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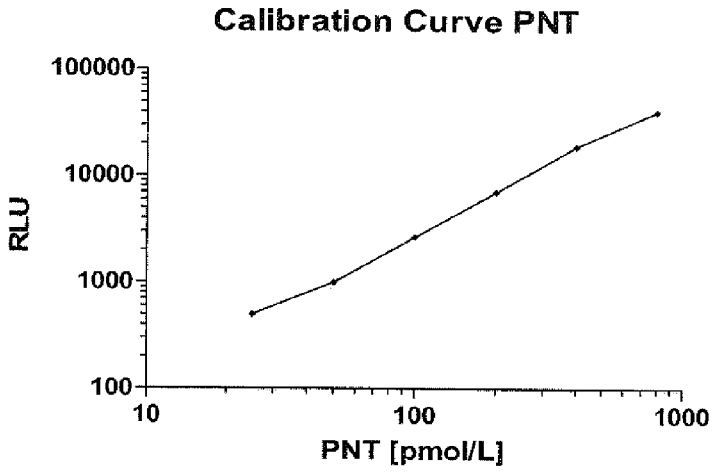
(54) Title: A METHOD FOR PREDICTING THE RISK OF GETTING A CARDIOVASCULAR EVENT IN A FEMALE SUBJECT

(57) Abstract: Subject of the present invention is a method for predicting the risk of getting a cardiovascular event in a female subject comprising 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 correlating said level of pro-neurotensin or fragments thereof with the a risk for getting a cardiovascular event, wherein an elevated level is predictive for an enhanced risk of getting a cardiovascular event.

FIGURES

Figure 1



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A method for predicting the risk of getting a cardiovascular event in a female subject

Subject of the present invention is a method for predicting the risk of getting a cardiovascular event in a female subject comprising:

- 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

- correlating said level of pro-neurotensin or fragments thereof with the a risk for getting a cardiovascular event, wherein an elevated level is predictive for an enhanced risk of getting a cardiovascular event.

The term "elevated level" means a level above a certain threshold level.

Neurotensin is a 13-amino acid neuropeptide derived from the prepro-neurotensin precursor and stochiometrically released together with the stable 117-amino acid peptide pro-neurotensin (P-NT) and the mature hormone binds to three different receptors, neurotensin receptor 1 and 2 (Ntsr1 and Ntsr2), which are G-protein coupled receptors and neurotensin receptor 3 (Ntsr3) which is non-G-protein coupled and also known as Sortillin-1 (SORT1).

Neurotensin is released peripherally from the small intestine as well as centrally from the hypothalamus. The peripheral secretion of neurotensin is stimulated by food-intake, especially by fat, and is known to regulate gastrointestinal motility and pancreatic and biliary secretion. Interestingly, neurotensin is implicated in appetite control as an anorectic hormone as it acutely reduces food intake following both central (intracerebroventricular) and peripheral (intraperitoneal) injection in rats, an effect which seems mainly mediated through the neurotensin-1 receptor (Ntsr1). In obese as compared to normal-weight human subjects, postprandial plasma neurotensin concentration was reduced following a liquid fatty meal (Widen et al 1992, Reg peptides; Plasma concentrations of regulatory peptides in obesity following modified sham feeding (MSF) and a liquid test meal), suggesting regulation of neurotensin secretion is disturbed in obesity. However, no large study has investigated if and how neurotensin is related to measures of obesity. Interestingly, P-NT significantly increases after gastric by-pass (Roux-en-Y), an operation shown to lead to normoglycemia in the majority of obese type 2 diabetes patients, but it is not known whether neurotensin is

implicated in the development diabetes mellitus in general. Furthermore, the neurotensin system has been implicated in development of coronary artery disease and myocardial infarction as variation of the Ntsr3 (SORT1) gene is one of the strongest common coronary artery diseases susceptibility genes known in humans.

The mechanistic link between obesity and cancer is largely unknown, however, one of the dominating theories is that excess of fat deposits leads to increased peripheral aromatization of androgens and thus elevated circulating estrogen levels. In addition, one of the hallmarks of obesity, hyperinsulinemia, has been shown to inhibit hepatic production of Sexual Hormone Binding Globulin (SHBG), thus increasing bioavailable levels of both estrogens and androgens suggesting ways through which obesity may increase the risk of common forms of sex-hormone driven forms of cancer such as breast and prostate cancer. Interestingly, both neurotensin and Ntsr1 expression is common in malignant ductal breast cancer tumors and experimentally pharmacological blockade or RNA silencing of the NTSR1 reduces tumour growth in mice.

The level of expression of neurotensin receptor 1 (NTSR1) in breast cancer cells has been used for determining the prognosis of a subject suffering from breast cancer (US 2011/0305633). Further, it is stated in by the same authors that no clear correlation has been described today between circulating neurotensin and the stages of pancreas, prostate, or medullar thyroid tumors probably due to rapid clearance by the liver. Interestingly, it was found that in a series of 51 patients with invasive ductal breast cancer 91 % of all tumors were positive for neurotensin receptor 1 (NTSR1) but only 31 % of all tumors were positive for neurotensin in said tissue (Souaze et. Al. Cancer Research 2006; 66: (12) pages 6243-6249.

There is some evidence that neurotensin and neurotensin receptors participate in cancer growth, in particular in lung cancer, pancreatic cancer and colon cancer (Carraway et al.; Peptides 27 (2006) 2445-2460). It has been reported that levels of NT in sera of patients with pancreatic cancer were significantly enhanced (Picheon et al, Anticancer Research 1999; 19; 1445-50).Interestingly this group found that NT levels fell with progression of the disease for both prostate an pancreatic cancer. In contrast, thereto, Meggiato et al; Tumori 1996; 82; 592-5; found that plasma levels of NT were normal in pancreatic cancer but elevated in case where pancreatitis was diagnosed.

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.

CRP and Pro-BNP are known predictors of cardiovascular events in the population (Melander et. Al, JAMA. 2009;302(1):49-57). There is now information about any gender difference of the predictive power CRP and Pro-BNP for CVD endpoints.

