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Bergmann

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(54) **METHOD FOR DIAGNOSING OR MONITORING KIDNEY FUNCTION OR DIAGNOSING KIDNEY DYSFUNCTION**

(71) Applicant: **SPHINGOTEC GMBH**, Henningsdorf (DE)

(72) Inventor: **Andreas Bergmann**, Berlin (DE)

(73) Assignee: **Sphingotec GmbH**, Henningsdorf (DE)

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G01N 33/53 (2006.01)
G01N 33/543 (2006.01)
G01N 33/68 (2006.01)

(52) **U.S. Cl.**
CPC ***G01N 33/74*** (2013.01); ***G01N 33/54366*** (2013.01); ***G01N 33/6893*** (2013.01); *G01N 2333/70* (2013.01); *G01N 2800/347* (2013.01); *G01N 2800/52* (2013.01)

(58) **Field of Classification Search**
CPC G01N 33/74; G01N 33/54366; G01N 33/6893; G01N 2333/70
See application file for complete search history.

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Primary Examiner — Jacob Cheu

(74) *Attorney, Agent, or Firm* — Millen White Zelano & Branigan P.C.

(57) **ABSTRACT**

A method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject or (d) predicting or monitoring the success of a therapy or intervention comprising

determining the level of Pro-Enkephalin (PENK) or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said subject; and correlating said level of Pro-Enkephalin or fragments thereof with

(a) kidney function in a subject or

(b) kidney dysfunction in said subject or

(c) enhanced risk of adverse events or

(d) success of a therapy or intervention in a diseased subject.

37 Claims, 20 Drawing Sheets

Fig. 1:

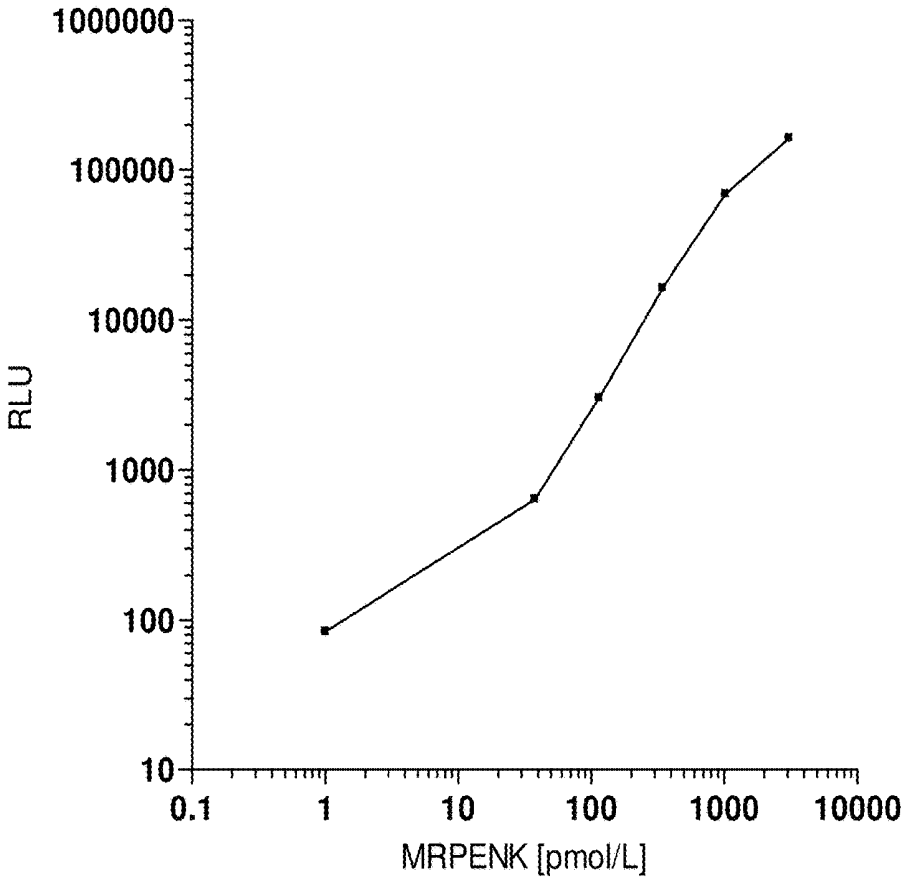

1000000
100000
10000
1000
100
10
RLU
0.1
1
10
100
1000
10000
MRPENK [pmol/L]

Fig. 2:

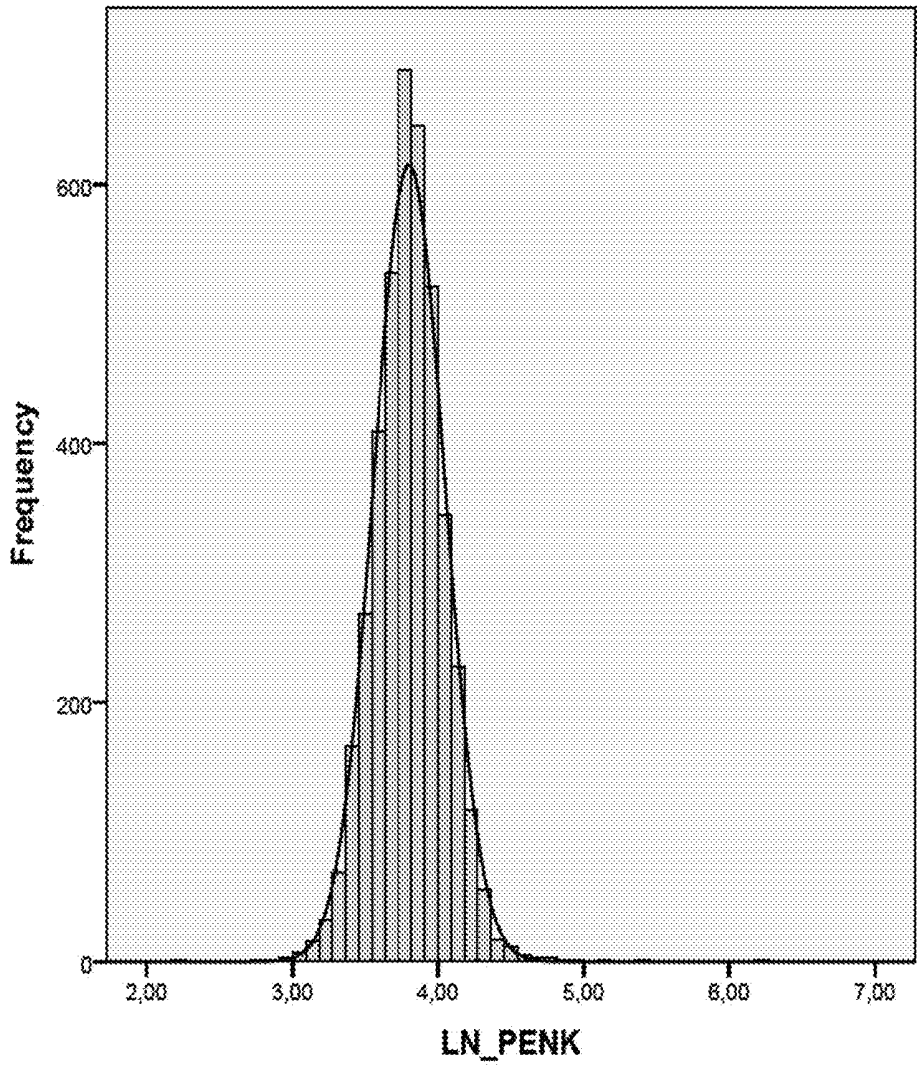


Fig. 3:

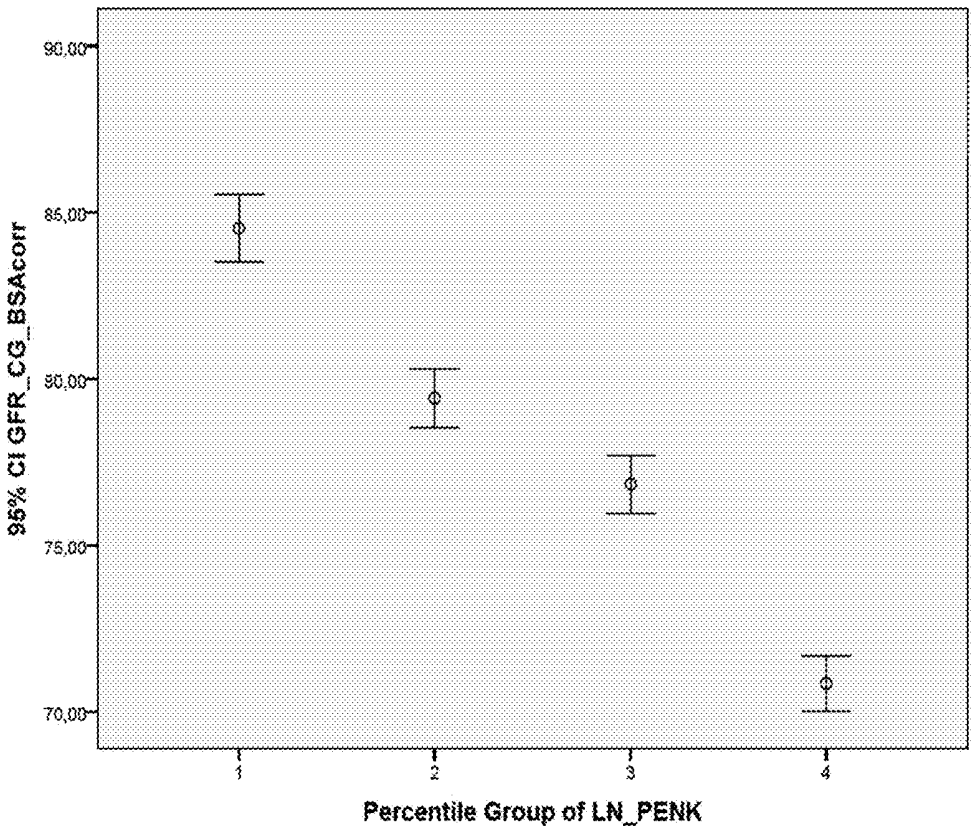

90,00
85,00
80,00
75,00
70,00
95% CI GFR_CG_BSAcorr
1
2
3
4
Percentile Group of LN_PENK

Fig. 4:

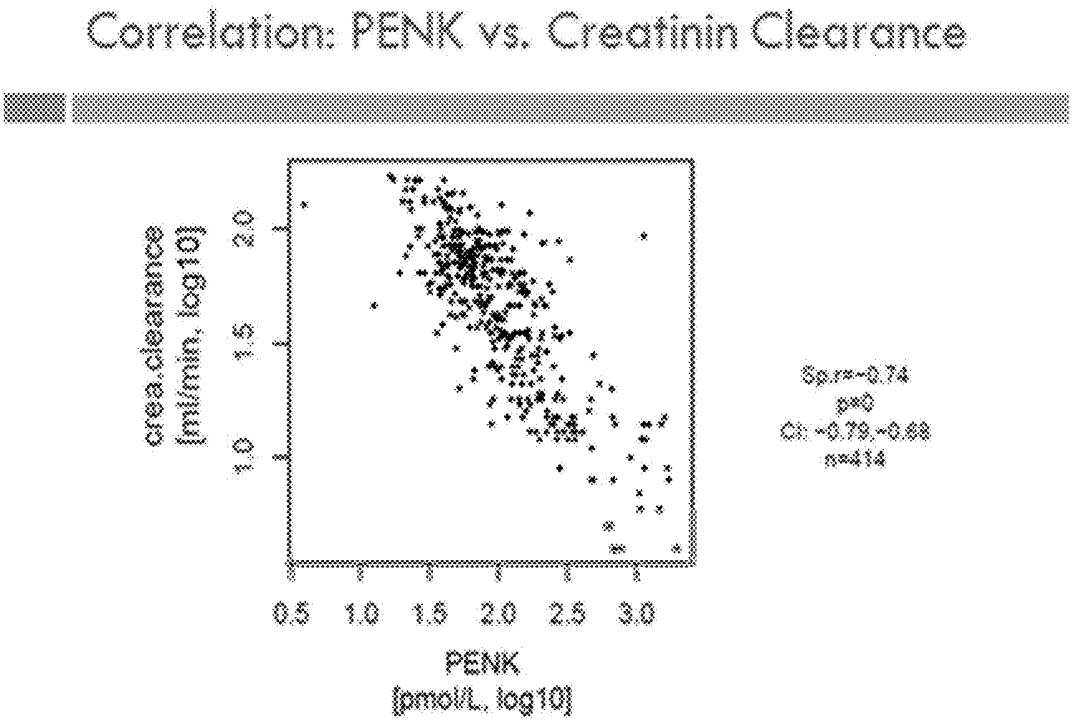

Correlation: PENK vs. Creatinin Clearance
crea.clearance [ml/min, log10]
1.0
1.5
2.0
0.5
1.0
1.5
2.0
2.5
3.0
PENK [pmol/L, log10]
Sp.rho=-0.74
p=0
CI: -0.79,-0.68
n=414

Fig. 5:

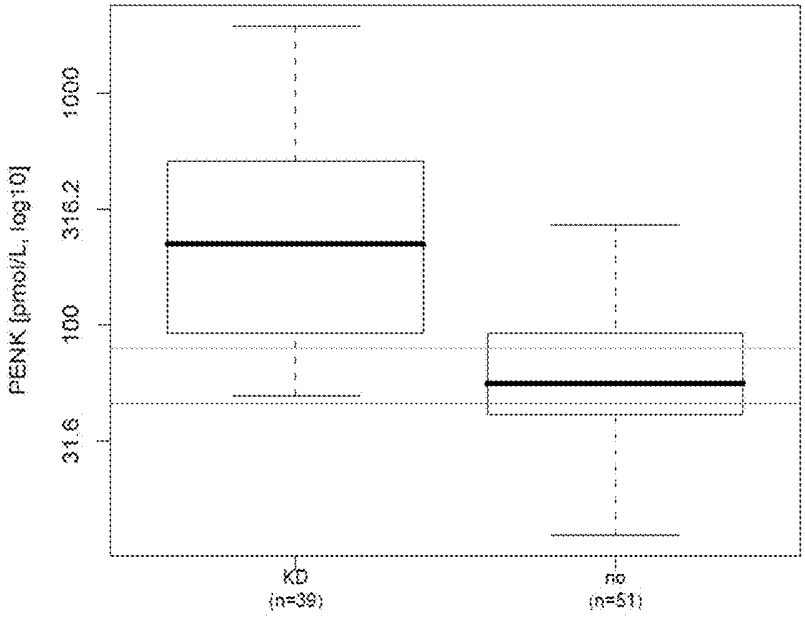

PENK [pmol/L, log10]
1000
316.2
100
31.6
KD
(n=39)
no
(n=51)

Fig. 6:

