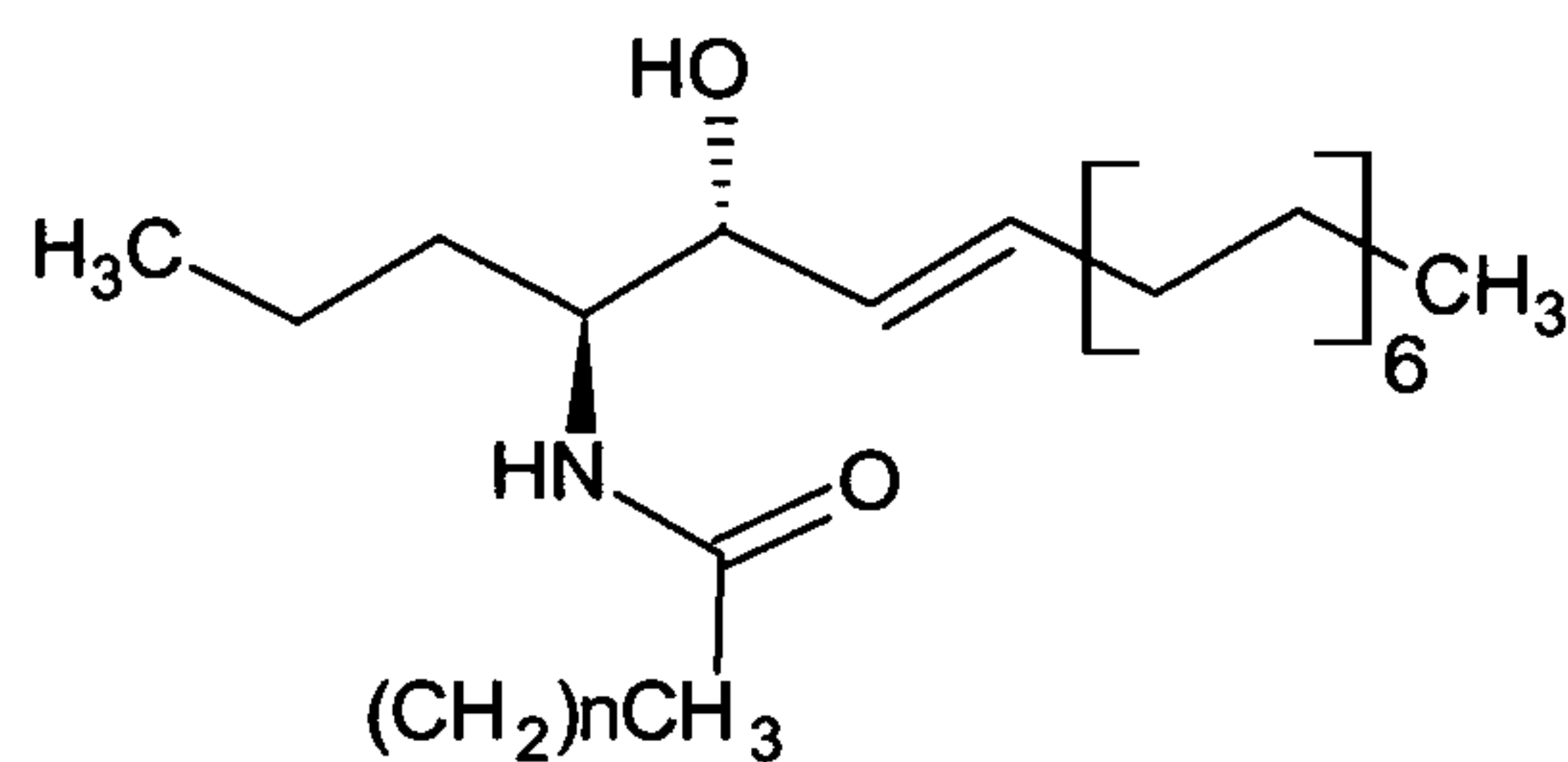
19. Use of mass spectrometric analysis for the detection of a biomarker, wherein the biomarker is selected from the group consisting of C26 ceramide, a medium ceramide and a long ceramide, wherein a medium ceramide is a ceramide of formula (2)



wherein n is any integer from: 8, 10, 12, 14 and 16 and

a long ceramide is a ceramide of formula (2)

a long ceramide is a ceramide of formula (2)



(2)

wherein n is any integer from 18, 20, 22, 24, 26 and 28, or

wherein the biomarker is selected from the group consisting of isoform 1 compound, isoform 2 compound or the total of isoform 1 compound and isoform 2 compound.