A subject of the present invention was to investigate the prognostic and diagnostic power of NT for predicting the risk of getting a cardiovascular event in a subject to address this issue, we measured stable fragments of pro-neurotensin in fasting plasma in said Swedish prospective cohort study (Malmö Diet and Cancer Study, see Melander et. al, JAMA. 2009;302(1):49-57) and related baseline level of this biomarker to cardiovascular events during 15 years of follow-up.

Surprisingly, it has been shown that neurotensin is a powerful and highly significant biomarker for woman for predicting the risk of getting a cardiovascular event

Thus, subject of the present invention is a method for predicting the risk of getting a cardiovascular event in a female subject comprising:

- 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
- correlating said level of pro-neurotensin or fragments thereof with the a risk for getting a cardiovascular event, wherein an elevated level is predictive for an enhanced risk of getting a cardiovascular event.

In a specific embodiment of the invention said cardiovascular event is an acute cardiovascular event selected from the group comprising myocardial infarction, stroke, acute heart failure and cardiovascular death related to myocardial infarction, stroke or acute heart failure.

In a specific embodiment of the invention subject matter of the invention is a method for predicting the risk of getting a cardiovascular event in a female subject comprising:

- determining the level of pro-neurotensin 1-117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid obtained from said female subject; and
- correlating said level of pro-neurotensin 1-117 or fragments thereof or pro-neurotensin 1-117 comprising peptides with the a risk for getting a cardiovascular event, wherein an elevated level is predictive for an enhanced risk of getting a cardiovascular event.

And wherein said cardiovascular event is an acute cardiovascular event selected from the group comprising myocardial infarction, stroke, acute heart failure and cardiovascular death related to myocardial infarction, stroke or acute heart failure.

The level of pro-neurotensin 1–117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid obtained from said female subject that is predictive for the risk of getting a cardiovascular event in said female subject may is released from the small intestine. The release of neurotensin from the small intestine is stimulated by food intake, especially by fat, and is known to regulate gastrointestinal motility and pancreatic and biliary secretion. Pro-neurotensin 1 -117 and fragments thereof or pro-neurotensin 1-117 comprising peptides are used as a surrogate marker for the released neurotensin as neurotensin and pro-neurotensin 1 -117 and fragments thereof or pro-neurotensin 1-117 comprising peptides are released in equimolar amounts from pro-neurotensin.

It is the surprising finding of the present invention that the peripheral secretion of neurotensin/pro-neurotensin 1–117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides is indicative for the susceptibility of a female subject to get a cardiovascular event. Thus, dietary measures as reduction of fat uptake may lower said risk in said female subject.

Data obtained in present study revealed also a correlation between the risk of getting a cardiovascular event in male subjects with the level of pro-neurotensin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said male subject; this correlation however, was not that significant for the present data. Thus, we assume that there is a value

for the method according to the invention also for male subjects but the observed effect was not that strong for males in the present study.

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

The correlation between the level of pro-neurotensin or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid obtained from said female subject and the risk of getting a cardiovascular event is continuous, i.e. the higher the level the higher the risk. This can be seen from the data e.g. in Table 17. In comparison to the first quartile the second, third and forth quartile exhibits higher Hazard Risks respectively.

For the sake of practicability the person skilled in the art may use threshold(s).

Thus, the term "elevated level" may mean a level above a threshold level.

In one embodiment of the invention the level of pro-neurotensin or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid is the fasting level of pro-neurotensin or fragments thereof of at least 5 amino acid or pro-neurotensin 1-117 comprising peptides. Fasting level means no food uptake 12 h prior blood sampling.

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

In one embodiment of a method according to the present invention said female subject has no history of diagnosis of an acute cardiovascular event at the time the sample of bodily fluid is taken from said female subject.

In another embodiment of a method according to the present invention said female subject has been diagnosed as having at a cardiovascular disease or diabetes wherein at the time the sample of bodily fluid is taken from said female subject.

In a specific embodiment said cardiovascular disease at the time the sample of bodily fluid is taken from said female subject may be selected from the group comprising heart failure, atherosclerosis, and hypertension.

The present data suggest a strong correlation between the level of pro-neurotensin or fragments thereof with a cardiovascular event in woman with no prevalent diabetes, no prevalent breast cancer and no prevalent cardiovascular disease.

The present data also suggest a strong correlation between the level of pro-neurotensin or fragments thereof with a cardiovascular event in hypertensive woman, which is a common high-risk group for cardiovascular disease.

Furthermore, the present data also suggest a strong correlation between the level of pro-neurotensin or fragments thereof with a cardiovascular event in normotensive woman. Further, the present data suggest a strong correlation between the level of pro-neurotensin or fragments thereof with a cardiovascular event in diabetic woman.

In another specific embodiment of the invention at the time the sample of bodily fluid is taken from said female subject, said female subject has been diagnosed as having at diabetes Typ II. In a specific embodiment of the invention the prediction of a first adverse event in a subject or the identification of a subject having an enhanced risk for getting a first adverse event is improved by additionally determining and using the level of at least one further marker selected from the group comprising: CRP, LpLA2, Cystatin C and natriuretic peptides of the A- and the B-type as well as their precursors and fragments thereof including ANP, proANP, NT-proANP, MR-proANP, BNP, proBNP, NT-proBNP triglycerides, HDL cholesterol or subfractions thereof, LDL cholesterol or subfractions thereof, GDF15, ST2.

In a very specific embodiment of the method according to the invention in addition to the level of pro-neurotensin or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides the level of the following marker is determined and used: proBNP or fragments or precursors thereof having at least 12 amino acids and/or CRP.

In another specific embodiment of the invention additionally at least one clinical parameter is determined selected from the group comprising: age, systolic blood pressure, diastolic blood

pressure, antihypertensive treatment, body mass index, presence of diabetes mellitus, current smoking.