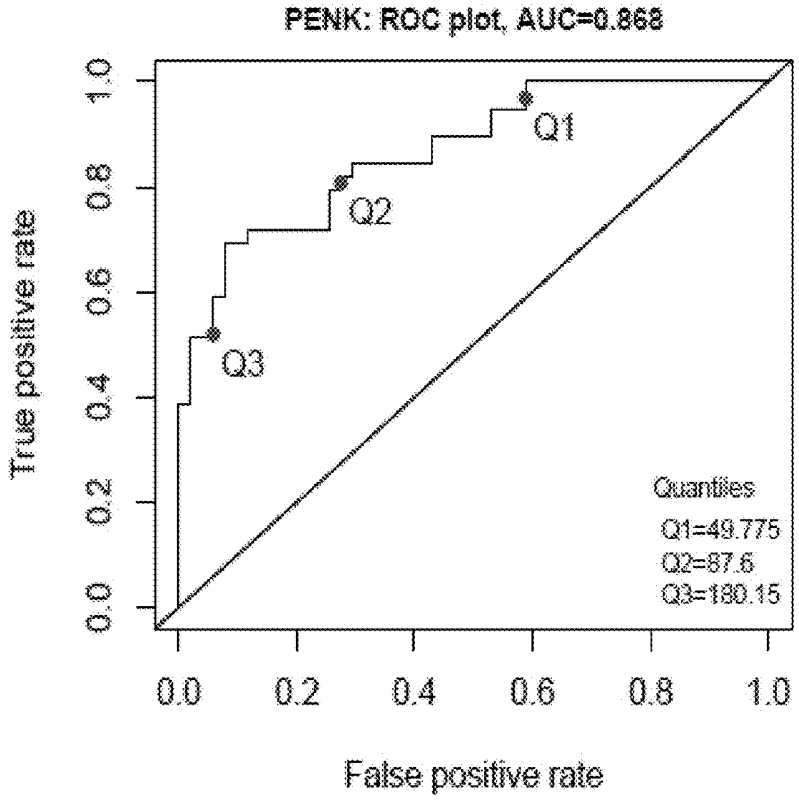

PENK: ROC plot, AUC=0.868
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Q1
Q2
Q3
Quantiles
Q1=49.775
Q2=87.6
Q3=180.15

Fig. 7:

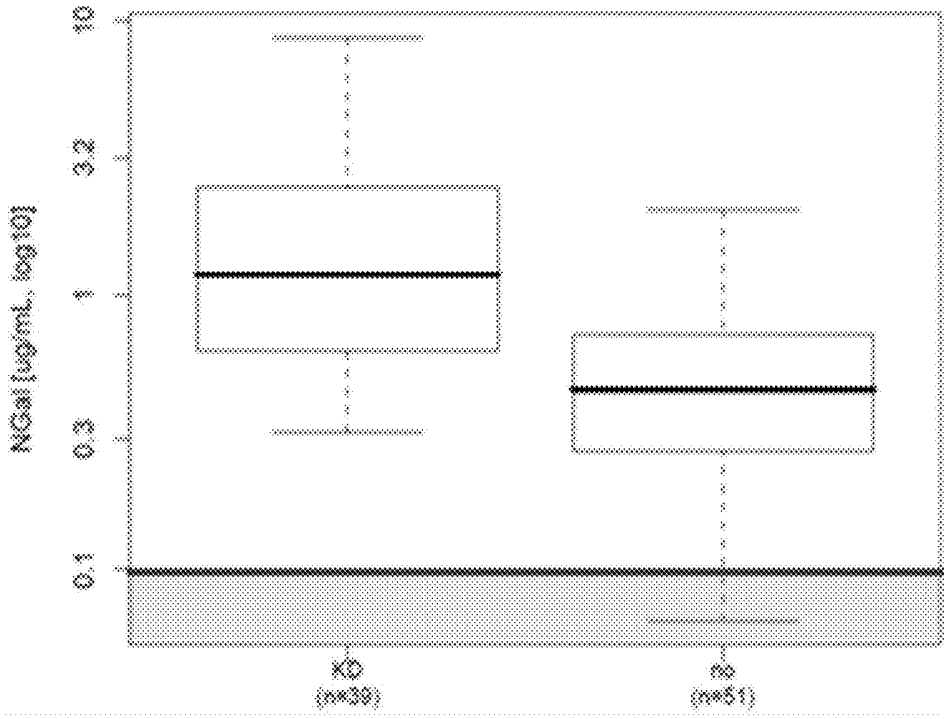

NGal [ug/mL, log10]
10
3.2
1
0.3
0.1
KD
(n=39)
(n=51)

Fig. 8:

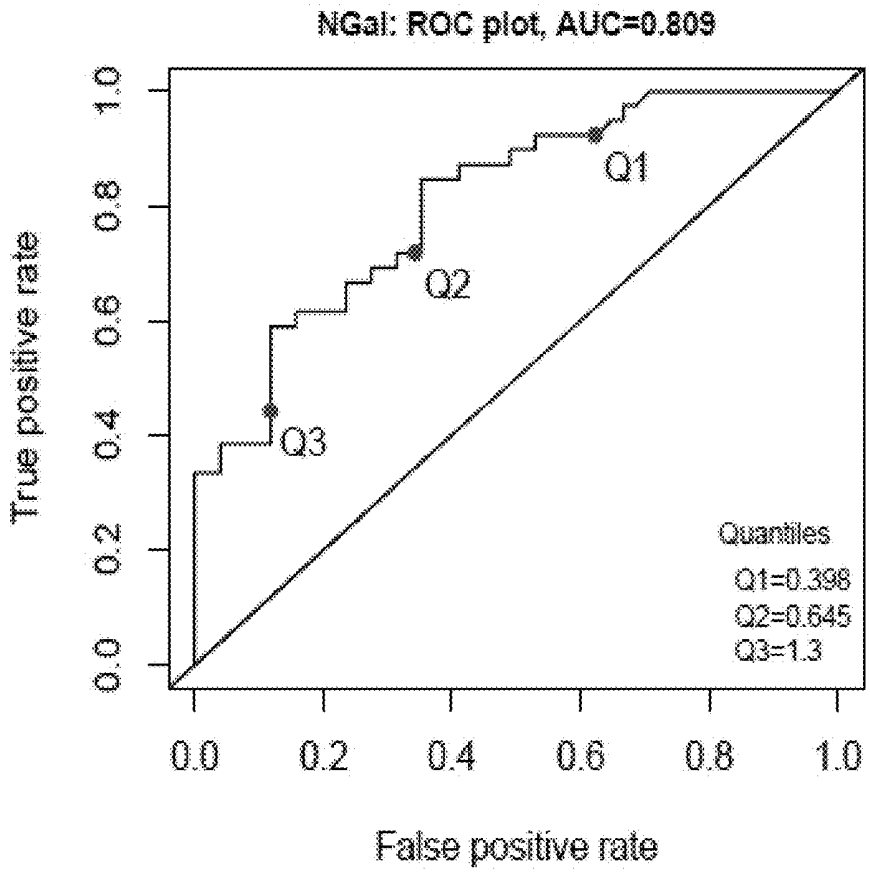
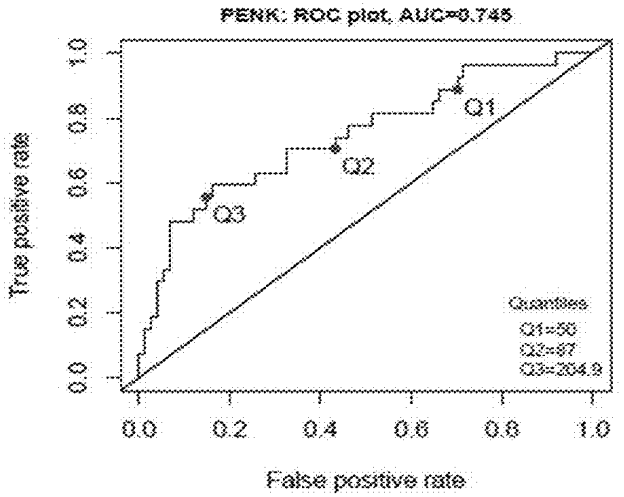

NGal: ROC plot, AUC=0.809
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Q1
Q2
Q3
Quantiles
Q1=0.398
Q2=0.645
Q3=1.3

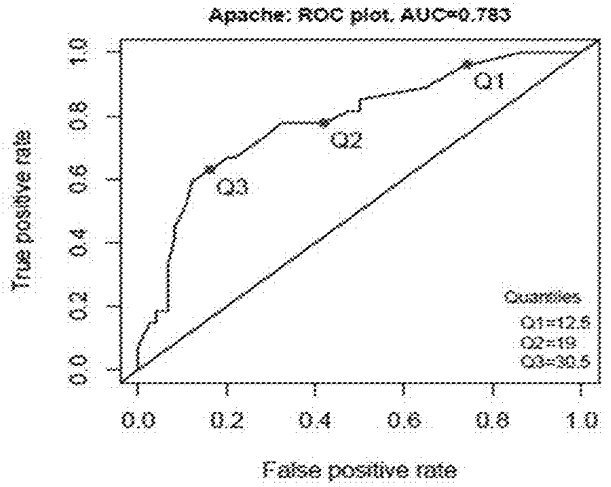
Fig. 9:

Predicting in-hospital mortality

Results from logistic regression:

Model	N	Events	Model Chi2	d.f.	LR p-value	C index [95-CI]
crea.clearance	98	24	4.02	1	0.04485	0.638 [0.503,0.772]
PENK	101	27	17.53	1	0.00003	0.744 [0.631,0.858]
APACHE	101	27	19.98	1	0.00001	0.783 [0.681,0.886]





Predicting in-hospital mortality

- PENK is independent from Apache and provides additional prognostic information:

	LR χ^2	d.f.	p value
adding APACHE (Apache) to PENK	7.07	1	0.0078
adding PENK (PENK) to APACHE	4.62	1	0.0316

- AUC(PENK plus APACHE): 0.794 (vs. 0.783 for APACHE alone)
- Chi^2: 26.4 (vs. 20.0 for APACHE)

Fig. 10:

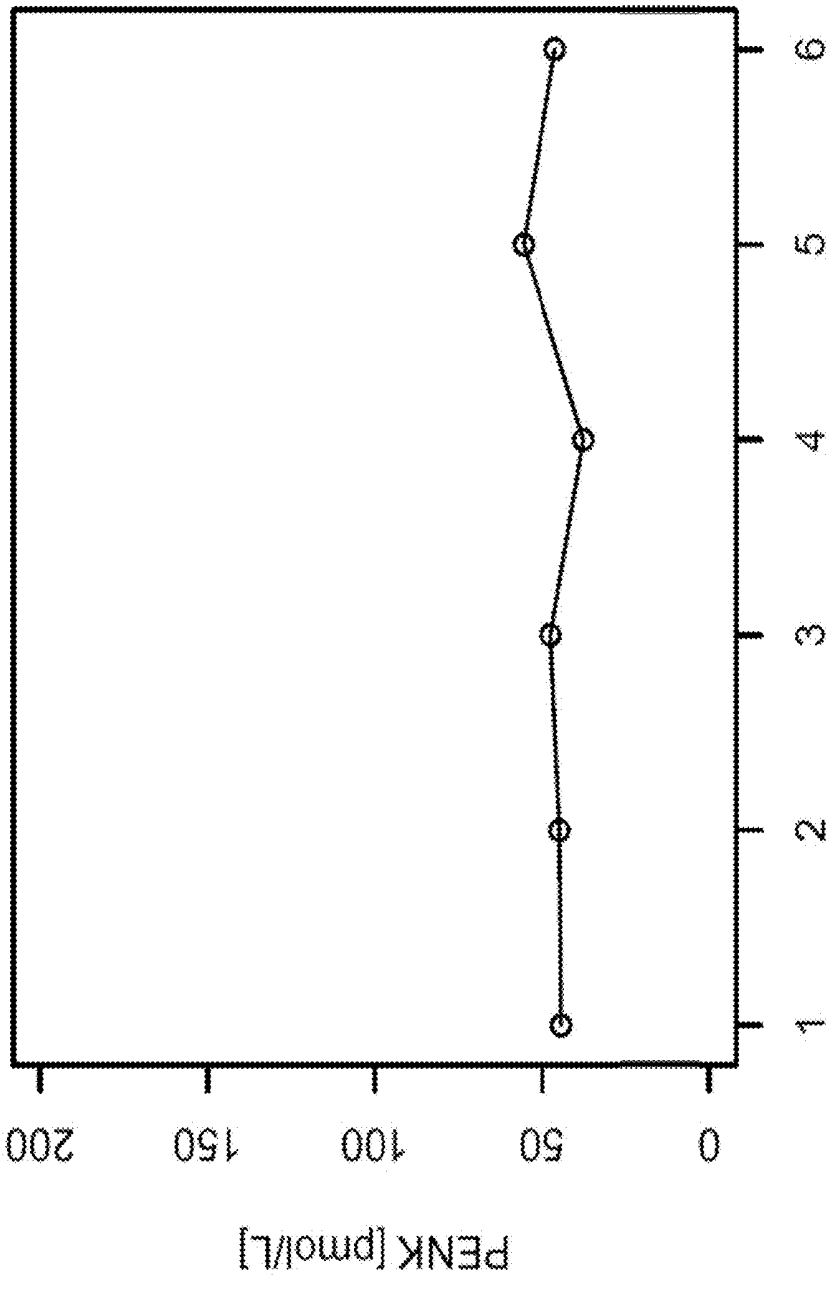


Fig. 11 a:

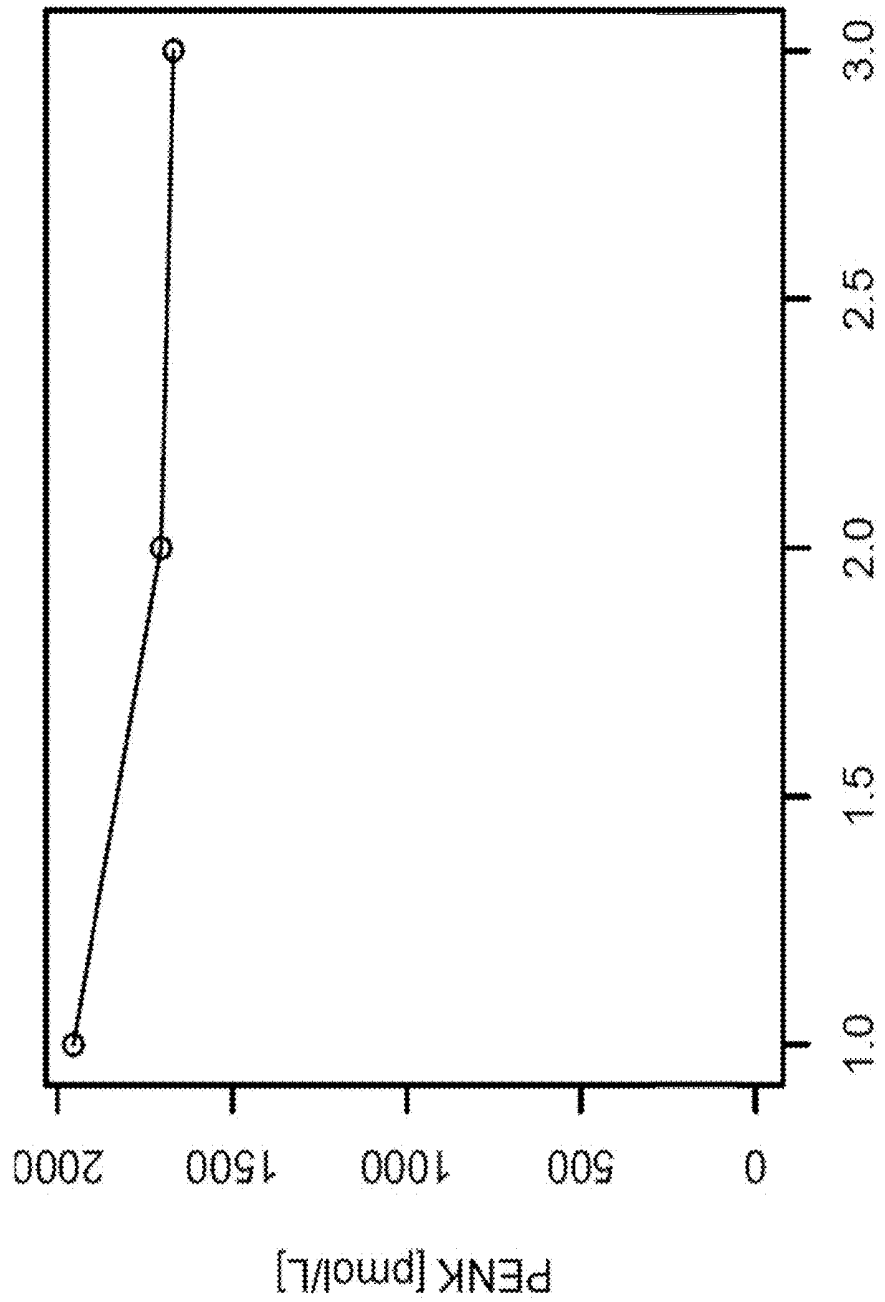


Fig. 11 b:

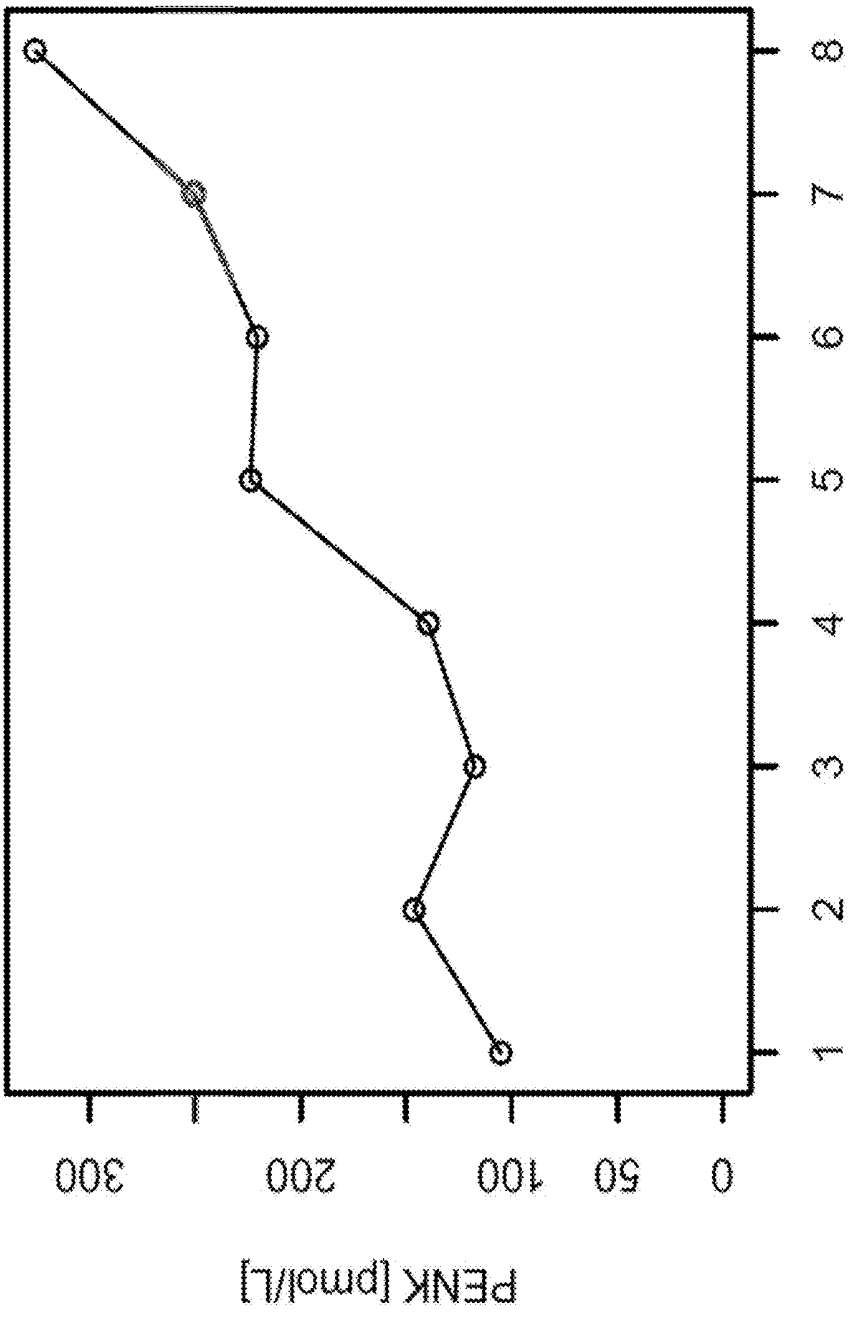


Fig. 11 c:

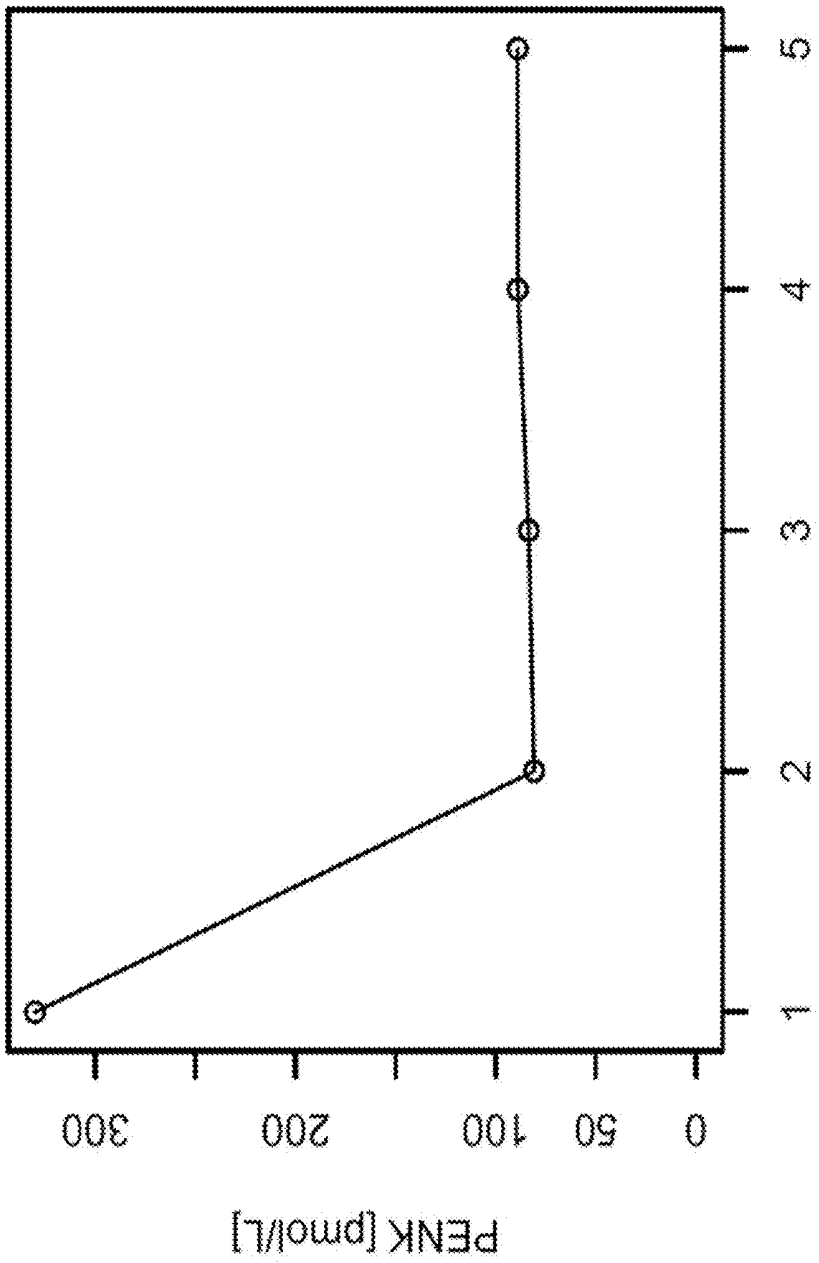


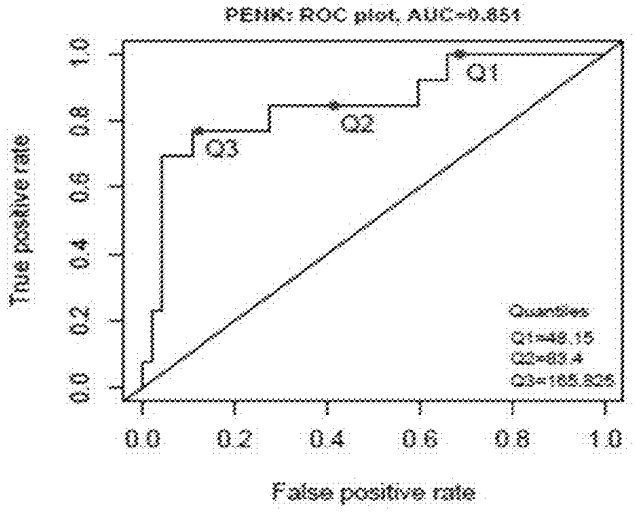
Fig. 11 d:

Fig. 12:

Predicting in-hospital mortality

Results from logistic regression:

Model	N	Events	Model Chi2	d.f.	LR p-value	C index [95-CI]
crea.clearance	58	11	5.26	1	0.02188	0.721 [0.528,0.913]
PENK	60	13	18.9	1	0.00001	0.849 [0.724,0.979]
APACHE	60	13	20.19	1	0.00001	0.837 [0.693,0.981]



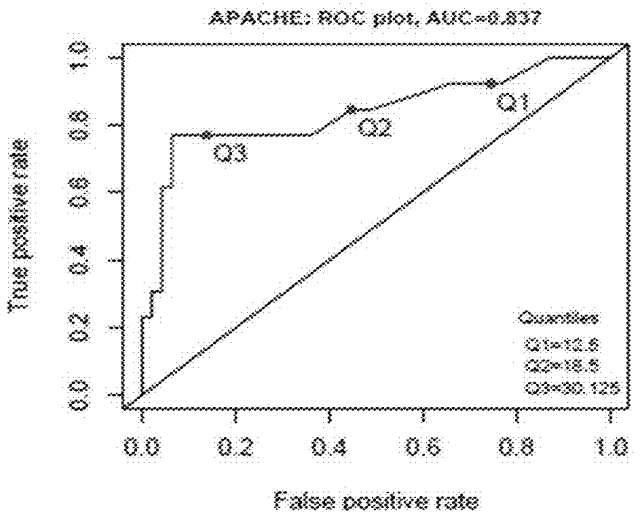


Fig. 13:

Predicting in-hospital mortality

- PENK is independent from Apache and provides additional prognostic information:

	LR χ^2	d.f.	p value
adding APACHE (Apache) to PENK	7.69	1	0.0056
adding PENK (PENK) to APACHE	6.4	1	0.0114

- AUC(PENK plus APACHE): 0.890 (vs. 0.837 for APACHE alone)
- Chi^2: 26.6 (vs. 20.2 for APACHE)

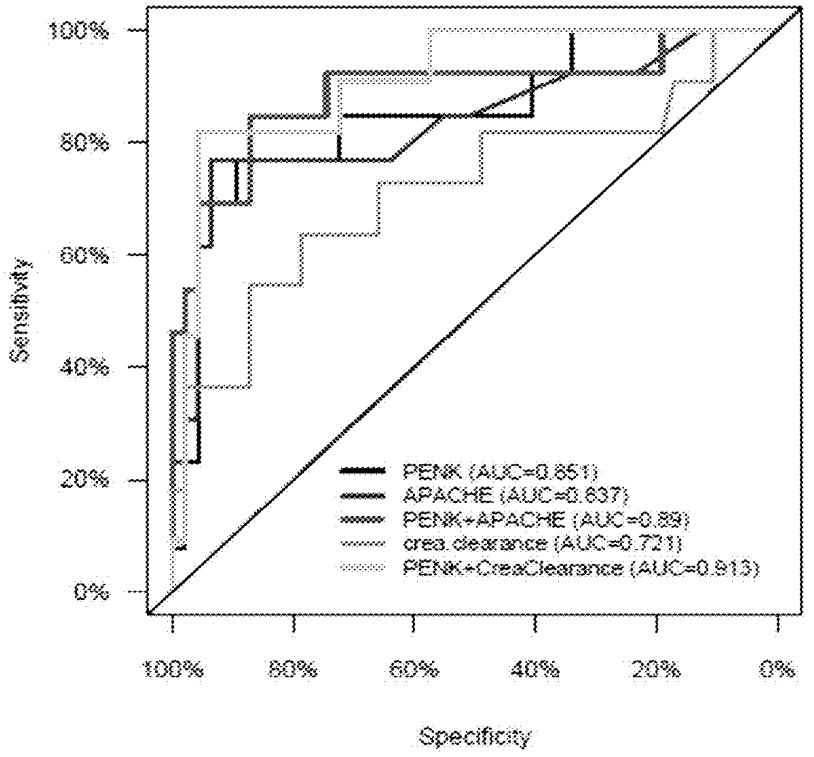


Fig. 14:

Predicting in-hospital mortality

- PENK is independent from Creatinine Clearance and provides superior prognostic information:

	LR χ^2	d.f.	p value
adding crea.clearance (crea.clearance) to PENK	3.31	1	0.069
adding PENK (PENK) to crea.clearance	18.23	1	<0.00001

- AUC(PENK plus Crea Cl): 0.913 (vs. 0.851 for PENK alone)

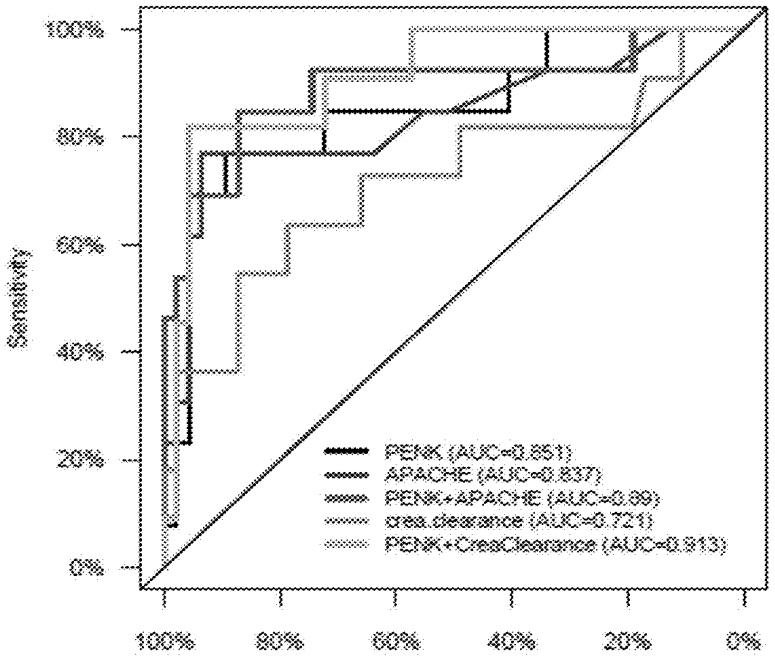


Fig. 15a:

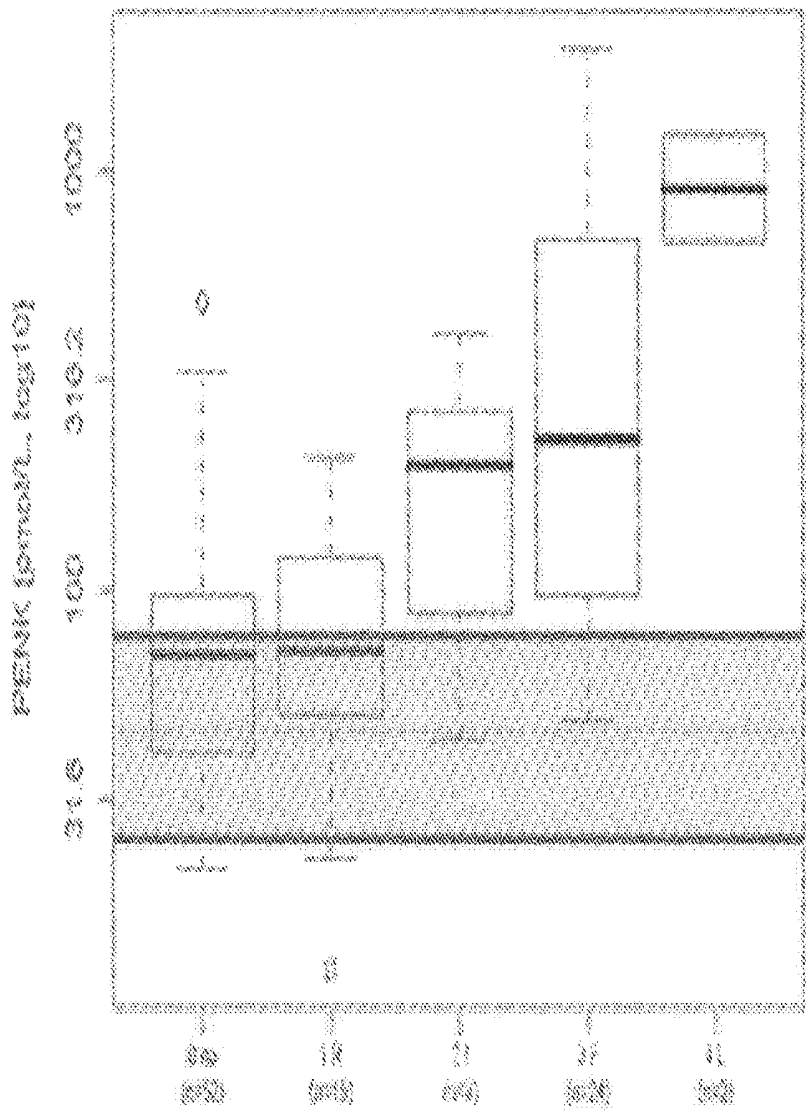


Fig. 15b:

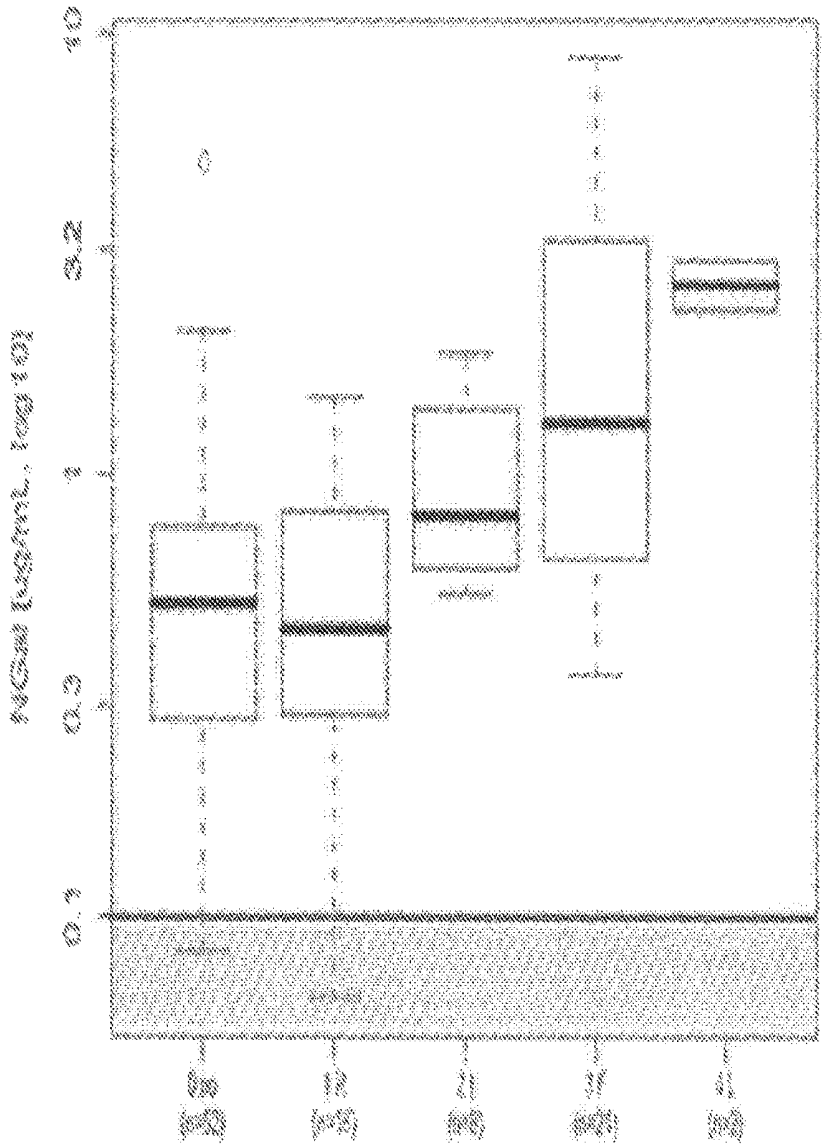


Fig. 16a:

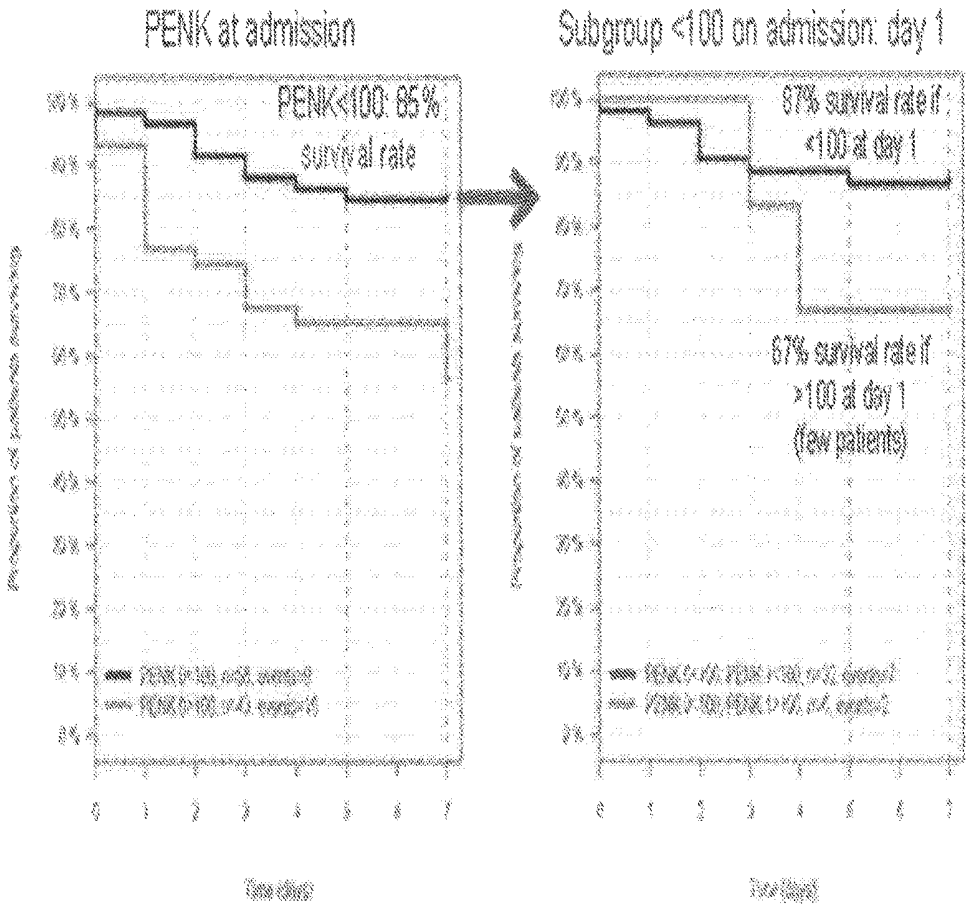
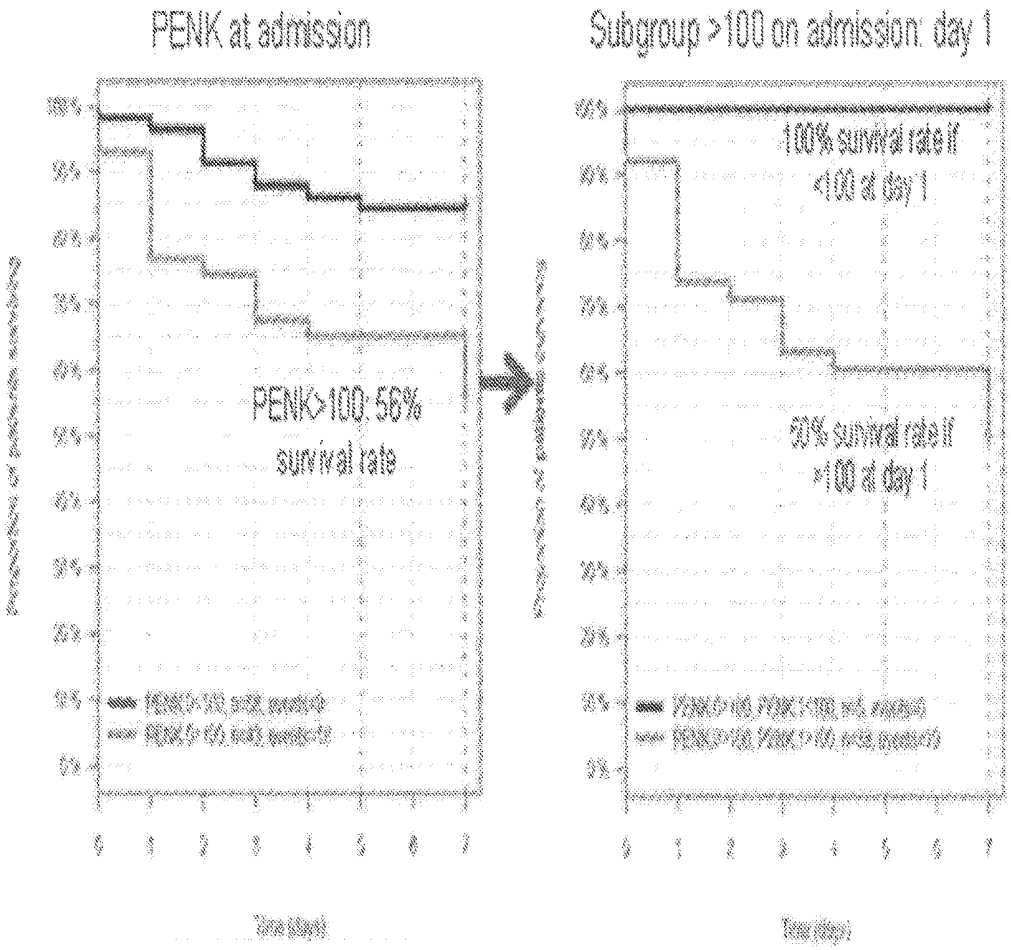

PENK at admission
PENK<100: 65% survival rate
Subgroup <100 on admission: day 1
67% survival rate if <100 at day 1
67% survival rate if >100 at day 1 (few patients)

Fig. 16b:


PENK at admission
PENK>100: 56% survival rate
Subgroup >100 on admission: day 1
100% survival rate if <100 at day 1
20% survival rate if >100 at day 1

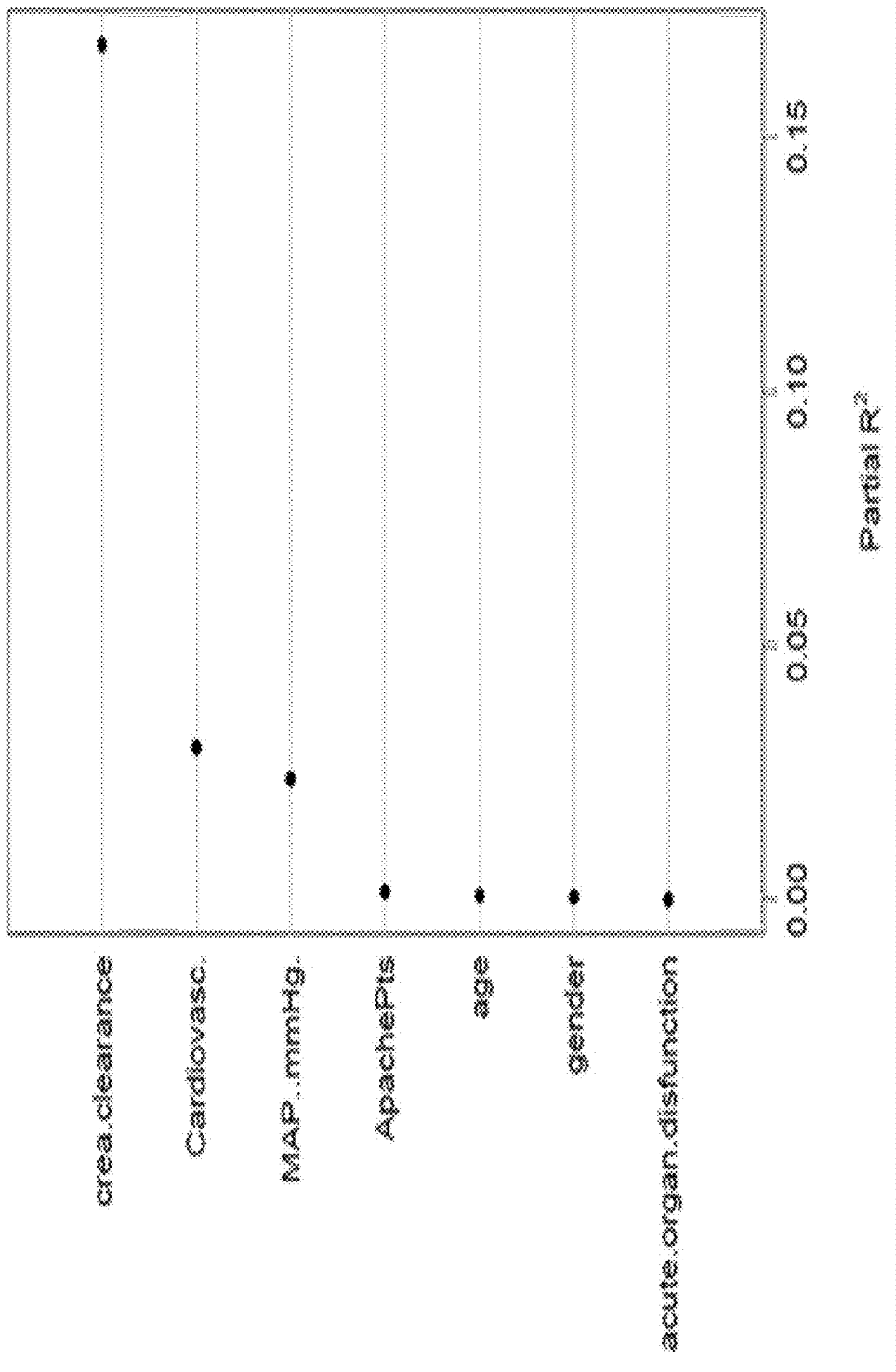

crea.clearance
Cardiovasc.
MAP..mmHg.
ApachePts
age
gender
acute.organ.disfunction
0.00
0.05
0.10
0.15
Partial R^2

Fig. 17:

METHOD FOR DIAGNOSING OR MONITORING KIDNEY FUNCTION OR DIAGNOSING KIDNEY DYSFUNCTION

Subject matter of the present invention is a method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising

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

(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in a subject or

(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject or

(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events or

(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a diseased subject, wherein a level below a certain threshold is predictive for a success of therapy or intervention.

