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(54) **METHOD FOR PREDICTING THE RISK OF GETTING CANCER OR DIAGNOSING CANCER IN A FEMALE SUBJECT**

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(57) **ABSTRACT**

Subject matter of the present invention is a method for predicting the risk of getting cancer in a female subject that does not suffer from cancer or alternatively diagnosing cancer in a female subject comprising:

determining the level of Pro-Enkephalin or fragments thereof including Leu-Enkephalin and Met-Enkephalin of at least 5 amino acids in a bodily fluid obtained from said female subject; and

correlating said level of Pro-Enkephalin or fragments thereof with a risk for getting cancer, wherein a reduced level is predictive for an enhanced risk of getting cancer or alternatively diagnosing cancer wherein an reduced level is correlated with the diagnosis of cancer.

Specification includes a Sequence Listing.

Fig. 1:

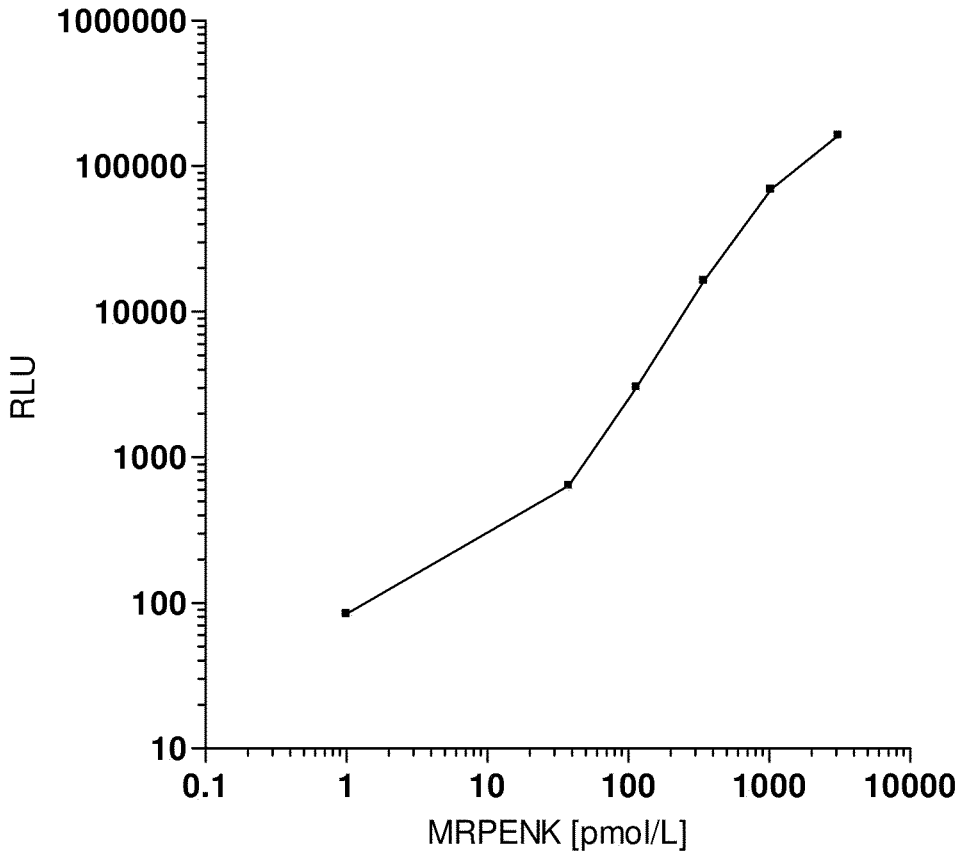

1000000
100000
10000
1000
100
10
RLU
0.1
1
10
100
1000
10000
MRPENK [pmol/L]

Fig. 2:

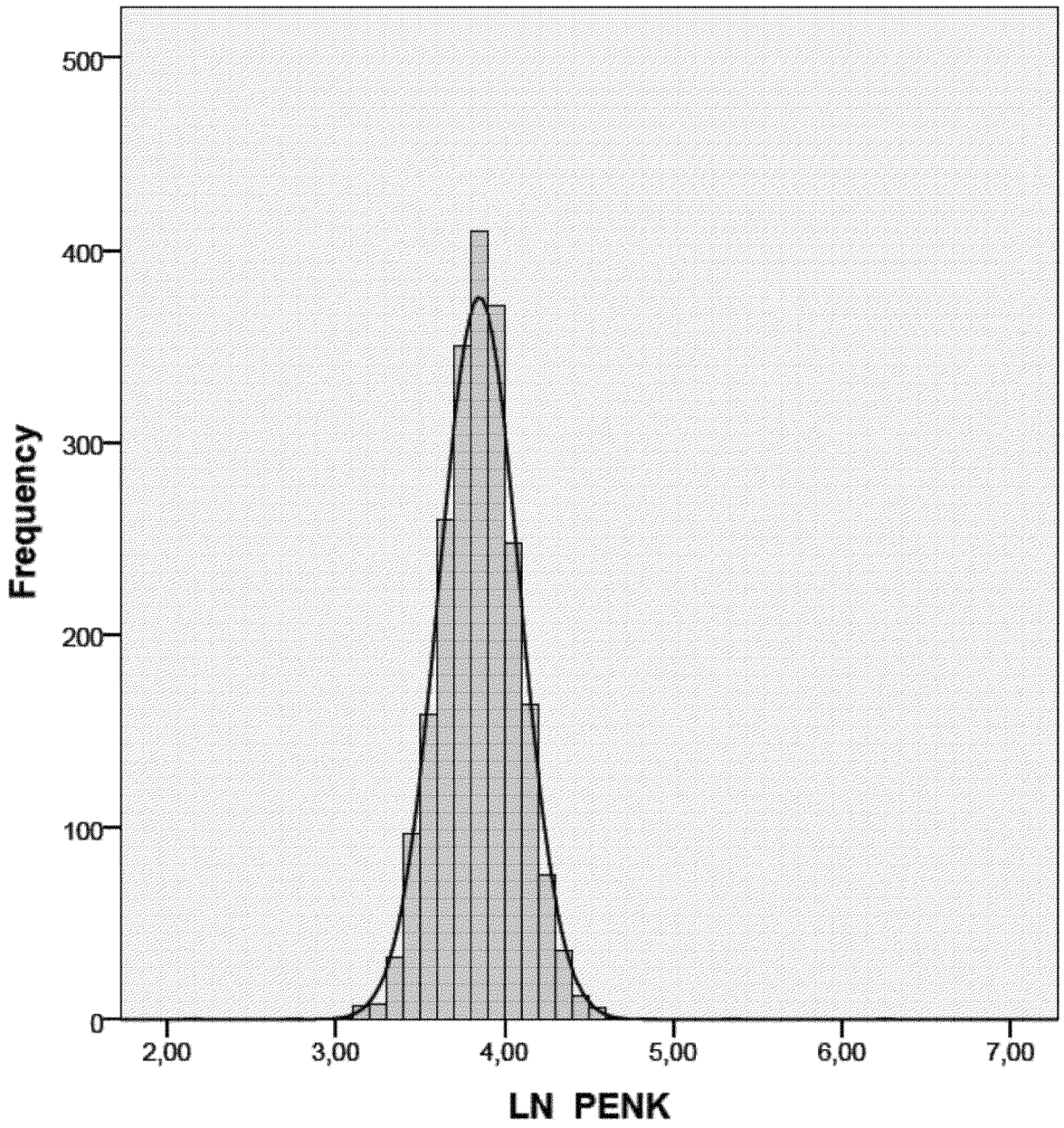
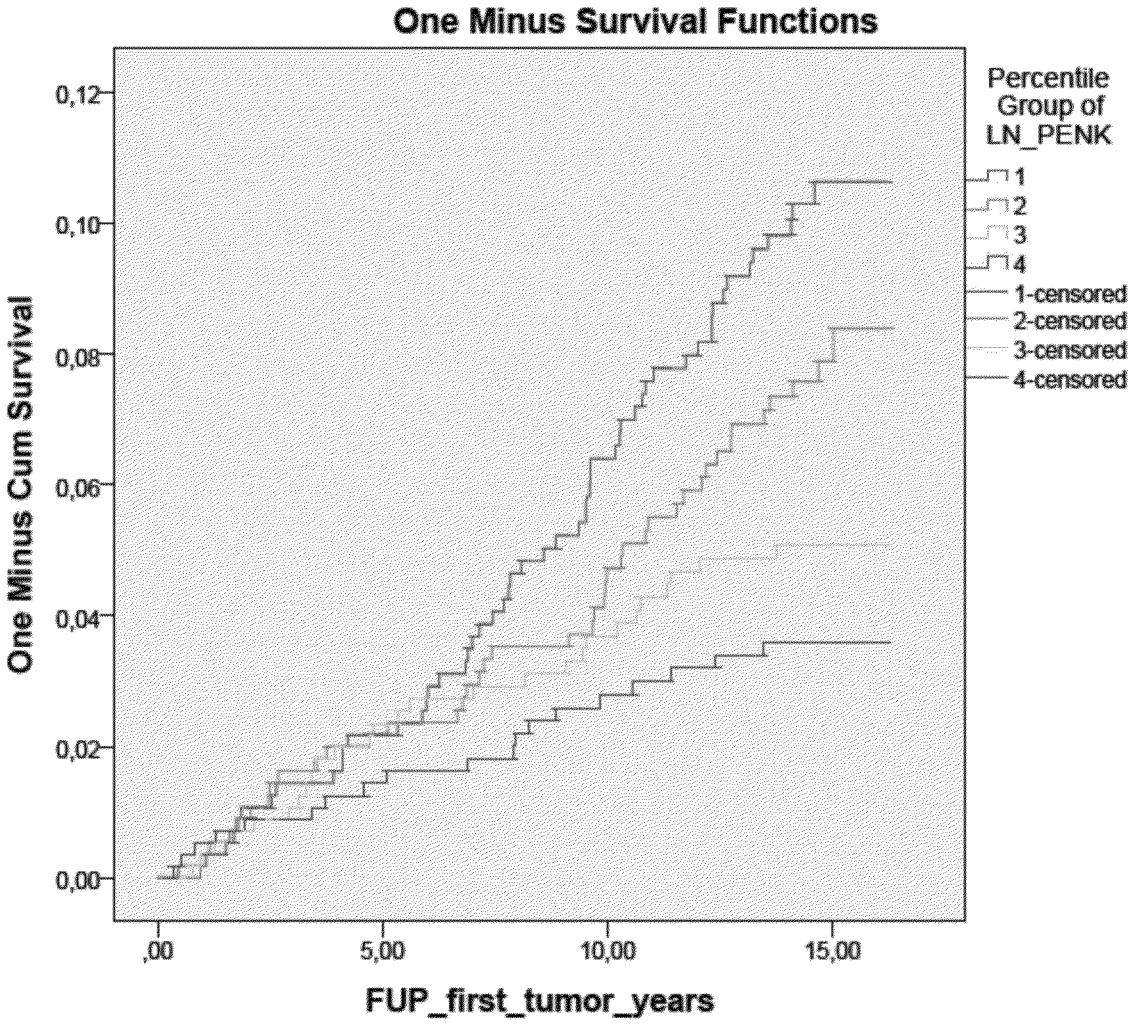