21. A kit for determining the presence of a biomarker in a sample from a subject, wherein the kit comprises

- a) an interaction partner of the biomarker;
- b) optionally a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds the biomarker; and
- c) instructions for using the solid support to detect the biomarker, wherein the biomarker is C26 ceramide, preferably cis-C26 ceramide.

22. The kit according to claim 21, wherein the kit is for

- a) use in a method for diagnosing cystic fibrosis;
- b) use in a method for determining the course of cystic fibrosis in a subject; and/or
- c) use in a method for determining the effectiveness of at least one treatment applied to a subject,

wherein preferably the method of a), b) and/or c) is a method according to any one of claims 1 to 18.

23. Use of a compound selected from the group consisting of N-lauroyl sphingosine, lyso-Gb2, a C17 ceramide, a C19 ceramide, a C21 ceramide, a C23 ceramide and a C25 ceramide as an internal standard in

- a) use in a method for diagnosing cystic fibrosis;
- b) use in a method for determining the course of cystic fibrosis in a subject; and/or
- c) use in a method for determining the effectiveness of at least one treatment applied to a subject,

wherein preferably the method of a), b) and/or c) is a method according to any one of claims 1 to 18.

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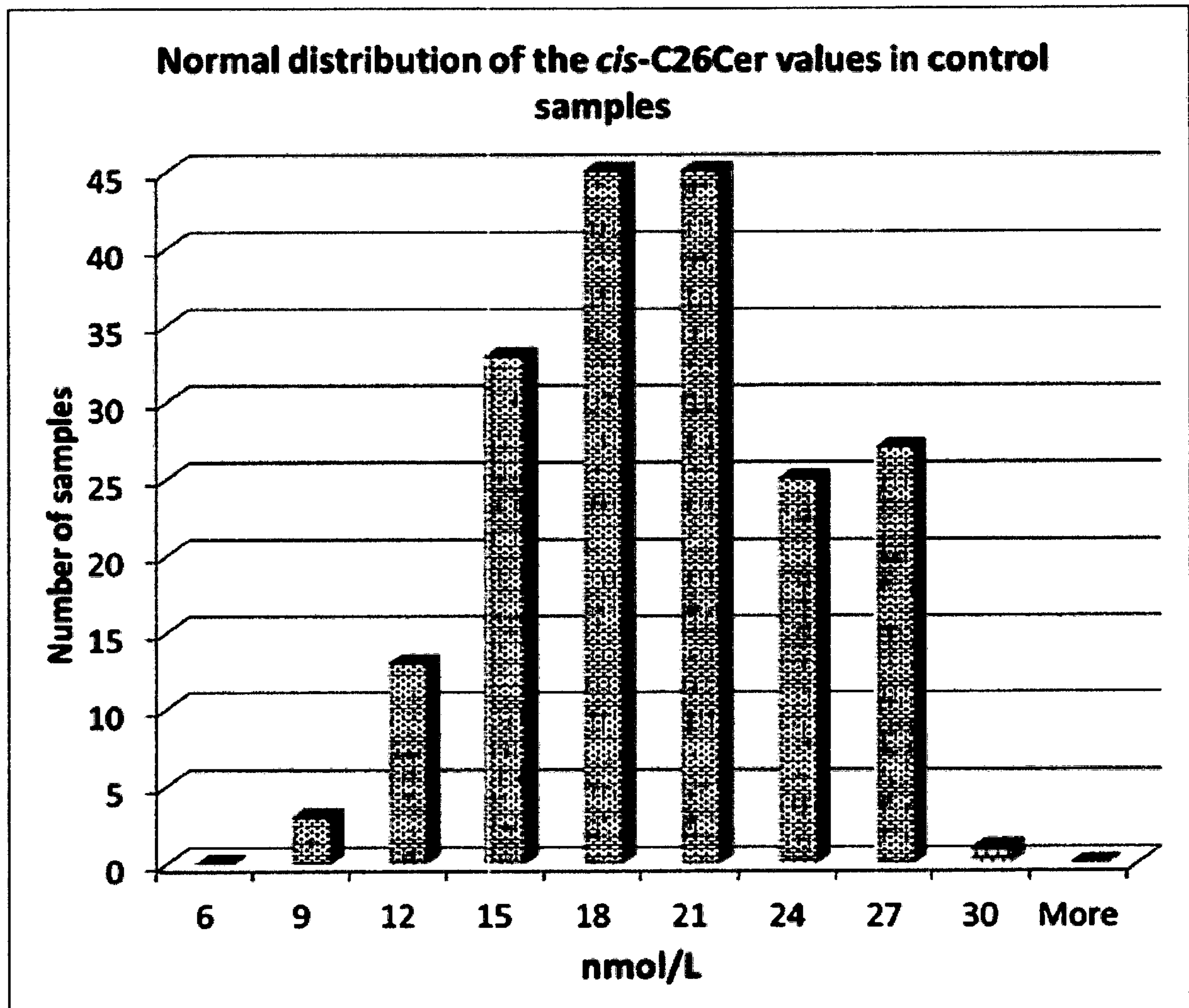
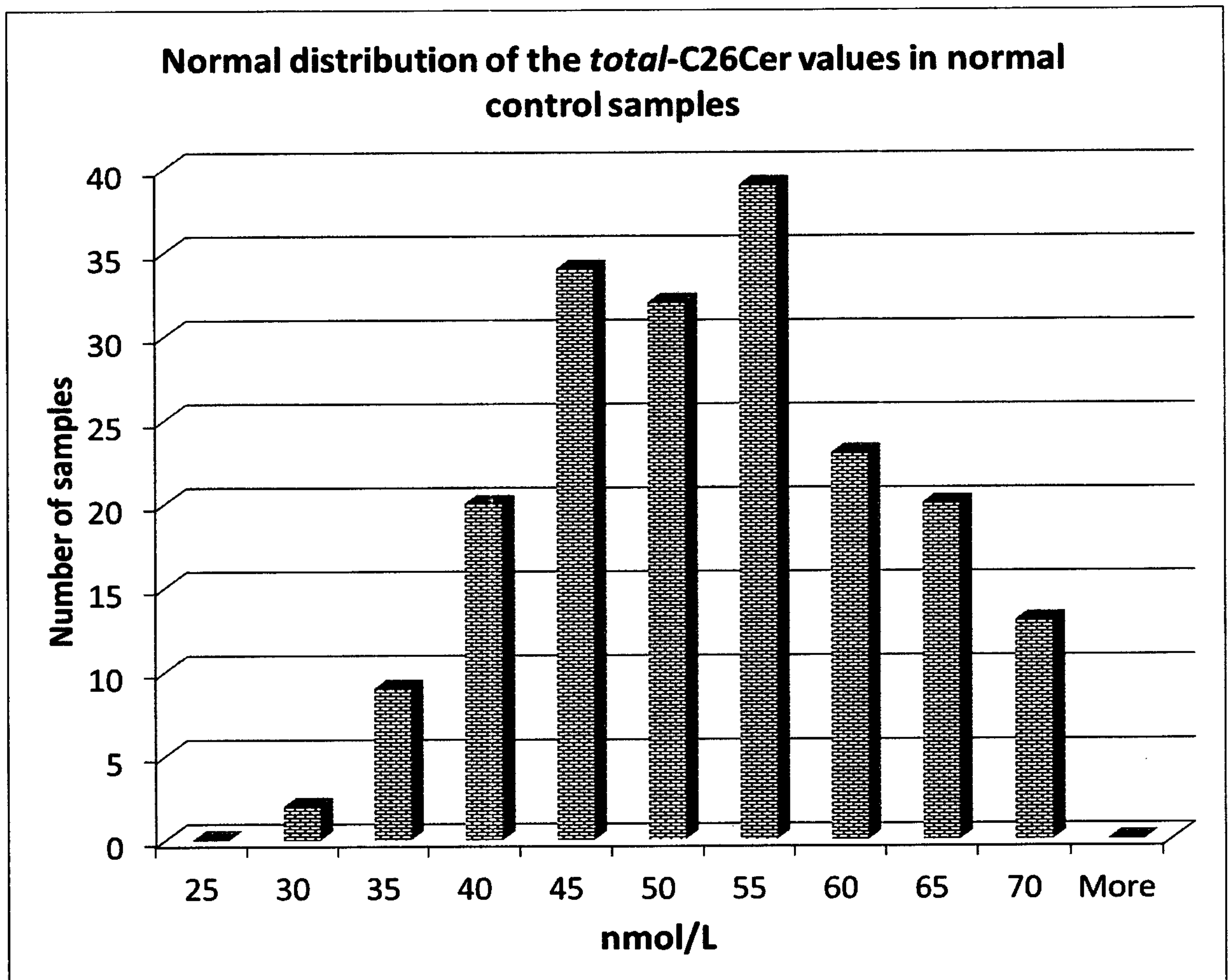
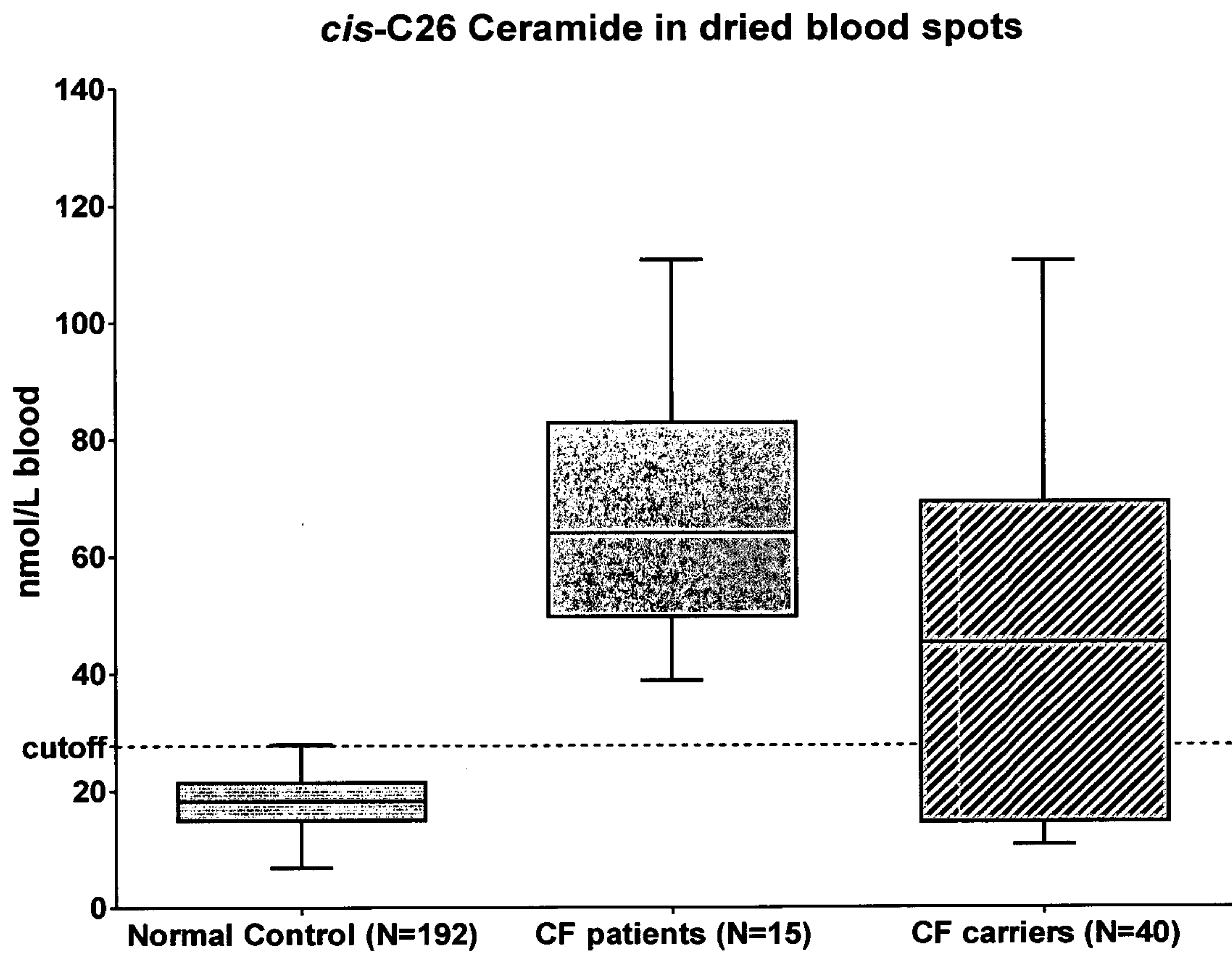