Met-Enkephalin, a 5 amino acid peptide derived from the Enkephalin precursor (Pre-Pro-Enkephalin), also named "Opioid Growth Factor" (OGF) is released together with Pro-Enkephalin-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). Pro-Enkephalin related peptides exhibiting antibiotic actions (Goumon et al., 1998). Pro-Enkephalin and Enkephalin exhibits anti tumor action and acting as pro-apoptotic agents (Tavish et al., 2007, Donahue et al., 2011, Zagon et al., 2009). Enkephalin was reported to be elevated in kidney dysfunction (Smith et al, 1985, Zoccali et al., 1987, Smith et al., 1981). Enkephalin is produced as the larger Pro-Enkephalin and converted by proteolysis to the mature pentapeptides. During maturation process a number of Pro-Enkephalin fragments are generated, which are co-released together with Enkephalin (Ernst et al., 2006).

Subject matter of the present invention is the use of Pro-Enkephalin (PENK) or fragments thereof as marker for kidney function and dysfunction and its clinical utility in healthy and diseased subjects. Subject matter of the present invention is a method for diagnosing or monitoring kidney function in subject or diagnosing kidney dysfunction in a subject or predicting the risk of death or adverse events in a diseased subject.

A subject of the present invention was also the provision of the prognostic and diagnostic power of PENK or fragments thereof for the diagnosis of kidney function, dysfunction and the prognostic value in diseased subjects.

Surprisingly, it has been shown that PENK or fragments are powerful and highly significant biomarker for kidney, its function, dysfunction, risk of death or adverse events and prognosis and monitoring success of therapy or intervention.

Subject matter of the present invention is method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising

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

(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in a subject or

(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject or

(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events or

(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a diseased subject, wherein a level below a certain threshold is predictive for a success of therapy or intervention.

According to the present invention said Pro-Enkephalin or fragments thereof is not leu-enkephalin and not met-enkephalin in one specific embodiment. In another specific embodiment said Pro-Enkephalin fragment is MR-Pro-Enkephalin (MRPENK) or a fragment thereof having at least 5 amino acids.

To put it in other words: Subject matter of the present invention is method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising

determining the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) in a bodily fluid obtained from said subject; and

(a) correlating said level of immunoreactive analyte with kidney function in a subject or

(b) correlating said level of immunoreactive analyte with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject or

(c) correlating said level of immunoreactive analyte with said risk of an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events or

(d) correlating said level of immunoreactive analyte with success of a therapy or intervention in a diseased subject, wherein a level below a certain threshold is predictive for a success of therapy or intervention.

According to the present invention said immunoreactive analyte is not leu-enkephalin and not met-enkephalin in a specific embodiment. In another specific embodiment said immunoreactive analyte is MR-Pro-Enkephalin (MRPENK) or a fragment thereof having at least 5 amino acids.

This means in case a binder is used in the methods of the present invention that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) in a bodily fluid then the terms "determining the level of Pro-Enkephalin (PENK) or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said subject" are equivalent to "determining the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) in a bodily fluid obtained from said subject". In a specific embodiment a binder is used in the methods of the present invention that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) in a bodily fluid. In a specific embodiment said binder used in the methods of the present invention does not bind to a region within the amino acid sequence of leu-enkephalin or met-enkephalin in a bodily fluid. In another specific embodiment of the present invention said at least one binder binds to MR-Pro-Enkephalin (MRPENK) or a fragment thereof having at least 5 amino acids.

The term "subject" as used herein refers to a living human or non-human organism. Preferably herein the subject is a human subject. The subject may be healthy or diseased if not stated otherwise. In one embodiment of the invention said subject has not suffered a stroke. In another embodiment said subject is not an acute stroke patient.

The term "elevated level" means a level above a certain threshold level. The term " elevated" level may mean a level above a value that is regarded as being a reference level.

Predicting or monitoring the success of a therapy or intervention may be e.g. the prediction or monitoring of success of renal replacement therapy using measurement of Pro-Enkephalin (PENK) or fragments thereof of at least 5 amino acids.

Predicting or monitoring the success of a therapy or intervention may be e.g. the prediction or monitoring of success of treatment with hyaluronic acid in patients having received renal replacement therapy using measurement of Pro-Enkephalin (PENK) or fragments thereof of at least 5 amino acids.

Predicting or monitoring the success of a therapy or intervention may be e.g. the prediction or monitoring recovery of renal function in patients with impaired renal function prior to and after renal replacement therapy and/or pharmaceutical interventions using measurement of PENK or fragments thereof of at least 5 amino acids.

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

Determination of Pro-Enkephalin or fragments thereof exhibit kidney function in a subject. An increased concentration of Pro-Enkephalin or fragments thereof indicates a reduced kidney function. During follow up measurements, a relative change of Pro-Enkephalin or fragments thereof correlates with the improvement (lowering Pro-Enkephalin or fragments thereof) and with the worsening (increased Pro-Enkephalin or fragments thereof) of the subjects kidney function.

Pro-Enkephalin or fragments thereof are diagnostic for kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject. During follow up measurements, a relative change of Pro-Enkephalin or fragments thereof correlates with the improvement (lowering Pro-Enkephalin or fragments thereof) and with the worsening (increased Pro-Enkephalin or fragments thereof) of the subjects kidney dysfunction.

Pro-Enkephalin or fragments thereof are superior in comparision to other markers for kidney function/dysfunction diagnosis and follow up (NGAL, blood creatinine, creatinine clearance, Cystatin C, Urea). Superiority means higher specificity, higher sensitivity and better correlation to clinical endpoints.

Correlating said level of Pro-Enkephalin or fragments thereof with a risk of death or an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of death or adverse events. Also in this aspect, Pro-Enkephalin or fragments thereof are superior to above mentioned clinical markers.

Risk according to the present invention correlates with the risk as defined by the RIFLE criteria, Venkatamaran and Kellum, 2006).

The diseased person may suffer from a disease selected from chronical kidney failure caused by immune responses to inflammation, acute kidney failure caused by decreased blood flow which may occur with extremely low blood pressure caused by trauma, traumatic patients, surgery, stroke, acute and chronic renal failure, patients with SIRS, Sepsis, Septic Shock, Stroke, acute- and post Myocardial Infarction, acute- and chronic Heart Failure, local and systemic bacterial and viral infections, autoimmune diseases, burned patients, cancer, liver diseases, lung diseases, patients receiving nephrotoxins such as cyclosporine, antibiotics including aminoglycosides and anticancer drugs such as cisplatin.

The therapy or intervention supporting or replacing kidney function may comprise various methods of renal replacement therapy including but not limited to hemodialysis, peritoneal dialysis, hemofiltration and renal transplantation.

An adverse event may be selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease (according to the RIFLE criteria, Venkatamaran and Kellum, 2006).

In one embodiment of the invention it should be understood that the term fragments of Pro-Enkephalin also include Leu-Enkephalin and Met-Enkephalin.

Subject matter according to the present invention is a method wherein the level of Pro-Enkephalin or fragments thereof of at least 5 amino acids is determined by using a binder, at least one binder, to Pro-Enkephalin or fragments thereof of at least 5 amino acids. In one embodiment of the invention said binder is selected from the group comprising an antibody, an antibody fragment or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof of at least 5 amino acids. In a specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. In a specific embodiment said binder do not bind to enkephalin peptides [Met]enkephalin SEQ ID No:3, and [Leu] enkephalin. SEQ ID No:4. In a specific embodiment said at

least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 5, 6, 7, 8, 9 and 10. In another specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 2, 5, 6, and 10. In another very specific embodiment said binder bind to Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK.

Pro-Enkephalin has the following sequence:

SEQ ID NO. 1
(Pro-Enkephalin (1-243)
ECSQDCATCSYRLVRPADINFLACVMECEGKLPSLKIWETCKELLQLSKP

ELPQDGTSTL

RENSKPEESHLLAKRYGGFMKRYGGFMKKMDELYPMEPEEEANGSEILAK

RYGGFMKKDAEEDDSLANSSDLLKELLETGDNRERSHHQDGSDNEEEVSK

RYGGFMRGLKRSPQLEDEAKELQKRYGGFMRRVGRPEWWMDYQKRYGGFL

KRFAEALPSDEEGESYSKEVPEMEKRYGGF MRF

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

SEQ ID NO. 2
(Synenkephalin, Pro-Enkephalin 1-73)
ECSQDCATCSYRLVRPADINFLACVMECEGKLPSLKIWETCKELLQLSKP

ELPQDGTSTL

RENSKPEESHLLA

SEQ ID NO. 3
(Met-Enkephalin)
YGGFM

SEQ ID NO. 4
(Leu-Enkephalin)
YGGFL

SEQ ID NO. 5
(Pro-Enkephalin 90-109)
MDELYPMEPEEEANGSEILA

SEQ ID NO 6: (Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK)
DAEEDDSLANSSDLLKELLETGDNRERSHHQDGSDNEEEVS

SEQ ID NO. 7
(Met-Enkephalin-Arg-Gly-Leu)
YGGFMRGL

SEQ ID NO. 8
(Pro-Enkephalin 172-183)
SPQLEDEAKELQ

SEQ ID NO. 9
(Pro-Enkephalin 193-203)
VGRPEWWMDYQ

SEQ ID NO. 10
(Pro-Enkephalin 213-234)
FAEALPSDEEGESYSKEVPEME

SEQ ID NO. 11
(Pro-Enkephalin 213-241)
FAEALPSDEEGESYSKEVPEMEKRYGGF M

SEQ ID NO. 12
(Met-Enkephalin-Arg-Phe)
YGGFMRF

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.

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.

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 above mentioned embodiments according to the invention to the specific embodiments of clinical relevance, e.g.

correlating said level of immunoreactive analyte with kidney function in a subject or

(b) correlating said level of immunoreactive analyte with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject or

(c) correlating said level of immunoreactive analyte with said risk of an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events or

(d) correlating said level of immunoreactive analyte with success of a therapy or intervention in a diseased subject, wherein a level below a certain threshold is predictive for a success of therapy or intervention.

Alternatively the level of any of the above analytes may be determined by other nalytical methods e.g. mass spectroscopy.

Thus, subject matter of the present invention is method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising

determining the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of a peptide selected from the group comprising the peptides and fragments of SEQ ID No. 1 to 12 in a bodily fluid obtained from said subject; and

(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in a subject or

(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject or

(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a diseased subject, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events or

(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a diseased subject, wherein a level below a certain threshold is predictive for a success of therapy or intervention.

In a specific embodiment the level of immunoreactive analyte is determined by using at least one binder that binds to a region within the amino acid sequence of a peptide selected from the group comprising Pro-Enkephalin or fragments thereof of at least 5 amino acids. In a specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. In a specific embodiment said binder do not bind to enkephalin peptides [Met]enkephalin SEQ ID No:3, and [Leu]enkephalin. SEQ ID No:4. In a specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 5, 6, 7, 8, 9 and 10. In another specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 2, 5, 6, and 10. In another very specific embodiment said binder binds to Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK. The before mentioned binder binds to said peptides in a bodily fluid obtained from said subject.

In one embodiment of the invention said binder is selected from the group comprising an antibody, an antibody fragment or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof of at least 5 amino acids.

In a more specific embodiment the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK (SEQ ID No. 6) in a bodily fluid obtained from said subject.

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 1. All thresholds and values have to be seen in correlation to the test and the calibration used according to Example 1. 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 1).

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.

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.

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/).

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.

In addition to antibodies other biopolymer scaffolds are well known in the art to complex a target molecule and have been used for the generation of highly target specific biopolymers. Examples are aptamers, spiegelmers, anticalins and conotoxins. Non-Ig scaffolds may be protein scaffolds and may be used as antibody mimics as they are capable to bind to ligands or antigenes. Non-Ig scaffolds may be selected from the group comprising 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).

The threshold for diagnosing kidney disease/dysfunction or for determining the risk of death or an adverse event may be the upper normal range (99 percentile, 80 pmol MRPENK/L, more preferred 100 pmol/L more preferred 120 pmol/L.) A threshold range is useful between 75 and 130 pmol MRPENK/L.

In one specific embodiment the level of Pro Enkephalin is measured with an immunoassay and said binder is an antibody, or an antibody fragment binding to Pro-Enkephalin or fragments thereof of at least 5 amino acids.

In one specific embodiment the assay used comprises two binders that bind to two different regions within the region of Pro-Enkephalin that is aminoacid 133-140 (LKELLETG, SEQ ID No. 13) and aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

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/L, preferably <10 pmol/L and more preferably L <6pmol/L.

Subject matter of the present invention is the use of at least one binder that binds to a region within the amino acid sequence of a peptide selected from the group comprising the peptides and fragments of SEQ ID No. 1 to 12 in a bodily fluid obtained from said subject in a method a for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention. In one embodiment of the invention said binder is selected from the group comprising an antibody, an antibody fragment or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof of at least 5 amino acids. In a specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. In a specific embodiment said binder do not bind to enkephalin peptides [Met]enkephalin SEQ ID No:3, and [Leu]enkephalin. SEQ ID No:4.

In a specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 1, 2, 5, 6, 7, 8, 9 and 10. In another specific embodiment said at least one binder binds to a region with the sequences selected from the group comprising SEQ ID No. 2, 5, 6, and 10. In another very specific embodiment said binder bind to Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK.

In a more specific embodiment the at least one binder binds to a region within the amino acid sequence of Pro-Enkephalin 119-159, Mid regional Pro-Enkephalin-fragment, MRPENK (SEQ ID No. 6) in a bodily fluid obtained from said subject, more specifically to to aminoacid 133-140 (LKELLETG, SEQ ID No. 13) and/or aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

Thus, according to the present methods the level of immunoreactivity of the above binder is determined in a bodily fluid obtained from said subject. Level of immunoreactivity means the concentration of an analyte determined quantitatively, semi-quantitatively or qualitatively by a binding reaction of a binder to such analyte, where preferably the binder has an affinity constant for binding to the analyte of at least 10^8 M^{-1}, and the binder may be an antibody or an antibody fragment or an non.IgG scaffold, and the binding reaction is an immunoassay.