Fig. 3:


One Minus Survival Functions
One Minus Cum Survival
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0,10
0,08
0,06
0,04
0,02
0,00
,00
5,00
10,00
15,00
FUP_first_tumor_years
Percentile Group of LN_PENK
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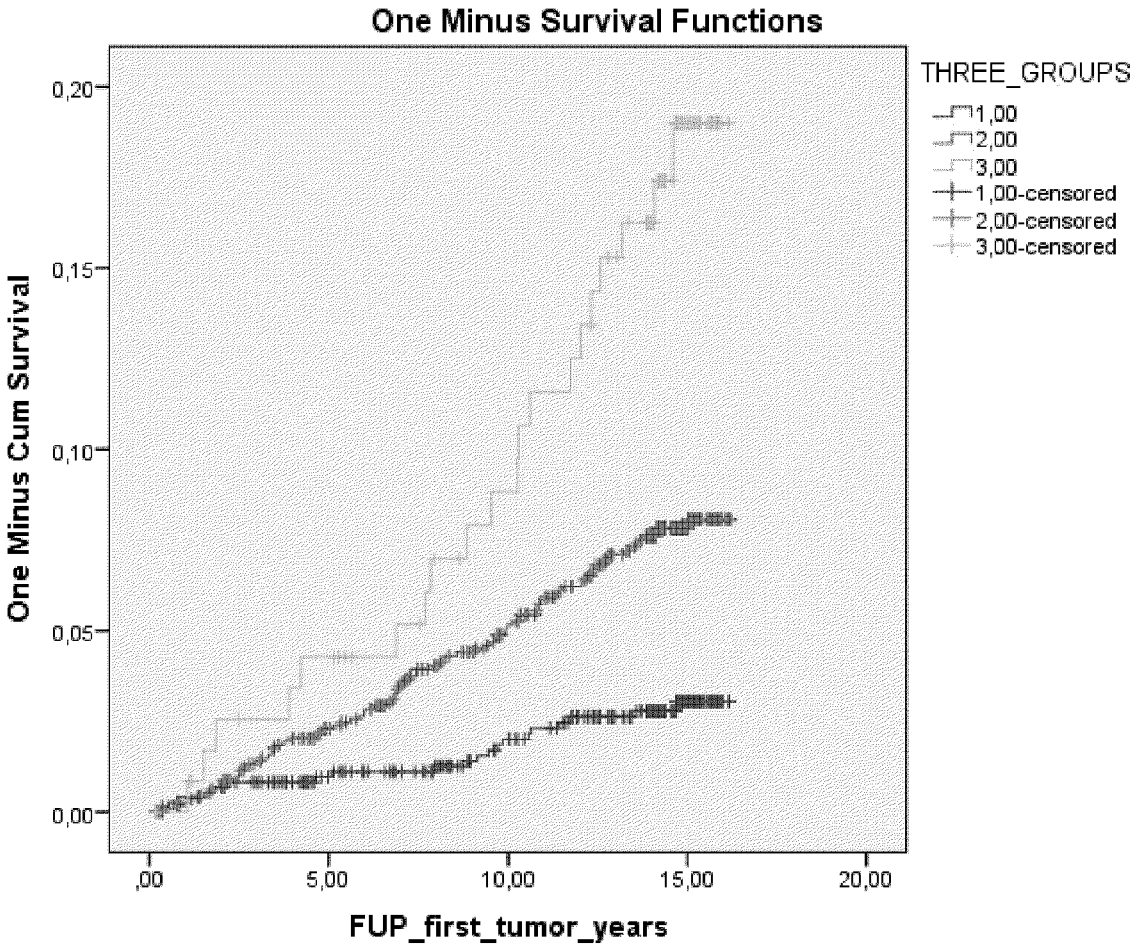
2
3
4
1-censored
2-censored
3-censored
4-censored

Fig. 4:


One Minus Survival Functions
One Minus Cum Survival
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0,15
0,10
0,05
0,00
,00
5,00
10,00
15,00
20,00
FUP_first_tumor_years
THREE_GROUPS
1,00
2,00
3,00
1,00-censored
2,00-censored
3,00-censored

METHOD FOR PREDICTING THE RISK OF GETTING CANCER OR DIAGNOSING CANCER IN A FEMALE SUBJECT

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on Dec. 14, 2022, is named BOEH-MERP-0221-C02_SL.xml and is 30,740 bytes in size.

[0002] Subject matter of the present invention is a method for predicting the risk of getting cancer in a female subject that does not suffer from cancer or alternatively diagnosing cancer in a female subject comprising:

[0003] determining the level of Pro-Enkephalin (PENK) or fragments thereof including Leu-Enkephalin and Met-Enkephalin of at least 5 amino acids in a bodily fluid obtained from said female subject; and

[0004] correlating said level of Pro-Enkephalin or fragments thereof with a risk for getting cancer, wherein a reduced level is predictive for an enhanced risk of getting cancer or alternatively diagnosing cancer wherein an reduced level is correlated with the diagnosis of cancer.

[0005] Met-Enkephalin, a 5 amino acid peptide derived from the Enkephalin precursor (PreProEnkephalin), also named "Opioid Growth Factor" (OGF) is released together with ProEnkephalin-fragments. The mature peptide binds to different opioid receptors (Koneru et al., 2009). Enkephalin (OGF) was found to have a number of physiological functions. In the CNS it down regulates Substance P associated pain signalling, it plays roles as cytokine (Plotnikoff et al., 1997). Proenkephalin related peptides exhibiting antibiotic actions (Goumon et al., 1998). Proenkephalin and Enkephalin exhibits anti tumor action and acting as pro-apoptotic agents (Tavish et al., 2007, Donahue et al., 2011, Zagon et al., 2009).

[0006] The use of vasoactive peptides for prediction of cancer risks in males has been reported by Belting et al., Cancer, Epidemiology, Biomarkes & Prevention. MR-pro-ANP, MR-pro-ADM and copeptin was measured in the fasting plasma from participants of the Malmö Diet and Cancer Study that were free from cancer prior to the baseline exam in 1991 to 1994 (1768 males and 2293 females). The authors stated that among females, there was no relationship between biomarkers and cancer incidence.

[0007] A subject of the present invention was to investigate the prognostic and diagnostic power of PENK for the prediction of cancer incidence and the prediction of the risk of reoccurrence of cancer. To address this issue, stable fragments of Pro-Enkephalin (Ernst et al., 2006) in fasting plasma were measured in said Swedish prospective cohort study (Malmö Diet and Cancer Study) and related baseline level of this biomarker to breast-cancer incidence during 15 years of follow-up.

[0008] Surprisingly, it has been shown that Pro-Enkephalin is a powerful and highly significant biomarker for woman for predicting the risk of getting cancer in a female subject that does not suffer from cancer or alternatively diagnosing cancer in a female subject.

[0009] Thus, subject matter of the present invention is a method for predicting the risk of getting cancer in a female subject that does not suffer from cancer or alternatively diagnosing cancer in a female subject comprising:

[0010] determining the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject; and

[0011] correlating said level of Pro-Enkephalin or fragments thereof with a risk for getting cancer, wherein an reduced level is predictive for an enhanced risk of getting cancer or alternatively diagnosing cancer wherein an reduced level is correlated with the diagnosis of cancer.

[0012] Examples of cancers may be selected from the group comprising breast cancer, lung cancer, pancreatic cancer and colon cancer.

[0013] Throughout the specification it should be understood that the term fragments of Pro-Enkephalin also include Leu-Enkephalin and Met-Enkephalin.

[0014] In a specific embodiment of the invention said cancer is breast cancer. In another specific embodiment of the invention said cancer is lung cancer.