Fig. 1a

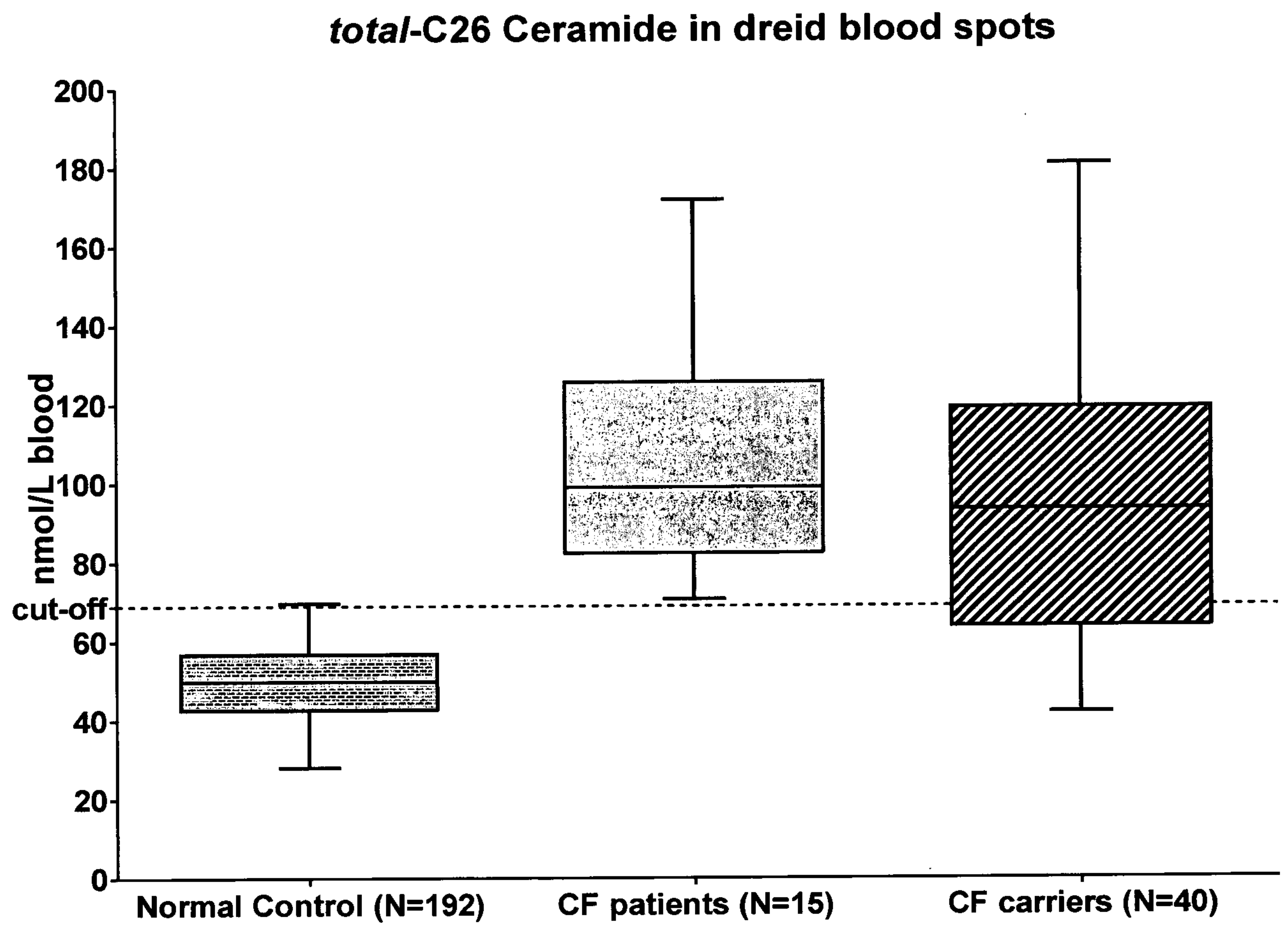
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**Fig. 1b**

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**Fig. 2**

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**Fig. 3**