The present methods using PENK and fragments thereof, especially MRPENK, are far superior over the methods and biomarkers used according to the prior art for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention. First of all PENK and fragments thereof as biomarker for the before mentioned uses is an inflammation independent marker. That is an important feature as most of the known kidney biomarker like NGAL and KIM are inflammation dependent, meaning if the subject has an inflammation, e.g. in sepsis, the elevation of NGAL or KIM may be either due to inflammation or to kidney function/dysfunction. Thus, no differential diagnosis may be conducted, at least not by using a simple cut-off value (meaning one (1) cut-off value), which is independent from the particular patient population investigated. For NGAL and KIM each and every patient has an "individual" threshold for kidney function/dysfunction depending on the inflammation status of said subject which makes clinical application of these kidney markers difficult in some diseases and impossible in others. In contrast thereto one single threshold that is independent of the inflammation status of the subject may be used according to the present methods for all subjects. This makes the present methods suitable for clinical routine in contrast to the before-mentioned marker.

PENK and fragments thereof as biomarker in the methods of the present invention, especially MRPENK reflects "real" kidney function in contrast to NGAL and KIM, they reflect kidney damage and inflammation.

Thus, subject matter of the present invention is method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention with the before mentioned steps and features wherein an inflammation status independent threshold is used.

Another advantage of the above methods and the use of PENK and fragments as biomarker in the methods for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention is that PENK and fragments as biomarker are very early biomarker for kidney function, kidney dysfunction, risk of an adverse event, success of a therapy or intervention. Very early means e.g. earlier than creatinin, earlier than NGAL.

One clear indication of the superiority of PENK over creatinine comes from an analysis of the association of the respective concentrations determined in critically ill patients on the day of admission with their 7 day mortality rate: PENK concentrations of survivors differ significantly from non-survivors, whereas this is not the case for creatinine clearance. Mortality in such patient population is mainly driven by loss of kidney function. Thus, the significant and much stronger association of PENK with mortality than of creatinine clearance supports the superiority of PENK over creatinine clearance as kidney dysfunction marker.

Subject of the present invention is also a method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a diseased subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction includ-

ing kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention supporting or replacing kidney function comprising various methods of renal replacement therapy including but not limited to hemodialysis, peritoneal dialysis, hemofiltration and renal transplantation 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 subject either alone or in conjunction with other prognostically useful laboratory or clinical parameters is used which may be selected from the following alternatives:

- 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 subject 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-Enkephalin or fragments thereof of at least 5 amino acids in a bodily fluid obtained from said subject 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).

Said additionally at least one clinical parameter may be determined selected from the group comprising: age, NGAL, Cystatin C, Creatinine Clearance, Creatinin, Urea and Apache Score.

In one embodiment of the invention said method is performed more than once in order to monitor the function or dysfunction or risk of said subject or in order to monitor the course of treatment of kidney and/or disease. In one specific embodiment said monitoring is performed in order to evaluate the response of said subject to preventive and/or therapeutic measures taken.

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

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. 13) and aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

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.

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 Pro-Enkephalin 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.

In one embodiment of the invention it may be a so-called POC-test (point-of-care), that is a test technology which allows performing the test within less than 1 hour near the patient without the requirement of a fully automated assay system. One example for this technology is the immunochromatographic test technology.

In one embodiment of the invention such an assay is a sandwich immunoassay using any kind of detection technology including but not restricted to enzyme label, chemiluminescence label, electrochemiluminescence label, preferably a fully automated assay. In one embodiment of the invention such an assay is an enzyme labeled sandwich assay. 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®.

A variety of immunoassays are known and may be used for the assays and methods of the present invention, these include: radioimmunoassays ("RIA"), homogeneous enzyme-multiplied immunoassays ("EMIT"), enzyme linked immunoadsorbent assays ("ELISA"), apoenzyme reactivation immunoassay ("ARIS"), dipstick immunoassays and immuno-chromotography assays.

In one embodiment of the invention at least one of said two binders is labeled in order to be detected.

The preferred detection methods comprise immunoassays in various formats such as for instance radioimmunoassay (RIA), chemiluminescence- and fluorescence-immunoassays, Enzyme-linked immunoassays (ELISA), Luminex-based bead arrays, protein microarray assays, and rapid test formats such as for instance immunochromatographic strip tests.

In a preferred embodiment said label is selected from the group comprising chemiluminescent label, enzyme label, fluorescence label, radioiodine label.

The assays can be homogenous or heterogeneous assays, competitive and non-competitive assays. In one embodiment, the assay is in the form of a sandwich assay, which is a non-competitive immunoassay, wherein the molecule to be detected and/or quantified is bound to a first antibody and to a second antibody. The first antibody may be bound to a solid phase, e.g. a bead, a surface of a well or other container, a chip or a strip, and the second antibody is an antibody which is labeled, e.g. with a dye, with a radioisotope, or a reactive or catalytically active moiety. The amount of labeled antibody bound to the analyte is then measured by an appropriate method. The general composition and procedures involved with "sandwich assays" are well-established and known to the skilled person (23).

In another embodiment the assay comprises two capture molecules, preferably antibodies which are both present as dispersions in a liquid reaction mixture, wherein a first labelling component is attached to the first capture molecule, wherein said first labelling component is part of a labelling system based on fluorescence- or chemiluminescence-quenching or amplification, and a second labelling component of said marking system is attached to the second capture molecule, so that upon binding of both capture molecules to the analyte a measurable signal is generated that allows for the detection of the formed sandwich complexes in the solution comprising the sample.

In another embodiment, said labeling system comprises rare earth cryptates or rare earth chelates in combination with fluorescence dye or chemiluminescence dye, in particular a dye of the cyanine type.

In the context of the present invention, fluorescence based assays comprise the use of dyes, which may for instance be selected from the group comprising FAM (5-or 6-carboxy-

fluorescein), VIC, NED, Fluorescein, Fluoresceinisothiocyanate (FITC), IRD-700/800, Cyanine dyes, auch as CY3, CY5, CY3.5, CY5.5, Cy7, Xanthen, 6-Carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), TET, 6-Carboxy-4',5'-dichloro-2',7'-dimethodyfluorescein (JOE), N,N,N',N'-Tetramethyl-6-carboxyrhodamine (TAMRA), 6-Carboxy-X-rhodamine (ROX), 5-Carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), Rhodamine, Rhodamine Green, Rhodamine Red, Rhodamine 110, BODIPY dyes, such as BODIPY TMR, Oregon Green, Coumarines such as Umbelliferone, Benzimides, such as Hoechst 33258; Phenanthridines, such as Texas Red, Yakima Yellow, Alexa Fluor, PET, Ethidiumbromide, Acridinium dyes, Carbazol dyes, Phenoxazine dyes, Porphyrine dyes, Polymethin dyes, and the like.

In the context of the present invention, chemiluminescence based assays comprise the use of dyes, based on the physical principles described for chemiluminescent materials in (24). Preferred chemiluminescent dyes are acridiniumesters.

As mentioned herein, an "assay" or "diagnostic assay" can be of any type applied in the field of diagnostics. Such an assay may be based on the binding of an analyte to be detected to one or more capture probes with a certain affinity. Concerning the interaction between capture molecules and target molecules or molecules of interest, the affinity constant is preferably greater than 10^8 M^{-1}.

In the context of the present invention, "binder molecules" are molecules which may be used to bind target molecules or molecules of interest, i.e. analytes (i.e. in the context of the present invention PENK and fragments thereof), from a sample. Binder molecules must thus be shaped adequately, both spatially and in terms of surface features, such as surface charge, hydrophobicity, hydrophilicity, presence or absence of lewis donors and/or acceptors, to specifically bind the target molecules or molecules of interest. Hereby, the binding may for instance be mediated by ionic, van-der-Waals, pi-pi, sigma-pi, hydrophobic or hydrogen bond interactions or a combination of two or more of the aforementioned interactions between the capture molecules and the target molecules or molecules of interest. In the context of the present invention, binder molecules may for instance be selected from the group comprising a nucleic acid molecule, a carbohydrate molecule, a PNA molecule, a protein, an antibody, a peptide or a glycoprotein. Preferably, the binder molecules are antibodies, including fragments thereof with sufficient affinity to a target or molecule of interest, and including recombinant antibodies or recombinant antibody fragments, as well as chemically and/or biochemically modified derivatives of said antibodies or fragments derived from the variant chain with a length of at least 12 amino acids thereof.

Chemiluminescent label may be acridinium ester label, steroid labels involving isoluminol labels and the like.

Enzyme labels may be lactate dehydrogenase (LDH), creatinekinase (CPK), alkaline phosphatase, aspartate aminotransferace (AST), alanine aminotransferace (ALT), acid phosphatase, glucose-6-phosphate dehydrogenase and so on.

In one embodiment of the invention at least one of said two binders is bound to a solid phase as magnetic particles, and polystyrene surfaces.

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.

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.

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.

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. 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.

In one embodiment subject matter of the present invention is a point-of-care device for performing a method according to the invention wherein said point of care device comprises at least one antibody or antibody fragment directed to either aminoacid 133-140 (LKELLETG, SEQ ID No. 13) or aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

In one embodiment subject matter of the present invention is a point-of-care device for performing a method according to the invention wherein said point of care device comprises at least two antibodies or antibody fragments directed to aminoacid 133-140 (LKELLETG, SEQ ID No. 13) and aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

In one embodiment subject matter of the present invention is a kit or performing a method according to the invention wherein said point of care device comprises at least one antibody or antibody fragment directed to either aminoacid 133-140 (LKELLETG, SEQ ID No. 13) or aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

In one embodiment subject matter of the present invention is a kit for performing a method according to the invention wherein said point of care device comprises at least two antibodies or antibody fragments directed to aminoacid 133-140 (LKELLETG, SEQ ID No. 13) and aminoacid 152-159 (SDNEEEVS, SEQ ID NO. 14) wherein each of said regions comprises at least 4 or 5 amino acids.

EXAMPLES

Example 1

Development of Antibodies

Peptides

Peptides were synthesized (JPT Technologies, Berlin, Germany).

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.

TABLE 1

Peptide for immunization	Pro-Enkephalin-sequence
(C) DAEEDD	119-125
(C) EEDDSLANSSDLLK	121-134
(C) LKELLETG	133-140
(C) TGDNRERSHHQDGSDNE	139-155
(C) SDNEEEVS	152-159

The antibodies were generated according to the following method:

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.

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.

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 (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.

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

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/1 potassiumphosphate, 100 mmol/1 NaCl, 10 mmol/1 Na-EDTA, 5 g/1 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):

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/1 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

TABLE 2

Peptide for immunization	Pre-Pro-Enkephalin-sequence	Antibody name
(C) DAEEDD	119-125	NT-MRPENK
(C) EEDDSLANSSDLLK	121-134	NM-MRPENK
(C) LKELLETG	133-140	MR-MRPENK
(C) TGDNRERSHHQDGSDNE	139-155	MC-MRPENK
(C) SDNEEEVS	152-159	CT-MRPENK

Antibody cross-reactivities were determined as follows:

lug 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 1m1) 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 Luminumeter 953. MRPENK-peptide was used as reference substance (100%).

The crossreactivities of the different antibodies are listed in table 3.

TABLE 3

Anti-body	DAEEDD	EEDDSLANSSDLLK	LKELLETG	TGDNRERSHHQDGSDNE	SDNEEEVS	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

All antibodies bound the MRPENK peptide, comparable to the peptides which were used for immunization. Except for NT-MRPENK-antibody (10% cross reaction with EEDDSLANSSDLLK), no antibody showed a cross reaction with MR-PENK fragments not used for immunization of the individual antibody.

Pro-Enkephalin Immunoassay:

50 µl of sample (or calibrator) was pipetted into coated tubes, after adding labeled antibody (200 µl), 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 953. Using a fixed concentration of 1000 pmol/of MRPENK. The signal (RLU at 1000 pmol MRPENK/1) 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

Labelled antibody \ Solid phase antibody	NT-MRPENK	NM-MRPENK	MR-MRPENK	MC-MRPENK	CT-MRPENK
NT-MRPENK	/	27	212	232	<1
NM-MRPENK	36	/	451	487	<1
MR-MRPENK	175	306	/	536	1050
MC-MRPENK	329	577	542	/	<1
CT-MRPENK	<1	615	1117	516	/

Calibration:

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.

FIG. **1** shows a typical Pro-Enkephalin dose/signal curve.

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

Creatinine Clearance

Creatinine clearance was determined using the MDRD formula (see Levey et al, 2009).

Example 2

PENK in healthy subjects

Healthy subjects (n=4211, average age 56 years) were measured using the MRPENK assay. The mean value was 44.7 pmol MRPENK/L, the lowest value was 9 pmol/L and the 99^{th}percentile was 80 pmol/L. Since the assay sensitivity was 5.5 pmol/L, 100% of all healthy subjects were detectable using the described MRPENK assay (see FIG. **2**).

Pro-Enkephalin correlates with Creatinine Clearance in healthy subjects with normal kidney function.

Surprisingly, Pro-Enkephalin was negatively correlated with Creatinine Clearance in healthy subjects ($r=-0.33$, $p<0.0001$), see FIG. **3**. The coefficient of correlation was slightly stronger in male than in females ($r=-0.34$ vs -0.29, both $p<0.0001$). These data indicating a strong association between PENK and kidney function.

FIG. **3**: correlation of creatinine clearance vs. PENK in healthy subjects. Y axis: quartiles of Creatinine Clearance, x axis: quartiles of PENK.

Example 3

Correlation of PENK and kidney function (creatinine clearance) in patients with chronic and acute diseases.

TABLE 5

Disease	r-value	p-value
Chronic Heart Failure N = 122	−0.55	<0.0001
Acute Heart Failure N = 149	−0.68	<0.0001
Acute Myocardial Infarction N = 78	−0.82	<0.0001
Sepsis N = 101	−0.74	<0.0001
SIRS N = 109	−0.79	<0.0001

PENK correlated always significantly with creatinine clearance, in acute diseases the correlation was stronger than in chronic diseases or in healthy subjects.

Example 4

PENK in critical ill patients

To investigate the diagnostic performance of PENK for diagnosis of kidney failure in acute clinical settings, we performed the following clinical study:

Clinical Study

101 ED patients fulfilling the definition of sepsis (Crit Care Med. 2008 Jan; 36(1):296-327.) were subsequently hospitalized (average 5 days of hospitalization) and received a standard of care treatment. EDTA-plasma was generated from day 1 (ED presentation) and one sample each day during hospital stay. The time to freeze samples for later analyte-measurement was less than 4 h.

