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(54) Title: METHOD OF DETECTING PROBNP WITH A MONOCLONAL ANTIBODY BINDING TO THE AMINO ACID 41-46

(57) Abstract: The present invention relates to antibodies binding to native proBNP, a method for specific detection of native proBNP, a method of correlating the level of native proBNP to the diagnosis of heart failure, a kit for detection of native proBNP and to a hybridoma cell line producing an antibody to native proBNP.

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METHOD OF DETECTING PROBNP WITH A MONOCLONAL ANTIBODY BINDING TO THE AMINO ACIDS 41-46

The present invention relates to antibodies specifically binding to a sub-population of total proBNP termed native proBNP, a method for specific detection of native proBNP, a method of correlating the level of native proBNP to the diagnosis of heart failure, a kit for detection of native proBNP and to a hybridoma cell line producing an antibody to native proBNP.

Heart failure is a widespread phenomenon especially in the western world. According to the Roche medical dictionary (Urban & Schwarzenberg, 1993) heart failure is the acute or chronic inability of the heart to generate the blood flow required for the metabolism during exercise or even at rest or to assure the venous reflux (backward and forward failure). Thus the pump function of the heart is weak. The causes of heart failure are very complex. Among others, inflammatory and degenerative modifications of the cardiac muscle, coronary perfusion disorder, coronary infarction and injuries are to be mentioned here. This leads to modifications of the peripheral bloodstream, disorders of the breathing system, renal function and electrolyte metabolism (oedema) and to a reduced performance of the muscular system of the skeleton.

According to the New York Heart Association (NYHA) heart failure is divided into the following NYHA classes using physical tests after effort: I means completely free from pain after normal physical effort, II means low limitation of the physical toughness, III means strong limitation of the physical toughness, IV means that with each physical activity the insufficiency symptoms increase which most of the time also exist at rest.

For an effective medicament treatment of heart failure by means of glycosides, vasodilators, ACE inhibitors and/or ß-blockers it is first of all necessary to exactly and correctly identify and diagnose heart failure, to classify it, if possible, according to the degree of severity, and to additionally monitor the course of treatment.

In the art some serum markers are discussed as marker candidates for an early diagnosis of heart failure as for example ANP (atrial natriuretic peptide hormone) and proANP, CNP (C-natriuretic peptide), adrenomedullin, neuropeptide Y, endotheline and BNP (brain natriuretic peptide). ANP and proANP theoretically

would represent suitable markers for the diagnosis of heart failure; in practice they are, however, not very stable or only have a short half life in blood which represents a serious drawback to routine diagnostic measurements (Buckley, M.G., et al., Clin. Sci. 95 (1998) 235-239; Cleland, J.G., et al., Heart 75 (1996) 410-413).

A frequently cited and meaningful marker is BNP (brain natriuretic peptide). Originally, BNP was identified in the brain of pigs. It is a cardiac hormone which structurally and functionally resembles ANP (atrial natriuretic peptide) (Sudoh, T., et al., Nature 332 (1988) 78-81). Human BNP, consisting of 32 amino acids, is mainly secreted by the heart ventricles and circulates in the human blood plasma. The use of BNP as a diagnostic marker is for example known from EP-A-0 542 255. BNP has an intramolecular disulfide bridge and is not a very stable analyte. This presumably is due to its physiological function as a hormone that must be broken down quickly. Therefore, its use as a diagnostic marker requires careful and special attention in sample collection and processing (Masuta, C., et al., Clin. Chem. 44 (1998) 130; Tsuji, T., et al., Clin. Chem. 40 (1994) 672-673).

The precursor molecule of BNP, i.e. proBNP consists of 108 amino acids. proBNP is cleaved into the aforementioned 32 C-terminal amino acids (77-108) called BNP and the N-terminal amino acids 1-76 called N-terminal proBNP (or NT-proBNP). BNP, N-terminal proBNP (1-76) as well as further breakdown products (Hunt, P.J., et al., Biochem. Biophys. Res. Com. 214 (1995) 1175-1183) circulate in blood. Whether the complete precursor molecule (proBNP 1-108) also occurs in the plasma is not completely resolved. It is however described (Hunt, P.J., et al, Peptides, Vol. 18, No. 10 (1997), 1475-1481) that a low release of proBNP (1-108) in plasma is detectable but that due to the very quick partial breakdown at the N-terminal end some amino acids are absent.

As known from the art, the N-terminal proBNP (1-76) is considered a marker of heart failure.

WO 93/24531 (US 5,786,163) describes an immunological method of identifying N-terminal proBNP and the antibodies used for it. In WO 93/24531 polyclonal antibodies against one single peptide derived from the N-terminal proBNP are produced. It is shown that the antibodies produced bind to the immunization peptide (amino acids 47-64) in the competitive test format.

In the competitive test performed in WO 93/24531 the peptide 47-64 in a labelled form competes as a tracer with proBNP in a sample or the unlabelled peptide standard 47-64 for binding to polyclonal antibodies from rabbit serum. Only a moderate competition is reached after 48 hours of incubation resulting in a lower limit of detection of approximately 250 fmol/ml. The long incubation times of this competitive test are not acceptable for routine measurements of the samples in automated laboratories.

Hunt, P.J., et al., Clinical Endocrinology 47 (1997) 287-296, also describe a competitive test for the detection of N-terminal proBNP. In this assay a complex extraction of the plasma sample is necessary before the measurement can be performed; this may lead to the destruction of the analyte and erroneous measurements. The antiserum used is produced analogously to WO 93/24531 by immunization with a synthetic peptide. Hunt et al. produce the antiserum by immunization with the N-terminal proBNP amino acids 1-13 and a peptide consisting of amino acids 1-21 is used as a standard. For this test long incubation times are necessary, too. After an incubation of 24 hours a lower detection limit of 1.3 fmol/ml is reached.

Ng, L., et al., WO 00/35951 describe a further method for diagnosing N-terminal proBNP. This method is based on use of antibodies raised against a synthetic peptide corresponding to amino acids 65 to 76 of human proBNP.

Hughes, D., et al., Clin. Sci. 96 (1999) 373-380, report on two different assays for N-terminal proBNP. In a first assay a polyclonal antibody generated with an immunogen comprising a synthetic peptide corresponding to amino acids 65-76 of proBNP is used whereas in a second assay the polyclonal antibody was generated in analogy but to amino acids 37 – 49. According to the data produced by Hughes, D., et al. an antibody generated and reactive with the peptide corresponding to amino acids 37 – 49 of proBNP does not react with intact endogenous proBNP. An assay based thereon does not discriminate patients with left ventricular dysfunction from normal controls. With the assay based on proBNP 65-76 the same patient groups could be clearly discriminated.

Goetze, J.P., et al., Clin. Chem. 48 (2002) 1035-1042, describe an assay for the most N-terminal amino acids (1-21) of N-terminal proBNP. Their assay is based on a

polyclonal antibody raised against a synthetic peptide corresponding to the same amino acids (1-21) of proBNP.

The assay of Goetze, J.P., et al., supra, requires complete digestion of the sample and the various proBNPs comprised therein. It is said that this assay also was efficient in reduction of non-specific binding.

Karl, J., et al., WO 00/45176, for the first time show that sensitive and rapid detection of N-terminal proBNP is possible in a sandwich immunoassay. Preferred epitopes, as described in WO 00/45176 are between amino acids 10 and 50 of N-terminal proBNP.

US 2003/0219734 refers to the fact that a plurality of different BNP-related polypeptides derived from proBNP (1-108), BNP (77-108) as well as from N-terminal proBNP (1-76) may be present in a sample.

Mair, J., et al., Clin. Chem. Lab. Med. 39 (2001) 571-588, have summarised the impact of cardiac natriuretic peptide determination on the diagnosis and management of heart failure. They stress that currently available commercial assays are not standardized, i.e. they have not been calibrated against common standards. In some assays even an extraction of plasma is needed. Consequently, the results obtained with assays from different manufacturers may differ markedly. Therefore, reference intervals and decision limits derived from clinical studies are only valid for the particular assay used and must not be extrapolated to other assays for N-terminal proBNP.

Along the same lines Goetze, J.P., et al., supra and Mair, J., Clin. Chem. 48 (2002) 977-978, note that the discrepancies in between different assays of N-terminal proBNP both with respect to the values obtained as well as with regard to their clinical implications represent a crucial problem to the widespread use of this marker candidate.

Obviously a great need exists to provide for an improved, e.g. more reproducible better standardized, better characterized or/and more clinically relevant assays for N-terminal proBNP.

It was a task of the present invention to develop a more specific assay for measurement of N-terminal proBNP, and/or for a clinically relevant fragment or sub-population of N-terminal proBNP.

The invention as described below and claimed in the appending claims at least partially solves one or more of the problems known in the art.

It has surprisingly been found that it is possible to specifically detect a sub-population of all proBNP species (total proBNP) present in the circulation. This sub-population is termed native N-terminal proBNP or simply "native proBNP". Strikingly, it appears that the sub-population of native proBNP is clinically more relevant as compared to the total proBNP.

In a first embodiment the present invention relates to an isolated antibody specifically binding to native proBNP.

The invention also relates to a method for specific detection of native proBNP, comprising the steps of contacting a sample suspected or known to contain proBNP with an antibody specifically binding to native proBNP under conditions allowing for the formation of an antibody to native proBNP-native proBNP complex and detecting the complex formed.

Further the invention discloses a method for diagnosing heart failure comprising detection of native proBNP and correlating the level of native proBNP to heart failure. Whereby this correlated value of native proBNP is indicative for the absence, the presence or the status of heart failure.

The invention also relates to a kit for measurement of native proBNP, comprising an antibody specifically binding to native proBNP and auxiliary reagents for detection of native proBNP. Also claimed are monoclonal antibodies as produced by hybridoma cell line MAB 1.21.3 producing a specific monoclonal antibody to native proBNP which have been deposited with the DSMZ.

During the course of our experiments leading to the present invention it has been found and established that at least two populations of proBNP exist in human blood. One population of proBNP appears to represent the majority of all proBNP molecules which can be detected by immunological methods. This population is

termed "total" proBNP. Our studies have shown that the proBNP molecules which may be summarized as total proBNP apparently have a central core structure in common which ranges from about amino acid position 10 to about amino acid position 66 of proBNP. Preferably the total proBNP detected by a method according to the present invention comprises amino acids 10 through 66. As the skilled artisan will appreciate, such total proBNP can be easily detected by immunological procedures either in the competitive or in a sandwich assay format. Preferably a sandwich assay is used to detect total proBNP. Such sandwich assay may be designed to comprise antibodies binding C- and N-terminal to the native proBNP epitope, respectively. However, it is also possible to e.g. detect total proBNP using antibodies capable of sandwich formation and both reactive with epitopes N-terminal to the native proBNP epitope. Obviously, in such competitive or in such sandwich determination of total proBNP an antibody to native proBNP must not be used.

The term "native proBNP" denotes any proBNP molecule on which the epitope as recognized by MAB 1.21.3 is present. According to the findings demonstrated further below this epitope is only present on a sub-population of all proBNP-molecules (i.e. a sub-population of "total" proBNP). We found and could establish that this sub-population of total proBNP termed "native proBNP" is detectable by specific binding partners, preferably by polyclonal and/or monoclonal antibodies.

The sub-population termed native proBNP must not necessarily be a uniform polypeptide fragment. The length of the native proBNP polypeptide(s) may vary. Most of the molecules recognized in a sandwich immunoassay for native proBNP are expected to represent N-terminal proBNP (1-76) or fragments thereof. Preferably an assay for native proBNP is set up in a manner appropriate for measurement of NT-proBNP fragments comprising amino acids 10 through 66. The characteristic property of native proBNP is the presence of a native proBNP epitope as recognized by MAB 1.21.3.

In one embodiment the present invention relates to an antibody specifically binding to native proBNP, wherein said antibody specifically binding to native proBNP is an antibody which in terms of the values for proBNP as determined in patient samples correlates with an r-value of at least r=0.95 or above to MAB 1.21.3.

Based on the findings, disclosure and deposits of the present invention the skilled artisan will have no problem in assessing whether an antibody is specifically binding to native proBNP or to total proBNP. MAB 1.21.3 is considered a prototype example of an antibody specifically binding to native proBNP, whereas MAB 18.4.34 is considered a prototype example of an antibody to total proBNP. Any binding agent to proBNP whatsoever can now be assessed for its binding specificity to native or total proBNP, respectively.

An antibody "specifically binding" to native proBNP is an antibody which in terms of the values for proBNP as determined in patient samples correlates with an r-value of at least r=0.95 or above to MAB 1.21.3. Binding specificity to native proBNP is assessed in relation to the binding properties of MAB 1.21.3 using relevant clinical samples. At least 15 and at most 25 serum samples with an NT-proBNP-level of 10 ng/ml to 150 ng/ml of native proBNP are used. Binding to proBNP is determined with the Biacore® 3000 system. The values measured are correlated to native proBNP- values as measured using MAB 1.21.3 and the statistical assessment is performed by linear regression analysis. A linear regression of the type y= ax + b preferably is fitted using MS-Excel and the coefficient of correlation r and the slope is calculated. Even more preferred such antibody will detect essentially the same native proBNP sub-population of total proBNP as bound by MAB 1.21.3, wherein a binding to the essentially the same sub-population results in a correlation of r=0.98 or higher according to the above procedures.

MAB 18.4.34 may be considered a prototype antibody for measurement of total proBNP. For any antibody specifically binding to native proBNP (using the same samples and procedures as described above) the correlation to MAB 18.4.34, i.e. to total proBNP typically will be lower as compared to the correlation to MAB 1.21.3. Preferably the correlation to MAB 18.4.34 for an antibody specifically binding to the native proBNP sub-fraction of proBNP will be r=0.94 or below. Even more preferred it will be 0.9 or below or as low as r=0.8 or below.

When comparing absolute amounts measured in such method comparisons, the assays detecting total proBNP consistently yield about 2- to 20-fold, in most cases about 2- to 5-fold higher values of proBNP, as compared MAB 1.21.3. In addition to the above specified correlation a preferred antibody specifically binding to native

proBNP will in the above method comparison also show a slope of less than 1.5. Most preferred the slope will be between 0.7 and 1.5.

The specific binding preferably occurs with a binding affinity of least 10^7L/mol. The specific binding agent more preferred has an affinity of 10^8L/mol or even more preferred of 10^9L/mol for native proBNP.

As explained above a very important and preferred characteristics of MAB 1.21.3 is the fact that only a variable fraction of between about 5% and about 50% of total proBNP as comprised in a typical clinical sample is bound by this antibody.

A prototype example of an antibody which specifically binds to native proBNP is the monoclonal antibody as produced by clone MAB 1.21.3 which has been deposited with the DSMZMAB 1.21.3 is a sheep monoclonal antibody and has been produced as described in the Examples section. The epitope on proBNP recognized by these and by other antibodies has been identified, characterized and mapped by use of short synthetic peptides corresponding to well-defined sequences of proBNP. This method is known and referred to as PepScan analysis.

In brief, 69 synthetic peptides comprising 8 consecutive amino acids of proBNP have been synthesized comprising an N-terminal cysteine, a spacer molecule and biotin. Each of these peptides was shifted by one amino acid from, the N- to the C-terminus. Peptide 1 thus comprises amino acids (aas) 1 to 8, peptide 2 aas from aas 2 to 9, etc., and peptide 69 aas 69 to 76.

MAB 1.21.3 has been found to significantly react with peptides number 39 (amino acids 38-46) to 42 (aas 42-49) which have the amino acid positions 42 to 46 of proBNP in common. It therefore can be concluded that MAB 1.21.3 reacts with an epitope essentially consisting of amino acids 42 to 46 of proBNP.

As the skilled artisan appreciates the presence or absence of an epitope may depend on tertiary structure, secondary modifications, complex formation, accessibility and so on. Obviously, MAB 1.21.3 and other antibodies to native proBNP have very specific requirements in this regard and do not react with the majority of proBNP-molecules present in a typical sample. Since the short synthetic PepScan peptides are unlikely to have a tertiary structure or secondary modifications the epitope

recognized by MAB 1.21.3 should by un-modified and the terminology "native" has therefore been considered appropriate.

An assay based on an antibody to native proBNP and an assay measuring total proBNP exhibit striking differences in reaction intensity once synthetic proBNP (1-76) and proBNP as comprised in a clinical sample, like human serum, respectively, are measured and compared. Using synthetic proBNP (1-76) detection procedures can easily be set up and standardized. Employing such assay synthetic proBNP (1-76) either in a synthetic matrix or supplemented to a native sample, like human serum, is measured to the same levels in both these assays. Surprisingly, however, striking differences are found, once proBNP as comprised in a native sample like human serum, is measured both these assays.

An assay employing an antibody (or antibodies, respectively) reactive to total proBNP, like monoclonal antibody MAB 17.3.1, 18.4.34, or 18.29.23, respectively, appears to detect all the proBNP molecules present in a serum sample, i.e. total proBNP. On the contrary an assay based on an antibody reactive with native proBNP, e.g. MAB 1.21.3, only detects a fraction of this total proBNP.

It can be demonstrated by the present invention that the native proBNP sub-population of total proBNP clinically shows a very good correlation to the biologically active BNP. Of course, in the measurement of BNP all the precautions for obtaining correct BNP values with respect to sampling and handling have been observed. For measurement of native proBNP routine sample processing without specific precautions proved satisfactory.

All the data established with the present invention clearly indicate that the epitope identified in the present invention and as specifically bound by MAB 1.21.3 is a major epitope for appropriate antibodies to native proBNP. Obviously, this native proBNP epitope as recognized by MAB 1.21.3 can undergo a natural modification or can become part of a protein complex which changes this epitope with the effect that MAB 1.21.3 binds to such modified or complexed proBNP to a lower extend or not at all. The proBNP carrying such modified "non-native" proBNP epitope is only significantly measured in assays for total proBNP.

Because of their intrinsic high reproducibility monoclonal antibodies are preferred tools to detect native proBNP. In a preferred embodiment the present invention therefore relates to a monoclonal antibody specifically binding to native proBNP.

As the skilled artisan will appreciate, other monoclonal antibodies may be found which compared to MAB 1.21.3 may show a slightly different pattern in reactivity to PepScan peptides numbers 39 to 42. A monoclonal antibody to native proBNP will not depart from the spirit of this invention as long as only a sub-population of total proBNP is detected, which correlates with an r-value of at least r=0.95 or above to the native proBNP sub-population comprised in the total proBNP population and as bound by MAB 1.21.3. Such correlation is determined using the Biacore® system and the statistical assessment as described above. Even more preferred such monoclonal antibody will detect essentially the same native proBNP sub-population of total proBNP as bound by MAB 1.21.3, wherein the essentially the same sub-population results in a correlation of r=0.98 or higher according to the above procedures.

In a preferred embodiment the present invention also relates to a method of producing a monoclonal antibody, the method comprising the steps of immunizing an appropriate non-human animal with proBNP, preferably a mouse, a rat or a sheep, obtaining B-cells producing antibodies thereto, fusing these B-cells to appropriate fusion partners and testing the antibodies produced by the hybridomas thus obtained for reactivity to native proBNP. Preferably only such monoclonal antibodies are selected and used in an immunoassay which in appropriate patient samples correlate to MAB 1.21.3 with an r-value of at least 0.95. Such correlation is assessed as described above. Preferably the immunization is performed with synthetic proBNP or a proBNP produced in a prokaryotic host or with a synthetic peptide or fragments of proBNP both at least comprising the amino acids 41 to 44 of proBNP. Sheep monoclonals to native proBNP with an affinity of 10^9L/mol or above represent a preferred embodiment according to the present invention.

Now that MAB 1.21.3 is available, it is of course also possible to produce, purify and identify polyclonal antibodies which can be used in the specific detection of native proBNP. It has, e.g., been found that a polyclonal antibody (PAB) to native proBNP can now be generated, purified and characterized by its correlation to MAB 1.21.3.

As the skilled artisan will appreciate there are various ways to produce a PAB which binds to native proBNP. Obviously it will e.g. be possible to use one or more synthetic peptides as an immunosorbent in various successful routes of immunopurification.

Polyclonal antibodies to native proBNP will not depart from the spirit of this invention as long as only a sub-population of total proBNP is detected, which correlates with an r-value of at least r=0.95 or above to the native proBNP sub-population comprised in the total proBNP population and as bound by MAB 1.21.3. Such correlation is determined using the Biacore® system and the statistical analysis as described above. Even more preferred such polyclonal antibody preparation will detect essentially the same native proBNP sub-population of total proBNP as bound by MAB 1.21.3, wherein such binding of the essentially the same sub-population results in a correlation of r=0.98 or higher according to the above procedures.

One way to obtain such PAB to native proBNP is to immunize with recombinant or synthetically produced proBNP, to purify the native proBNP-specific antibodies therefrom by affinity purification and to assess the polyclonal antibody thus obtained via patient samples as described above.

In a preferred embodiment the present invention therefore relates to a method of producing polyclonal antibodies, the method comprising the steps of immunizing an appropriate non-human animal with proBNP, obtaining polyclonal antibodies thereto and testing the antibodies thus obtained for reactivity to native proBNP. Preferably only such polyclonal antibodies are selected and used in an immunoassay which in appropriate patient samples correlate to MAB 1.21.3 with an r-value of at least 0.95. Such correlation is assessed as described above.

For any polyclonal antibody specifically binding to native proBNP (using the same samples and procedures) the correlation to MAB 18.4.34, i.e. to total proBNP will be significantly lower as compared to the correlation to MAB 1.21.3. Since polyclonal antibody preparations may always contain individual antibodies with different properties preferably the correlation to MAB 18.4.34 for a polyclonal antibody specifically binding to the native proBNP sub-fraction of total proBNP will be r=0.94 or below. Even more preferred it will be as low as 0.9 or below. Preferably polyclonal antibody preparations specifically binding to native proBNP

will correlate to MAB 1.21.3 with r=0.98 or above and to MAB 18.4.34 with r=0.94 or below, or even more preferably with r=0.9 or below to MAB 18.4.34.

Preferably the immunization for obtaining polyclonal antibodies to native proBNP is performed with synthetic proBNP or a proBNP produced in a prokaryotic host or with a synthetic peptide or fragments of proBNP both at least comprising the amino acids 41 to 44 of proBNP.

In the course of our experiments a large variety of immunological reagents has been produced, analyzed, combined in various sandwich assays formats and used in the detection of proBNP. These various combinations of immunological reagents revealed that the majority of assays appears to measure total proBNP.

The total proBNP assays investigated have been found to exhibit a reasonable correlation to BNP, which goes hand in hand with reasonable diagnostic accuracy for diagnosis of heart failure, cf. e.g., Mair, J. supra.

It could, however, now be established that an assay only detecting native proBNP as compared to an assay detecting total proBNP better differentiates between patients in NYHA class 0 or I and patients in the NYHA classes II, III, or IV, respectively. This also leads to an improved clinical discrimination of heart failure patients.

In a preferred embodiment the present invention therefore relates to a method for specific detection of native proBNP, comprising the steps of contacting a sample suspected or known to contain proBNP with an antibody specifically binding to native proBNP under conditions allowing for the formation of an antibody to native proBNP - native proBNP complex and detecting the complex formed. Preferably said method for specific detection of native proBNP is used to differentiate NYHA stages 0 and I from NYHA stages II, III, or IV.

The "antibody to native proBNP – native proBNP complex" may also simply be termed "antibody – native proBNP complex".

The term "antibody" relates to mono- or polyclonal antibodies, chimeric or humanized or other antibodies obtainable by genetic engineering, as well as all antibody fragments known to the expert such as $F(ab')_2$, Fab' or Fab fragments. Other binding agents with appropriate specificity for native proBNP can be used to

substitute for antibodies or antibody fragments. Only the specific binding to native proBNP, in analogy to MAB 1.21.3 must be ensured.

As the skilled artisan appreciates there are numerous ways to detect native proBNP employing an antibody specifically binding thereto, which all are described in detail in relevant textbooks (cf., e.g. Tijssen, P., Practice and theory of enzyme immunoassays 11 (1990) Elsevier, Amsterdam, or Diamandis, et al., eds. (1996) Immunoassay, Academic Press, Boston).

In the context of the present invention many reagents and reagent combinations for detection of total proBNP or native proBNP have been analyzed by the Biacore® system, some results of which are shown in the Examples section.

In clinical routine diagnostics frequently methods based on a heterogeneous immunoassay format are used. In a preferred embodiment according to the present invention the method for detection of native proBNP is a competitive immunoassay.

Even more preferred are immunoassays according to the sandwich assay principle, in which an antibody – antigen – antibody complex, also called a sandwich is formed.

In a preferred embodiment according to the present invention the method for specific detection of native proBNP is a sandwich immunoassay, wherein a first antibody to native proBNP and a second antibody to total proBNP are used and wherein said second antibody to proBNP and the first antibody to native proBNP both bind to native proBNP at different epitopes thus forming a (first) anti-native proBNP antibody - native proBNP - (second) anti-proBNP antibody complex.

As the skilled artisan will appreciate, a sandwich assay for detection of native proBNP can also be set up using the antibody to total proBNP as a first (capture) antibody and the anti-native proBNP antibody as a (tracer, detection or labelled) second antibody.

Preferably, such a sandwich method for determination of the native proBNP comprises the following steps:

a) mixing of the sample with the first native proBNP-specific antibody carrying a group suitable for binding to a solid phase or mixing the sample with the first native proBNP-specific antibody which is already bound to a solid phase,

b) mixing of this solution with the second antibody to total proBNP binding to an epitope outside the native proBNP epitope, which is present on both native proBNP and total proBNP and carrying a label under conditions that a first antibody-native proBNP-second antibody complex is formed,

c) binding of the immune complex formed to a solid phase

d) separation of the solid phase from the liquid phase

e) detection of the label in one or both phases.

In a quantitative determination the same measurement is carried out with a defined amount of native proBNP as a standard and after the determination of the sample a step f) is performed, i.e. the measuring values of the standard or standard curve are compared to those obtained with the sample, and the corresponding concentration of native proBNP is extrapolated.

The first antibody specific for native proBNP can be bound directly to the solid phase or indirectly via a specific binding pair system. The direct binding of this antibody to the solid phase follows methods known to the expert, for example in an adsorptive way. If the binding is indirect via a specific binding pair system the first antibody is a conjugate consisting of an antibody against native proBNP and a first partner of the specific binding pair system. A specific binding pair system means two partners which can react specifically with each other. This binding can be based on an immunological binding or on an alternative specific binding. Preferred combinations are biotin and avidin, streptavidin or anti-biotin, respectively, hapten and anti-hapten, Fc-fragment of an antibody and antibodies against this Fc-fragment or carbohydrate and lectin. Preferably, a combination of biotin and avidin, or of biotin and streptavidin is used as a specific binding pair system.

The second partner of the specific binding pair system is coated to a solid phase. Streptavidin or avidin are used preferably. The binding of this partner of the specific binding pair system to an insoluble carrier material can be performed according to standard procedures known to the expert. Here a covalent as well as an adsorptive binding is suitable.

As a solid phase test tubes or microtiter plates made of polystyrene or similar plastics are suitable which are coated with the second partner of the specific binding pair system. Further suitable and particularly preferred are particulate substances such as latex particles, magnetic particles, molecular sieve materials, and glass corpuscles. Paper or nitrocellulose can also be used as carriers. Use of magnetic beads coated with the second partner of the specific binding pair system as described above is particularly preferred. After completion of the immunological reaction and binding of the immunological complex formed to the solid phase, these microparticles can be separated from the liquid phase for example by filtration, centrifugation or in the case of the magnetic particles via a magnet. Detection of label bound to the solid phase (or of the label remaining in the liquid phase or of both) is then performed according to standard procedures.

The second antibody binding to total proBNP, binds to an epitope outside the native proBNP epitope, which is present on both native proBNP and total proBNP Simultaneous binding of both antibodies to these two epitopes on the native proBNP molecule must be possible, because otherwise no sandwich complex would form.

The investigators of the present invention have also identified epitopes on proBNP which are very appropriate for the sandwich assay described above.

A large number of monoclonal antibodies has been generated. It could be established that not all epitopes recognized on recombinant proBNP are equally appropriate to measure proBNP in a patient sample.

Three epitopes essentially consisting of amino acids 13 – 16, 27 – 31, and 64-67, respectively, as recognized by MABs 17.3.1, 18.4.34, and 18.29.23, respectively appear to be present on the vast majority of (N-terminal) proBNP molecules. i.e. are epitopes of total proBNP. These hybridomas have been deposited with the

DSMZ on 07.05.03. The antibodies produced by these hybridomas represent ideal tools for measurement of total proBNP. If used alone in a competitive assay format or in combination with each other or a PAB reacting with total proBNP in a sandwich assay total proBNP can be easily measured.

The preferred hybridoma cell lines according to the invention MAB<NT-proBNP> 1.21.3(= MAK<NT-proBNP> 1.21.3= MAB 1.21.3), MAB<NT-proBNP>17.3.1, MAB<NT-proBNP>18.4.34 and MAB<NT-proBNP>18.29.23 were deposited, under the Budapest Treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure, with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany:

Cell line	Deposition No.	Date of Deposit
MAK<NT-proBNP>17.3.1	DSM ACC 2591	07.05.2003
MAK<NT-proBNP>18.4.34	DSM ACC 2592	07.05.2003
MAK<NT-proBNP>18.29.23	DSM ACC 2593	07.05.2003
MAK<NT-proBNP>1.21.3	DSM ACC 2650	27.04.2004

The antibodies obtainable from said cell lines are preferred embodiments of the invention.

In the detection of native proBNP, preferably a monoclonal antibody to total proBNP as described above is used in a sandwich assay in combination with an antibody specifically binding to native proBNP. Such sandwich then results in an assay specifically detecting only the native proBNP sub-population of total proBNP. Preferred antibodies to total proBNP in such sandwich for measurement of native proBNP are antibodies essentially binding to amino acids 13 – 16, 27 – 31, and 64-67, respectively. These epitopes for example are recognized by MABs 17.3.1, 18.4.34, and 18.29.23, respectively. Most preferably an antibody binding to amino acids 27 to 31, like MAB 18.4.34 is used in such sandwich assay for native proBNP.

All biological liquids known to the expert can be used as a sample in a method for specific detection of native proBNP in vitro. The preferred samples for in vitro diagnosis are body fluids like whole blood, blood serum, blood plasma, urine or saliva. The use of serum or plasma, respectively, is particularly preferred.

Besides the so-called wet tests as described above, with test reagents in a liquid phase, all standard dry test formats suitable for the detection of antigens, haptens, peptides, proteins, antibodies etc. can be used too. These dry tests or test strips, as for instance described in EP-A-0 186 799, combine all test components on one single carrier – except the sample to be analyzed.

In a preferred embodiment the present invention relates to a method for diagnosing heart failure comprising detection of native proBNP and correlating the level of native proBNP to the presence of heart failure. As the skilled artisan will appreciate the level of native proBNP can also be used to assert the absence or the severity of heart failure.

It is also preferred to use a measurement of native proBNP in the follow-up of patients with heart failure and in the monitoring of treatment.

A further preferred embodiment relates to a kit for measurement of native proBNP, comprising an antibody specifically binding to native proBNP and auxiliary reagents for detection of native proBNP.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1: Epitope identification for MAB 1.21.3

The reactivity profile of MAB 1.21.3 has been analyzed by use of 69 different biotinylated 8-mer peptides derived from the sequence of proBNP (1-76), each shifted by one amino acid thus covering the complete sequence of proBNP (1-76). Extinction is given in mE-units. Strong reactivity has been found to peptides numbers 39 to 42.

Figure 2: Instrument settings used in the Biacore analyses

The specificity for native proBNP of the various antibodies to proBNP has been assessed using the mode of operation of the Biacore® 3000 analyzer as given in this Figure.

Figures 3 to 7: Correlation of MAB 1.21.3 to various mono- and polyclonal anti-proBNP antibodies

20 human sera with a concentration of proBNP of about 10 mg/ml and above (as determined by using MAB 1.21.3 and synthetic proBNP as a calibrator) have been analyzed in a sandwich assay using the Biacore 3000 analyzer. Values measured with MAB 1.21.3 are given on the x-axis. The corresponding values determined with the antibody used in the method comparison are given in the y-axis. Correlations of MAB 1.21.3 to MAB 18.4.34, MAB 18.29.23, PAB 30-38, PAB 44-51 and PAB 41-46 are given in Figures 3, 4, 5, 6, and 7, respectively.

Example 1

Method of production of recombinant N-terminal proBNP (1-76)

1. Cloning of the recombinant N-terminal proBNP

The nucleotide sequence of the N-terminal proBNP (amino acid sequence 1-76) was produced my means of genetic synthesis. To obtain an optimum expression of the gene in *E.coli* the DNA sequence was suited to the codons most frequently used in *E.coli*. The sequences of the oligonucleotides used for the production of the gene are the following:

Pro5' (SEQ ID NO: 1):

5'CCGGATCCCACCCGCTG3'

Pro1hum (SEQ ID NO: 2):

5'CGGGATCCCACCCGCTGGGTTCCCCGGGTTCCGCTTCCGACCTGGAAAC CTCCGGTCTGCAGGAACAGCGTAACCACCT3'

Pro2hum (SEQ ID NO: 3):

5'CGGTTCCAGGGAGGTCTGTTCAACCTGCAGTTCGGACAGTTTACCCTGCA GGTGGTTACGCTGTTCCTGC3'

Pro3hum (SEQ ID NO: 4):

5'CAGACCTCCCTGGAACCGCTGCAGGAATCCCCGCGTCCGACCGGTGTTT GGAAATCCCGTGAAGTTGCTAC 3'

Pro4hum (SEQ ID NO: 5):

5'CCCAAGCTTAACGCGGAGCACGCAGGGTGTACAGAACCATTTTACGGTG ACCACGGATACCTTCGGTAGCAACTTCACGGGATTTCC3'

Pro3' (SEQ ID NO: 6):

5'CCCAAGCTTAACGCGGAGC3'

The production of the gene was carried out with these primers using PCR (polymerase chain reaction). The amplified gene was cloned in a suitable vector like for example the vector pUC19 and then sequenced. For the cloning of the gene in the expression vector pQE8 the gene was cut out of the vector pUC19 via the restriction cutting points *Bam* Hi and *Hind* III and then ligated in the vector pQE8 allowing an expression of proteins with N-terminal Histidin-Tag and transformed in *E.coli* M15 [pREP4].

2. Expression of the N-terminal proBNP in *E.coli*

For the expression of the gene in *E.coli* an over-night culture of a recombinant *E.coli* clone was transfected 1/60 in Luria-Broth (with 100µg/ml ampicillin and 50µg/ml kanamycin) and induced at an OD 550 of 1 with IPTG (isopropylthiogalactoside; 1 mM final concentration). After the induction the cultures were further incubated for 4 hours at 37°C. The cultures were then centrifuged and the cell pellet gathered in 50 mM Na-phosphate buffer, pH 8.0; 300 mM NaCl. After decomposition of the cell suspension via ultrasound the suspension was centrifuged and the supernatant applied on a Ni-NTA (nitrilo-triacetate) column. After a washing step with 50 mM

Na phosphate buffer, pH 8.0; 300 mM NaCl; 20 mM imidazole the histidin-tagged N-terminal proBNP was eluted with 50 mM Na-phosphate buffer, pH 8.0; 300 mM NaCl; 300 mM imidazole. The eluted fractions were gathered and dialysed against 50 mM Tris pH 8.0. To separate impurities the dialysate was applied to a Q-sepharose column. The mass of the purified N-terminal proBNP was determined via MALDI-TOF. This preparation (= recombinant proBNP) was found to contain proBNP 1-76 and proBNP 1-66, the later most likely representing a degradation product.

Example 2

Synthesis of NTproBNP(1-76)amide

NTproBNP(1-76)amide (swissprot: accession no. P16860; aa 27 to aa 134) was synthesized by an optimized solid phase peptide synthesis protocol (Merrifield (1962) Fed. Proc. Fed. Amer. Soc Exp. Biol. 21, 412) on an ABI 433 peptide synthesizer. In brief, the peptide was assembled on a Rink-Linker modified polystyrene solid phase by repeatedly conjugating an eightfold excess of amino acids each protected by temporary piperidine labile Fmoc- and permanent acid labile tBu-, BOC-, OtBu-, Trt- or Pmc-groups depending on the side chain function. To get an oxidative stabile material the methionine at position 10 was exchanged by the equivalent amino acid norleucine. Further, to stabilize against proteolytic degradation the C-terminus was amidated by using the Rink linkage. After the assembly the fully protected peptide was removed from the solid phase and the permanent protecting groups were released by treatment with trifluoracetic acid in a mixture of suitable cation scavengers and finally isolated by a preparative reverse phase HPLC purification. Three 125 µmol scale syntheses yielded 16.0, 17.1 and 18.0 mg RP-HPLC single peak pure material (lyophilisate), respectively. The identity was proven by MALDI- and ESI-mass spectroscopy [8439.4].

Example 3

Production of and screening for monoclonal antibodies against total or native proBNP, respectively

1. Obtaining monoclonal antibodies against N-terminal proBNP

Balb/c mice, 8-12 weeks old, are subjected to intraperitoneal immunization with 100µg N-terminal proBNP antigen, with complete Freund's adjuvant. Recombinant

as well as proBNP (1-76) produced synthetically by peptide synthesis, respectively, has been used as an antigen in mice. After 6 weeks three further immunizations are performed at 4-week intervals. One week after the last immunization blood was taken and the antibody titre was determined in the serum of the test animals. From the spleen of positively reacting mice B-lymphocytes are obtained and subjected to fusion with a permanent myeloma cell line. The fusion is carried out according to the well-known method of Köhler and Millstein (Nature 256, 1975, p. 495-497). The primary cultures of the positive hybridomas are cloned in a usual way for example by using the commercially available cell sorter or by "limiting dilution".

For the production of ascites 5 x 10^6 hybridoma cells are intraperitoneally injected in Balb/c mice which had been treated 1-2 times with 0.5 ml Pristan before. After 2-3 weeks ascites liquid can be obtained from the abdominal region of the mice. From this, the antibodies can be isolated in the usual way.

2. Screening test for monoclonal antibodies against proBNP peptides, synthetic proBNP, and proBNP in human serum, respectively

To identify the presence of antibodies against proBNP in the culture supernatant of the hybridoma cells, supernatants were evaluated according to three screening assay formats.

a) Reactivity with synthetic N-terminal proBNP

Microtitre plates (Nunc, Maxisorb) are bound with 2.5 µg/ml synthetic NT-proBNP as an antigen in a loading buffer (Coating buffer, Cat.No. 0726 559, Scil Diagnostics, GmbH) 100 µl/well, for 1 hour at room temperature under stirring. The post-loading is carried out in PBS buffer (phosphate buffered saline, Oxid , Code-BR 14a) and 1% Byco C, for 30 minutes. Subsequently, washing is performed with washing buffer (0.9 sodium chloride solution, 0.05% Tween 20). The antibody sample incubation is carried out with 100 µl/well for 1 hour at room temperature under stirring. A further washing step with washing solution takes place twice then. Afterwards, a further incubation is carried out with the detection antibody PAB<M-Fcy> Goat-F(ab')$_2$-peroxidase conjugate (Chemicon, Cat. No. AQ127P), 100 mU/ml, 100 µl/well, for 1 hour at room temperature under stirring. After a further washing step with washing buffer the peroxidase activity is established in the

usual way (for example with ABTS®, for 30 minutes at room temperature, the extinction difference is read in mU at 405 nm by means of an ELISA reader.

b) Epitope characterization using synthetic peptides for epitope analysis

For epitope analysis streptavidin-loaded microtitre plates are incubated with peptide biotin conjugates derived from the sequence of proBNP (1-76). The complete proBNP-sequence was scanned by applying 69 8-mer peptides shifted through the sequence in single amino acid steps i.e. 1-8, 2-9, 3-10, 4-11 till 66-73, 67-74, 68-75, and 69-76, respectively. Additional biotinylated sequences have been tested comprising the amino acid positions 1-10, 8-18, 1-21, 16-30, 30-38, 32-43, 39-50, 47-57, 50-63, 62-70 and 64-76, respectively. The individual antigenic peptides have been dissolved to 250 ng/ml in PBS buffer (phosphate buffered saline, Oxid, Code-BR 14a) with 0.5% Byco C. For peptide coating 100 µl of each solution has been distributed in distinct wells of the microtitre plates which were then gently agitated for 1 hour under room temperature. Subsequently, washing was carried out with washing buffer (0.9m sodium chloride solution, 0.05% Tween 20). The antibody sample incubation and the detection reaction are performed as described in a). Due to their reactivity with certain NT-proBNP peptides the position of the epitope as recognized by a mono- or polyclonal antibody could be delineated.

An example of a PepScan is shown in Figure 1. The monoclonal antibody secreted by hybridoma 1.21.3 strongly reacts with peptides numbers 39 through 42. This corresponds to a shared epitope consisting of the amino acids 41 through 46 (SEQ ID NO: 11) of proBNP.

c) Reactivity with proBNP in a patient sample

Wells of microtitre plates (Nunc, Maxisorb) are coated with 5 µg/ml PAB<human proBNP>S-IgG (IS, (1-21) or (30-38)S-IgG in loading buffer (Coating buffer, Cat.No. 0726 559, Scil Diagnostics, GmbH), 100 µl/well, for 1 hour at room temperature under stirring. The post-loading is carried out in PBS buffer (phosphate buffered saline, Oxid , Code-BR 14a) and 1% Byco C, for 30 minutes. Subsequently, washing is performed with washing buffer (0.9 sodium chloride solution, 0.05% Tween 20). The incubation with native antigen in patient plasma, diluted in PBS buffer, is carried out with 100 µl/well for 1 hour at room temperature under stirring. After a further washing step the hybridoma supernatant

incubation is performed with 100 µl/well for 1 hour at room temperature under stirring. Subsequently, washing is carried out twice with washing solution and a further incubation with the detection antibody PAB<M-Fcy> Goat-F(ab')$_2$-peroxidase conjugate (Chemicon, Cat. No. AQ127P), 100 mU/ml, 100 µl/well, for 1 hour at room temperature under stirring. After a further washing step with washing buffer the peroxidase activity is established in the usual way (for example with ABTS®, for 30 minutes at room temperature, the extinction difference is read in mU at 405 nm by means of an ELISA reader).

Only those hybridoma cultures have been further processed which reacted positively with synthetically produced N-terminal proBNP, or with proBNP in human serum.

Example 4

Production of sheep monoclonal antibodies against pro BNP

1. Obtaining monoclonal sheep antibodies against N-terminal proBNP

Sheep were immunized with recombinant N-terminal proBNP in complete Freund's adjuvant. The dose was 0.1 mg per animal. The immunizations were repeated at 4-week intervals in a period of 10 months. 6 weeks after the first immunization and afterwards once a month the serum samples were obtained and tested for their sensitivity and titre.

The lymphocytes for fusions were obtained from lymph nodes of immunized sheep. Therefore 3 days before removal the lymph node a final boost injection with recombinant N-terminal proBNP was done, directly into a inguinale sheep lymph node.

After surgical removal of the lymph node, the lymphocytes were prepared and isolated under sterile condition. About 2×10^9 cells from one lymph node will be stored in liquid nitrogen at a density of 1×10^8 cells/vial.

For fusions a vial of 1×10^8 frozen lymph node lymphocytes were thawed and mixed with a hypoxanthine and thymidine sensitive myeloma/heteromyeloma cells (mouse NS1 x sheep lymphocytes, clone 1 C 10, Bioventix, Inc.) in a ratio of 2 : 1 with fusing agent polyethylene glycol (PEG).

Several 96-well plates were seeded with 1-3 x 10^4 cells (or heteromyelomas) per well and cultivated in selection medium. After 8-10 days examination and screening of the hybridoma cells for reactivity with N-terminal pro BNP were done by ELISA assay.

The primary cultures of the postive hybridomas were cloned in the usual way by using the commercially available cell sorter or by "limiting dilution".

2. Screening test for monoclonal antibodies against proBNP peptides, synthetic proBNP, and proBNP in human serum, respectively

To identify the presence of antibodies against proBNP in the culture supernatant of the hybridoma cells, supernatants were evaluated according to three screening assay formats.

a) Reactivity with synthetic N-terminal proBNP

Microtitre plates (Nunc, Maxisorb) are coated with 2.5 µg/ml synthetic NT-proBNP as an antigen in a loading buffer (Coating buffer, Cat.No. 0726 559, Scil Diagnostics, GmbH) 100 µl/well, for 1 hour at room temperature under stirring. The post-loading is carried out in PBS buffer (phosphate buffered saline, Oxid, Code-BR 14a) and 1% Byco C, for 30 minutes. Subsequently, washing is performed with washing buffer (0.9 sodium chloride solution, 0.05% Tween 20). The antibody sample incubation is carried out with 100 µl/well for 1 hour at room temperature under stirring. A further washing step with washing solution takes place twice then. Afterwards, a further incubation is carried out with the detection antibody peroxidase-conjugated AffiniPure Donkey Anti-Sheep IgG (Dianova Code Number 713-035-147), diluted 1: 40 000 in PBS buffer, 100 µl/well, for 1 hour at room temperature under stirring. After a further washing step with washing buffer the peroxidase activity is established in the usual way (for example with ABTS®, for 30 minutes at room temperature, the extinction difference is read in mU at 405 nm by means of an ELISA reader.

b) Epitope characterization using synthetic peptides for epitope analysis

For epitope analysis streptavidin-loaded microtitre plates are incubated with peptide biotin conjugates derived from the sequence of proBNP(1-76).

The complete proBNP-sequence was scanned by applying 69 8-mer peptides shifted through the sequence in single amino acid steps i.e. 1-8, 2-9, 3-10, 4-11 till 66-73, 67-74, 68-75, and 69-76, respectively. Additional biotinylated sequences have been tested comprising the amino acid positions 1-10, 8-18, 1-21, 16-30, 30-38, 32-43, 39-50, 47-57, 50-63, 62-70 and 64-76, respectively. The individual antigenic peptides have been dissolved to 250 ng/ml in PBS buffer (phosphate buffered saline, Oxid, Code-BR 14a) with 0.5% Byco C. For peptide coating 100 µl of each solution has been distributed in distinct wells of the microtitre plates which were then gently agitated for 1 hour under room temperature. Subsequently, washing was carried out with washing buffer (0.9m sodium chloride solution, 0.05% Tween 20). The antibody sample incubation and the detection reaction are performed as described in a). Due to their reactivity with certain NT-proBNP peptides the position of the epitope as recognized by a mono- or polyclonal antibody could be delineated.

c) Reactivity with proBNP in a patient sample

Wells of microtitre plates (Nunc, Maxisorb) are coated with 5 µg/ml MAB<human proBNP>M-18.4.34-IgG in loading buffer (Coating buffer, Cat.No. 0726 559, Scil Diagnostics, GmbH), 100 µl/well, for 1 hour at room temperature under stirring. The post-loading is carried out in PBS buffer (phosphate buffered saline, Oxid, Code-BR 14a) and 1% Byco C, for 30 minutes. Subsequently, washing is performed with washing buffer (0.9 sodium chloride solution, 0.05% Tween 20). The incubation with native antigen in patient plasma, diluted in PBS buffer, is carried out with 100 µl/well for 1 hour at room temperature under stirring. After a further washing step the hybridoma supernatant incubation is performed with 100 µl/well for 1 hour at room temperature under stirring. Subsequently, washing is carried out twice with washing solution and a further incubation with the detection antibody Peroxidas-conjugated AffiniPure Donkey Anti-Sheep IgG (Dianova Code Number 713-035-147), diluted 1: 40 000 in PBS buffer, 100 mU/ml, 100 µl/well, for 1 hour at room temperature under stirring. After a further washing step with washing buffer the peroxidase activity is established in the usual way (for example with ABTS®, for 30 minutes at room temperature, the extinction difference is read in mU at 405 nm by means of an ELISA reader).

Only those hybridoma cultures have been further processed which reacted positively with synthetically produced N-terminal proBNP, or with proBNP in human serum.

Example 5

Production of polyclonal antibodies against N-terminal proBNP

1. Immunization

Sheep were immunized with recombinant N-terminal proBNP (see Example 1) in complete Freund's adjuvant. The dose was 0.1 mg per animal. The immunizations were repeated at 4-week intervals in a period of 10 months. 6 weeks after the first immunization and afterwards once a month the serum samples were obtained and tested for their sensitivity and titre.

2. Purification of polyclonal antibodies from sheep serum

Starting from the raw serum of a sheep immunized with recombinant N-terminal proBNP lipid components were removed by delipidation with aerosil® (1.5 %). Afterwards the immunoglobulins were separated by ammonium sulphate precipitation (2 M). The dissolved precipitate was dialysed against 15 mM KPO_4, 50 mM NaCl pH 7.0 and chromatographed on DEAE sepharose. The IgG fraction (= PAB<NT-proBNP>S-IgG(DE)) was obtained in the flow through.

3. Affinity chromatography for the production of polyclonal antibodies specific for total proBNP

For the affinity purification of polyclonal antibodies binding specifically to total proBNP (= PAB<NT-proBNP>S-IgG(IS,1-21), or briefly PAB<1-21>) the peptide HPLGSPGSASDLETSGLQEQR-C ((1-21)21-Cys, SEQ ID NO: 7) was used. The affinity matrix was produced by covalently binding of 1 mg of the peptide (1-21)21-Cys to 2 ml of maleimide activated EAH-Sepharose 4B (Amersham Biosciences, Product No 17-0569-01).

With 10 ml of the affinity matrix a column was packed and equilibrated with 50 mM KPO_4, 150 mM NaCl pH 7.5 (PBS). 2g of PAB<NT-proBNP>S-IgG(DE) were applied to the column. The column was washed with PBS and 20 mM KPO_4, 500 mM NaCl, 0.1 % Triton X-100, 0.5 % Na-deoxicholic acid pH 7.5. The IgG specifically bound to the affinity matrix was eluted with ImmunoPure® Gentle Ag/Ab elution buffer (Pierce, Product N° 21013) and is referred to asPAB<1-21>.

The affinity matrix was regenerated with 1 M propionic acid and conserved in PBS/NaN_3.

A similar procedure was applied to generate affinity purified polyclonal antibodies PAB<NT-proBNP>S-IgG(IS,30-38), or briefly PAB<30-38>) specific for total proBNP (Karl, J. et al., WO 00/45176).

4. Affinity chromatography for the production of polyclonal antibodies specific for native proBNP

The polyclonal antibody to native proBNP (= PAB<NT-proBNP>S-IgG(IS,41-46), or briefly PAB<41-46>) was obtained by sequential affinity chromatography. In the same way as described above, 3 individual peptides, CEUEU-SLEPLQE ((37-43)37-Cys, SEQ ID NO: 8), CEUEU-SPRPTGVW ((44-51)44-Cys, SEQ ID NO: 9) and C-EPLQESPRPTG ((39-50)39-Cys, SEQ ID NO: 10) (EUEU merely functions as an extended linker for the peptide following behind) were used for the production of 3 individual affinity matrices. PAB<NT-proBNP>S-IgG(DE) was first applied to the affinity matrix comprising peptide (37-43)37-Cys to remove all polyclonal antibody primarily binding to the NT-proBNP sequence 37-43. The flow through was then applied to the second affinity matrix comprising peptide (44-51)44-Cys to capture polyclonal antibodies primarily binding to the NT-proBNP sequence 44-51. The bound antibodies were eluted and collected as described above (=PAB<44-51>). Finally, the flow through of the second affinity purification was passed over the third affinity matrix comprising peptide (39-50)39-Cys. The bound antibodies were eluted and collected as described above. As determined by the method known and referred to as PepScan analysis, the eluted antibodies from the third affinity matrix are specific for epitopes in the sequence 41-46 (=PAB<41-46>) which represent the remaining epitopes of in the overlapping sequence between 37-43 and 44-51.

Example 6

Biacore analysis of monoclonal and polyclonal antibodies to proBNP

The specificity of monoclonal and polyclonal antibodies to native NT-proBNP was determined by surface plasmon resonance using a Biacore 3000 analyzer. All surface plasmon resonance measurements were performed at 25 °C using the Biacore 3000 equipped with a research-grade CM5 sensor chip. The running buffer

was HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005 % P20 (=Polysorbat) at pH 7.4)

1. Immobilization of the ligand PAB<NT-proBNP,1-21>S-IgG

The ligand which was used as capture antibody for total NT-proBNP was immobilized using amine-coupling chemistry. Before coupling, the sensor chip was preconditioned at a flow rate of 20 µl/min by 10 µl injections of 0.1 % SDS, 50 mM NaOH, 10 mM HCl and 100 mM phosphoric acid. The surfaces of all flow cells were activated for 5 min with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.1 M EDC (3-(N,N-dimethyl-amino)propyl-N-ethylcarbodiimide) at a flow rate of 20 µl/min. The ligand at a concentration of 30 µg/ml in 10 mM sodium acetate, pH 5.0, was injected in all 4 flow cells for 5 min. The surfaces were blocked with a 5 min injection of 1 M ethanolamine, pH 8.0, followed by 30 s injections of HBSwash (100mM HEPES pH 7.4, 1.5 M NaCl, 3.4mM EDTA, 0.05% P20 (=Polysorbat), 2 % DMSO), 100 mM HCl and 2 x 100 mM phosphoric acid to remove noncovalently bound ligand. The density of the ligand was about 16.000 RU.

2. Concentration measurements of NT-proBNP in patient samples

To perform the following method in Biacore 3000 the program as attached in Figure 2 was used. Synthetic NT-proBNP(1-76)amid in concentrations of 0, 2.5, 5, 10, 20 and 40 nM in 20 % horse serum (horse serum diluted 1:5 with HBS + 1 mg/ml carboxymethyldextran) was used as calibrator. The addition of carboxymethyldextran was used to suppress non-specific binding of serum components to the surface of the sensor chip).

Patient samples with >10 ng/ml native NT-proBNP were diluted 1:5 with HBS also containing 1 mg/ml carboxymethyldextran.

Calibrator and patient samples were injected at a flow rate of 10 µl/min for 10 min over all four flow cells followed by a 30 s injection of HBS at a flow rate of 100 µl/min to remove non-specifically bound serum components. The antibody whose specificity had to be determined was injected in a concentration of 500 nM in HBS for 3 min at a flow rate of 10 µl/min. Antibody 1 in flow cell 1, antibody 2 in flow cell 2 and so on. The binding data of the antibodies in RU were determined as

difference between the response 10 s before the injection of an antibody and the response 10 s before the injection of the next antibody or HBS, respectively.

For the calculation of the NT-proBNP concentrations in the patient samples BIAevaluation software Vers. 4.1 was used. For each antibody a calibration curve of synthetic NT-proBNP(1-76)amid was generated using a spline fit and the corresponding concentrations of the 1:5 diluted patient samples were calculated. The concentrations were multiplied by 5 to get the concentrations of NT-proBNP in undiluted sera.

3. Determination of the specificity of an antibody

In order to determine if an antibody binds to native or total NT-proBNP in human serum, the concentrations of NT-proBNP determined with the antibody in question (y-axis) were plotted against the concentrations of the corresponding sample determined with the reference antibody MAB 1.21.3 (x-axis). A linear regression curve of the type y= ax + b was fitted using MS-Excel and the coefficient of correlation r and the slope were calculated.

Table 1: Characteristics of various anti-proBNP antibodies

Antibody	Epitope recognized	synthetic proBNP	Patient sample proBNP
MAB 17.3.1	Amino acids 13-16	+++	+++
MAB 18.4.34	Amino acids 27-31	+++	+++
MAB 18.29.23	Amino acids 62-76	+++	+++
MAB 1.21.3	Amino acids 42-46	+++	+
PAB <1-21>	Amino acids 1-21	+++	+++
PAB <44-51>	Amino acids 44-51	+++	++
PAB <41-46>	Amino acids 41-46	+++	+

+++ indicates that both synthetic proBNP and proBNP in a patient sample are recognized very well and to a similar extend

+ indicates a reaction in the range of 15% with proBNP in a patient sample as compared to the value obtained with synthetic proBNP

From Table 1 it is readily evident that the vast majority of proBNP epitopes appears to be present on synthetic proBNP and proBNP as comprised in a patient sample in the same manner. This is exemplified by MAB 17.3.1, MAB 18.4.34, MAB 28.29.13 and PAB <1-21>, respectively.

One epitope, however, appears not to be present on synthetic proBNP and proBNP as comprised in a patient sample in the same manner. This epitope essentially consists of amino acids 41-44 and is recognized by MAB 1.21.3 as well as by PAB <41-46>. It appears, that using these immunological reagents, only a sub-population of the total proBNP as present in a patient sample is recognized.

This leads to strikingly different results when measuring proBNP in a patient sample with an assay for total proBNP or an assay for native proBNP, respectively. Only this sub-population of total proBNP appears to carry an epitope characteristic for native proBNP.

As is obvious from Figures 3 to 7, that PAB<41-46> shows a very good correlation to MAB1.21.3, whereas the antibodies to total proBNP, i.e. MAB 18.4.34, MAB 18.29.23 and PAB 30-38 show a much lower correlation to MAB 1.21.3. PAB <44-51> interestingly appears to be of mixed reactivity and would not qualify as an antibody specifically binding to native proBNP because it correlates to less than r=0.95 to MAB 1.21.3.

Example 7

Clinical comparison of assays for native and total proBNP, respectively

In a clinical study 246 patient samples classified according to their NYHA-status have been analyzed by sandwich immunoassays for native proBNP and total proBNP, respectively. The results of this study are given in Table 2.

Table 2: Comparative analysis of native proBNP and total proBNP in patient samples

NYHA	n 246	native proBNP arbitrary units	NYHA X / NYHA 0	total proBNP pg/ml	NYHA X / NYHA 0
0	119	337	1,0	638	1,0
1	32	355	1,1	717	1,1
2	62	655	1,9	1072	1,7
3	30	2947	8,7	3609	5,6
4	3	12755	38	15902	25

Clinically very important is the differentiation of patients with no or very mild disease (HYHA-classes 0 and 1) as compared to patients with disease progression (NYHA X = classes 2 or more). As can be seen from Table 2, there is a significant increase from class 0/1 to class 2 and higher classes. This increase for all the classes 2, 3, and 4 is more pronounced for native proBNP as compared to total proBNP. This translates to a better sensitivity/specificity profile and clinical utility for native proBNP as compared to total proBNP.

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US 2003/0219734

WO 00/35951

WO 00/45176

WO 93/24531

Patent Claims

1. An antibody specifically binding to native proBNP, wherein said antibody specifically binding to native proBNP is an antibody which in terms of the values for proBNP as determined in patient samples correlates with an r-value of at least r=0.95 or above to MAB 1.21.3.

2. The antibody according to claim 1, wherein said antibody is a monoclonal antibody.

3. The antibody according to claim 2, wherein said monoclonal antibody is produced by hybridoma cell line MAB 1.21.3.

4. The antibody according to claim 1, wherein said antibody is an isolated polyclonal antibody.

5. A method for specific detection of native proBNP, comprising the steps of contacting a sample suspected or known to contain proBNP with an antibody to native proBNP according to any of claims 1 to 4 under conditions allowing for the formation of an antibody to native proBNP-native proBNP complex and detecting the complex formed.

6. The method according to claim 5, wherein said detection is performed by a competitive immunoassay.

7. The method according to claim 5, wherein said detection is performed by a sandwich immunoassay, wherein a second antibody to proBNP is also used and wherein said second antibody to proBNP and the antibody to native proBNP both bind to native proBNP thus forming a second anti-proBNP antibody-native proBNP-anti-native proBNP antibody complex.

8. A method for diagnosing heart failure comprising specific detection of native proBNP and correlating the level of native proBNP to heart failure.

9. A kit for measurement of native proBNP, comprising an antibody according to any of claims 1 to 4 and auxiliary reagents for detection of native proBNP.

10. The hybridoma cell line MAB 1.21.3 as deposited with the DSMZ.

Fig. 1

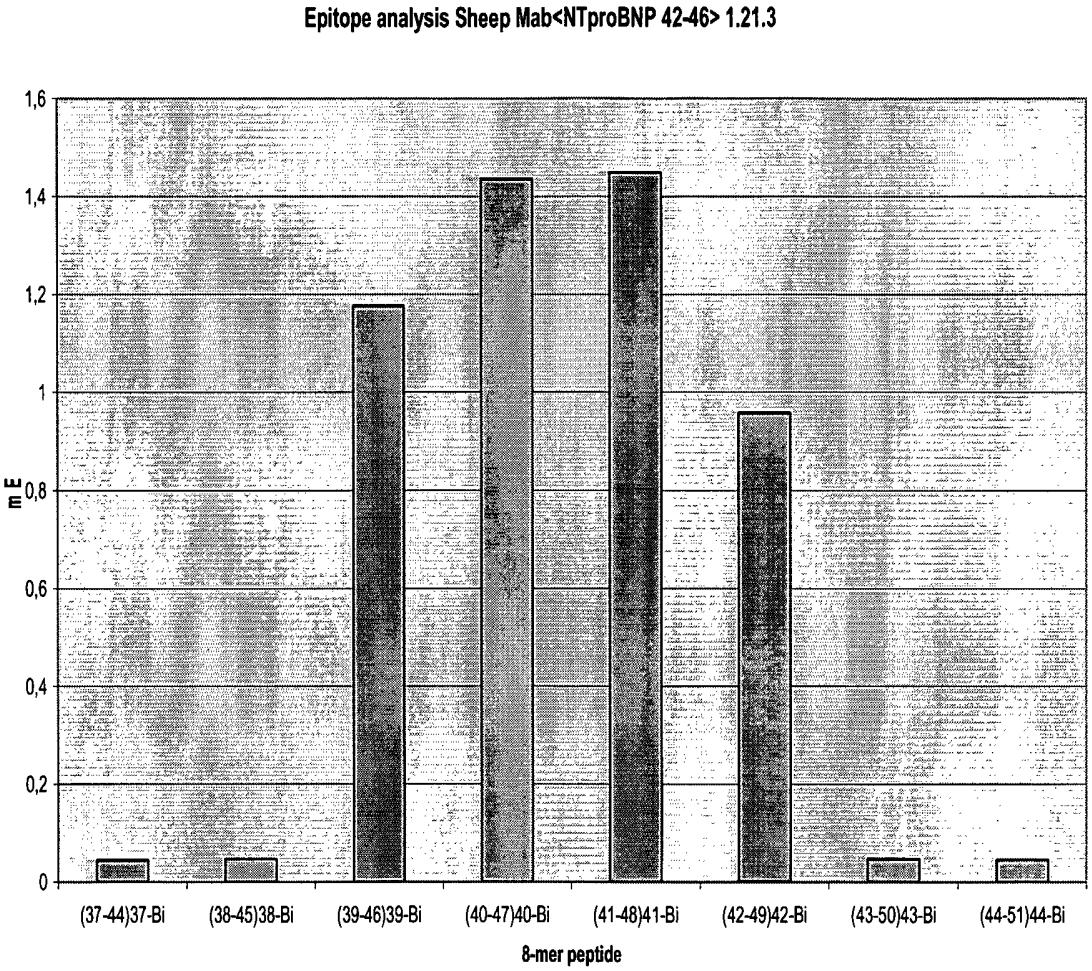

Epitope analysis Sheep Mab<NTproBNP 42-46> 1.21.3
mE
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
(37-44)37-Bi
(38-45)38-Bi
(39-46)39-Bi
(40-47)40-Bi
(41-48)41-Bi
(42-49)42-Bi
(43-50)43-Bi
(44-51)44-Bi
8-mer peptide

Fig. 2a

Program for the determination of NT-proBNP in 20 patient samples with 8 different antibodies in Biacore written and to be executed with BIACORE 3000 Control Software Version. 4.1

```
DEFINE APROG Sandwich
PARAM %apo %anr %aco %bnr %bco %c1po %c1nr %c1co %c2po %c2nr %c2co %c3po %c3nr %c3co %c4po
%c4nr %c4co

 KEYWORD anr %anr
 KEYWORD aco %aco
 KEYWORD bnr %bnr
 KEYWORD bco %bco
 KEYWORD c1nr %c1nr
 KEYWORD c1co %c1co
 KEYWORD c2nr %c2nr
 KEYWORD c2co %c2co
 KEYWORD c3nr %c3nr
 KEYWORD c3co %c3co
 KEYWORD c4nr %c4nr
 KEYWORD c4co %c4co

 CAPTION Sandwich: AG: %anr %aco mit %bnr %bco, AB: %c1nr, %c2nr, %c3nr, %c4nr

      FLOW      10 -f
      FLOWPATH  1,2,3,4

      DIPNEEDLE  r2e1
   *  QUICKINJECT %apo 100      !calibrator/human serum
-0:10 RPOINT     -b BL_start

      FLOW      100
   *  QUICKINJECT r2f6 50       !HBS

      FLOW      10 -f

      FLOWPATH  1
      DIPNEEDLE  r2e2
   *  QUICKINJECT %c1po 30      !AB1/5
-0:10 RPOINT     -b AG

      FLOWPATH  2
      DIPNEEDLE  r2e3
   *  QUICKINJECT %c2po 30      !AB2/6
-0:10 RPOINT     -b AB1

      FLOWPATH  3
      DIPNEEDLE  r2e4
   *  QUICKINJECT %c3po 30      !AB3/7

-0:10 RPOINT     -b AB2

      FLOWPATH  4
      DIPNEEDLE  r2e5
   *  QUICKINJECT %c4po 30      !AB4/8
-0:10 RPOINT     -b AB3
```

Fig. 2b

```
      FLOWPATH  1,2,3,4
      FLOW      100

   * QUICKINJECT r2f7 50      !HBS
-0:10 RPOINT    -b AB4

      FLOW      20

   * QUICKINJECT r2e10 5      !HBSwash
   * QUICKINJECT r2f3 10      !100 mM HCl
   * QUICKINJECT r2f4 10      !100 mM phosphoric acid
   * QUICKINJECT r2f5 10      !100 mM phosphoric acid
     EXTRACLEAN
 3:30 RPOINT    BL_end       !baseline after regen.cycle
END

DEFINE APROG Regen
 CAPTION regeneration cycle
      FLOW      20
      FLOWPATH  1,2,3,4
   * QUICKINJECT r2e10 5      !HBSwash
-0:10 RPOINT    -b BL_start
   * QUICKINJECT r2f3 10      !100 mM HCl
   * QUICKINJECT r2f4 10      !100 mM phosphoric acid
   * QUICKINJECT r2f5 10      !100 mM phosphoric acid
 3:30 RPOINT    BL_end       !baseline after regen.cycle
END

DEFINE LOOP AG
 LPARAM %apo  %anr     %aco  %bnr     %bco
 TIMES 1
!     %apo %anr     %aco  %bnr     %bco
      r2a1  NT-proBNP 40nM  HoSer/CMD 20%/1mg/mL
      r2a2  NT-proBNP 20nM  HoSer/CMD 20%/1mg/mL
      r2a3  NT-proBNP 10nM  HoSer/CMD 20%/1mg/mL
      r2a4  NT-proBNP 5nM   HoSer/CMD 20%/1mg/mL
      r2a5  NT-proBNP 2.5nM HoSer/CMD 20%/1mg/mL
      r2a6  NT-proBNP 0nM   HoSer/CMD 20%/1mg/mL
      r2b1  HuSer1    1:5   HBS/CMD   1mg/mL
      r2b2  HuSer2    1:5   HBS/CMD   1mg/mL
      r2b3  HuSer3    1:5   HBS/CMD   1mg/mL
      r2b4  HuSer4    1:5   HBS/CMD   1mg/mL
      r2b5  HuSer5    1:5   HBS/CMD   1mg/mL
      r2b6  HuSer6    1:5   HBS/CMD   1mg/mL
      r2b7  HuSer7    1:5   HBS/CMD   1mg/mL
      r2b8  HuSer8    1:5   HBS/CMD   1mg/mL
      r2b9  HuSer9    1:5   HBS/CMD   1mg/mL
      r2b10 HuSer10   1:5   HBS/CMD   1mg/mL
      r2c1  HuSer11   1:5   HBS/CMD   1mg/mL
      r2c2  HuSer12   1:5   HBS/CMD   1mg/mL
      r2c3  HuSer13   1:5   HBS/CMD   1mg/mL
      r2c4  HuSer14   1:5   HBS/CMD   1mg/mL
      r2c5  HuSer15   1:5   HBS/CMD   1mg/mL
```

Fig. 2c

```
      r2c6  HuSer16   1:5      HBS/CMD   1mg/mL
      r2c7  HuSer17   1:5      HBS/CMD   1mg/mL
      r2c8  HuSer18   1:5      HBS/CMD   1mg/mL
      r2c9  HuSer19    1:5     HBS/CMD   1mg/mL
      r2c10 HuSer20   1:5      HBS/CMD   1mg/mL
END

DEFINE LOOP AB
 LPARAM %c1po %c1nr %c1co %c2po %c2nr %c2co %c3po %c3nr %c3co %c4po %c4nr %c4co
 TIMES 1
      r1a1  AB1   500nM r1a2  AB2   500nM r1a3  AB3   500nM r1a4  AB4   500nM
      r1b1  AB5   500nM r1b2  AB6   500nM r1b3  AB7   500nM r1b4  AB8   500nM
END

MAIN
  RACK     2 Thermo_a
  RACK     1 Thermo_c

 detection  1,2,3,4

 LOOP AB ORDER
       APROG     Regen
       unclog
    LOOP AG ORDER
       APROG     Sandwich %apo %anr %aco %bnr %bco %c1po %c1nr %c1co %c2po %c2nr %c2co
                 %c3po %c3nr %c3co %c4po %c4nr %c4co
    ENDLOOP
 ENDLOOP

   APROG     Regen
   APPEND    continue
END
```

Fig. 3

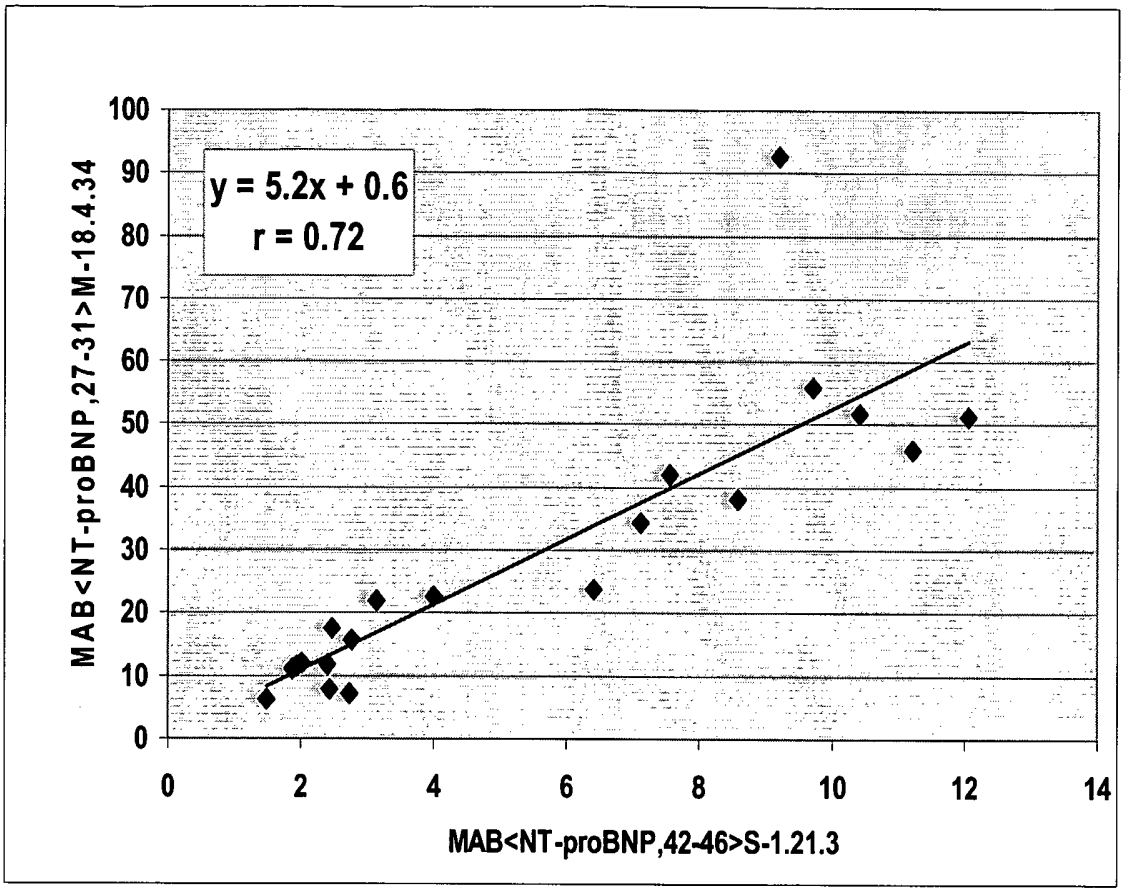

y = 5.2x + 0.6
r = 0.72
MAB<NT-proBNP,27-31>M-18.4.34
MAB<NT-proBNP,42-46>S-1.21.3
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14

Fig. 4

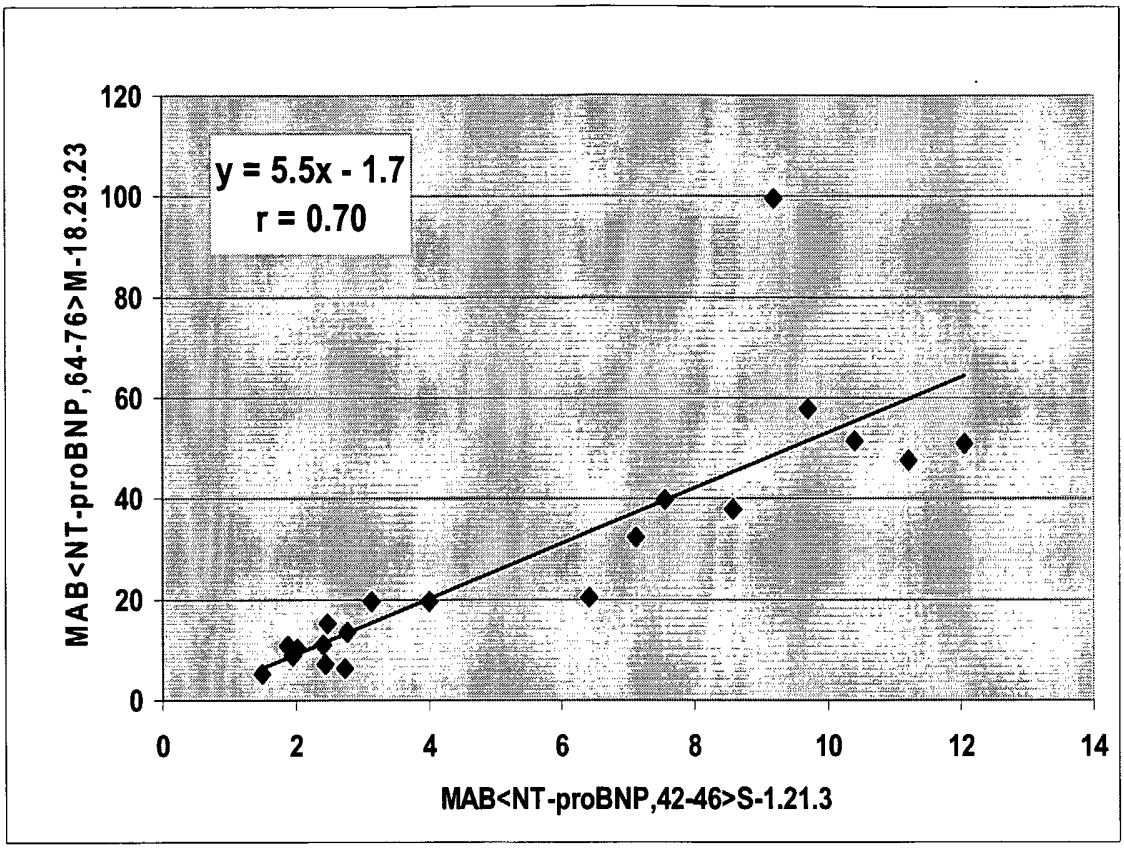

y = 5.5x - 1.7
r = 0.70
MAB<NT-proBNP,64-76>M-18.29.23
MAB<NT-proBNP,42-46>S-1.21.3
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20
40
60
80
100
120
0
2
4
6
8
10
12
14

Fig. 5

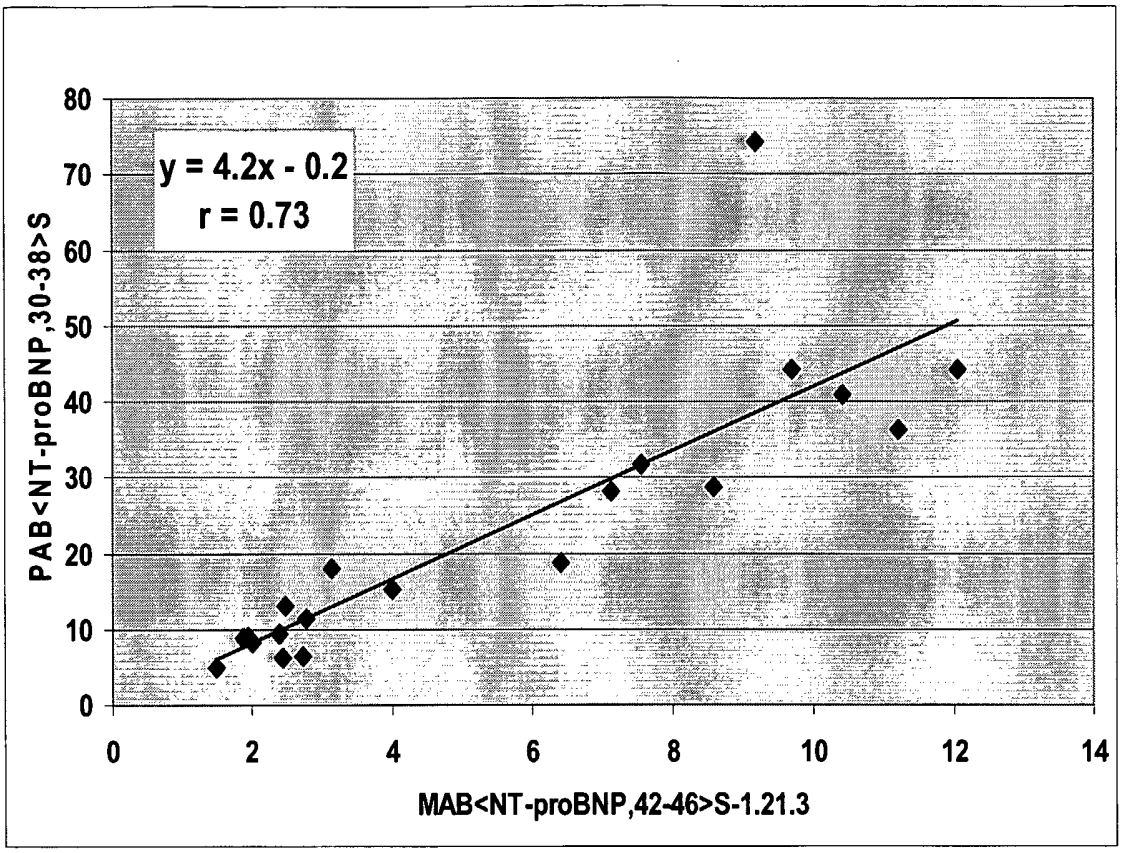

y = 4.2x - 0.2
r = 0.73
PAB<NT-proBNP,30-38>S
MAB<NT-proBNP,42-46>S-1.21.3
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14

Fig. 6

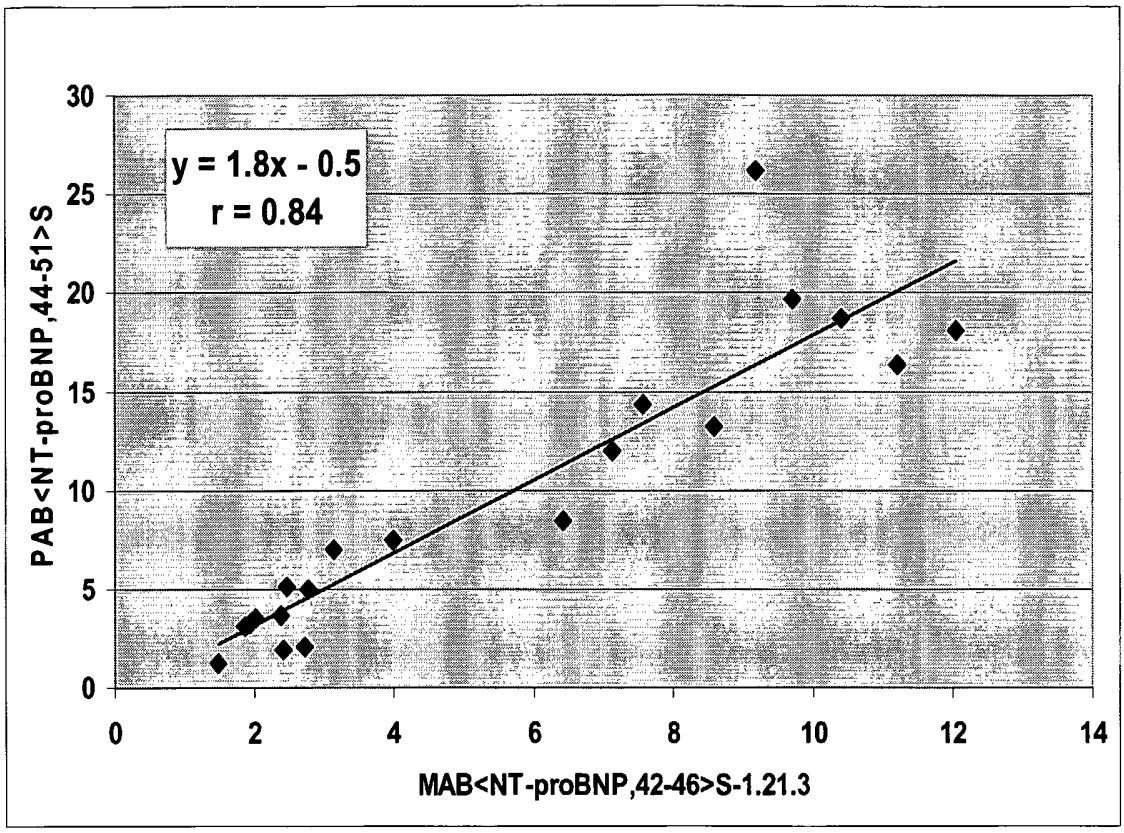
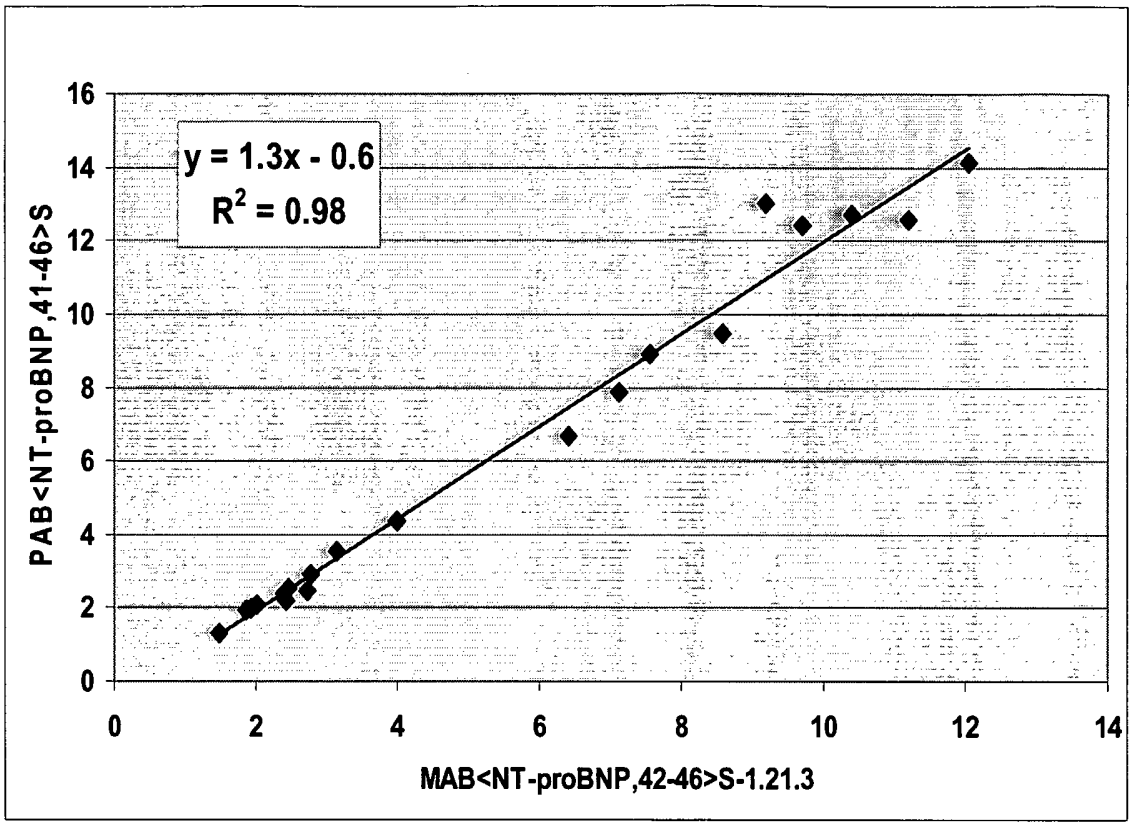

y = 1.8x - 0.5
r = 0.84
PAB<NT-proBNP,44-51>S
MAB<NT-proBNP,42-46>S-1.21.3
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14

Fig. 7


y = 1.3x - 0.6
R2 = 0.98
PAB<NT-proBNP,41-46>S
MAB<NT-proBNP,42-46>S-1.21.3
0
2
4
6
8
10
12
14
16

Sequence Listing

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F. Hoffmann-La Roche AG

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<130> 22564 WO

<150> EP 03010591.0
<151> 2003-05-12

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1 5 10

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1 5 10

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<400> 11

Leu Gln Glu Ser Pro Arg
1 5

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 16, line table.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depositary institution
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
07.05.2003	DSM ACC2591

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☒

Indications relating to the Expert Solution in respect of the deposited biological material DSM ACC2591, DSM ACC2592, DSM ACC2593 and DSM ACC 2650

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

CA (Canada), EP (European Patent), SG (Singapore)

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☒ This sheet was received by the International Bureau on: 13 SEPTEMBER 2004 (13.09.2004)
Authorized officer	Authorized officer A. BOUVIER.

B. IDENTIFICATION OF DEPOSIT (additional sheet)

Applicants	:	Roche Diagnostics GmbH, et al.
Applicants file reference	:	22564 WO-WN
International application No.	:	PCT/EP2004/005092

Further Deposits

The indications made below relate to the deposited cell cultures referred to in the description on page 16, table.

All mentioned cell lines were deposited with:

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)

Mascheroder Weg 1b

D-38124 Braunschweig

Germany

Date of deposit	Accession Number	Name, given by Depositor
07.05.2003	DSM ACC2592	MAK <NT-proBNP>18.4.34
07.05.2003	DSM ACC2593	MAK <NT-proBNP>18.29.23
27.04.2004	DSM ACC2650	MAK <NT-proBNP>1.21.3

C. ADDITIONAL INDICATIONS (additional sheet)

Applicants	:	Roche Diagnostics GmbH, et al.
Applicants file reference	:	22564 WO-WN
International application No.	:	PCT/EP2004/005092

Indications Relating to the Expert Solution in Respect of Deposited Biological Material Referred to in the Description

The indications relating to deposited biological material are all contained in the description. The following additional indications are not required to be part of the description and should be treated as "separate indications." They relate only to the expert solution.

The additional indications made below relate to the deposited biological material referred to as:

MAK <NT-proBNP>17.3.1	DSM ACC2591
MAK <NT-proBNP>18.4.34	DSM ACC2592
MAK <NT-proBNP>18.29.23	DSM ACC2593
MAK <NT-proBNP>1.21.3	DSM ACC2650

in the description on page 16.

The additional indications are:

For CA (Canada) designation:

In respect of the designation of Canada, samples of the deposited biological material will be made available until the grant of the Canadian patent, or until the date on which the application is refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, as provided in Rules 107 and 108 of the Patent Rules under the Canadian Patent Act, only by the issue of a sample to an independent expert nominated by the Commissioner (Rule 104(4))

For EP (European Patent) designation:

In respect of the designation of the EPO, samples of the deposited biological material will be made available until the publication of the mention of the grant of the European patent, or until 20 years from the date of filing if the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC, only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC).

For SG (Singapore) designation:

Applicants hereby give notice of our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005092

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/26 G01N33/531 G01N33/68 G01N33/74 C12N5/20

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUGHES D ET AL: "An immunoluminometric asay for N-terminal pro-brain natriuretic peptide: develop,ment of a test for left venticular dysfunction" CLINICAL SCIENCE, BIOCHEMICAL SOCIETY AND THE MEDICAL RESEARCH SOCIETY, LONDON,, GB, vol. 96, no. 4, April 1999 (1999-04), pages 373-380, XP002136252 ISSN: 0143-5221 cited in the application page 374, left-hand column, line 4 - right-hand column, line 3	1-10
X	WO 00/35951 A (NG LEONG LOKE ;UNIV LEICESTER (GB)) 22 June 2000 (2000-06-22) example 1	1-10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 2004	09/08/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Le Flao, K

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005092

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PEMBERTON C J ET AL: "AMINO-TERMINAL PROBNP IN OVINE PLASMA: EVIDENCE FOR ENHANCED SECRETION IN RESPONSE TO CARDIAC OVERLOAD" AMERICAN JOURNAL OF PHYSIOLOGY, AMERICAN PHYSIOLOGICAL SOCIETY, BETHESDA, MD, US, vol. 275, no. 4, PART 2, 1998, pages H1200-H1208, XP000913587 ISSN: 0002-9513 page H1201, left-hand column, line 16 - line 48 page H1203, left-hand column, line 16 - line 25 page H1204, right-hand column, line 1 - page H1205, right-hand column, line 5 page H1207, left-hand column, paragraph 2 - right-hand column, paragraph 1	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/005092

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5-8
because they relate to subject matter not required to be searched by this Authority, namely:

Considering the claims 5-8, the search has been carried out as if the method is not containing any step performed on the human or animal body (Article 52(4) EPC).

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0035951 A	22-06-2000	AU 1670300 A	03-07-2000
		WO 0035951 A1	22-06-2000