Cardiovascular events (CVD) were defined as coronary events or fatal or nonfatal stroke. Events were identified through linkage of the 10-digit personal identification number of each Swedish citizen with 3 registries: the Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Stroke in Malmö register. Myocardial infarction was defined on the basis of International Classification of Diseases, 9th and 10th revisions (ICD-9 and ICD-10) codes 410 and I21, respectively. Fatal or nonfatal stroke was defined using codes 430, 431, 434, and 436 (ICD-9) and I60, I61, I63, and I64 (ICD-10).

The definition of diabetes is as follows: a history of physician diagnosis or being on anti-diabetic medication or having a fasting whole blood glucose >/= 6.1 mmol/l (note this is = 7.0 mmol/l in plasma) at the baseline examination.

The definition of normotensive/ high blood pressure (HBP) is as follows:
HBP: Systolic BP>/=140 mmHg Diastolic BP >/=90 mmHg or being on antihypertensive medications. Subjects having normal blood pressure (BP) are all other subjects, i.e subjects with Systolic BP<140 mmHg or Diastolic BP <90 mmHg or not being on antihypertensive medications.

Fragments of pro-neurotensin that may be determined in a bodily fluid may be e.g. selected from the group of the following fragments:

SEQ ID NO: 1 (pro-neurotensin 1-147)

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

SEQ ID NO: 2 (pro-neurotensin 1-125 (large neuromedin N))

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

SEQ ID NO: 3 (neuromedin N:)

KIPYIL

SEQ ID NO: 4 (neurotensin)

pyroQLYENKPRRP YIL

SEQ ID NO: 5 (pro-neurotensin 1-117)

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

SEQ ID NO: 6 (pro-neurotensin 1-132)

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

Seq ID No 7: (pro-neurotensin 1-125)

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

SEQ ID NO: 8 (pro-neurotensin 120-140)

KIPYILKRQL YENKPRRPYI L

SEQ ID NO: 9 (pro-neurotensin 120-147)

KIPYILKRQL YENKPRRPYIL KRDSYYY

SEQ ID NO: 10 (pro-neurotensin 128-147)

QLYENKPRRP YILKRDSYYY

In a more specific embodiment of the method according to the present invention the level of pro-neurotensin 1-117 is determined.

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 27 (2006) 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 P-NT-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).

The threshold for determining the risk of getting a cardiovascular event 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 a cardiovascular event.

In a specific embodiment of the method according to the invention the prediction of the risk of the subject for contracting cardiovascular events is improved by additionally determining and using the level of at least one laboratory parameter or further marker selected from the group comprising fasting blood or plasma glucose, triglycerides, HDL cholesterol or subfractions thereof, LDL cholesterol or subfractions thereof, Cystatin C, Insulin, CRP, vasopressin or its precursors or fragments thereof and BNP or its precursors or fragments thereof.

In a specific embodiment of the method according to the invention additionally at least one clinical parameter is determined selected from the group comprising age, gender, systolic

blood pressure, diastolic blood pressure, antihypertensive treatment (AHT), body mass index, waist circumference, waist-hip-ratio, current smoker, diabetes heredity and previous cardiovascular disease (CVD).

Subject matter of the present invention is further a method for predicting the risk of getting a cardiovascular event in a subject or identifying a subject having an enhanced risk for getting a cardiovascular event according to the invention, wherein the level of pro-neurotensin or fragments thereof of at least 5 amino acids 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 a cardiovascular event by a method which may be selected from the following alternatives:

- Comparison with the median of the level of pro-neurotensin or fragments thereof or pro-neurotensin 1-117 comprising peptides in an ensemble of pre-determined samples in a population of "healthy" or "apparently healthy" subjects,
- Comparison with a quantile of the level of pro-neurotensin or fragments thereof or pro-neurotensin 1-117 comprising peptides in an ensemble of pre-determined samples in a population of "healthy" or "apparently healthy" subjects,
- 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).

In one embodiment of the method according to the invention said a method is performed more than once in order to monitor the risk of getting a cardiovascular event in a female subject.

In another embodiment of the method according to the invention said monitoring is performed in order to evaluate the response of said female subject to preventive and/or therapeutic measures taken.

In another embodiment of the method according to the in order invention the method is used to stratify said female subjects into risk groups.

Also encompassed by the present invention is a point-of-care device for performing a method according to the invention.

Also encompassed by the present invention is an assay and/or kit for performing a method according to the invention.

Subject matter of the invention is also a binder to neurotensin or to a neurotensin receptor, for the use in prevention or therapy of a cardiovascular event in a female subject.

In one embodiment of the invention the binder reduces the bioactivity of neurotensin to 70 % or less.

According to the invention the binder to neurotensin 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.

According to the invention the binder to a neurotensin receptor 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, or a peptide antagonist e.g. [D-Trp^{11}]-Neurotensin, [Tyr(Me)^{11}]-Neurotensin (e.g. described by Quiron et al.), or a non-peptide antagonist, e.g. Levocabastine, SR-48692 (NTS1 selective), SR-142948 (unselective), SR-142948A, CP 96345, [3H]SR-48692, SR 48692, SR-48527 and SR-49711, or a binder scaffold e.g. tetranectin-based non-Ig scaffolds (e.g. described in US 2010/0028995), fibronectin scaffolds (e.g. described in EP 1266 025; lipocalin-based scaffolds ((e.g. described in WO 2011/154420); ubiquitin scaffolds (e.g. described in WO 2011/073214), transferring scaffolds (e.g. described in US 2004/0023334), protein A scaffolds (e.g. described in EP 2231860), ankyrin repeat based scaffolds (e.g. described in WO 2010/060748) , microproteins preferably microproteins forming a cystine knot) scaffolds (e.g. described in EP 2314308), Fyn SH3 domain based scaffolds (e.g. described in WO 2011/023685) EGFR-A-domain based scaffolds (e.g. described in WO 2005/040229) and Kunitz domain based scaffolds (e.g. described in EP 1941867).

Examples

Example 1

Development of Antibodies

Peptides/ conjugates for Immunization:

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.

Labelled antibody (LA) peptide (P-NT 1-19):

H-CSDSEEEMKALEADFLTNMH-NH2

Solid phase antibody (SPA) peptide (P-NT 44-62):

H-CNLNSPAEETGEVHEEELVA-NH2

The antibodies were generated according to the following method:
A BALB/c mouse were 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 100µl saline, given as one intraperitoneal and one intra venous injection.

Splenocytes 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.

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.

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

Example 2

Immunoassay for the quantification of human pro-neurotensin

The technology used was a sandwich coated tube luminescence immunoassay, based on Akridinium ester labelling.

Labelled compound (tracer): 100 ug (100 ul) LA (1 mg/ml in PBS, pH 7.4, was mixed with 10 ul Akridinium NHS-ester (1 mg/ml in acetonitrile, InVent GmbH, Germany) (EP 0353971) and incubated for 20min 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. Akridiniumester chemiluminescence was measured by using an AutoLumat LB 953 (Berthold Technologies GmbH & Co. KG,().

Solid phase: Polystyrene tubes (Greiner Bio-One International AG, Austria) were coated (18h 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:

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

The standards were calibrated by use of the human P-NT-calibrator (ICI-Diagnostics, Berlin, Germany). Alternatively, the assay may be calibrated by synthetic or recombinant P-NT 1-117 or fragments thereof (see also Ernst et. al, 2006).

P-NT Immunoassay:

50 µl of sample (or calibrator) was pipetted into SPA coated tubes, after adding labelled LA (200µl), 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 mM PBS, pH 7.4, 0.1 % Triton X-100).

Tube-bound LA was measured by using the LB 953

Figure 1 shows a typical P-NT dose/ signal curve.

Example 3: Population study

Methods

We measured P-NT in fasting plasma from 4362 participants of the population based Malmö Diet and Cancer Study baseline exam in 1991-1994 (men 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 P-NT (hazard ratio per each standard deviation increase of log-transformed P-NT) 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.

Clinical characteristics of the total study population

Table 1

Descriptive Statistics

	N	Mean	Std. Deviation
Age at MDCS screening	4362	57.643	5.9797
Systolic blood pressure (mmHg)	4362	141.91	19.158
Diastolic blood presure (mmHg)	4362	87.02	9.501
body-mass-index (weight/kgxkg)	4362	25.7642	3.91173
WAIST (cm)	4361	83.56	12.791
Glucose (mmol/l)	4362	5.1826	1.33736
Triglycerides (mmol/l)	4362	1.3142	.63660
High density lipoprotein (mmol/l)	4362	1.3908	.37068
Low density lipoprotein (mmol/l)	4362	4.1632	.98774
P-INSULIN	4280	7.889	7.6975
PNT (pmol/l)	4362	123.01743	76.746549
Valid N (listwise)	4279		

Table 2

gender

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	male	1803	41.3	41.3	41.3
	woman	2559	58.7	58.7	100.0
	Total	4362	100.0	100.0	

Table 3

Q+Diary: Anti Hypertension Treatment (C02,C03,C07,C08,C09) at baseline according to questionnaire or diary book

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	No	3684	84.5	84.5	84.5
	Yes	678	15.5	15.5	100.0
	Total	4362	100.0	100.0	

Table 4

DIAB MELL (fb >6.0 or pos Q DM)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	no	3993	91.5	91.5	91.5
	yes	369	8.5	8.5	100.0
	Total	4362	100.0	100.0	

Table 5

current_smoker0

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	.00	3212	73.6	73.6	73.6
	1.00	1150	26.4	26.4	100.0
	Total	4362	100.0	100.0	

Table 6

Clinical characteristics of females in the study

Descriptive Statistics

	N	Mean	Std. Deviation
Age at MDCS screening	2559	57.554	5.9403
Systolic blood pressure (mm Hg)	2559	140.50	19.311
Diastolic blood presure (mm Hg)	2559	85.65	9.117
body-mass-index (weight/kgxkg)	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	.58404
High density lipoprotein (mmol/l)	2559	1.5123	.36949
Low density lipoprotein (mmol/l)	2559	4.2016	1.04762
P-INSULIN	2512	7.223	5.4223
PNT [pmol/L]	2559	125.60633	77.681673
Valid N (listwise)	2512		

Table 7

Q+Diary: Anti Hypertension Treatment (C02,C03,C07,C08,C09) at baseline according to questionnaire or diary book

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	No	2173	84.9	84.9	84.9
	Yes	386	15.1	15.1	100.0
	Total	2559	100.0	100.0	

Table 8

DIAB MELL (fb >6.0 or pos Q DM)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	no	2396	93.6	93.6	93.6
	yes	163	6.4	6.4	100.0
	Total	2559	100.0	100.0	

Table 9

current_smoker0

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	.00	1906	74.5	74.5	74.5
	1.00	653	25.5	25.5	100.0
	Total	2559	100.0	100.0	

Table 10

Clinical characteristics of males in the study

Descriptive Statistics

	N	Mean	Std. Deviation
Age at MDCS screening	1803	57.769	6.0345
Systolic blood pressure (mm Hg)	1803	143.90	18.766
Diastolic blood presure (mm Hg)	1803	88.95	9.698
body-mass-index (weight/kgxkg)	1803	26.1113	3.44882
WAIST (cm)	1802	92.89	9.932
Glucose (mmol/l)	1803	5.3825	1.46780
Triglycerides (mmol/l)	1803	1.4416	.68477
High density lipoprotein (mmol/l)	1803	1.2183	.29669
Low density lipoprotein (mmol/l)	1803	4.1087	.89336
P-INSULIN	1768	8.835	10.0090
PNT [pmol/l]	1803	119.34300	75.268054
Valid N (listwise)	1767		

Table 11

Q+Diary: Anti Hypertension Treatment (C02,C03,C07,C08,C09) at baseline according to questionnaire or diary book

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	No	1511	83.8	83.8	83.8
	Yes	292	16.2	16.2	100.0
	Total	1803	100.0	100.0	

Table 12

DIAB MELL (fb >6.0 or pos Q DM)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	no	1597	88.6	88.6	88.6
	yes	206	11.4	11.4	100.0
	Total	1803	100.0	100.0	

Table 13

current_smoker0

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	.00	1306	72.4	72.4	72.4
	1.00	497	27.6	27.6	100.0
	Total	1803	100.0	100.0	

Results

Cross sectional relationship between cardiometabolic risk factors and P-NT

The baseline characteristics of the study population are shown in Table 1. Women had slightly but significantly higher P-NT [median (interquartile range)] than men [109 (79-150) versus 99 (71-144) pmol/l] ($P<0.001$). The cross sectional relationship between P-NT and measures of obesity, cardiovascular risk factors and diabetes risk factors was generally weak with the strongest correlation being that with fasting insulin concentration in both gender (Supplementary Table 1). In a linear regression model with backward elimination and a retention P-value of 0.10, significant independent determinants of P-NT were smoking and fasting concentrations of insulin, glucose, and HDL (all positive) in women and smoking and fasting concentrations of insulin and HDL (positively related) and age and LDL (negatively related) in men (Table 2).

Table 14

QUARTILES OF PNT IN ALL:

PNT [pmol/l]

Percentile Group of PNTpmolL	N	Median	Minimum	Maximum
1	1091	60.22000	3.270	75.740
2	1090	89.29000	75.790	104.600
3	1092	122.67000	104.640	147.610
4	1089	190.03000	147.660	1154.520
Total	4362	104.62000	3.270	1154.520

Table 15

QUARTILES OF PNT IN WOMEN:

PNT [pmol/l]

Percentile Group of PNTpmolL	N	Median	Minimum	Maximum
1	639	62.37000	5.100	78.580
2	639	92.07000	78.610	108.770
3	641	125.07000	108.960	150.000
4	640	194.38500	150.050	1154.520
Total	2559	108.96000	5.100	1154.520

Table 16

QUARTILES OF PNT IN MEN:

PNT [pmol/l]

Percentile Group of PNTpmolL	N	Median	Minimum	Maximum
1	450	58.02000	3.270	70.800
2	451	85.88000	70.970	98.820
3	451	118.18000	98.880	143.940
4	451	186.39000	144.180	1057.360
Total	1803	98.88000	3.270	1057.360

Table 17

CARDIOVASCULAR DISEASE							
	HR per 1 SD	**P-value**	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**	**P for trend**
All (4362 / 519)	1.17 (1.07-1.27)	<0.001	1.0 (ref)	1.09 (0.84-1.42)	1.39 (1.09-1.78)	1.37 (1.07-1.75)	0.003
Women (2559 / 224)	1.33 (1.17-1.51)	<0.001	1.0 (ref)	0.91 (0.59-1.41)	1.58 (1.08-2.30)	1.65 (1.13-2.41)	0.001
Men (1803 /295)	1.06 (0.95-1.19)	0.310	1.0 (ref)	1.25 (0.90-1.74)	1.26 (0.90-1.76)	1.21 (0.87-1.69)	0.278

CARDIOVASCULAR MORTALITY							
	HR per 1 SD	**P-value**	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**	**P for trend**
All (4362 / 174)	1.29 (1.12-1.49)	0.001	1.0 (ref)	0.95 (0.59-1.53)	1.41 (0.91-2.17)	1.73 (1.14-2.61)	0.003
Women (2559 / 75)	1.50 (1.21-1.87)	<0.001	1.0 (ref)	1.02 (0.47-2.22)	1.53 (0.76-3.08)	2.18 (1.13-4.20)	0.008
Men (1803 / 99)	1.16 (0.96-1.41)	0.132	1.0 (ref)	1.06 (0.58-1.93)	1.36 (0.76-2.42)	1.43 (0.82-2.51)	0.147

P-NT and prediction of cardiovascular disease, cardiovascular mortality and all-cause mortality

Among subjects without cardiovascular disease prior to the baseline exam, 519 suffered a first cardiovascular disease event during 14.4 ± 4.4 years of follow-up. After full adjustment for baseline levels of cardiovascular risk factors (age, gender, antihypertensive treatment, systolic blood pressure, body mass index, diabetes mellitus, HDL, LDL and smoking) each SD increase of P-NT was associated with 17% increased risk of incident cardiovascular disease (Table 3). There was a strong interaction between P-NT and female gender (P<0.001) and gender stratified analyses revealed that each SD increase of baseline P-NT was strongly associated with a 33% increased risk of incident cardiovascular disease in women, whereas there was no significant relationship among men (Table 3). Quartile analyses revealed that the top versus the bottom quartile was associated with a 37% increased risk for incident cardiovascular disease in the total population and 65% increased risk in women (Table 3).

Additional adjustment for fasting insulin concentration, i.e. the strongest cross-sectional correlate of P-NT, did not affect the results (not shown).

We then assessed the relationship between total and cardiovascular mortality in the entire population as well as in men and women separately in models fully adjusted for all cardiovascular risk factors. Each SD increase of P-NT was associated with a significant 8% increase in the risk of all-cause mortality in the total population and a 13% risk of all-cause mortality among women whereas there was no such increased risk related to P-NT in men (Table 3). The excess risk of death in appeared to be mainly accounted for by cardiovascular deaths with 29% per SD increase in the risk of cardiovascular death in the total population and 50% excess risk in females. Female subjects belonging to the top as compared to the bottom quartile of P-NT had a more than 2-fold increased risk of suffering cardiovascular death (Table 3).

Multivariate Cox proportional Hazards models for baseline P-NT versus incidence of cardiovascular disease, all-cause- and cardiovascular mortality

Head-to-head comparison between P-NT, N-BNP and CRP

In order to compare the statistical strength and the effect estimates on the studied endpoints between P-NT and established plasma biomarkers, we entered P-NT simultaneously with N-BNP and CRP in fully adjusted models (CRP and N-BNP was measured as described by Melander et al., JAMA. 2009;302(1):49-57). As seen below, P-NT performed equally well as N-BNP and CRP for most endpoints in the total population and in females P-NT performed clearly better than N-BNP and CRP (CRP alone was not significant in females). Combining (see Melander et al., JAMA. 2009;302(1):49-57) N-BNP and P-NT further improved the predictive power for CVD in females from 33% HR per 1SD (P-NT alone to 34,8% per 1SD ($p < 0,001$) (combination of P-NT and N-BNP.

Table 18

Incident CVD- ALL SUBJECTS

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
AGE	.079	.010	68.888	1	.000	1.082	1.062	1.103
SEX	-.476	.106	20.042	1	.000	.621	.505	.765
AHT_B	.231	.116	3.991	1	.046	1.260	1.004	1.581
SBP_B	.015	.003	35.264	1	.000	1.015	1.010	1.020
BMI_B	-.012	.014	.775	1	.379	.988	.962	1.015
DM_B	.544	.130	17.618	1	.000	1.723	1.336	2.221
HDL_B	-.851	.169	25.479	1	.000	.427	.307	.594
LDL_B	.152	.048	9.800	1	.002	1.164	1.058	1.280
current_smoker 0	.495	.106	21.876	1	.000	1.640	1.333	2.017
ZLN_PNT	**.133**	**.046**	**8.336**	**1**	**.004**	**1.142**	**1.044**	**1.250**
ZLN_BNP	**.132**	**.049**	**7.168**	**1**	**.007**	**1.141**	**1.036**	**1.257**
ZLN__CRP	**.147**	**.050**	**8.523**	**1**	**.004**	**1.158**	**1.049**	**1.278**

Table 19

INCIDENT CVD- FEMALE SUBJECTS

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
AGE	.080	.015	28.703	1	.000	1.084	1.052	1.116
SEX			.	0[a]	.			
AHT_B	.466	.168	7.664	1	.006	1.593	1.146	2.215
SBP_B	.014	.004	13.454	1	.000	1.014	1.007	1.022
BMI_B	-.038	.019	3.908	1	.048	.962	.927	1.000
DM_B	.925	.202	21.035	1	.000	2.522	1.699	3.745
HDL_B	-.926	.231	16.088	1	.000	.396	.252	.623
LDL_B	.116	.069	2.801	1	.094	1.123	.980	1.286
current_smoker 0	.740	.155	22.725	1	.000	2.095	1.546	2.840
Z_LNBNP	**.154**	**.071**	**4.755**	**1**	**.029**	**1.167**	**1.016**	**1.340**
ZLN__CRP	**.112**	**.077**	**2.123**	**1**	**.145**	**1.119**	**.962**	**1.301**
ZLN_PNT	**.224**	**.070**	**10.217**	**1**	**.001**	**1.251**	**1.091**	**1.435**

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
AGE	.080	.015	28.703	1	.000	1.084	1.052	1.116
SEX			.	0[a]	.			
AHT_B	.466	.168	7.664	1	.006	1.593	1.146	2.215
SBP_B	.014	.004	13.454	1	.000	1.014	1.007	1.022
BMI_B	-.038	.019	3.908	1	.048	.962	.927	1.000
DM_B	.925	.202	21.035	1	.000	2.522	1.699	3.745
HDL_B	-.926	.231	16.088	1	.000	.396	.252	.623
LDL_B	.116	.069	2.801	1	.094	1.123	.980	1.286
current_smoker	.740	.155	22.725	1	.000	2.095	1.546	2.840
0								
Z_LNBNP	**.154**	**.071**	**4.755**	**1**	**.029**	**1.167**	**1.016**	**1.340**
ZLN__CRP	**.112**	**.077**	**2.123**	**1**	**.145**	**1.119**	**.962**	**1.301**
ZLN_PNT	**.224**	**.070**	**10.217**	**1**	**.001**	**1.251**	**1.091**	**1.435**

Fig 2: Kaplan Meier Graph for illustrating the cumulative CVD-events in women, cut off >< median = 109 pmol/l P-NT.
The prediction of CVD-events by baseline P-NT was given for the complete observation period.

Subgroup analysis

Using the same variables in the equation, we investigated different subgroups for prediction of CVD, mortality, CVD mortality. Subjects with precious CVD-events were excluded.

Table 20

Prediction of CVD events

Subgroup	No of subjects	No of events	Hazard risk per 1SD PNT	Significance (p-value)
all	4361	519	16,5%	<0,001
women	2559	224	33,2%	<0,001
male	1802	295	6%	0,31 (n.s.)
Diabetic women	163	40	42,9%	0,05
nonDiabetic women	2396	184	33,6%	<0,001
HBP women	1545	178	30,6%	<0,001
Normal BP Women	1014	46	40,8%	0,014
Women w/o history of cancer, diabetes and CVD events	2022	144	30,6%	0,001

Prediction of CVD events was only related to females. The predictive power of P-NT was similar in completely healthy and in high risk subgroups like diabetic women or HBP women.

Table 21

Prediction of CVD mortality

Subgroup	No of subjects	No of events	Hazard risk per 1SD PNT	Significance (p-value)
all	4361	174	28,7%	0,001
women	2559	75	50%	<0,001
male	1803	99	16%	0.132 (n.s.)
Diabetic women	163	14	141%	0,006
Non Diabetic women)	2396	61	39,6%	0,006
HBP women	1545	63	35,1%	0,016
Normal BP Women	1014	12	125,7%	0,001
Women w/o history of cancer, diabetes and CVD events	2022	48	36%	0,025

Prediction of CVD mortality by P-NT was strong in women and not significant in male. The predictive power of P-NT was given in healthy women and in high risk women (diabetic or HBP).

Reclassification of woman into risk groups

Methods:

We calculated model c-statistics and reclassification across 10-year predicted risk categories for the different events (<5%, >=5-10%, >=10-20% and >=20%, respectively) with Net Reclassification Improvement (NRI) for models with and without P-NT.30-32 All analyses were performed with Stata software version 11 (StataCorp, College Station, Texas). A two-sided P-value of <.05 was considered statistically significant.

For cardiovascular mortality, there was a borderline significant increase of the over-all NRI of 11%. P-NT correctly reclassified 19% of females who actually suffered cardiovascular death to a higher category of risk but only reclassified 5% of women who did not suffer cardiovascular death to a lower category of risk (Table 5). Among women at intermediate (10-20%) 10-year risk, i.e. the group in which biomarker support has been suggested to be particularly important for clinical decision making regarding initiation of primary preventive therapy (and reclassification thus referred to as "clinical NRI"),38 addition of P-NT to traditional cardiovascular risk factors resulted in a significant clinical NRI of 40% for cardiovascular mortality, with reclassification of 21% women who died a cardiovascular death to a higher category of risk and 30% of women who did not suffer cardiovascular death to a lower category of risk.

Literature:

Pencina MJ, D'Agostino RB. Overall C as a measure of discrimination in survival analysis: model specific population value and confidence interval estimation. Stat Med. Jul 15 2004;23(13):2109-2123.

Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med. Jan 30 2008;27(2):157-172; discussion 207-112.

Ridker PM, Buring JE, Rifai N, Cook NR. Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: the Reynolds Risk Score. Jama. Feb 14 2007;297(6):611-619.

Figure description:

Figure 1 shows a typical P-NT dose/ signal curve

Figure 2: Kaplan Meier Graph for illustrating the cumulative CVD-events in women, cut off >< median = 109 pmol/l P-NT

CLAIMS

1. A method for predicting the risk of getting a cardiovascular event in a female subject comprising:

   - determining the level of pro-neurotensin 1-117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid obtained from said female subject; and
   - correlating said level of pro-neurotensin 1-117 or fragments thereof or pro-neurotensin 1-117 comprising peptides with the a risk for getting a cardiovascular event, wherein an elevated level is predictive for an enhanced risk of getting a cardiovascular event.

   and wherein said cardiovascular event is an acute cardiovascular event selected from the group comprising myocardial infarction, stroke, acute heart failure and cardiovascular death related to myocardial infarction, stroke or acute heart failure.

2. A method according to claim 1, wherein the level of pro-neurotensin 1-117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides in a bodily fluid is the fasting level.

3. A method according to claims 1 or 2, wherein said female subject has no history of diagnosis of an acute cardiovascular event at the time the sample of bodily fluid is taken from said female subject.

4. A method according to claims 1 to 3, wherein at the time the sample of bodily fluid is taken from said female subject, said female subject has been diagnosed as having at a cardiovascular disease or diabetes.

5 A method according to claim 4, wherein at the time the sample of bodily fluid is taken from said female subject, said cardiovascular disease may be selected from the group comprising heart failure, atherosclerosis, hypertension.

6. A method according to claims 4, wherein at the time the sample of bodily fluid is taken from said female subject, said female subject has been diagnosed as having at diabetes type II.

7. A method according to claims 1 to 6 wherein in addition the level of the following marker is determined and used: pro-BNP or fragments or precursors thereof having at least 5 amino acids and/or C-reactive protein (CRP).

8. A method according to claims 1 to 7, wherein additionally at least one clinical parameter is determined selected from the group comprising: age, systolic blood pressure, diastolic blood pressure, antihypertensive treatment, body mass index, presence of diabetes mellitus, current smoking.

9. A method according to any of the preceding claims, wherein the level of pro-neurotensin 1-117 is determined.

10. A method according to any of the preceding claims, wherein the level of pro-neurotensin 1-117 or fragments thereof or pro-neurotensin 1-117 comprising peptides is measured with an immunoassay.

11. A method according to any of claims 1 – 10 wherein said a method is performed more than once in order to monitor the risk of getting a cardiovascular event in a female subject.

12. A method according to claim 11 wherein said monitoring is performed in order to evaluate the response of said female subject to preventive and/or therapeutic measures taken.

13. A method according to any of claims 1 to 12 in order to stratify said female subjects into risk groups.

14. Use of a device, e.g. point-of-care device for performing a method according to any of claims 1-13.

15. Use of a device for predicting the risk of getting a cardiovascular event comprising
    - a binder against proBNP or fragments or precursors thereof having at least 5 amino acids and
    - a binder against pro-neurotensin 1-117 or fragments thereof of at least 5 amino acids or pro-neurotensin 1-117 comprising peptides and/or a binder against CRP.

16. Use of a device for predicting the risk of getting a cardiovascular event wherein said binder is selected from the group comprising an antibody, an antibody fragment according to claim 15 and a non-Ig scaffold.

17. A binder to neurotensin or to a neurotensin receptor, for the use in prevention or therapy of a cardiovascular event in a female subject.

18. A binder according to claim 17, which reduces the bioactivity of neurotensin to 70 % or less.

19. The binder to neurotensin according to claim 17 or 18 wherein the binder 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.

20. The binder to a neurotensin receptor according to any of claims 17 to 20 wherein the binder 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, or a peptide antagonist e.g. [D-Trp^{11}]-Neurotensin, [Tyr(Me)^{11}]-Neurotensin, or a non-peptide antagonist, e.g. Levocabastine, SR-48692 (NTS1 selective), SR-142948 (unselective), SR-142948A, CP 96345, [3H]SR-48692, SR-48527 and SR-49711, or a binder scaffold e.g. tetranectin-based non-Ig scaffolds, fibronectin scaffolds, lipocalin-based scaffolds, ubiquitin scaffolds, transferring scaffolds, protein A scaffolds, ankyrin repeat based scaffolds, microproteins, preferably microproteins forming a cystine knot scaffolds, Fyn SH3 domain based scaffolds, EGFR-A-domain based scaffolds and Kunitz domain based scaffolds.

FIGURES

Figure 1

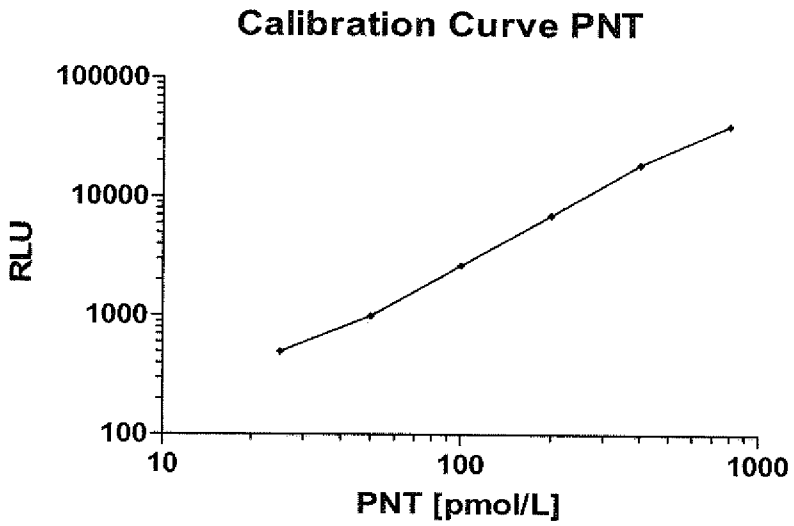
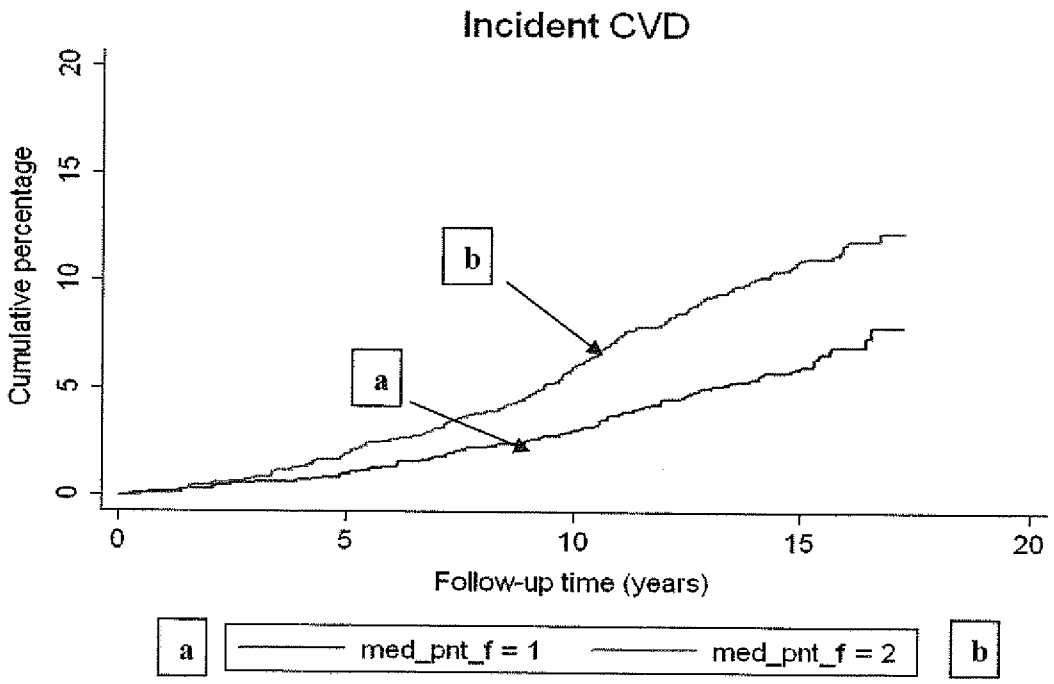


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/054799

Box No. 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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 No. 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:

see additional sheet

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 fees, this Authority did not invite payment of additional fees.

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.:

1-14

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/054799

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/74
ADD.

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)
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 practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ERNST A ET AL: "Proneurotensin 1-117, a stable neurotensin precursor fragment identified in human circulation", PEPTIDES, ELSEVIER, AMSTERDAM, vol. 27, no. 7, 1 July 2006 (2006-07-01), pages 1787-1793, XP027957440, ISSN: 0196-9781 [retrieved on 2006-07-01] the whole document page 1789, column 1 page 1791, column 1 ----- -/--	1-14

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent 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
8 May 2013	22/07/2013

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, Fax: (+31-70) 340-3016	Jenkins, Gareth

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/054799

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBASE [Online] ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL; October 2004 (2004-10), LIU R -M ET AL: "Correlation of plasma neuropeptide Y and neurotensin with cardiac functional damage and prognosis in essential hypertension patients", XP002677074, Database accession no. EMB-2005023506 abstract & CHINESE JOURNAL OF CLINICAL REHABILITATION 200410 CN, vol. 8, no. 30, October 2004 (2004-10), pages 6794-6795, ISSN: 1671-5926	1-8, 10-14

X	WO 2010/128071 A1 (BRAHMS AG [DE]; BERGMANN ANDREAS [DE]; STRUCK JOACHIM [DE]; HARTMANN O) 11 November 2010 (2010-11-11) the whole document claims 1,5,13 page 3, paragraph 1	1-8, 10-14

X	US 2005/130230 A1 (DAVALOS ANTONI [ES] ET AL) 16 June 2005 (2005-06-16) the whole document claims 1,11	1-8, 10-14

X,P	MELANDER OLLE ET AL: "Plasma Proneurotensin and Incidence of Diabetes, Cardiovascular Disease, Breast Cancer, and Mortality", JAMA THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, AMERICAN MEDICAL ASSOCIATION, US, vol. 308, no. 14, 1 October 2012 (2012-10-01), pages 1469-1475, XP009165659, ISSN: 0098-7484 the whole document abstract	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/054799

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010128071 A1	11-11-2010	CN 102428368 A	25-04-2012
		EP 2427764 A1	14-03-2012
		JP 2012526271 A	25-10-2012
		US 2012142120 A1	07-06-2012
		WO 2010128071 A1	11-11-2010
US 2005130230 A1	16-06-2005	EP 1792178 A2	06-06-2007
		US 2005130230 A1	16-06-2005
		WO 2006036220 A2	06-04-2006

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14

   Method according to claim 1.

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2. claims: 15, 16(partially)

   Use of a device according to claim 15 wherein the binders are against proBNP and proNT.

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3. claims: 15, 16(partially)

   Use of a device according to claim 15 wherein the binders are against proBNP and CRP.

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4. claims: 17-20

   Composition-for-use according to claim 17.