Patient characteristics are summarized in table 6:

TABLE 6

Variable	all (n = 101)	in hospital deaths (n = 27)	discharged (n = 74)	p-value
Demographics				
Gender—male	60 (60)	13 (48)	47 (64)	0.163
Age—median [IQR]	78 [72-72]	77 [71.25-83]	80 [75-84.5]	0.142

TABLE 6-continued

Variable	all (n = 101)	in hospital deaths (n = 27)	discharged (n = 74)	p-value
Examination variables				
BP systolic (mmHg)—median [IQR]	115 [100-100]	120 [106.25-138.75]	105 [80-120]	0.001
BP diastolic (mmHg)—median [IQR]	65 [60-60]	65 [60-85]	60 [50-70]	0.002
HR—median [IQR]	100 [94-94]	100 [94-114.75]	100 [93.5-107.5]	0.407
RR—median [IQR]	24 [22-22]	24 [22-28]	26 [24-28]	0.069
MAP (mmHg)—median [IQR]	83.3 [74-74]	83.3 [77.62-100.75]	81.6 [63.5-89]	0.026
concomitant diseases				
Cardiovascular—yes	26 (25.7)	9 (33.3)	17 (23)	0.311
Hypertensive—yes	47 (46.5)	13 (48.1)	34 (45.9)	1.000
Diabetes—yes	35 (34.7)	9 (33.3)	26 (35.1)	1.000
Cancere—yes	13 (12.9)	3 (11.1)	10 (13.5)	1.000
routine labaratory variables				
Blood culture—yes	31 (31)	5 (19)	26 (35)	0.246
negative	15 (16.3)	2 (8)	13 (19.4)	
positive	16 (17.4)	3 (12)	13 (19.4)	
Creatinine clearance (ml/min)—median [IQR]	48 [23.25-23.25]	56 [29.25-80]	31.5 [14.75-66]	0.043
Creatinine—median [IQR]	1.3 [0.9-0.9]	1.25 [0.9-2.08]	1.8 [1-3.15]	0.080
UREA—median [IQR]	36 [21-21]	31.5 [20-53.25]	51 [42-87]	0.004
GCS—median [IQR]	15 [10-10]	15 [12.5-15]	8 [8-11]	<0.001
Pcr—median [IQR]	16 [6.6-6.6]	14.5 [6.7-23.7]	17.35 [6.6-28.05]	0.846
Gluco—median [IQR]	113.5 [94.5-94.5]	110 [95.5-144]	128 [94-160.5]	0.400
biliru—median [IQR]	0.9 [0.71-0.71]	0.9 [0.7-1.03]	0.91 [0.77-1.18]	0.534
GR—median [IQR]	3.8 [3.3-3.3]	3.8 [3.2-4.3]	3.7 [3.4-4.2]	0.684
GB—median [IQR]	12700 [6774-6774]	13100 [8115-17565]	11920 [25.55-18790]	0.343
PLT—median [IQR]	213 [150-150]	217 [154.75-301]	185 [130-236.5]	0.113
HCT—median [IQR]	32 [28-28]	31.5 [28-37]	34 [31.25-39.5]	0.149
Leuco/Neutr (%)—median [IQR]	87 [80-80]	86 [78.25-89.95]	91 [87-93.05]	0.001
HB—median [IQR]	10.4 [9.47-9.47]	10.15 [9.3-12.4]	10.85 [9.9-12.67]	0.220
Na—median [IQR]	137 [134-134]	137 [133-141]	139 [134-144.5]	0.204
K—median [IQR]	3.9 [3.5-3.5]	3.9 [3.6-4.3]	3.9 [3.3-5.1]	0.982
INR—median [IQR]	1.19 [1.1-1.1]	1.19 [1.1-1.4]	1.18 [1.04-1.36]	0.731
TC—median [IQR]	38.4 [36-36]	38.5 [38.12-38.7]	36 [35.55-38.5]	<0.001
SAO2—median [IQR]	94 [90-90]	95 [90.25-97]	93 [88.5-95.5]	0.119
pH—median [IQR]	7.45 [7.38-7.38]	7.46 [7.4-7.5]	7.4 [7.24-7.4]	<0.001
PO2—median [IQR]	67 [56-56]	66.5 [56-78]	67 [56.5-79.5]	0.806
PCO2—median [IQR]	36 [32-32]	37.5 [33-43.75]	34 [30-41]	0.245
Lact—median [IQR]	1.5 [1-1]	1.3 [0.83-1.9]	2.5 [1.4-4.15]	<0.001
Bic—median [IQR]	23.5 [21-21]	24.25 [21.43-28]	21 [17.35-23.25]	0.001
FiO2 (%)—median [IQR]	21 [21-21]	21 [21-23.25]	24 [21-45]	<0.001
other				
Acute organ disfunction—yes	39 (43.3)	16 (64)	23 (35.4)	0.021
Apache score (%)—median [IQR]	19 [12.5-12.5]	14.65 [12.12-20.38]	32 [20-39]	<0.001
Days hospitalized—median [IQR]	5 [2-2]	6 [4-7]	2 [1-6]	0.003
treatment at baseline				
Diuresis (cc)—median [IQR]	900 [600-600]	1000 [700-1200]	450 [200-1025]	<0.001
Steroids—yes	16 (15.8)	4 (14.8)	12 (16.2)	1.000
Vasopressors—yes	18 (17.8)	13 (48.1)	5 (6.8)	<0.001
Antibiotics—yes	101 (100)	27 (100)	74 (100)	1.000
Fluid therapy—yes	101 (100)	27 (100)	74 (100)	1.000

26.7% of all patients died during hospital stay and are counted as treatment non responder, 73.3% of all patients survived the sepsis and are counted as treatment responder.

53% off all patients presenting with sepsis had an non-normal PENK value >80 pmol/L (99 percentile), indicating PENK not to be a marker for the infection.

Results of Clinical Study

PENK highly correlated to creatinine clearance (r=−0.74, p <0.0001, FIG. **4**).

PENK diagnoses kidney dysfunction:

Kidney dysfunction was defined based on the RIFLE criteria (Venkatamaran and Kellum, 2007). Patients were counted as kidney dysfunction if any of the RIFLE classification factors was fulfilled. Within the study cohort, we determined the RIFLE within 90 subjects at day 1 (presentation at ED), 39 patients fulfilled RIFLE classification (had risk of kidney disease, kidney injury, kidney failure loss of kidney function or end-stage kidney disease) and 51 patients had no kidney dysfunction. Increased PENK was significantly (p=<0.0001) correlated with kidney dysfunction (AUC: 0.868). (FIGS. **5** and **6**)

To compare the diagnostic value for kidney dysfunction, we used NGAL as reference marker (Soni et al, 2010). NGAL was measured, using a commercially ELISA (NGAL Elisa kit, Bioporto, Gentofte, Denmark).

NGAL, like PENK, was significantly increased in patients with kidney dysfunction (p<0.0001), the AUC for diagnosis of kidney dysfunction was 0.809. (FIGS. **7** and **8**)

Comparing PENK and NGAL showed a strong superiority of PENK vs NGAL for diagnosis of kidney dysfunction: the Chi2 value of PENK was 45.32 vs. 32.21 for NGAL, indicating a 40% improvement of diagnostic quality (specificity and sensitivity) by PENK. (Table 7)

TABLE 7

Model	N	Events	Model Chi2	d.f.	LR p-value	C index [95-CI]
PCT	76	34	13.02	1	0.00031	0.721 [0.602,0.839]
Apache	90	39	28.58	1	<0.00001	0.778 [0.681,0.874]
NGal	90	39	32.21	1	<0.00001	0.809 [0.723,0.896]
PENK	90	39	45.32	1	<0.00001	0.868 [0.796,0.94]

Initial PENK is highly prognostic.

We correlated the initial PENK value with the in hospital mortality and compared PENK with APACHE 2 sepsis score (see Knaus et al, 1985, 2001) and creatinine clearance. PENK is highly prognostic for sepsis outcome (see FIG. **9**) and comparable to APACHE 2 score (AUC/C index 0.744 (PENK) and 0.783 (Apache). There is a significant added information if PENK and APACHE 2 are combined (combined AUC: 0.794 FIG. **10**). PENK is substantially stronger in prognosis than the creatinine clearance (AUC 0.638). Surprisingly, the prognostic value of PENK was stronger after the first day of ICU-treatment (AUC 0.79).

Cut Off-Analysis for in hospital death prognosis using baseline sample and 1 sample after 1 day of ICU treatment.

Since the prognostic power of PENK was further improved one day after starting ICU treatment, we analyzed the PENK in serial measurements of day before ICU-treatment and 1 day after starting ICU treatment. To illustrate the clinical performance, we used a simple cut off analysis at a cut off value of 100 pmol/L.

If patients are below the cut off at hospital presentation and remain below the cut off after initiating ICU treatment, the mortality was 11% (well treated before and during hospitalization). If PENK was above the cut off at both time points, the mortality was about 5 times higher (52.5%) (not responding to treatment) and if patients present with PENK values above 100 pmol/1 and reducing their PENK levels below 100 pmol/1 during ICU treatment the mortality was 0 (treatment responder). These data indicate a strong association of PENK and treatment success, supporting its use for therapy follow up (serial testing).

TABLE 8

	mortality	N patients died vs all
PENK >100 pmol/l presentation and first day after ICU treatment	52.5%	21/40
PENK >100 pmol/l at presentation and <100 pmol/l first day after ICU treatment	0%	0/7
PENK <100 pmol/l at presentation and first day of ICU treatment	11%	6/54

FIG. **11***a-d:* examples of patient follow up measurements.

a) A patient (survivor) with initial PENK <100 pmol/L and remained <100 pmol/L during hospital stay.

b) A patient (died during hospital stay) with initial PENK >100 pmol/L and was not reduced to values <100 pmol/L.

c) A patient (died during hospital stay) with initial PENK >100 pmol/L and was not reduced to values <100 pmol/L.

d) A patient (survivor) with initial PENK>100 pmol/L, the PENK value declined to values <100 pmol/L within one day of ICU treatment.

Example 5

The use of serial measurement of PENK

In the patient population described in example 4 (patients with sepsis, severe sepsis or septic shock) plasma PENK was measured on the day of admission and on the following day (day 1). Using a simple cut-off value of 100 pmol/L, which is close to the 99th percentile of the normal range, the population was segmented in two groups (above and below 100 pmol/L) and the corresponding 7 day survival rates were depicted in Kaplan-Meier-Plots (FIGS. **16** *a*) and *b*)). Patients with a PENK concentration below 100 pmol/L on the day of admission, whose PENK concentration remained below 100 pmol/L on day 1, had a high survival rate of 87%, whereas, when their PENK concentration increased over 100 pmol/L on day 1, the survival rate was lowered to 67%. In contrast, patients with a PENK concentration above 100 pmol/L on the day of admission, whose PENK concentration remained above 100 pmol/L on day 1, had a poor survival rate of only 50%, whereas, when their PENK concentration decreased below 100 pmol/L on day 1, the survival rate was 100%.

Example 6

Using the plasma PENK concentrations determined in the patient population described in example 4 (patients with sepsis, severe sepsis or septic shock) on the day of admission, it was analyzed by multivariable linear regression analysis, which parameters/variables determine to which extent the PENK concentrations. In FIG. **17** the partial R2 are depicted. The analysis demonstrates that measures of kidney function (in the case shown creatinine clearance) are by far the strongest determinants for PENK concentrations.

TABLE 9

Association of variables determined in the patient population as described in example 4 on the day of admission with the 7 day mortality.

	Variable—median [IQR]			
	all (n = 101)	deaths within 7 days (n = 28)	7 day survivor (n = 73)	p-value
PENK (pmol/L)	87 [50-205]	209 [77-499]	75 [47-124]	<0.001
Creatinine clearance (ng/mL)	48 [23-77]	33 [15-69]	56 [29-81]	0.071
Apache score (points)	16 [13-21]	23 [18-27]	14 [12-18]	<0.001

PENK in males

Using PENK as prognostic marker, PENK at first day (presentation at ED) was even stronger in prognosis of in hospital death in the male population (AUC 0.849, FIG. **12**), a combination of PENK and Apache resulted in an AUC of 0.89 vs 0.837 Apache alone (FIG. **13**). The combination of PENK and creatinine clearance generated a superior prognostic value of AUC 0.91 vs 0.721 for creatinine clearance alone (FIG. **14**). As for the whole patient population, the prognostic value of PENK was stronger after the first day of ICU-treatment (day 2, AUC 0.872).

BRIEF DESCRIPTION OF FIGURES

FIG. **1**: shows a typical ProEnkephalin dose/signal curve. Standard curve proEnkephalin

FIG. **2**: frequence distribution of Pro Enkephalin in a healthy population (n=4211) The mean value of PENK was 44.7 pmol/L, standard deviation=1.27, the 99percentile (upper normal range) was 80 pmol PENK/L. FIG. **2** shows the LN values of PENK.

FIG. **3**: Correlation of creatinine clearance vs. PENK in healthy subjects. Y axis: quartiles of Creatinine Clearance, x axis: quartiles of PENK.

FIG. **4**: PENK highly correlated to creatinine clearance (r=−0.74, p <0.0001).

FIG. **5**: Increased PENK was significantly correlated with kidney dysfunction.

FIG. **6**: A receiver/operator curve (ROC) for Pro-Enkephalin and the diagnosis of Kidney Dysfunction according the RIFLE criteria (see above). The area under the curve (AUC) was 0.868, indicating a strong diagnostic power of Pro-Enkephalin for Kidney Dysfunction.

FIG. **7**: Increased NGAL was significantly increased in patients with kidney dysfunction. The normal ranges of NGAL (range 0.037-0.106 μg/mL; http://www.bioporto.com/products/bioporto_diagnostics/ngal_elisa_kits/ngl_rapid_elisa_kit_ce_ivd) is indicated by a shadowed area in the graph.

FIG. **8**: A receiver/operator curve (ROC) for NGAL and the diagnosis of Kidney Dysfunction according the RIFLE criteria (see above). We used NGAL as a reference marker for Kidney dysfunction. The AUC was 0.809, substantially lower than for Pro-Enkephalin (AUC 0.868, FIG. **6**), indicating the incremental value of Pro-Enkephalin.

FIG. **9**: PENK is highly prognostic for sepsis outcome

FIG. **10**: There is a significant added information if PENK and APACHE 2 are combined

FIG. **11***a*): A patient (survivor) with initial PENK <100 pmol/L and remained <100 pmol/L during hospital stay

FIG. **11***b*): A patient (died during hospital stay) with initial PENK >100 pmol/L and was not reduced to values <100 pmol/L

FIG. **11***c*): A patient (died during hospital stay) with initial PENK >100 pmol/L and was not reduced to values <100 pmol/L

FIG. **11***d*): A patient (survivor) with initial PENK >100 pmol/L, the PENK value declined to values <100 pmol/L within one day of ICU treatment

FIG. **12**: PENK at first day (presentation at ED) was even stronger in prognosis of in hospital death in the male population

FIG. **13**: A combination of PENK and Apache resulted in an AUC of 0.89 vs. 0.837 Apache alone

FIG. **14**: The combination of PENK and creatinine clearance generated a superior prognostic value of AUC 0.91 vs. 0.721 for creatinine clearance alone

FIG. **15**: FIG. **15** A/B: Concentrations of plasma PENK (A) and NGAL (B), respectively, in septic patients categorized by grade of acute kidney dysfunction. 0=no kidney dysfunction; R=Risk; I=Injury; F=Failure; L=Loss. The categories are defined as follows (http://en.wikipedia.org/wiki/Acute_kidney_injury): Risk: GFR decrease >25%, serum creatinine increased 1.5 times or urine production of <0.5 ml/kg/hr for 6 hours; Injury: GFR decrease >50%, doubling of creatinine or urine production <0.5 ml/kg/hr for 12 hours; Failure: GFR decrease >75%, tripling of creatinine or creatinine >355 μmol/l (with a rise of >44) (>4 mg/dl) OR urine output below 0.3 ml/kg/hr for 24 hours; Loss: persistent AKI or complete loss of kidney function for more than 4 weeks. Normal ranges of PENK (see FIG. **2**) and NGAL (range 0.037-0.106 μg/mL; http://www.bioporto.com/products/bioporto_diagnostics/ngal_elisa_kits/ngal_rapid_elisa_kit_ce_ivd) concentrations are indicated by shadowed areas in the graphs. The figure demonstrates that NGAL concentrations are massively elevated in septic patients even when they have no kidney dysfunction, whereas this is not the case for PENK.