[0015] Thus, subject matter of the present invention is the determination of susceptibility of a woman to aquire cancer, e.g. breast cancer, lung cancer etc

[0016] Data obtained in the present study revealed also a correlation between the risk of getting cancer in male subjects with the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said male subject; this correlation however, was not that statistically significant for the present data set although there was a clear trend for an increased cancer risk at reduced levels of PENK also in males. Thus, there is a value for the method according to the invention also for male subjects but in the present study the observed effect was not as strong for males as compared to females. This may be primarily due to the low number of cancer incidents in the male population.

[0017] Further, data obtained in the present study revealed also a correlation between the risk of getting cancer in female subjects with the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject, wherein said cancer was not lung cancer or breast cancer. Due to the small number of incidents in this particular population this correlation however, was not that statistically significant for the present data set. Although it was not significant there was a clear trend. It is furthermore credible that the present data suggest such a correlation also in other cancers due to the known proapoptotic effect of Enkephalin, a fragment of PENK. Starting from the prior art it is surprising that Pro-Enkephalin or fragments thereof may be predictive for cancer. Starting from the present data that are statistically highly relevant for breast cancer and lung cancer it is to be expected and credible that it may be prognostic for other types of cancer as well.

[0018] The term "subject" as used herein refers to a living human or non-human organism. Preferably herein the subject is a human subject.

[0019] The term "reduced level" means a level below a certain threshold level.

[0020] A bodily fluid may be selected from the group comprising blood, serum, plasma, urine, cerebro spinal liquid (csf), and saliva.

[0021] In one embodiment of the invention said female subject has never had a diagnosed cancer at the time the sample of bodily fluid is taken from said female subject.

[0022] In another embodiment said female subject has been diagnosed before with having cancer and has been cured at the time the sample of bodily fluid is taken from said female subject and the risk of reoccurrence of getting cancer is determined or alternatively the re-occurrence of cancer is predicted.

[0023] Pro-Enkephalin has the following sequence:

[0024] SEQ ID NO. 1 (Pro-Enkephalin (1-243)

ECSQDCATCSYRLVRPADINFLACVMECEGKLPSLKIWETCKELLQLSK
PELPQDGTSTLRENSKPEESHLLAKRYGGFMKRYGGFMKKMDELYPME
PEEEANGSEILAKRYGGFMKKDAEEDDSLANSSDLLKELLETGDN
RERSHHQDGSDNEEEVSKRYGGFMRGLKRSPQLEDEAKELQK
RYGGFMRRVGRPEWWMDYQKRYGGFLKRFAEALPSDEEGESYSKEVPE
MEKRYGGF MRF

[0025] Fragments of Pro-Enkephalin that may be determined in a bodily fluid may be e.g. selected from the group of the following fragments:

[0026] SEQ ID NO. 2 (Synenkephalin, Pro-Enkephalin 1-73)

ECSQDCATCSYRLVRPADINFLACVMECEGKLPSLKIWETCKELLQLSK
PELPQDGTSTLRENSKPEESHLLA

[0027] SEQ ID NO. 3 (Met-Enkephalin)

YGGFM

[0028] SEQ ID NO. 4 (Leu-Enkephalin)

YGGFL

[0029] SEQ ID NO. 5 (ProEnkephalin 90-109)

MDELYPMEPEEEANGSEILA

[0030] SEQ ID NO. 6 (Pro Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK)

DAEEDDSLANSSDLLKELLETGDNRERSHHQDGSDNEEEVS

[0031] SEQ ID NO. 7 (Met-Enkephalin-Arg-Gly-Leu)

YGGFMRGL

[0032] SEQ ID NO. 8 (Pro-Enkephalin 172-183)

SPQLEDEAKELQ

[0033] SEQ ID NO. 9 (Pro-Enkephalin 193-203)

VGRPEWWMDYQ

[0034] SEQ ID NO. 10 (Pro-Enkephalin 213-234)

FAEALPSDEEGESYSKEVPEME

[0035] SEQ ID NO. 11 (Pro-Enkephalin 213-241)

FAEALPSDEEGESYSKEVPEMEKRYGGF M

[0036] SEQ ID NO. 12 (Met-Enkephalin-Arg-Phe)

YGGFMRF

[0037] Determining the level of Pro-Enkephalin including Leu-Enkephalin and Met-Enkephalin or fragments thereof may mean that the immunoreactivity towards Pro-Enkephalin or fragments thereof including Leu-Enkephalin and Met-Enkephalin is determined. A binder used for determination of Pro-Enkephalin including Leu-Enkephalin and Met-Enkephalin or fragments thereof depending of the region of binding may bind to more than one of the above displayed molecules. This is clear to a person skilled in the art.

[0038] Thus, according to the present invention the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of any of the above peptide and peptide fragments, (i.e. Pro-Enkephalin (PENK) and fragments according to any of the sequences 1 to 12), is determined in a bodily fluid obtained from said subject; and correlated to the specific embodiments of clinical relevance.

[0039] In a more specific embodiment of the method according to the present invention the level of MRPENK is determined (SEQ ID NO. 6: Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK). In a more specific embodiment the level of immunoreactive analyte by using at least one binder that binds to MR-PENK is determined and is correlated to the specific embodiments of clinical relevance according to the invention.

[0040] Determining the level of Pro-Enkephalin or fragments thereof including Leu-Enkephalin and Met-Enkephalin or fragments thereof may mean that the immunoreactivity towards Pro-Enkephalin or fragments thereof including Leu-Enkephalin and Met-Enkephalin is determined. A binder used for determination of Pro-Enkephalin including Leu-Enkephalin and Met-Enkephalin or fragments thereof depending of the region of binding may bind to more than one of the above displayed molecules. This is clear to a person skilled in the art. In another embodiment of the invention the fragment is not Leu-Enkephalin or Met-Enkephalin, In another embodiment of the invention the immunoreactivity towards Pro-Enkephalin or fragments thereof not including Leu-Enkephalin and Met-Enkephalin is determined.

[0041] In a more specific embodiment of the method according to the present invention the level of MRPENK. (SEQ ID NO. 6 (Pro Enkephalin 119-159, Mid regional

Pro-Enkephalin-fragment, MRPENK, DAEEDD-SLANSSDLLKELLETGDNRERSHHQDGSDNEEEVS) is determined.

[0042] Alternatively the level of any of the above analytes may be determined by other analytical methods e.g. mass spectroscopy.

[0043] In a specific embodiment the level of Pro-Enkephalin or fragments thereof are measured with an immunoassay using antibodies or fragments of antibodies binding to Pro-Enkephalin or fragments thereof. An immunoassay that may be useful for determining the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids may comprise the steps as outlined in Example 2. All thresholds and values have to be seen in correlation to the test and the calibration used according to Example 2. A person skilled in the art may know that the absolute value of a threshold might be influenced by the calibration used. This means that all values and thresholds given herein are to be understood in context of the calibration used in herein (Example 2). According to the invention the diagnostic binder to Pro-Enkephalin is selected from the group consisting of antibodies e.g. IgG, a typical full-length immunoglobulin, or antibody fragments containing at least the F-variable domain of heavy and/or light chain as e.g. chemically coupled antibodies (fragment antigen binding) including but not limited to Fab-fragments including Fab minibodies, single chain Fab antibody, monovalent Fab antibody with epitope tags, e.g. Fab-V5Sx2; bivalent Fab (mini-antibody) dimerized with the CH3 domain; bivalent Fab or multivalent Fab, e.g. formed via multimerization with the aid of a heterologous domain, e.g. via dimerization of dHLX domains, e.g. Fab-dHLX-FSx2; F(ab′)2-fragments, scFv-fragments, multimerized multivalent or/and multispecific scFv-fragments, bivalent and/or bispecific diabodies, BITE® (bispecific T-cell engager), trifunctional antibodies, polyvalent antibodies, e.g. from a different class than G; single-domain antibodies, e.g. nanobodies derived from camelid or fish immunoglobulines.

[0044] In a specific embodiment the level of Pro-Enkephalin or fragments thereof are measured with an assay using binders selected from the group comprising aptamers, non-Ig scaffolds as described in greater detail below binding to Pro-Enkephalin or fragments thereof.

[0045] Binder that may be used for determining the level of Pro-Enkephalin or fragments thereof exhibit an affinity constant to Pro-Enkephalin of at least 10^7 M^{-1}, preferred 10^8 M^{-1}, preferred affinity constant is greater than 10^9 M^{-1}, most preferred greater than 10^{10} M^{-1}. A person skilled in the art knows that it may be considered to compensate lower affinity by applying a higher dose of compounds and this measure would not lead out-of-the-scope of the invention. Binding affinity may be determined using the Biacore method, offered as service analysis e.g. at Biaffin, Kassel, Germany (http://www.biaffin.com/de/).

[0046] A human Pro-Enkephalin-control sample is available by ICI-Diagnostics, Berlin, Germany http://www.ici-diagnostics.com/. The assay may also be calibrated by synthetic (for our experiments we used synthetic MRPENK, SEQ ID NO. 6) or recombinant Pro-Enkephalin or fragments thereof.

[0047] The threshold for determining the risk of getting breast cancer in a female subject or diagnosing breast cancer in a female subject according to the methods of the present invention is below 100 pmol/l PENK, preferred below 50 pmol/l, more preferred below 40.4 pmol/l. In a specific embodiment said threshold is about 40.4 pmol/l. These thresholds are related to the above mentioned calibration method. A PENK value below said threshold means that the subject has an enhanced risk of getting cancer or has already cancer.

[0048] In one embodiment of the invention said method is performed more than once in order to monitor the risk of getting breast cancer in a female subject or in order to monitor the course of treatment. In one specific embodiment said monitoring is performed in order to evaluate the response of said female subject to preventive and/or therapeutic measures taken.

[0049] In one embodiment of the invention the method is used in order to stratify said female subjects into risk groups.

[0050] Subject of the present invention is also a method for predicting the risk of getting cancer in a female or identifying a female subject having an enhanced risk for getting cancer according to any of the preceding embodiments, wherein the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject either alone or in conjunction with other prognostically useful laboratory or clinical parameters is used for the prediction of a subject's risk for getting an adverse event by a method which may be selected from the following alternatives:

[0051] Comparison with the median of the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject in an ensemble of predetermined samples in a population of "healthy" or "apparently healthy" subjects,

[0052] Comparison with a quantile of the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject in an ensemble of predetermined samples in a population of "healthy" or "apparently healthy" subjects,

[0053] Calculation based on Cox Proportional Hazards analysis or by using Risk index calculations such as the NRI (Net Reclassification Index) or the IDI (Integrated Discrimination Index).

[0054] In one embodiment of the invention subject of the present invention is also a method for predicting the risk of getting cancer in a female or identifying a female subject having an enhanced risk for getting cancer according to any of the preceding embodiments, wherein the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject either alone or in conjunction with other prognostically useful biomarker. Such a useful biomarker may be Pro-Neurotensin and fragments thereof of at least 5 amino acids.

[0055] In a more specific embodiment of the method according to the present invention the level of Pro-Neurotensin 1-117 is determined in addition to the determination of Pro-Enkephalin an fragments thereof.

[0056] Thus, subject matter of the present invention is also a method for predicting the risk of getting cancer in a female subject that does not suffer from cancer or alternatively diagnosing cancer in a female subject comprising:

[0057] determining the level of Pro-Enkephalin or fragments thereof including Leu-Enkephalin and Met-

Enkephalin of at least 5 amino acids in a bodily fluid obtained from said female subject; and

[0058] determining the level of Pro-Neurotensin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said female subject; and

[0059] correlating said level of Pro-Enkephalin or fragments thereof and Pro-Neurotensin or fragments thereof of at least 5 amino acids with a risk for getting cancer, wherein an reduced level of Pro-Enkephalin is predictive for an enhanced risk of getting cancer or alternatively diagnosing cancer wherein an reduced level is correlated with the diagnosis of cancer and wherein an increased level of Pro-Neurotensin is predictive for an enhanced risk of getting cancer or alternatively diagnosing cancer wherein an increased level is correlated with the diagnosis of cancer.

[0060] SEQ ID NO. 13 (Pro-Neurotensin 1-147)

SDSEEEMKAL EADFLTNMHT SKISKAHVPS WKMTLLNVCS LVNNLNSPAE ETGEVHEEEL VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVIKRK IPYILKRQLY ENKPRRPYIL KRDSYYY

[0061] SEQ ID NO. 14 (Pro-Neurotensin 1-125 (large neuromedin N))

SDSEEEMKAL EADFLTNMHT SKISKAHVPS WKMTLLNVCS LVNNLNSPAE ETGEVHEEEL VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVI KR KIPYIL

[0062] SEQ ID NO. 15 (neuromedin N)

KIPYIL

[0063] SEQ ID NO. 16 (neurotensin)

pyroQLYENKPRRP YIL

[0064] SEQ ID NO. 17 (Pro-Neurotensin 1-117)

SDSEEEMKAL EADFLTNMHT SKISKAHVPS WKMTLLNVCS LVNNLNSPAE ETGEVHEEEL VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVI

[0065] SEQ ID NO. 18 (Pro-Neurotensin 1-132)

SDSEEEMKAL EADFLTNMHT SKISKAHVPS WKMTLLNVCS LVNNLNSPAE ETGEVHEEEL VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVIKRK IPYILKRQLY EN

[0066] SEQ ID NO. 19 (Pro-Neurotensin 120-140)

KIPYILKRQL YENKPRRPYI L

[0067] SEQ ID NO. 20 (Pro-Neurotensin 120-147)

KIPYILKRQL YENKPRRPYIL KRDSYYY

[0068] SEQ ID NO. 21 (Pro-Neurotensin 128-147)

QLYENKPRRP YILKRDSYYY

[0069] In a specific embodiment the level of Pro-Neurotensin is measured with an immunoassay. More specifically an immunoassay is used as described in Ernst et al. (Peptides (2006), (27) 1787-1793). An immunoassay that may be useful for determining the level of Pro-Neurotensin or fragments thereof of at least 5 amino acids may comprise the steps as outlined in Example 2. All thresholds and values have to be seen in correlation to the test and the calibration used according to Example 2. A person skilled in the art may know that the absolute value of a threshold might be influenced by the calibration used. This means that all values and thresholds given herein are to be understood in context of the calibration used in herein (Example 2). A human Pro-Neurotensin-calibrator is available by ICI-Diagnostics, Berlin, Germany. Alternatively, the assay may also be calibrated by synthetic or recombinant P—NT 1-117 or fragments thereof (see also Ernst et al, 2006).

[0070] Binder that may be used for determining the level of Pro-Neurotensin or fragments thereof exhibit an affinity constant to Pro-Neurotensin of at least 10^7 M^{-1}, preferred 10^8 M^{-1}, preferred affinity constant is greater than 10^9 M^{-1}, most preferred greater than 10^{10} M^{-1}. A person skilled in the art knows that it may be considered to compensate lower affinity by applying a higher dose of compounds and this measure would not lead out-of-the-scope of the invention. Binding affinity may be determined using the Biacore method, offered as service analysis e.g. at Biaffin, Kassel, Germany (http://www.biaffin.com/de/).The threshold for determining the risk of getting breast cancer in a female subject or diagnosing breast cancer in a female subject according to the methods of the present invention is above 78 pmol/l PNT, preferred 100 pmol/l, more preferred 150 pmol/l. In a specific embodiment said threshold is about 100 pmol/l. These thresholds are related to the above mentioned calibration method. A P-NT value above said threshold means that the subject has an enhanced risk of getting cancer or has already cancer.

[0071] In one embodiment of the invention said method is performed more than once in order to monitor the risk of getting breast cancer in a female subject or in order to monitor the course of treatment. In one specific embodiment said monitoring is performed in order to evaluate the response of said female subject to preventive and/or therapeutic measures taken.

[0072] In one embodiment of the invention the method is used in order to stratify said female subjects into risk groups.

[0073] In one embodiment of the invention the cancer is selected from the group comprising breast cancer, and lung cancer. Subject matter of the invention is further an assay for determining Pro-Enkephalin and Pro-Enkephalin fragments in a sample comprising two binders that bind to two different regions within the region of Pro-Enkephalin that is aminoacid 133-140 (LKELLETG, SEQ ID NO. 22) and aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 23) wherein each of said regions comprises at least 4 or 5 amino acids.

[0074] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention the assay sensitivity of said assay is able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is < 15 pmol/, preferably < 10 pmol/l and more preferably L < 6 pmol/L.

[0075] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention said binder exhibits an binding affinity to its binding partner of at least 10^7 M^{-1}, preferred 10^8 M^{-1}, preferred affinity constant is lower than 10^9 M^{-1}, most preferred lower than 10^{10} M^{-1}. A person skilled [K1] in the art knows that it may be considered to compensate lower affinity by applying a higher dose of compounds and this measure would not lead out-of-the-scope of the invention binding affinity may be determined as described above.

[0076] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention such assay is a sandwich assay, preferably a fully automated assay. It may be an ELISA fully automated or manual. It may be a so-called POC-test (point -of-care). Examples of automated or fully automated assay comprise assays that may be used for one of the following systems: Roche Elecsys®, Abbott Architect®, Siemens Centauer®, Brahms Kryptor®, Biomerieux Vidas®, Alere Triage®. Examples of test formats are provided above.

[0077] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention at least one of said two binders is labeled in order to be detected. Examples of labels are provided above.

[0078] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention at least one of said two binders is bound to a solid phase. Examples of solid phases are provided above.

[0079] In one embodiment of the assays for determining Pro-Enkephalin or Pro-Enkephalin fragments in a sample according to the present invention said label is selected from the group comprising chemiluminescent label, enzyme label, fluorescence label, radioiodine label.

[0080] A further subject of the present invention is a kit comprising an assay according to the present invention wherein the components of said assay may be comprised in one or more container.

EXAMPLES

Example 1

Development of Antibodies

Peptides

[0081] Peptides were synthesized (JPT Technologies, Berlin, Germany).

Peptides/Conjugates for Immunization

[0082] Peptides for immunization were synthesized (JPT Technologies, Berlin, Germany) with an additional N-terminal Cystein residue for conjugation of the peptides to bovine serum albumin (BSA). The peptides were covalently linked to BSA by using Sulfo-SMCC (Perbio-science, Bonn, Germany). The coupling procedure was performed according to the manual of Perbio.

TABLE 1

Peptide for immunization	Pro-Enkephalin sequence
(C)DAEEDD (SEQ ID NO: 26)	119-125
(C)EEDDSLANSSDLLK (SEQ ID NO: 27)	121-134
(C)LKELLETG (SEQ ID NO: 28)	133-140
(C)TGDNRERSHHQDGSDNE (SEQ ID NO: 29)	139-155
(C)SDNEEEVS (SEQ ID NO: 30)	152-159

[0083] The antibodies were generated according to the following method:

[0084] A BALB/c mouse was immunized with 100 µg peptide-BSA-conjugate at day 0 and 14 (emulsified in 100 µl complete Freund's adjuvant) and 50 µg at day 21 and 28 (in 100 µl incomplete Freund's adjuvant). Three days before the fusion experiment was performed, the animal received 50 µg of the conjugate dissolved in 100 µl saline, given as one intraperitonal and one intravenous injection.

[0085] Spenocytes from the immunized mouse and cells of the myeloma cell line SP2/0 were fused with 1 ml 50% polyethylene glycol for 30 s at 37° C. After washing, the cells were seeded in 96-well cell culture plates. Hybrid clones were selected by growing in HAT medium [RPMI 1640 culture medium supplemented with 20% fetal calf serum and HAT-supplement]. After two weeks the HAT medium is replaced with HT Medium for three passages followed by returning to the normal cell culture medium.

[0086] The cell culture supernatants were primary screened for antigen specific IgG antibodies three weeks after fusion. The positive tested microcultures were transferred into 24-well plates for propagation. After retesting the selected cultures were cloned and recloned using the limiting-dilution technique and the isotypes were determined.

[0087] (Lane, R.D. "A short-duration polyethylene glycol fusiontechnique for increasing production of monoclonal antibody-secreting hybridomas", J. Immunol. Meth. 81: 223-228; (1985), Ziegler, B. et al. "Glutamate decarboxylase (GAD) is not detectable on the surface of rat islet cells examined by cytofluorometry and complement-dependent antibody-mediated cytotoxicity of monoclonal GAD antibodies", Horm. Metab. Res. 28: 11-15, (1996)).

Monoclonal Antibody Production

[0088] Antibodies were produced via standard antibody production methods (Marx et al., Monoclonal Antibody Production (1997), ATLA 25, 121) and purified via Protein A-chromatography. The antibody purities were > 95% based on SDS gel electrophoresis analysis.

Labelling and Coating of Antibodies

[0089] All antibodies were labelled with acridinium ester according the following procedure:

[0090] Labelled compound (tracer): 100 µg (100 µl) antibody (1 mg/ml in PBS, pH 7.4, was mixed with 10 µl Acridinium NHS-ester (1 mg/ml in acetonitrile, InVent GmbH, Germany) (EP 0353971) and incubated for 20 min at room temperature. Labelled antibody was purified by gel-filtration HPLC on Bio-Sil SEC 400-5 (Bio-Rad Laboratories, Inc., USA) The purified labelled antibody was diluted in (300 mmol/l potassiumphosphate, 100 mmol/l NaCl, 10 mmol/l Na-EDTA, 5 g/l bovine serum albumin, pH 7.0). The final concentration was approx. 800.000 relative light units (RLU) of labelled compound (approx. 20 ng labeled antibody) per 200 µl. Acridiniumester chemiluminescence was measured by using an AutoLumat LB 953 (Berthold Technologies GmbH & Co. KG).

Solid Phase Antibody (Coated Antibody)

[0091] Solid phase: Polystyrene tubes (Greiner Bio-One International AG, Austria) were coated (18 h at room temperature) with antibody (1.5 µg antibody/0.3 ml 100 mmol/l NaCl, 50 mmol/l Tris/HCl, pH 7.8). After blocking with 5% bovine serum albumine, the tubes were washed with PBS, pH 7.4 and vacuum dried.

Antibody Specificity

[0092] The crossreactivities of the different antibodies are listed in table 2.

TABLE 2

Peptide for immunization	Pre-Pro-Enkephalin-sequence	Antibody name
(C)DAEEDD (SEQ ID NO: 26)	119-125	NT-MRPENK
(C)EEDDSLANSSDLLK (SEQ ID NO: 27)	121-134	NM-MRPENK
(C)LKELLETG (SEQ ID NO: 28)	133-140	MR-MRPENK
(C)TGDNRERSHHQDGSDNE (SEQ ID NO: 29)	139-155	MC-MRPENK
(C)SDNEEEVS (SEQ ID NO: 30)	152-159	CT-MRPENK

[0093] Antibody cross-reactivities were determined as follows:

[0094] 1ug peptide in 300 µl PBS, pH 7.4 was pipetted into Polystyrene tubes and incubated for 1 h at room temperature. After incubation the tubes were washed 5 times (each 1 ml) using 5% BSA in PBS, pH 7.4. Each of the labelled antibodies were added (300 µl in PBS, pH 7.4, 800.000 RLU/ 300 µl) an incubated for 2 h at room temperature, After washing 5 times (each 1 ml of washing solution (20 mmol/l PBS, pH 7.4, 0.1 % Triton X 100), the remaining luminescence (labelled antibody) was quantified using the AutoLumat LB 953. MRPENK-peptide was used as reference substance (100%).

TABLE 3

antibody peptide	DAEEDD (SEQ ID NO: 31)	EEDDSLANSSD LLK (SEQ ID NO: 32)	LKELLETG (SEQ ID NO: 22)	TGDNRERSH HQDGSDNE (SEQ ID NO: 33)	SDNEEEVS (SEQ ID NO: 23)	MRPENK (SEQ ID NO. 6)
NT-MRPENK	121	10	<1	<1	<1	100
NM-MRPENK	<1	98	<1	<1	<1	100
MR-MRPENK	<1	<1	105	<1	<1	100
MC-MRPENK	<1	<1	<1	115	<1	100
CT-MRPENK	<1	<1	<1	<1	95	100

[0095] All antibodies bound the MRPENK peptide, compareable to the peptides which were used for immunization. Except for NT-MRPENK-antibody (10% cross reaction with EEDDSLANSSDLLK (SEQ ID NO: 32)) no antibody showed a cross reaction with MR-PENK peptides not used for immunization of the antibody.

Pro-Enkephalin Immunoassay

[0096] 50 µl of sample (or calibrator) was pipetted into coated tubes, after adding labeled antibody (200 ul), the tubes were incubated for 2 h at 18-25° C. Unbound tracer was removed by washing 5 times (each 1 ml) with washing solution (20 mmol/l PBS, pH 7.4, 0.1% Triton X-100). Tube-bound labelled antibody was measured by using the Luminumeter LB 953 and a fixed concentration of 1000 pmol/l of MRPENK. The signal (RLU at 1000 pmol MRPENK/l) to noise (RLU without MRPENK) ratio of different antibody combinations is given in table 4. All antibodies were able to generate a sandwich complex with any other antibody. Surprisingly, the strongest signal to noise ratio (best sensitivity) was generated by combining the MR-MRPENK and CT-MRPENK antibody. Subsequently, we used this antibody combination to perform the MRPENK-immunoassay for further investigations. MR-MRPENK antibody was used as coated tube antibody and CT-MRPENK antibody was used as labelled antibody.

TABLE 4

	Solid phase antibody	NT-MRPENK	NM-MRPENK	MR-MRPENK	MC-MRPENK	CT-MRPENK
Labelled antibody						
NT-MRPENK		/	27	212	232	<1
NM-MRPENK		36	/	451	487	<1

TABLE 4-continued

Solid phase antibody	NT-MRPENK	NM-MRPENK	MR-MRPENK	MC-MRPENK	CT-MRPENK
MR-MRPENK	175	306	/	536	1050
MC-MRPENK	329	577	542	/	<1
CT-MRPENK	<1	615	1117	516	/

Calibration

[0097] The assay was calibrated, using dilutions of synthetic MRPENK, diluted in 20 mM K2PO4, 6 mM EDTA, 0.5% BSA, 50 μM Amastatin, 100 μM Leupeptin, pH 8.0. Pro-Enkephalin control plasma is available at ICI-diagnostics, Berlin, Germany.

[0098] FIG. **1** shows a typical Pro-Enkephalin dose/ signal curve. Standard curve Pro-Enkephalin.

[0099] The assay sensitivity was 20 determinations of 0-calibrator (no addition of MRPENK) + 2SD) 5.5 pmol/L.

Population Study

Methods

[0100] We measured Pro-Enkephalin in fasting plasma from 2559 female participants of the population based Malmö Diet and Cancer Study baseline exam in 1991-1994 (age 58 ± 6 years and 59% females). We used multivariable adjusted (all traditional cardiovascular risk factors, diabetes risk factors and in analyses of cancer also heredity for cancer) Cox proportional hazards models to relate baseline PENK (hazard ratio per each standard deviation increase of log-transformed PENK) to the time to the first event of each of the studied endpoints during a median follow-up time of more than 12 years. Endpoints were retrieved through the Swedish National Hospital Discharge Registry, the Swedish Myocardial Infarction Registry, the Stroke in Malmö Registry and the Swedish Cancer Registry. Retrieval of endpoints through these registries has been validated and found to be accurate (see also Belting et al. Cancer Epidemiol Biomarkers Prev; 1-10. 2012 AACR).

[0101] Clinical characteristics of females in the study

TABLE 5

Descriptive Statistics	N	Mean	Std. Deviation
Age at MDCS screening	2559	57.554	5.9403
Systolic blood pressure (mmHg)	2559	140.50	19.311
Diastolic blood pressure (mmHg)	2559	85.65	9.117
body-mass-index (weight/kg x kg)	2559	25.5196	4.19083
WAIST (cm)	2559	76.99	10.245
Glucose (mmol/l)	2559	5.0418	1.21798
Triglycerides (mmol/l)	2559	1.2245	0.58404
High density lipoprotein (mmol/l)	2559	1.5123	0.36949
Low density lipoprotein (mmol/l)	2559	4.2016	1.04762
P-INSULIN	2512	7.223	5.4223

[0102] FIG. **2**: frequence distribution of Pro Enkephalin in the females population:

[0103] The mean value was 47.2 pmol/L, standard deviation= 1.2 pmol/L. The x axis is the Logarithmus Naturalis (LN) of the PENK concentration. All results were within the measurement of the assay, the lowest PENK concentration was 9 pmol/L. These results indicating the suitability of the used assay (assay sensitivity 5.5 pmol/L).

PENK and Prediction of Breast Cancer

[0104] We assessed the relationship between Pro-Enkephalin and breast cancer (Table 6). There was a strong relationship between Pro-Enkephalin and breast cancer in females. In a fully adjusted model each SD increase of Pro-Enkephalin was associated with a 28.6% risk reduction or each SD of decrease of Pro-Enkephalin (revPENK) was associated with a 40% increased risk of future breast cancer (table 5) and the top versus bottom quartile of Pro-Enkephalin identified a more than 3-fold difference in risk of breast cancer (see table 7 and FIG. **3**).

TABLE 6

Variables in the Equation°	B	SE	Wald	df	Sig.	Exp(B)	95.0% Cl for Exp(B) Lower	Upper
AGE	0.007	0.016	0.228	1	0.633	1.007	0.977	1.039
SEX				0[a]				
BMI_B	0.026	0.025	1.139	1	0.286	1.027	0.978	1.077
DM_B	-0.242	0.407	0.352	1	0.553	0.785	0.354	1.744
HDL_B	0.044	0.252	0.031	1	0.860	1.045	0.638	1.714
LDL_B	-0.001	0.090	0.000	1	0.988	0.999	0.837	1.191
current_smoker	0.330	0.195	2.886	1	0.089	1.392	0.950	2.037
HER_CANCER_0	0.034	0.176	0.038	1	0.846	1.035	0.733	1.461
LNINS	-0.288	0.197	2.127	1	0.145	0.750	0.509	1.104
ZscoreLNPENK_ females_noCa	-0.337	0.082	16.858	1	0.000	0.714	0.608	0.839

TABLE 7

	BREAST CANCER						
	HR per 1 SD	P-value	Quartile 4	Quartile 3	Quartile 2	Quartile 1	P for trend
Women (2140 / 135)	1.40 (13-1.6)	<0.001	1.0 (ref)	1.50 (0.81-2.1)	2.7(1.7-3.4)	3.6 (2.7-4.9)	<0.001

Multivariate Cox proportional Hazards models for baseline Pro-Enkephalin versus incidence of breast cancer.

[0105] FIG. **3**: Kaplan Meier graphs, illustrating the cumulative breast cancer diagnosis in women Quartile (Q) 1 (below 40.4 pmol/l) quartile 2 (40.4-47.1 pmol/l), quartile 3 (47.2-54.1 pmol/l), quartile 4 (above 54.1 pmol/l). Decreased PENK indicates a long term increased risk of breast cancer development. Since any women with cancer history at day of baseline (blood sampling) were excluded, Pro-Enkephalin is highly predictive for future breast cancer development. Over all, women from Q 1 have a 3.6 times higher risk to develop breast cancer than women from Q 4.

COMBINATION PRO ENKEPHALIN AND PRO NEUROTENSIN

[0106] Since increasing Pro-Neurotensin recently was shown to be highly predictive for breast cancer, we combined both biomarkers for breast cancer prediction.

EXAMPLES

Pro-Neurotensin Assay

[0107] Antibodies were generated as described above. The antibody for labelling (LA) was generated against P—NT 119 (H-CSDSEEEMKALEADFLTNMH (SEQ ID NO: 24)) and the solid phase antibody (SPA) was generated against peptide P—NT 44—62 (CNLNSPAEETGEV-HEEELVA (SEQ ID NO: 25)).

Immunoassay for the Quantification of Human Pro-Neurotensin

[0108] The technology used was a sandwich coated tube luminescence immunoassay, based on Acridinium ester labelling.

[0109] Labelled compound (tracer): 100 µg (100 µl) LA (1 mg/ml in PBS, pH 7.4, was mixed with 10 µl Acridinium NHS-ester (1 mg/ml in acetonitrile, InVent GmbH, Germany) (EP 0353971) and incubated for 20 min at room temperature. Labelled LA was purified by gel-filtration HPLC on Bio-Sil SEC 400-5 (Bio-Rad Laboratories, Inc., USA) The purified LA was diluted in (300 mmol/l potassiumphosphate, 100 mmol/l NaCl, 10 mmol/l Na-EDTA, 5 g/l bovine serum albumin, pH 7.0). The final concentration was approx. 800.000 relative light units (RLU) of labelled compound (approx. 20 ng labeled antibody) per 200 µl. Acridiniumester chemiluminescence was measured by using an AutoLumat LB 953 (Berthold Technologies GmbH & Co. KG).

[0110] Solid phase: Polystyrene tubes (Greiner Bio-One International AG, Austria) were coated (18 h at room temperature) with SPA (1.5 µg SPA/0.3 ml 100 mmol/l NaCl, 50 mmol/l Tris/HCl, pH 7.8). After blocking with 5 % bovine serum albumine, the tubes were washed with PBS, pH 7.4 and vakuum dried.

Calibration

[0111] The assay was calibrated, using dilutions of Pro-Neurotensin containing human serum. A pool of human sera with high Pro-Neurotensin immunoreactivity (InVent Diagostika, Hennigsdorf, Germany) was diluted with horse serum (Biochrom AG, Deutschland) (assay standards).

[0112] The standards were calibrated by use of the human Pro-Neurotensin-calibrator (ICI-Diagnostics, Berlin, Germany). Alternatively, the assay may be calibrated by synthetic or recombinant P—NT 1117 or fragments thereof (see also Ernst et al., 2006).

ProNT Immunoassay

[0113] 50 µl of sample (or calibrator) was pipetted into SPA coated tubes, after adding labeleld LA (200 ul), the tubes were incubated for 16-22 h at 18-25° C. Unbound tracer was removed by washing 5 times (each 1 ml) with washing solution (20 mmol/l PBS, pH 7.4, 0.1% Triton X-100). Tube-bound LA was measured by using the Luminumeter LB 953. Results were calculated from the calibration curve.

[0114] Combined analysis of Pro-Enkephalin and PNT in the female population: There was no significant correlation between Pro-Enkephalin and Pro-Neurotensin (p= 0.56). In a combined model using both biomarkers, we found them both independent in breast cancer prediction.

[0115] In a fully adjusted model each SD increase of PNT was associated with a 49.9% risk increase of future breast cancer. Surprisingly, after adding PNT to the equation, PENK was even stronger than without PNT and showed for each SD increase of Pro-Enkephalin a 30.8 % risk reduction or each SD of decrease of Pro-Enkephalin (revPENK) was associated with a 44.5% increased risk of future breast cancer (table 8).

TABLE 8

combined analysis of PNT and PENK for breast cancer prediction

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% Cl for Exp(B) Lower	Upper
AGE	-0.003	0.019	0.020	1	0.888	0.997	0.960	1.036
current_smoker0	0.434	0.204	4.505	1	0.034	1.543	1.034	2.304
BMI_B	0.001	0.027	0.001	1	0.979	1.001	0.948	1.056
GFR_CG_BSAcorr	-0.005	0.008	0.357	1	0.550	0.995	0.979	1.011
hrt_curr	0.730	0.201	13.146	1	0.000	2.075	1.399	3.079
PNT	0.405	0.091	19.731	1	0.000	1.499	1.254	1.793
PENK	-0.368	0.088	17.416	1	0.000	0.692	0.582	0.823

[0116] Highest vs. lowest quartile PNT indicated a 2.56 fold risk for breast cancer development and Pro Enkephalin on top of PNT lowest vs highest quartile (rev=reversed quartiles Q1=Q4, Q2=Q3, Q3=Q2, Q4=Q1)) an independent 3.6 fold risk (table 9).

[0117] Combining highest quartile of PNT and lowest Pro-Enkephalin quartile vs. lowest PNT- and highest Pro -Enkephalin quartile showed a combined risk of 6.17 (see FIG. **3**).

[0118] Table 9: combined analysis of PNT and PENK for breast cancer prediction.

TABLE 9

Variables in the Equation

	B	95.0% Cl SE	Wald	df	Sig.	Exp(B)	Lower
AGE	-0.022	0.018	1.468	1	0.226	0.976	0.943
current_smoker0	0.391	0.200	3.808		1.051	1.478	0.998
Nt_curr	0.652	0.195	11.145	1	0.001	1.920	1.309
BMI_B	0.012	0.025	0.247	1	0.619	1.012	0.964
GFR_CG_BSAcurr	-0.012	0.008	2.279	1	0.131	0.968	0.972
NLN_PNT			13.898	3	0.003		
NLN_PNT(1)	0.353	0.301	1.378	1	0.241	1.424	0.789
NLN_PNT(2)	0.604	0.286	4.452	1	0.035	1.630	1.044
NLN_PNT(3)	0.942	0.269	12.280	1	0.000	2.566	1.514
Q_PENK_rev			23.381	3	0.000		
Q_PENK_rev(1)	0.410	0.331	1.534	1	0.215	1.507	0.787
Q_PENK_rev(2)	0.979	0.306	10.299	1	0.001	2.663	1.464
Q_PENK_rev(3)	1.284	0.300	18.315	1	0.000	3.610	2.005

[0119] FIG. **4**: Illustration example of combined analysis of Pro-Enkephalin for breast cancer prediction:

[0120] We combined the women with lowest Pro-Enkephalin (1^{st}) quartile and highest (4^{th}) Pro-Neurotensin quartile (group 3). Within that high risk group about 19.02% of women developed breast cancer within the following 15 years.

[0121] Group 2 is a combination of women with 3^{nd} quartile of Pro-Neurotensin and 2^{nd} quartile of Pro-Enkephalin plus 2^{nd} quartile of Pro-Neurotensin and 3th quartile of Pro-Enkephalin. Within that medium risk group about 7.48% of women developed breast cancer within the following 15 years.

[0122] Group 1 is a combination of women with 1^{st} quartile of Pro-Neurotensin and 4^{th} quartile of Pro-Enkephalin. Within that low risk group about 3.08% of women developed breast cancer within the following 15 years. The Hazard risk between group 1 and group 3 is about 6.17.

LUNG CANCER

[0123] Pro-Enkephalin also predicts lung cancer in females.

[0124] 40 women developed lung cancer during the observation period. Pro-Enkephalin is not different in smoking and not smoking women (p=0.44). As expected, smoking is a strong risk prediction marker for lung cancer (p<0.0001). Surprisingly, although smoking is part of the equation, low Pro-Enkephalin indicated a 3.2 fold risk of developing lung cancer (table 10 a and 10 b).

[0125] Table 10 a and 10 b: PENK in the prediction of lung cancer in females. The women were grouped in tertiles (see table 10 a) and than analyzed for lung cancer development (see table 10 b). rev = highest tertile (tertile 3), rev (1)= tertile 2 and rev(2) = lowest tertile (tertile 1).

TABLE 10 A

	PENK [pmol/L]		
Percentile Group of PENKpmolL	Median	Minimum	Maximum
1	37.80000	9.000	42.800
2	47.20000	42.900	51.300
3	58.30000	51.400	518.100
Total	47.25000	9.000	518.100

TABLE 10 B

Variables in the Equation	B	SE	Wald	df	Sig.	Exp(B)
AGE	0.045	0.040	1.251	1	0.263	1.046
current_smokerD	1.897	0.427	19.761	1	0.000	6.667
BMI_B	-0.034	0.063	0.287	1	0.592	0.967
GFR_CG_BSAcorr	-0.024	0.019	1.592	1	0.207	0.976
T_PENK_females_rev			6.698	2	0.035	
T_PENK_females_rev(1)	0.208	0.580	0.128	1	0.721	1.231
T_PENK_females_rev(2)	1.168	0.511	5.220	1	0.022	3.214

FIGURE DESCRIPTION

[0126] FIG. **1**: shows a typical Pro-Enkephalin dose/ signal curve. Standard curve Pro-Enkephalin.

[0127] FIG. **2**: frequence distribution of Pro-Enkephalin in the females population:

[0128] FIG. **3**: Kaplan Meier graphs, illustrating the cumulative breast cancer diagnosis in women quartile (Q) 1 (below 40.4 pmol/l) quartile 2 (40.4-47.1 pmol/l), quartile 3 (47.2-54.1 pmol/l), quartile 4 (above 54.1 pmol/l). Decreased PENK indicates a long term increased risk of breast cancer development. Since any women with cancer history at day of baseline (blood sampling) were excluded, Pro-Enkephalin is highly predictive for future breast cancer development. Over all, women from Q 1 have a 3.6 times higher risk to develop breast cancer than women from Q 4.

[0129] FIG. **4**: Illustration example of combined analysis of Pro-Enkephalin for breast cancer prediction:

SEQUENCE LISTING

Sequence total quantity: 33

SEQ ID NO: 1 moltype = AA length = 243
FEATURE Location/Qualifiers
source 1..243
mol_type = protein
organism = Homo sapiens
SEQ ID NO: 1
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RENSKPEESH LLAKRYGGFM KRYGGFMKKM DELYPMEPEE EANGSEILAK RYGGFMKKDA 120
EEDDSLANSS DLLKELLETG DNRERSHHQD GSDNEEEVSK RYGGFMRGLK RSPQLEDEAK 180
ELQKRYGGFM RRVGRPEWWM DYQKRYGGFL KRFAEALPSD EEGESYSKEV PEMEKRYGGF 240
MRF 243

SEQ ID NO: 2 moltype = AA length = 73
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RENSKPEESH LLA 73

SEQ ID NO: 3 moltype = AA length = 5
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mol_type = protein
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SEQ ID NO: 3
YGGFM 5

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SEQ ID NO: 4
YGGFL 5

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SEQ ID NO: 5
MDELYPMEPE EEANGSEILA 20

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SPQLEDEAKE LQ                                                     12

SEQ ID NO: 9         moltype = AA  length = 11
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FEATURE              Location/Qualifiers
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                     organism = Homo sapiens
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SEQ ID NO: 11        moltype = AA  length = 29
FEATURE              Location/Qualifiers
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                     organism = Homo sapiens
SEQ ID NO: 11
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SEQ ID NO: 12        moltype = AA  length = 7
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                     organism = Homo sapiens
SEQ ID NO: 12
YGGFMRF                                                           7
SEQ ID NO: 13        moltype = AA  length = 147
FEATURE              Location/Qualifiers
source               1..147
                     mol_type = protein
                     organism = Homo sapiens
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VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVIKRK  120
IPYILKRQLY ENKPRRPYIL KRDSYYY                                     147
SEQ ID NO: 14        moltype = AA  length = 125
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IPYIL                                                             125
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SEQ ID NO: 15
KIPYIL                                                            6
SEQ ID NO: 16        moltype = AA  length = 13
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                    organism = Homo sapiens
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VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVI     117
SEQ ID NO: 18       moltype = AA  length = 132
FEATURE             Location/Qualifiers
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                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 18
SDSEEEMKAL EADFLTNMHT SKISKAHVPS WKMTLLNVCS LVNNLNSPAE ETGEVHEEEL  60
VARRKLPTAL DGFSLEAMLT IYQLHKICHS RAFQHWELIQ EDILDTGNDK NGKEEVIKRK  120
IPYILKRQLY EN                                                   132
SEQ ID NO: 19       moltype = AA  length = 21
FEATURE             Location/Qualifiers
source              1..21
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 19
KIPYILKRQL YENKPRRPYI L                                         21

SEQ ID NO: 20       moltype = AA  length = 28
FEATURE             Location/Qualifiers
source              1..28
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 20
KIPYILKRQL YENKPRRPYI LKRDSYYY                                  28

SEQ ID NO: 21       moltype = AA  length = 20
FEATURE             Location/Qualifiers
source              1..20
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 21
QLYENKPRRP YILKRDSYYY                                           20

SEQ ID NO: 22       moltype = AA  length = 8
FEATURE             Location/Qualifiers
source              1..8
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 22
LKELLETG                                                        8

SEQ ID NO: 23       moltype = AA  length = 8
FEATURE             Location/Qualifiers
source              1..8
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 23
SDNEEEVS                                                        8

SEQ ID NO: 24       moltype = AA  length = 20
FEATURE             Location/Qualifiers
source              1..20
                    mol_type = protein
                    organism = Homo sapiens
SEQ ID NO: 24
CSDSEEEMKA LEADFLTNMH                                           20

SEQ ID NO: 25       moltype = AA  length = 20
```

-continued

FEATURE	Location/Qualifiers	
source	1..20	
	mol_type = protein	
	organism = Homo sapiens	
SEQ ID NO: 25		
CNLNSPAEET GEVHEEELVA		20
SEQ ID NO: 26	moltype = AA length = 7	
FEATURE	Location/Qualifiers	
source	1..7	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 26		
CDAEEDD		7
SEQ ID NO: 27	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 27		
CEEDDSLANS SDLLK		15
SEQ ID NO: 28	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
source	1..9	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 28		
CLKELLETG		9
SEQ ID NO: 29	moltype = AA length = 18	
FEATURE	Location/Qualifiers	
source	1..18	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 29		
CTGDNRERSH HQDGSDNE		18
SEQ ID NO: 30	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
source	1..9	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 30		
CSDNEEEVS		9
SEQ ID NO: 31	moltype = AA length = 6	
FEATURE	Location/Qualifiers	
source	1..6	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 31		
DAEEDD		6
SEQ ID NO: 32	moltype = AA length = 14	
FEATURE	Location/Qualifiers	
source	1..14	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 32		
EEDDSLANSS DLLK		14
SEQ ID NO: 33	moltype = AA length = 17	
FEATURE	Location/Qualifiers	
source	1..17	
	mol_type = protein	
	organism = synthetic construct	
SEQ ID NO: 33		
TGDNRERSHH QDGSDNE		17

1-20. (canceled)

21. A method for predicting a risk of getting breast cancer or lung cancer in a female subject that does not suffer from breast cancer or lung cancer comprising:

measuring the level of Pro-Enkephalin (SEQ ID No. 1), or one or more fragments thereof having at least 5 amino acids, in a sample of bodily fluid obtained from said female subject using an immunoassay that has antibodies or fragments of antibodies that bind to Pro-Enkephalin (SEQ ID No. 1) or said one or more fragments thereof; and

correlating said level of Pro-Enkephalin (SEQ ID No. 1), or of said one or more fragments thereof, with risk for getting breast cancer or lung cancer, wherein a reduced level of Pro-Enkephalin (SEQ ID No. 1), or of said one or more fragments thereof is predictive for an enhanced risk of getting breast cancer or lung cancer in comparison to a normal female subject;

wherein said one or more fragments thereof is selected from SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 and SEQ ID No. 11,

wherein the reduced level of Pro-Enkephalin or fragments thereof is a level below 100 pmol/l,wherein the immunoassay comprises:

a) bringing said serum or plasma sample into contact with a solid phase comprising a bound first antibody or first antibody fragment that binds to Pro-Enkephalin (SEQ ID No. 1) or said one or more fragments thereof whereby Pro-Enkephalin (SEQ ID No. 1) or said one or more fragments thereof within said sample react with said bound first antibody or antibody fragment to form a complex bound to said solid phase,

b) contacting said solid phase with the bound complex with a second antibody or second antibody fragment, wherein said second antibody or second antibody fragment is labelled with a detectable label, and whereby the labelled second antibody or second antibody fragment binds to said complex, and

c) measuring the level of Pro-Enkephalin (SEQ ID No. 1) or said one or more fragments thereof in said sample by measuring the amount of labelled second antibody or second antibody fragment bound to the complex on said solid phase.

22. The method according to claim **21**, wherein said cancer is-lung cancer.

23. The method according to claim **21**, wherein said female subject has never had a history of diagnosis of breast cancer or lung cancer at the time said sample of bodily fluid is taken from said female subject.

24. The method according to claim **21**, wherein said female subject has had a history of diagnosis of breast cancer and has been cured at the time said sample of bodily fluid is taken from said female subject and the risk of reoccurrence of getting breast cancer is determined.

25. The method according to claim **21**, wherein at the time said sample of bodily fluid is taken from said female subject, said female subject has been diagnosed as having a cardiovascular disease or diabetes.

26. The method according to claim **21**, further comprising determining at least one clinical parameter selected from: age, presence of diabetes mellitus, and currently smoking.

27. The method according to claim **21**, wherein said method is performed more than once in order to monitor the risk of getting breast cancer in said female subject.

28. The method according to claim **21**, wherein said a method is performed to monitor the response of said female subject to preventive and/or therapeutic measures taken following an assessment of the risk of getting breast cancer or lung cancer.

29. The method according to claim **21**, wherein said a method is performed in order to stratify female subjects into risk groups.

30. The method according to claim **21**, wherein said method further comprises:

measuring the level of Pro-Neurotensin 1-117 (SEQ ID No. 17) in a sample of bodily fluid obtained from said female subject; and

correlating said level of Pro-Neurotensin 1-117 (SEQ ID No. 17) with risk for getting breast cancer or lung cancer, wherein an increased level of Pro-Neurotensin 1-117 (SEQ ID No. 17) is predictive for an enhanced risk of getting breast cancer or lung cancer in comparison to a normal female subject.

31. The method according to claim **21**, wherein said reduced level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof is a level below 50 pmol/l.

32. The method according to claim **30**, wherein said increased level of Pro-Neurotensin 1-117 (SEQ ID No. 17) is a level above 78 pmol/l.

33. The method according to claim **21**, wherein said bodily fluid is blood, plasma or serum.

34. The method according to claim **21**, wherein the level of a fragment of Pro-Enkephalin (SEQ ID No. 1) is measured and said fragment is MR- Pro-Enkephalin (SEQ ID No. 6).

35. A kit for determining Pro-Enkephalin (SEQ ID No. 1) and/or one or more fragments thereof in a sample comprising a first and second binder, wherein the first binder binds to a region of Pro-Enkephalin (SEQ ID No. 1) that is within amino acid sequence 133-140 (LKELLETG, SEQ ID NO. 22), and the second binder binds to a region of Pro-Enkephalin (SEQ ID No. 1) that is within amino acid sequence 152-159 (SDNEEEVS, SEQ ID NO. 23), wherein each of said regions comprises at least 4 or 5 amino acids.

36. The kit according to claim **35**, wherein said kit is sufficiently sensitive to quantify the level of Pro-Enkephalin (SEQ ID No. 1) or said one or more fragments thereof of healthy subjects and has an analytical assay sensitivity < 15 pmol/L.

37. A method for predicting a risk of getting cancer in a female subject that does not suffer from breast cancer or lung cancer, and optionally for stratification of female subjects into risk groups, said method comprising:

measuring the level of Pro-Enkephalin (SEQ ID No. 1), or of one or more fragments thereof, in a sample of bodily fluid obtained from a female subject using said kit according to claim **35**;

correlating said level of Pro-Enkephalin, or of one or more fragments thereof, with risk for getting breast cancer or lung cancer, wherein a reduced level of Pro-Enkephalin (SEQ ID No. 1), or of said one or more fragments thereof is predictive for an enhanced risk of getting breast cancer or lung cancer in comparison to a normal female subject; and

optionally using said method to stratify female subjects into risk groups,

wherein the reduced level of Pro-Enkephalin or fragments thereof is a level below 100 pmol/l.

38. The method according to claim **37**, wherein said method is used for stratification of female subjects into a group that should obtain opioid growth factor (OGF) therapy.

39. The method according to claim **21**, wherein said cancer is breast cancer.

40. The method according to claim **30**, wherein said cancer is breast cancer.

41. The method according to claim **21**, wherein said correlating is performed by:

(a) comparing the level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof in said sample with the median of the level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof in an ensemble of pre-determined samples in a population of "healthy" or "apparently healthy" subjects,

(b) comparing the level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof in said sample with a quantile of the level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof in an ensemble of pre-determined samples in a population of "healthy" or "apparently healthy" subjects, or

(c) calculating the risk by Cox Proportional Hazards analysis or by Risk index calculations using the level of Pro-Enkephalin (SEQ ID No. 1) or of said one or more fragments thereof in said sample.

42. A method according to claim **21**, wherein

one of either (a) said bound first antibody or first antibody fragment, or (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 133-140 of Pro-Enkephalin (SEQ ID No. 1), and

the other of (a) said bound first antibody or first antibody fragment, and (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 152-159 of Pro-Enkephalin (SEQ ID No. 1).

43. A method according to claim **30**, wherein

one of either (a) said bound first antibody or first antibody fragment, or (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 133-140 of Pro-Enkephalin (SEQ ID No. 1), and

the other of (a) said bound first antibody or first antibody fragment, and (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 152-159 of Pro-Enkephalin (SEQ ID No. 1).

44. A method according to claim **30**, wherein said measuring of the level of Pro-Neurotensin 1-117 (SEQ ID No. 17) comprises:

a) bringing said sample of bodily fluid into contact with a solid phase comprising a bound third antibody or third antibody fragment that binds to Pro-Neurotensin 1-117 (SEQ ID No. 17) whereby Pro-Neurotensin 1-117 (SEQ ID No. 17) within said sample reacts with said bound third antibody or third antibody fragment to form a complex bound to said solid phase,

b) bringing said solid phase with the bound complex into contact with a fourth antibody or fourth antibody fragment, wherein said fourth antibody or fourth antibody fragment is labelled with a detectable label, and whereby the labelled fourth antibody or fourth antibody fragment binds to said complex,

c) measuring the level of Pro-Neurotensin 1-117 (SEQ ID No. 17) in said sample by measuring the amount of labelled fourth antibody or fourth antibody fragment bound to the complex on said solid phase.

45. A method according to claim **44**, wherein

one of either (a) said bound third antibody or third antibody fragment, or (b) said labelled fourth antibody or fourth antibody fragment, binds to a peptide consisting of amino acid sequence 1-19 of Pro-Neurotensin 1-117 (SEQ ID No. 17), and

the other of (a) said bound third antibody or third antibody fragment, and (b) said labelled fourth antibody or fourth antibody fragment, binds to a peptide consisting of amino acid sequence 44-62 of Pro-Neurotensin 1-117 (SEQ ID No. 17).

46. A method according to claim **45**, wherein

one of either (a) said bound first antibody or first antibody fragment, or (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 133-140 of Pro-Enkephalin (SEQ ID No. 1), and

the other of (a) said bound first antibody or first antibody fragment, and (b) said labelled second antibody or second antibody fragment, binds to a peptide consisting of amino acid sequence 152-159 of Pro-Enkephalin (SEQ ID No. 1).

47. The method according to claim **21**, wherein said measuring of the level of Pro-Enkephalin (SEQ ID No. 1), or one or more fragments thereof of at least 5 amino acids, is performed using a kit for determining Pro-Enkephalin (SEQ ID No. 1) and/or one or more fragments thereof in a sample comprising a first and second binder, wherein the first binder binds to a region of Pro-Enkephalin (SEQ ID No. 1) that is within amino acid sequence 133-140 (LKELLETG, SEQ ID NO. 22), and the second binder binds to a region of Pro-Enkephalin (SEQ ID No. 1) that is within amino acid sequence 152-159 (SDNEEEVS, SEQ ID NO. 23), wherein each of said regions comprises at least 4 or 5 amino acids.

48. The method according to claim **21**, wherein said antibodies or fragments of antibodies that bind to Pro-Enkephalin or said one or more fragments thereof are prepared by:

immunizing a mouse with a peptide-BSA-conjugate, wherein BSA is bovine serum albumin and said peptide is an amino acid sequence selected from SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 and SEQ ID No. 11 in which the amino acid sequence is provided with an additional N-terminal cysteine residue for conjugation to bovine serum albumin;

fusing spenocytes obtained from the immunized mouse with cells of a myeloma cell line; and

producing said antibodies or fragments of antibodies from the resultant fused cells.

49. A method comprising:

obtaining a sample of bodily fluid from a female subject that does not suffer from breast cancer or lung cancer;

combining said sample with a binder that binds to the amino acid sequence of SEQ ID No. 6;

measuring the level of bound binder within said sample to be below 100 pmol/1; and

subjecting said female subject to measures for preventing breast cancer or lung cancer, or subjecting said female subject to measures for treating breast cancer or lung cancer.

50. A method comprising:

subjecting a female subject to measures for preventing breast cancer or lung cancer, or subjecting a female subject to measures for treating breast cancer or lung cancer,

wherein said female subject that does not suffer from breast cancer or lung cancer and, prior to subjecting said female subject to measures for preventing or treating breast cancer or lung cancer, a sample of bodily fluid obtained from said female subject has been combined with a binder that binds to the amino acid sequence of SEQ ID No. 6 and the level of bound binder within said sample has been determined to be below 100 pmol/l.

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