FIG. **16**: Survival rates of critically ill patients depending on their plasma PENK concentrations on the day of admission and on the next day (day 1). Panel A: On the left hand side, the Kaplan-Meier-Plot is shown for those patient subpopulations with a PENK concentration on admission of above and below 100 pmol/L, respectively. On the right hand side the Kaplan-Meier-Plot is shown for those patient subpopulations with a PENK concentration on day 1 of above and below 100 pmol/L, respectively, who had a PENK concentration below 100 pmol/L on the day of admission. Panel B: On the left hand side, the

Kaplan-Meier-Plot is shown for those patient subpopulations with a PENK concentration on admission of above and below 100 pmol/L, respectively. On the right hand side the Kaplan-Meier-Plot is shown for those patient subpopulations with a PENK concentration on day 1 of above and below 100 pmol/L, respectively, who had a PENK concentration above 100 pmol/L on the day of admission.

FIG. **17**: Multivariable linear regression predicting PENK. Note: BP, Creatinine and Urea were left out due to high correlation with MAP or creatinine clearance. The linear regression was calculated using the variables listed as follows: Log(PENK) =a*CreaClearance+b* Cardiovasc+c*MAP+etc . Partial R2 gives the degree up to which each variable contributes to PENK, i.e. Crea Clearance is strongest and has a partial R2 of slightly above 0.15, i.e. crea clearance accounts for about 15% of the variability that you observe in PENK. Importantly, age, gender, etc. do not have a significant influence on PENK concentrations.

SEQUENCE LISTING

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Pro Ser Leu Lys Ile Trp Glu Thr Cys Lys Glu Leu Leu Gln Leu Ser
        35                  40                  45

Lys Pro Glu Leu Pro Gln Asp Gly Thr Ser Thr Leu Arg Glu Asn Ser
```

-continued

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    50                  55                  60

Lys Pro Glu Glu Ser His Leu Leu Ala Lys Arg Tyr Gly Gly Phe Met
65                  70                  75                  80

Lys Arg Tyr Gly Gly Phe Met Lys Lys Met Asp Glu Leu Tyr Pro Met
                85                  90                  95

Glu Pro Glu Glu Glu Ala Asn Gly Ser Glu Ile Leu Ala Lys Arg Tyr
            100                 105                 110

Gly Gly Phe Met Lys Lys Asp Ala Glu Glu Asp Asp Ser Leu Ala Asn
        115                 120                 125

Ser Ser Asp Leu Leu Lys Glu Leu Leu Glu Thr Gly Asp Asn Arg Glu
    130                 135                 140

Arg Ser His His Gln Asp Gly Ser Asp Asn Glu Glu Glu Val Ser Lys
145                 150                 155                 160

Arg Tyr Gly Gly Phe Met Arg Gly Leu Lys Arg Ser Pro Gln Leu Glu
                165                 170                 175

Asp Glu Ala Lys Glu Leu Gln Lys Arg Tyr Gly Gly Phe Met Arg Arg
            180                 185                 190

Val Gly Arg Pro Glu Trp Trp Met Asp Tyr Gln Lys Arg Tyr Gly Gly
        195                 200                 205

Phe Leu Lys Arg Phe Ala Glu Ala Leu Pro Ser Asp Glu Glu Gly Glu
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225                 230                 235                 240

Met Arg Phe


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Pro Ser Leu Lys Ile Trp Glu Thr Cys Lys Glu Leu Leu Gln Leu Ser
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Gly Ser Asp Asn Glu Glu Glu Val Ser
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Glu Val Pro Glu Met Glu

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<400> SEQUENCE: 16

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<400> SEQUENCE: 17

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<400> SEQUENCE: 18

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1 5 10 15

Glu

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1 5

The invention claimed is:

1. A method for (a) diagnosing or monitoring kidney function in a subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse event in a subject with kidney disease wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention in a subject with kidney disease comprising:

determining the level of Pro-Enkephalin or fragments thereof in a bodily fluid obtained from said subject; and

(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in a subject, or

(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a subject with a kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or

(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a subject with a kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 1 or SEQ ID No. 6,

wherein the level of Pro-Enkephalin or fragments thereof is determined by using an assay comprising two binders that bind to two different regions within the region of Pro-Enkephalin that is amino acid 133-140 (LKELLETG, SEQ ID NO. 13) and amino acid 152-159 (SDNEEEVS, SEQ ID No. 14), wherein each of said regions comprises at least 4 amino acids.

2. A method according to claim **1** wherein the binders are antibodies, antibody fragments or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof.

3. A method according to claim **1** comprising

determining the level of immunoreactive analyte by using an assay comprising two binders that bind to two different regions within the region of Pro-Enkephalin that is amino acid 133-140 (LKELLETG, SEQ ID NO. 13) and amino acid 152-159 (SDNEEEVS, SEQ ID No. 14), wherein each of said regions comprises at least 4 amino acids; and

(a) correlating said level of immunoreactive analyte with kidney function in a subject, or

(b) correlating said level of immunoreactive analyte with kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

(c) correlating said level of immunoreactive analyte with said risk of an adverse event in a subject with a kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or

(d) correlating said level of immunoreactive analyte with success of a therapy or intervention in a subject with a kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 1 or SEQ ID No. 6.

4. A method according to claim **1**, wherein said binders bind to a region within an amino acid sequence which is one or more of SEQ ID No. 1 or 6.

5. A method according to claim **1**, wherein said threshold level of Pro-Enkephalin or fragments thereof is 80 pmol/L.

6. A method according to claim **1**, wherein the level of Pro-Enkephalin is measured with an immunoassay and each of said binders is an antibody, or an antibody fragment or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof.

7. A method according to claim **1**, wherein an assay is used for determining the level of Pro-Enkephalin or fragments thereof and wherein the assay sensitivity of said assay is able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is <15 pmol/L.

8. A method according to claim 1, wherein said bodily fluid may be selected from the group comprising blood, serum, plasma, urine, cerebrospinal liquid (csf), and saliva.

9. A method according to claim 1, wherein at least one additional clinical parameter is determined selected from the group comprising: age, BUN, NGAL, Creatinine Clearance, Creatinine and Apache Score.

10. A method according to claim 1, wherein said determination of Pro-Enkephalin or fragments thereof is performed more than once in one subject.

11. A method according to claim 1, wherein said monitoring is performed in order to evaluate the response of said subject to preventive and/or therapeutic measures taken.

12. A method according to claim 1, in order to stratify said subjects into risk groups.

13. A method according to claim 1, wherein said level of Pro-Enkephalin or fragments thereof are correlated with a risk of death or an adverse event in a subject with a kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of death or adverse events and wherein said subject with a kidney disease is male.

14. A point-of-care device for performing assays near a subject, wherein said point-of-care device is capable of performing a method of claim 1 and comprises at least two antibodies or antibody fragments directed to amino acid 133-140 (LKELLETG, SEQ ID No. 13) and amino acid 152-159 (SDNEEEVS, SEQ ID NO. 14).

15. A kit which comprises: at least two antibodies or antibody fragments directed to either amino acid 133-140 (LKELLETG, SEQ ID No. 13) or amino acid 152-159 (SDNEEEVS, SEQ ID NO. 14).

16. A method according to claim 1, wherein said binders do not bind to enkephalin peptides [Met]enkephalin SEQ ID No:3, and [Leu]enkephalin. SEQ ID No: 4.

17. A method according to claim 1, wherein said binders bind to SEQ ID No. 6.

18. A method according to claim 1, wherein said binders bind to a region within an amino acid sequence SEQ ID No. 6.

19. A method comprising

determining the level of Pro-Enkephalin fragments in a bodily fluid obtained from a subject using a binder to said Pro-Enkephalin fragments; and

correlating said level of said Pro-Enkephalin fragments with

kidney function in said subject, or

kidney dysfunction in said subject, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or

success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin fragments is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11.

20. A method according to claim 19 additionally comprising

determining the level of immunoreactive analyte by using at least one binder that binds to a region within the amino acid sequence of Pro-Enkephalin fragments in a bodily fluid obtained from said subject; and

correlating said level of immunoreactive analyte with

kidney function in said subject, or

kidney dysfunction in said subject, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

risk of an adverse event in said subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or

success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin fragment is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11.

21. A method for (a) diagnosing or monitoring kidney function in a subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a subject, wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising

determining the level of Pro-Enkephalin or fragments thereof in a bodily fluid obtained from said subject; and

(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in said subject, or

(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or

(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin or fragments thereof is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11,

wherein the level of Pro-Enkephalin or fragments thereof is determined by using an assay comprising a binder, wherein the binder is selected from the group consisting of an antibody, an antibody fragment, or a non-Ig-Scaffold that does not bind to enkephalin peptides [Met]enkephalin SEQ ID No:3.

22. A method according to claim 21 wherein the binder is selected from the group consisting of an antibody, an antibody fragment, or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof that also does not bind to [Lu]enkephalin. SEQ ID No: 4.

23. A method according to claim 21 further comprising

determining the level of immunoreactive analyte in a bodily fluid obtained from said subject by using an assay comprising a binder that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) or fragments thereof but does not bind to enkephalin peptides [Met]enkephalin SEQ ID No: 3; and

correlating said level of immunoreactive analyte with
kidney function in a subject, or
kidney dysfunction, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or
risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse event, or
success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,
wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11.

24. A method according to claim **23** wherein the binder is selected from the group consisting of an antibody, an antibody fragment, or a non-Ig-Scaffold binding to Pro-Enkephalin or fragments thereof that also does not bind to [Leu]enkephalin SEQ ID No: 4.

25. A method according to claim **21**, wherein said threshold level of Pro-Enkephalin or fragments thereof is 80 pmol/L.

26. A method according to claim **21**, wherein an assay is used for determining the level of Pro-Enkephalin or fragments thereof and wherein the assay sensitivity of said assay is able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is <15 pmol/L.

27. A point-of-care device for performing assays near a subject, wherein said point-of-care device is capable of performing a method of claim **21** and comprises an antibody or antibody fragment that does not bind to enkephalin peptides [Met]enkephalin SEQ ID No: 3.

28. A kit which comprises:
a) a point-of-care device for performing assays near a subject, capable of performing a method of claim **21** and
b) an antibody or antibody fragment that does not bind to enkephalin peptides [Met]enkephalin SEQ ID No: 3.

29. A method for (a) diagnosing or monitoring kidney function in subject or (b) diagnosing kidney dysfunction in a subject or (c) predicting or monitoring the risk of an adverse events in a subject wherein said adverse event is selected from the group comprising worsening of kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or death due to kidney dysfunction including kidney failure, loss of kidney function and end-stage kidney disease or (d) predicting or monitoring the success of a therapy or intervention comprising
determining the level of Pro-Enkephalin or fragments thereof in a bodily fluid obtained from said subject; and
(a) correlating said level of Pro-Enkephalin or fragments thereof with kidney function in a subject, or
(b) correlating said level of Pro-Enkephalin or fragments thereof with kidney dysfunction, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or
(c) correlating said level of Pro-Enkephalin or fragments thereof with said risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse events, or
(d) correlating said level of Pro-Enkephalin or fragments thereof with success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,
wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11,
wherein the level of Pro-Enkephalin or fragments thereof is determined by using an assay with an assay sensitivity able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is <15 pmol/L.

30. A method according to claim **29** additionally comprising
determining the level of immunoreactive analyte in a bodily fluid obtained from said subject by using an assay with at least one binder that binds to a region within the amino acid sequence of Pro-Enkephalin (PENK) or fragments thereof with an assay sensitivity able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is <15 pmol/L; and
correlating said level of immunoreactive analyte with:
kidney function in a subject, or
kidney dysfunction, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or
risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse event, or
success of a therapy or intervention in a subject with kidney disease, wherein a level below a certain threshold is predictive for a success of therapy or intervention,
wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11.

31. A method for diagnosing or monitoring kidney function using a kit or assay to detect Pro-Enkephalin or fragments thereof in a subject, wherein said kit or assay comprises at least one antibody capable of binding Pro-Enkephalin or fragments thereof in a sample of bodily fluid obtained from said subject, said method comprising:
determining the level of Pro-Enkephalin or fragments thereof in a sample of bodily fluid obtained from a subject using an assay comprising an antibody that binds to Pro-Enkephalin or fragments thereof;
wherein said Pro-Enkephalin or fragment is one or more of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10 or SEQ ID No. 11
correlating said level of Pro-Enkephalin or fragments thereof with
kidney function in a subject, or
kidney dysfunction wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or
risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse event, or
success of a therapy or intervention in a subject with kidney disease,
wherein a level below a certain threshold is predictive for a success of therapy or intervention.

32. A method of using a kit or assay according to claim 31 additionally comprising

determining the level of immunoreactive analyte by using an assay comprising an antibody that binds to a region within the amino acid sequence of Pro-Enkephalin or fragments thereof in a bodily fluid obtained from said subject; and

correlating said level of immunoreactive analyte with

kidney function in a subject, or

kidney dysfunction in a subject, wherein an elevated level above a certain threshold is predictive or diagnostic for kidney dysfunction in said subject, or

risk of an adverse event in a subject with kidney disease, wherein an elevated level above a certain threshold is predictive for an enhanced risk of said adverse event, or

success of a therapy or intervention in a subject with kidney disease,

wherein a level below a certain threshold is predictive for a success of therapy or intervention,

wherein said Pro-Enkephalin or fragment is selected from the group consisting of SEQ ID No. 1, 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.

33. A method according to claim 31, wherein the antibody of said kit or assay does not bind to enkephalin peptides [Me]enkephalin SEQ ID No: 3, and [Leu]enkephalin. SEQ ID No: 4.

34. A method according to claim 31, wherein the antibody of said kit or assay binds to SEQ ID No. 6.

35. A method according to claim 31, wherein the antibody of said kit or assay binds to a region within an amino acid sequence selected from the group consisting of SEQ ID No. 1, 2, 5, 6, 7, 8, 9 and 10.

36. A method according to claim 31, wherein the antibody of said kit or assay binds to a region within an amino acid sequence selected from the group consisting of SEQ ID No. 2, 5, 6, and 10.

37. A method according to claim 31, wherein the sensitivity of the assay used for determining the level of Pro-Enkephalin or fragments thereof is able to quantify the Pro-Enkephalin or Pro-Enkephalin fragments of healthy subjects and is <15 pmol/L.

* * * * *

专利名称(译)	诊断或监测肾功能或诊断肾功能障碍的方法		
公开(公告)号	US9664695	公开(公告)日	2017-05-30
申请号	US14/432957	申请日	2013-10-01
[标]申请(专利权)人(译)	思芬构技术有限公司		
申请(专利权)人(译)	SPHINGOTEC GMBH		
当前申请(专利权)人(译)	SPHINGOTEC GMBH		
[标]发明人	BERGMANN ANDREAS		
发明人	BERGMANN, ANDREAS		
IPC分类号	G01N33/74 G01N33/53 G01N33/543 G01N33/68		
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优先权	2012187051 2012-10-02 EP 2013170327 2013-06-03 EP		
其他公开文献	US20150260732A1		
外部链接	Espacenet USPTO		

摘要(译)

（a）诊断或监测受试者的肾功能或（b）诊断受试者的肾功能障碍或（c）预测或监测患病受试者中的不良事件的风险或（d）预测或监测受试者的成功的方法治疗或干预包括 确定从所述受试者获得的体液中至少5个氨基酸的前脑啡肽（PENK）或其片段的水平;并将所述Pro-Enkephalin或其片段的水平与（a）受试者的肾功能或（b）所述受试者的肾功能不全或（c）不良事件风险增加或（d）治疗或干预患病受试者的成功。

Fig. 1: