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- (71) **Applicant (for DE only):** **ROCHE DIAGNOSTICS GMBH** [DE/DE]; Sandhofer Strasse 116, 68305 Mannheim (DE).
- (71) **Applicant (for all designated States except DE, US):** **F. HOFFMANN-LA ROCHE AG** [—/CH]; Grenzacher Strasse 124, CH-4070 Basel (CH).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** **HESS, Georg** [DE/DE]; Oppenheimer Str. 82, 55130 Mainz (DE). **ZDUNEK, Dietmar** [DE/DE]; Elly-Ney Straße 3, 82327 Tutzing (DE). **HORSCH, Andrea** [DE/DE]; Christian Morgenstern Straße 11, 68259 Mannheim (DE).
- (74) **Agent:** **DICK, Alexander**; Herzog Fiesser & Partner, Patentanwälte, Dudenstrasse 46, 68167 Mannheim (DE).

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(54) **Title:** DIAGNOSIS OF KIDNEY INJURY AFTER SURGERY

(57) **Abstract:** The present invention relates to the field of laboratory diagnostics. Specifically, means and methods for diagnosing acute kidney injury associated with a major surgery based on the biomarker sFlt-1 are disclosed. Moreover, the present invention relates to means and methods for monitoring a major surgery.



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Diagnosis of kidney injury after surgery

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The present invention relates to the field of laboratory diagnostics. Specifically, means and methods for diagnosing acute kidney injury associated with a major surgery based on the biomarker sFlt-1 are disclosed.

10 Patients with advanced coronary artery disease may suffer from stable and/or unstable angina. This is caused by advanced coronary artery disease which can be classified into 1, 2 or 3 vessel disease depending on the number of affected coronary arteries. Patients with coronary artery disease may benefit from percutaneous cardiovascular intervention (PCI) or from coronary artery bypass graft surgery (CABG) as recommended from the
15 ACC/AHA 2004 guideline update for coronary artery bypass graft surgery: a report of the American College of Cardiology/American Heart Association Task Force of Practice Guidelines (Committee to Update the 1999 Guidelines for coronary Artery Bypass Graft Surgery) (Circulation 110 (14) e 340 – e437).

20 While PCI is primarily useful in patients with single stenosis of main arteries or with a limited number of stenoses in major vessels, CABG is primarily useful in multiple vessel disease and multiple stenoses.

Coronary bypass surgery is, however associated with a significant risk of complications.

25 The incidence of acute kidney injury (AKI) after coronary bypass surgery ranges from 10 to 20 % (Mehta et al, Circulation 2006, 114: 2208 – 2216). One to 5 percent of these individuals require postoperative dialysis. The pathogenesis of postoperative AKI is multifactorial and its association with increased morbidity and long term mortality after cardiac surgery is well established. (Brown et al, Annals of Thoracic Surgery 2008, 86: 4 – 11:
30 Kourliouros et al, European Journal of Cardiothoracic Surgery 2009, in press).

AKI may be prevented in risk patients. Prevention includes careful fluid balance during and after surgery, avoidance of low cardiopulmonary bypass /CPB) perfusion temperatures (Kourliouros et al), avoidance or discontinuation of potentially nephrotoxic drugs prior to surgery or application of drugs such as erythropoietin after surgery (Song et al American Journal of Nephrology 2009, 30: 253 – 260).

Soluble fms-like tyrosine kinase-1 (sFlt-1 or sVEGFR-1) is a splice variant of VEGF receptor 1 (FLT-1) which is produced by a variety of tissues. It is a tyrosine kinase protein that disables proteins that cause blood vessel growth.

Ataga et al. (Eur J Haematol. 2010 Sep;85(3):257-63) discloses that in sickle cell disease patients, sFlt-1 serum levels correlate with the magnitude of albuminuria which in turn indicates the degree of renal impairment.

Marco et al. (J Am Soc Nephrol 20: 2235-2245) discloses that increased sFlt-1 (serum) levels are associated with impairment of renal function in an animal model of chronic kidney disease. It also discloses that sFlt-1 levels rise early in chronic kidney disease patients and that sFlt-1 possesses antiangiogenic effects which may contribute to the impairment of renal function.

Acute kidney injury can only be diagnosed by assessing the serum creatinine level. An absolute increase in the serum creatinine concentration of larger than 0.3 mg/dL (26.4 micromol/L) from baseline, or a more than 50 percent increase in the serum creatinine in a period of 48 hours is indicative for the diagnosis of acute kidney injury. The determination of serum creatinine, however, has the disadvantage that the diagnosis of acute kidney injury can be only diagnosed at late stage of acute kidney injury (approximately 2 days after the acute event).

The biomarker sFlt-1 is frequently measured in combination with the biomarker PlGF (Placental Growth Factor) in order for assessing preeclampsia. In particular, the ratio of sFlt-1 to PlGF has been shown to be a reliable tool for predicting preeclampsia. Studies show that the determination of the ratio is a much better predictor of preeclampsia than the determination of sFlt-1 or PlGF alone (Hirashima C, et al. Establishing Reference Values

for Both Total Soluble Fms-Like Tyrosine Kinase 1 and Free Placental Growth Factor in Pregnant Woman. Hypertens Res 2005;28:727-732).

5 It is of high importance to diagnose acute kidney injury in subjects undergoing a major surgery as early as possible, since the early detection of acute kidney injury allows for an early treatment of acute kidney injury.

10 Consequently, the technical problem underlying the present invention could be seen as the provision of means and methods for the identification of individuals suffering from kidney injury associated with a major surgery. The problem is solved by the embodiments of the present invention described in the claims and in the specification below.

Accordingly, the present invention relates to a method for diagnosing acute kidney injury associated with major surgery in a subject, said method comprising the steps of

- 15 a) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a first sample of said subject obtained prior to major surgery;
- b) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a second sample of said subject, wherein said second sample has been obtained during or after said major surgery, and
- 20 c) comparing the amount of sFlt-1 in said second sample to the amount of sFlt-1 in said first sample, whereby acute kidney injury is diagnosed.

Preferably, acute kidney injury is diagnosed by carrying out the further step of d) diagnosing acute kidney injury based on the result of the comparison carried out in step c).

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The method of the present invention, preferably, is an ex vivo or in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate to sample pre-treatments or evaluation of the results obtained by the method. The method may be carried out manually or assisted by automation. Preferably, step (a), (b) and/or (c) may in total or in part be assisted by automation, e.g., by a suitable robotic and sensory equipment for the determination in step (a), or (b), or a computer-implemented comparison and/or diagnosis based on said comparison in step (b).

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The term “diagnosing” as used herein, preferably, means assessing whether a subject as referred to herein has developed acute kidney injury associated with major surgery, or, whether a subject is at risk of developing acute kidney injury associated with major surgery. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for 100% of the subjects to be diagnosed. The term, however, requires that the assessment is correct for a statistically significant portion of the subjects (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistical evaluation tools, e.g., determination of confidence intervals, p-value determination, Student’s t-test, Mann-Whitney test etc.. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001.

The expression “diagnosis of the risk” as used herein refers to assessing the probability according to which a subject will suffer from acute kidney injury within a certain time window, i.e. the predictive window. In accordance with the present invention, the predictive window, preferably, is within about 3 hours, about 6 hours, about 12 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days or about 1 week after completion of the surgery, preferably after the respiration support (e.g. heart-lung machine) was disconnected, after the patient left the operation room or after the subject arrived at the intensive-care unit. The term, preferably, relates to predicting whether or not there is an increased risk for acute kidney injury compared to the average risk for developing acute kidney injury in a population of subjects rather than giving a precise probability for the said risk.

Preferably, the term "about" as used herein encompasses a range of + and — 20% relative to the specific value, amount, concentration, level, time period etc., e.g., indication of a value of "about 100" is meant to encompass a value of a numerical range of 100 +/- 20%, i.e. a value range from 80 to 120. Preferably, the encompasses a range of + and - 10 % relative to the specific value, amount, concentration, level, time period etc. and, more preferably, a range of + and — 10 % relative to the specific value, amount, concentration, lev-

el, time period etc. Most preferably, the term "about" refers to the exact value, amount, concentration, level, etc.

5 The term "subject" as used herein relates to animals, preferably mammals, and, more preferably, humans. The subject according to the present invention, preferably, shall undergo major surgery. Preferably, the subject undergoes major surgery after obtaining said first sample.

10 It is, however, contemplated that the subject shall not exhibit an acute coronary syndrome (ACS) at the time at which the sample(s) as referred to herein is (are) obtained. Moreover, the subject, preferably, is not pregnant.

In is preferred that the subject shall not exhibit impaired renal function prior to major surgery (for an explanation of this term see above). How to assess whether a subject exhibits
15 impaired renal function is well known in the art. Renal disorders can be diagnosed by any means known and deemed appropriate. Particularly, renal function can be assessed by means of the glomerular filtration rate (GFR). For example, the GFR may be calculated by the Cockcroft-Gault or the MDRD formula (Levey 1999, Annals of Internal Medicine, 461-470). GFR is the volume of fluid filtered from the renal glomerular capillaries into the
20 Bowman's capsule per unit time. Clinically, this is often used to determine renal function. The GFR was originally estimated (the GFR can never be determined, all calculations derived from formulas such as the Cockcroft Gault formula of the MDRD formula deliver only estimates and not the "real" GFR) by injecting inulin into the plasma. Since inulin is not reabsorbed by the kidney after glomerular filtration, its rate of excretion is directly
25 proportional to the rate of filtration of water and solutes across the glomerular filter. In clinical practice however, creatinine clearance is used to measure GFR. Creatinine is an endogenous molecule, synthesized in the body, which is freely filtered by the glomerulus (but also secreted by the renal tubules in very small amounts). Creatinine clearance (CrCl) is therefore a close approximation of the GFR. The GFR is typically recorded in milliliters
30 per minute (mL/min). The normal range of GFR for males is 97 to 137 mL/min, the normal range of GFR for females is 88 to 128 ml/min. Thus, it is particularly contemplated that the GFR of a subject who does not exhibit impaired renal function is within this range. Moreover, said subject preferably, has a blood creatinine level (in particular a serum creatinine

level) of lower than 0.9 mg/dl, more preferably of lower than 1.1 mg/dl and most preferably of lower than 1.3 mg/dl.

It is further contemplated that the subject shall have a risk of suffering from acute kidney injury associated with major surgery. A “subject having a risk of suffering from acute kidney injury associated with major surgery” (herein also referred to a “risk subject”) according to the present application is a patient who will suffer from acute kidney injury following said major surgery with a statistically significant increased probability compared to the incidence of said acute kidney injury in a population, preferably a control population or a randomized population, of subjects subjected to the intervention. Preexisting underlying disorders increase the risk that the subject will suffer from kidney injury following a surgical procedure. Preferably, the risk subject is a subject suffering from heart failure, hypertension, a cardiovascular disease (such as coronary artery disease) and/or diabetes. More preferably, the risk subject suffers from cardiovascular atherosclerosis that requires treatment by coronary artery bypass surgery (CABP). In a subject suffering from cardiovascular artherosclerosis accompanying diseases, preferably diabetes and cardiovascular disease, the risk of the patient to suffer from acute kidney injury increases. Diabetes is, preferably, type 1 or, more preferably, type 2 diabetes.

The subject to be tested in the context of the present invention may exhibit a pre-existing disorder of the renal tubules (for an explanation of this term, see below) before the major surgery. However, it is preferred that the subject does not exhibit a pre-existing disorder of the renal tubules. It has been shown in the context of the present invention that patients with AKI associated with major surgery without pre-existing disorders of the renal tubules had the highest levels of sFlt-1.

The term “major surgery” as used in the context of the present invention, preferably, refers to any surgery that involves anesthesia and/or respiratory assistance. Preferably, the anesthesia is localized anesthesia, more preferably, the anesthesia is global anesthesia.

Accordingly, the term “major surgery”, preferably, includes interventions on inner organs (in particular on the liver, kidney, bowel, stomach, lung, without being exhaustive) as long as it involves anesthesia and/or respiratory assistance. Preferably, the term “major surgery”

also includes trauma surgery and burn surgery as long as it involves anesthesia and/or respiratory assistance. In particular, the term includes interventions on the heart (in particular, on the valve or any part of the myocard) that involves anesthesia and/or respiratory assistance, herein also referred to as cardiac surgery.

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Cardiac surgery is, preferably, coronary artery bypass graft (CABG) surgery (frequently also referred to as “aortocoronary bypass” or “coronary artery bypass surgery”). CABG is indicated if a patient suffers from stenosis of the coronary arteries which cannot be treated successfully with other methods such percutaneous coronary intervention (PCI). This is typically the case if multiple vessels are affected or if the stenosis is not clearly localized. In this surgery, arteries or veins are grafted to the coronary arteries. Thereby, it is possible to bypass atherosclerotic narrowings and improve the blood supply to the myocardium. CABG is either performed “on-pump” i.e. the heart is stopped and does not beat during surgery, or “off-pump”, i.e. the heart continues to beat during the procedure. It is particularly preferred that CABP is performed “on-pump”. Further preferred is a bybass of obstructed coronary vessels using vessels preferably veins from other parts of the body (preferably the legs) or an implanation of the arteria thoracica interna into the heart to bridge obstructed vessels or to provide additional blood supply to the heart.

The term “acute kidney injury” or “AKI” as used herein refers to a rapid loss of kidney function. Preferably, said rapid loss of kidney function is caused by damage to the kidney(s). Preferably, said AKI is post-renal, intrinsic and, more preferably, pre-renal. The endpoint acute kidney injury within said window will become apparent, e.g. by an increase of the serum creatinine as defined elsewhere in this specification. Preferably, AKI is characterized by an increase of serum creatinine of at least 0.3 mg/dl within 48 hours after surgery or by an increase of at least 50 % from baseline (preferably within 48 hours after surgery). Methods and criteria to diagnose AKI have been described in Mehta et al. (2007) Critical Care (London, England) 11 (2): R31. doi:10.1186/cc5713. PMC 2206446. PMID 17331245).

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AKI may lead to various complications such metabolic acidosis, high potassium levels, uremia, changes in body fluid balance. Said changes in fluid balance may result in heart failure (or may worsen heart failure). Moreover, it may effect other organ systems. In se-

vere cases of acute kidney injury, renal replacement therapy (including e.g. hemodialysis or renal transplantation) may be required. Early detection of AKI is of high importance, since it allows for an early treatment, thereby minimizing the risks associated with kidney injury. In particular, early recognition of AKI allows for taking measures to avoid necessity of dialysis or complications that might be associated with fluid retention and volume overload such as heart failure.

It has been shown in the context of the present invention that the determination of sFlt-1 allows for an early assessment of AKI, i.e. even before AKI can be diagnosed via the determination of the aforementioned increase of serum creatinine. Accordingly, sFlt-1 is an earlier marker for AKI than creatinine. Thus, by determining the amount of sFlt-1 an increase of creatinine can be predicted. Preferably, an increase of serum creatinine of at least 0.3 mg/dl or by an increase of at least 50 % within 48 hours after surgery can be predicted. Accordingly, the term “diagnosis of AKI” as used herein, preferably, also refers to the prediction of an increase of serum creatinine of at least 0.3 mg/dl or by an increase of at least 50 % within 48 hours after surgery.

It is to be understood that the AKI shall be associated with the major surgery. The term “associated with” as used herein, preferably, refers to a statistical, temporal and/or causal relationship between the AKI and the major surgery. The person skilled in the art understands what is meant if AKI is considered to be associated with a major surgery. Particularly, the AKI shall be considered to be associated with a major surgery, if it is caused by said major surgery. Indication for such causal connection is e.g. a close time-relationship as well as a close statistical relationship between said major surgery and the incidence of AKI. In particular, the AKI is associated with said major surgery, if the AKI arises/occurs during or immediate after a major surgery (and, thus, was not present prior to said major surgery).

The term “sample” refers to a sample of a body fluid, to a sample of separated cells or to a sample from a tissue or an organ. Samples of body fluids can be obtained by well known techniques and include, preferably, samples of blood, plasma, serum, or urine, more preferably, samples of blood, plasma or serum. Tissue or organ samples may be obtained from any tissue or organ by, e.g., biopsy. Separated cells may be obtained from the body fluids

or the tissues or organs by separating techniques such as centrifugation or cell sorting. Preferably, cell-, tissue- or organ samples are obtained from those cells, tissues or organs which express or produce the peptides referred to herein.

- 5 The first sample is, preferably, obtained prior to major surgery, i.e. before the subject undergoes the surgical procedure. More preferably, the sample has been obtained not more than two weeks, not more than one week, or, more preferably, not more than 3 days, and even more preferably, not more than one day before said surgery. It is particularly contemplated that the first sample has been obtained not more than 6 hours, not more than 3 hours,
10 not more than 2 hours, and, more preferably, 1 hour before the major surgery.

The “second sample” is, preferably, understood as a sample which is obtained in order to reflect a change of the amount of the sFlt-1 as compared to the amount of the respective marker in the first sample. The second sample shall be obtained after the first sample. Preferably, the second sample is not obtained too early after the first sample (in order to observe a sufficiently significant change to allow for monitoring). Preferably, the second sample has been obtained during or after a major surgery. It is particularly contemplated that the second sample has been obtained after the surgical procedure has been completed, preferably, within a time period of not later than 1, 2 or 3 days after surgery. It is more
15 preferred that the second sample has been obtained immediately or not later than 1 day after completion of the surgery. It is most preferred that the sample has been obtained immediately after surgery. The term “immediately after surgery”, preferably, to a sample, that has been obtained not later than about 0.5, not later than about 1, not later than about 2, not later than about 3 or not later than about 6 hours after surgery, most preferably not later
20 than 0.5 hours after surgery. Accordingly, the sample is preferably obtained within a period of 0 to 6 hours, 0 to 3 hours, 0 to 2 hours, or 0 to 1 hour after the surgery (or more precisely after completion of the surgery). It is most preferred that the second sample has been obtained at the end of surgery. Preferably, the time “X hours after surgery” is calculated from the time point when the respiration support (e.g. heart-lung machine) is disconnected,
25 when the patient leaves the operation room or when the subject arrives at the intensive-care unit.
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Preferably, at least one further sample is obtained in order to further monitor the change of the amount of sFlt-1 to herein. Such further sample may be obtained, preferably, 1 to 12 hours, 2 to 9 hours, and, more preferably, 3 to 6 hours after said second sample.

- 5 The term “soluble Flt-1” or “sFlt-1” (abbreviation for Soluble fms-like tyrosine kinase-1) as used herein refers to polypeptide which is a soluble form of the VEGF receptor Flt1. It was identified in conditioned culture medium of human umbilical vein endothelial cells. The endogenous soluble Flt1 (sFlt-1) receptor is chromatographically and immunologically similar to recombinant human sFlt-1 and binds [125I] VEGF with a comparable high affinity.
- 10 Human sFlt-1 is shown to form a VEGF-stabilized complex with the extracellular domain of KDR/Flk-1 in vitro. Preferably, sFlt-1 refers to human sFlt-1 as described in Kendall 1996, Biochem Biophys Res Commun 226(2): 324-328 (for amino acid sequences, see, e.g., also P17948, GI: 125361 for human and BAA24499.1, GI: 2809071 for mouse sFlt-1). The term also encompasses variants of the aforementioned human sFlt-1 polypeptides.
- 15 Variants of a polypeptide have at least the same essential biological and immunological properties as said polypeptide. In particular, they share the same essential biological and immunological properties if they are detectable by the same specific assays referred to in this specification, e.g., by ELISA assays using polyclonal or monoclonal antibodies specifically recognizing the said polypeptides. Moreover, it is to be understood that a variant as
- 20 referred to in accordance with the present invention shall have an amino acid sequence which differs due to at least one amino acid substitution, deletion and/or addition wherein the amino acid sequence of the variant is still, preferably, at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identical with the amino sequence of the specific sFlt-1 polypeptide, preferably over the entire length of the human sFlt-1, respectively.
- 25 Preferably, the degree of identity is to be determined by comparing two optimally aligned sequences over a comparison window, where the fragment of amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment. The percentage is calculated by determining the number of positions at
- 30 which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conduct-

ed by the local homology algorithm of Smith and Waterman *Add. APL. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (USA)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment and, thus, the degree of identity. Preferably, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. Variants referred to above may be allelic variants or any other species specific homologs, paralogs, or orthologs. Moreover, the variants referred to herein include fragments or subunits of the specific sFlt-1 polypeptides or the aforementioned types of variants as long as these fragments have the essential immunological and biological properties as referred to above. Such fragments may be, e.g., degradation products of the sFlt-1 polypeptides. Further included are variants which differ due to posttranslational modifications such as phosphorylation or myristylation.

Determining the amount of a peptide or polypeptide referred to in this specification relates to measuring the amount or concentration, preferably, semi-quantitatively or quantitatively. Measuring can be done directly or indirectly. Direct measuring relates to measuring the amount or concentration of the peptide or polypeptide based on a signal which is obtained from the peptide or polypeptide itself and the intensity of which directly correlates with the number of molecules of the peptide present in the sample. Such a signal – sometimes referred to herein as intensity signal -may be obtained, e.g., by measuring an intensity value of a specific physical or chemical property of the peptide or polypeptide. Indirect measuring includes measuring of a signal obtained from a secondary component (i.e. a component not being the peptide or polypeptide itself) or a biological read out system, e.g., measurable cellular responses, ligands, labels, or enzymatic reaction products.

In accordance with the present invention, determining the amount of a peptide or polypeptide can be achieved by all known means for determining the amount of a peptide in a sample. Said means comprise immunoassay and methods which may utilize labeled molecules in various sandwich, competition, or other assay formats. Such assays are, preferably,

based on detection agents such as antibodies which specifically recognize the peptide or polypeptide to be determined. The detection agents shall be either directly or indirectly capable of generating a signal indicating the presence or absence of the peptide or polypeptide. Moreover, the signal strength can, preferably, be correlated directly or indirectly (e.g. reverse- proportional) to the amount of polypeptide present in a sample. Further suitable methods comprise measuring a physical or chemical property specific for the peptide or polypeptide such as its precise molecular mass or NMR spectrum. Said methods comprise, preferably, biosensors, optical devices coupled to immunoassays, biochips, analytical devices such as mass- spectrometers, NMR- analyzers, or chromatography devices. Further, methods include micro-plate ELISA-based methods, fully-automated or robotic immunoassays (available for example on ElecsysTM analyzers), CBA (an enzymatic Cobalt Binding Assay, available for example on Roche-HitachiTM analyzers), and latex agglutination assays (available for example on Roche-HitachiTM analyzers).

Preferably, determining the amount of a peptide or polypeptide comprises the steps of (a) contacting a cell capable of eliciting a cellular response the intensity of which is indicative of the amount of the peptide or polypeptide with the said peptide or polypeptide for an adequate period of time, (b) measuring the cellular response. For measuring cellular responses, the sample or processed sample is, preferably, added to a cell culture and an internal or external cellular response is measured. The cellular response may include the measurable expression of a reporter gene or the secretion of a substance, e.g. a peptide, polypeptide, or a small molecule. The expression or substance shall generate an intensity signal which correlates to the amount of the peptide or polypeptide.

Also preferably, determining the amount of a peptide or polypeptide comprises the step of measuring a specific intensity signal obtainable from the peptide or polypeptide in the sample. As described above, such a signal may be the signal intensity observed at an m/z variable specific for the peptide or polypeptide observed in mass spectra or a NMR spectrum specific for the peptide or polypeptide.

Determining the amount of a peptide or polypeptide may, preferably, comprises the steps of (a) contacting the peptide with a specific ligand, (b) (optionally) removing non-bound ligand, (c) measuring the amount of bound ligand. The bound ligand will generate an intensity signal. Binding according to the present invention includes both covalent and non-covalent binding. A ligand according to the present invention can be any compound, e.g., a peptide, polypeptide, nucleic acid, or small molecule, binding to the peptide or polypeptide described herein. Preferred ligands include antibodies, nucleic acids, peptides or polypeptides such as receptors or binding partners for the peptide or polypeptide and fragments thereof comprising the binding domains for the peptides, and aptamers, e.g. nucleic acid or peptide aptamers. Methods to prepare such ligands are well-known in the art. For example, identification and production of suitable antibodies or aptamers is also offered by commercial suppliers. The person skilled in the art is familiar with methods to develop derivatives of such ligands with higher affinity or specificity. For example, random mutations can be introduced into the nucleic acids, peptides or polypeptides. These derivatives can then be tested for binding according to screening procedures known in the art, e.g. phage display. Antibodies as referred to herein include both polyclonal and monoclonal antibodies, as well as fragments thereof, such as Fv, Fab and F(ab)₂ fragments that are capable of binding antigen or hapten. The present invention also includes single chain antibodies and humanized hybrid antibodies wherein amino acid sequences of a non-human donor antibody exhibiting a desired antigen-specificity are combined with sequences of a human acceptor antibody. The donor sequences will usually include at least the antigen-binding amino acid residues of the donor but may comprise other structurally and/or functionally relevant amino acid residues of the donor antibody as well. Such hybrids can be prepared by several methods well known in the art. Preferably, the ligand or agent binds specifically to the peptide or polypeptide. Specific binding according to the present invention means that the ligand or agent should not bind substantially to ("cross-react" with) another peptide, polypeptide or substance present in the sample to be analyzed. Preferably, the specifically bound peptide or polypeptide should be bound with at least 3 times higher, more preferably at least 10 times higher and even more preferably at least 50 times higher affinity than any other relevant peptide or polypeptide. Non-specific binding may be tolerable, if it can still be distinguished and measured unequivocally, e.g. according to its size on a Western Blot, or by its relatively higher abundance in the sample. Binding of the ligand can be measured by any method known in the art. Preferably, said method is semi-quantitative or quantita-

tive. Further suitable techniques for the determination of a polypeptide or peptide are described in the following.

First, binding of a ligand may be measured directly, e.g. by NMR or surface plasmon resonance. Second, if the ligand also serves as a substrate of an enzymatic activity of the peptide or polypeptide of interest, an enzymatic reaction product may be measured (e.g. the amount of a protease can be measured by measuring the amount of cleaved substrate, e.g. on a Western Blot). Alternatively, the ligand may exhibit enzymatic properties itself and the "ligand/peptide or polypeptide" complex or the ligand which was bound by the peptide or polypeptide, respectively, may be contacted with a suitable substrate allowing detection by the generation of an intensity signal. For measurement of enzymatic reaction products, preferably the amount of substrate is saturating. The substrate may also be labeled with a detectable label prior to the reaction. Preferably, the sample is contacted with the substrate for an adequate period of time. An adequate period of time refers to the time necessary for an detectable, preferably measurable, amount of product to be produced. Instead of measuring the amount of product, the time necessary for appearance of a given (e.g. detectable) amount of product can be measured. Third, the ligand may be coupled covalently or non-covalently to a label allowing detection and measurement of the ligand. Labeling may be done by direct or indirect methods. Direct labeling involves coupling of the label directly (covalently or non-covalently) to the ligand. Indirect labeling involves binding (covalently or non-covalently) of a secondary ligand to the first ligand. The secondary ligand should specifically bind to the first ligand. Said secondary ligand may be coupled with a suitable label and/or be the target (receptor) of tertiary ligand binding to the secondary ligand. The use of secondary, tertiary or even higher order ligands is often used to increase the signal. Suitable secondary and higher order ligands may include antibodies, secondary antibodies, and the well-known streptavidin-biotin system (Vector Laboratories, Inc.). The ligand or substrate may also be "tagged" with one or more tags as known in the art. Such tags may then be targets for higher order ligands. Suitable tags include biotin, digoxigenin, His-Tag, Glutathion-S-Transferase, FLAG, GFP, myc-tag, influenza A virus haemagglutinin (HA), maltose binding protein, and the like. In the case of a peptide or polypeptide, the tag is preferably at the N-terminus and/or C-terminus. Suitable labels are any labels detectable by an appropriate detection method. Typical labels include gold particles, latex beads, acridan

ester, luminol, ruthenium, enzymatically active labels, radioactive labels, magnetic labels ("e.g. magnetic beads", including paramagnetic and superparamagnetic labels), and fluorescent labels. Enzymatically active labels include e.g. horseradish peroxidase, alkaline phosphatase, beta-Galactosidase, Luciferase, and derivatives thereof. Suitable substrates for detection include di-amino-benzidine (DAB), 3,3'-5,5'-tetramethylbenzidine, NBT-BCIP (4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, available as ready-made stock solution from Roche Diagnostics), CDP-Star™ (Amersham Biosciences), ECF™ (Amersham Biosciences). A suitable enzyme-substrate combination may result in a colored reaction product, fluorescence or chemoluminescence, which can be measured according to methods known in the art (e.g. using a light-sensitive film or a suitable camera system). As for measuring the enzymatic reaction, the criteria given above apply analogously. Typical fluorescent labels include fluorescent proteins (such as GFP and its derivatives), Cy3, Cy5, Texas Red, Fluorescein, and the Alexa dyes (e.g. Alexa 568). Further fluorescent labels are available e.g. from Molecular Probes (Oregon). Also the use of quantum dots as fluorescent labels is contemplated. Typical radioactive labels include ^{35}S , ^{125}I , ^{32}P , ^{33}P and the like. A radioactive label can be detected by any method known and appropriate, e.g. a light-sensitive film or a phosphor imager. Suitable measurement methods according to the present invention also include precipitation (particularly immunoprecipitation), electrochemiluminescence (electro-generated chemiluminescence), RIA (radioimmunoassay), ELISA (enzyme-linked immunosorbent assay), sandwich enzyme immune tests, electrochemiluminescence sandwich immunoassays (ECLIA), dissociation-enhanced lanthanide fluoro immuno assay (DELFI), scintillation proximity assay (SPA), turbidimetry, nephelometry, latex-enhanced turbidimetry or nephelometry, or solid phase immune tests. Further methods known in the art (such as gel electrophoresis, 2D gel electrophoresis, SDS polyacrylamid gel electrophoresis (SDS-PAGE), Western Blotting, and mass spectrometry), can be used alone or in combination with labeling or other detection methods as described above.

The amount of a peptide or polypeptide may be, also preferably, determined as follows: (a) contacting a solid support comprising a ligand for the peptide or polypeptide as specified above with a sample comprising the peptide or polypeptide and (b) measuring the amount peptide or polypeptide which is bound to the support. The ligand, preferably chosen from

the group consisting of nucleic acids, peptides, polypeptides, antibodies and aptamers, is preferably present on a solid support in immobilized form. Materials for manufacturing solid supports are well known in the art and include, inter alia, commercially available column materials, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass and/or silicon chips and surfaces, nitrocellulose strips, membranes, sheets, duracytes, wells and walls of reaction trays, plastic tubes etc. The ligand or agent may be bound to many different carriers. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for the purposes of the invention. Suitable methods for fixing/immobilizing said ligand are well known and include, but are not limited to ionic, hydrophobic, covalent interactions and the like. It is also contemplated to use "suspension arrays" as arrays according to the present invention (Nolan 2002, Trends Biotechnol. 20(1):9-12). In such suspension arrays, the carrier, e.g. a microbead or microsphere, is present in suspension. The array consists of different microbeads or microspheres, possibly labeled, carrying different ligands. Methods of producing such arrays, for example based on solid-phase chemistry and photo-labile protective groups, are generally known (US 5,744,305).

The term "amount" as used herein encompasses the absolute amount of a polypeptide or peptide, the relative amount or concentration of the said polypeptide or peptide as well as any value or parameter which correlates thereto or can be derived therefrom. Such values or parameters comprise intensity signal values from all specific physical or chemical properties obtained from the said peptides by direct measurements, e.g., intensity values in mass spectra or NMR spectra. Moreover, encompassed are all values or parameters which are obtained by indirect measurements specified elsewhere in this description, e.g., response levels determined from biological read out systems in response to the peptides or intensity signals obtained from specifically bound ligands. It is to be understood that values correlating to the aforementioned amounts or parameters can also be obtained by all standard mathematical operations.

The expressing "comparing the amount in said first sample as compared to the amount in said second sample" as used herein in the context of the encompasses comparing the

amount of a sFlt-1 in a first sample with an amount of said marker in a second sample. The terms "first sample" and "second sample" are specified herein above. It is to be understood that comparing as used herein refers to a comparison of corresponding parameters or values. It is to be understood that an amount of a marker in a first sample is compared to an amount of the amount of the same marker, in a second sample. The comparison referred to in the context of the method of the present invention may be carried out manually or computer assisted. For a computer assisted comparison, the value of the determined amount in the second sample may be compared to a value of the determined amount in a first sample which is stored in a database by a computer program. The computer program may further evaluate the result of the comparison, i.e. automatically provide the desired assessment in a suitable output format. Based on the comparison of the amount of a biomarker as referred to herein in a first sample to the amount in a second sample it is possible to monitor major surgery and, thus, to diagnose whether AKI has occurred during said surgery or not.

As set forth above, the diagnosis of AKI is, preferably, based on the comparison of the amount of a sFlt-1 in a first sample to the amount of the sFlt-1 in a second sample obtained after said first sample (or vice versa).

Preferably, an increased amount, more preferably, a significantly increased, and most preferably, a statistically significant increase of the amount of sFlt-1 in the second sample as compared to the amount of sFlt-1 in said first sample is indicative for the diagnosis of AKI or for the risk of developing AKI associated with major surgery in the subject.

Moreover, an increase of the amount in the second sample as compared to the amount in the first sample which is considered to be significant and which is, thus, indicative for the diagnosis of AKI associated with major surgery or the risk thereof, is an increase which is equal or larger than an increase of the amount in the second sample as compared to the first sample in a control subject or a group of control subjects known to have suffered from AKI associated with major surgery. Preferably, said increase is a significant increase.

Preferably, the period of time between obtaining the first and the second sample as well as the time points for obtaining said samples in the test subject corresponds to the period of

time between obtaining the first and the second sample and to the time points for obtaining said samples in the control subject(s). Of course, the control subject shall have undergone major surgery.

- 5 Preferred significant increases of the amount of sFlt-1 which have been found in the course of the invention which are indicative for the diagnosis of AKI (or risk thereof) associated with major surgery.

10 According to the invention, an increase of the amount of sFlt-1 in the second sample compared to the amount in the first sample, preferably, of at least about 50 %, more preferably of at least about 75 % and, even more preferably, of at least about 100 %, and most preferably of at least about 150% or about 200% is considered to be significant and, thus, to be indicative for the diagnosis of AKI (or risk thereof) associated with major surgery.

15 Moreover, an increase of the amount of sFlt-1 in the second sample compared to the amount in the first sample, preferably, of at least 100 pg/ml, more preferably of at least 150 pg/ml and even, more preferably, of at least 200 pg/ml or 250 pg/ml, and most preferably of at least 300 pg/ml is considered to be significant and, thus, to be indicative for the diagnosis of AKI (or risk thereof) associated with major surgery.

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The aforementioned increases, preferably, apply to a first sample that has been obtained prior the major surgery (see above) and to a second sample that has been obtained within 0 to 3 or within 0 to 6 hours after a major surgery. Preferably, the second sample is obtained at the end of the surgery. Preferred changes, i.e. increases or decreases for other time inter-
25 vals can be determined by the person skilled in the art without further ado.

Advantageously, the method of the present invention allows for the early diagnosis of acute kidney injury in a subject who undergoes or who has undergone a major surgery. This is based on the surprising finding that the amount of sFlt-1 is increased in patients
30 suffering from AKI already during and immediately after surgery, or in patients having which are at risk of suffering from AKI after a major surgery. From the diagnosis of AKI, or risk thereof, in a patient practical consequences can be drawn immediately. Thus, in a preferred embodiment of the method of the present invention, the method further compris-

es the step of recommending a therapy. Preferably, nephrotoxic medication in particular of NSIADs (nonsteroidal anti-inflammatory drugs) , COX 2 inhibitors, ACE inhibitors and Angiotensin II receptor blockers shall be avoided once the diagnosis has been made. In particular, it is also envisaged to monitor the blood pressure once the diagnosis of AKI has been made and/or to increase the intake of fluid. In case of increased blood pressure, blood pressure lowering medicaments shall be administered. Moreover, careful fluid balance is important in subject for which the diagnosis of AKI has been established.

As set forth herein above, sFlt-1 is frequently measured in combination with the biomarker PlGF (Placental Growth Factor) in pregnant woman. The ratio of sFlt-1 to PlGF is reliable predictor of preeclampsia. In the context of the present invention, however, it has been surprisingly shown that the determination of sFlt-1 alone, i.e. without determining PlGF, allows for reliably diagnosing acute kidney injury associated with a major surgery.

Thus, in an aspect of the invention, the method does not encompass the determination of the biomarker PlGF. In particular, the method does not encompass the determination of the biomarker PlGF in the first and second sample from the subject. It is further envisaged, that the method does not encompass the determination of ratio of sFlt-1 to PlGF or vice versa.

The term "PlGF (Placental Growth Factor)" as used herein refers to a placenta derived growth factor which is a polypeptide having 149 amino acids in length and being highly homologous (53% identity) to the platelet-derived growth factor-like region of human vascular endothelial growth factor (VEGF). Like VEGF, PlGF has angiogenic activity in vitro and in vivo. For example, biochemical and functional characterization of PlGF derived from transfected COS-1 cells revealed that it is a glycosylated dimeric secreted protein which is able to stimulate endothelial cell growth in vitro (Maqlione1993, Oncogene 8(4):925-31). Preferably, PlGF refers to human PlGF, more preferably, to human PlGF having an amino acid sequence as shown in Genebank accession number P49763.2, GI: 17380553. Furthermore, the term encompasses variants of said specific human PlGF. Such variants have at least the same essential biological and immunological properties as the specific PlGF polypeptide. Variants are deemed to share the same essential biological and immunological properties if they are detectable by the same specific assays referred to in

this specification, e.g., by ELISA assays using polyclonal or monoclonal antibodies specifically recognizing the said PLGF polypeptides. A preferred assay is described in the accompanying Examples. Moreover, it is to be understood that a variant as referred to in accordance with the present invention shall have an amino acid sequence which differs due to at least one amino acid substitution, deletion and/or addition wherein the amino acid sequence of the variant is still, preferably, at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identical with the amino sequence of the specific PLGF polypeptides. The degree of identity between two amino acid sequences can be determined by algorithms well known in the art. Preferably, the degree of identity is to be determined by comparing two optimally aligned sequences over a comparison window, where the fragment of amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (USA) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment and, thus, the degree of identity. Preferably, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. Variants referred to above may be allelic variants or any other species specific homologs, paralogs, or orthologs. Moreover, the variants referred to herein include fragments of the specific PLGF polypeptides or the aforementioned types of variants as long as these fragments have the essential immunological and biological properties as referred to above. Such fragments may be, e.g., degradation products or splice variants of the PLGF polypeptides. Further included are variants

which differ due to posttranslational modifications such as phosphorylation or myristylation.

According to some embodiments of the instant disclosure, portions of some steps of methods disclosed and described herein may be performed by a computing device. A computing device may be a general purpose computer or a portable computing device, for example. It should also be understood that multiple computing devices may be used together, such as over a network or other methods of transferring data, for performing one or more steps of the methods disclosed herein. Exemplary computing devices include desktop computers, laptop computers, personal data assistants ("PDA"), such as BLACKBERRY brand devices, cellular devices, tablet computers, servers, and the like. In general, a computing device comprises a processor capable of executing a plurality of instructions (such as a program of software).

A computing device has access to a memory. A memory is a computer readable medium and may comprise a single storage device or multiple storage devices, located either locally with the computing device or accessible to the computing device across a network, for example. Computer-readable media may be any available media that can be accessed by the computing device and includes both volatile and non-volatile media. Further, computer readable-media may be one or both of removable and non-removable media. By way of example, and not limitation, computer-readable media may comprise computer storage media. Exemplary computer storage media includes, but is not limited to, RAM, ROM, EEPROM, flash memory or any other memory technology, CD-ROM, Digital Versatile Disk (DVD) or other optical disk storage, magnetic cassettes, magnetic tape, magnetic disk storage or other magnetic storage devices, or any other medium which can be used for storing a plurality of instructions capable of being accessed by the computing device and executed by the processor of the computing device.

According to embodiments of the instant disclosure, software may include instructions which, when executed by a processor of the computing device, may perform one or more steps of the methods disclosed herein. Some of the instructions may be adapted to produce signals that control operation of other machines and thus may operate through those control signals to transform materials far removed from the computer itself. These descriptions and representations are the means used by those skilled in the art of data processing,

for example, to most effectively convey the substance of their work to others skilled in the art.

The plurality of instructions may also comprise an algorithm which is generally conceived to be a self-consistent sequence of steps leading to a desired result. These steps are those requiring physical manipulations of physical quantities. Usually, though not necessarily, these quantities take the form of electrical or magnetic pulses or signals capable of being stored, transferred, transformed, combined, compared, and otherwise manipulated. It proves convenient at times, principally for reasons of common usage, to refer to these signals as values, characters, display data, numbers, or the like as a reference to the physical items or manifestations in which such signals are embodied or expressed. It should be borne in mind, however, that all of these and similar terms are to be associated with the appropriate physical quantities and are merely used here as convenient labels applied to these quantities.

The computing device may also have access to an output device. Exemplary output devices include fax machines, displays, printers, and files, for example. According to some embodiments of the present disclosure, a computing device may perform one or more steps of a method disclosed herein, and thereafter provide an output, via an output device, relating to a result, indication, ratio or other factor of the method.

The present disclosure describes some embodiments comprising various markers. Some of these markers are exemplified and described as comprising specific amino acid chains of specific lengths. However, it should be understood that it is within the scope of the present disclosure that these markers, exemplified herein as comprising specific amino acid sequences, may also comprise variant amino acid sequences (and variant or equivalent nucleotide sequences coding for the exemplified and variant amino acid sequences within the scope of the instant disclosure). For example, according to the instant disclosure, an equivalent amino acid sequence may have amino acid homology with a marker exemplified herein, such that the amino acid sequence identity will typically be greater than 75%. According to embodiments of the instant disclosure, the amino acid identity may be greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, and even greater than greater than 99% in some embodiments. According to some embodiments, the amino acid sequence identity will typically be highest in critical

regions of the marker which (in general) account for the biological activity, or are involved in the determination of three-dimensional configuration, ultimately responsible for the biological activity of the marker. As such, it should be understood that certain amino acid substitutions, deletions, or additions are acceptable and can be expected, for example, if these substitutions (deletions or additions) are in regions which are not critical to activity or do not affect the three-dimensional configuration of the marker molecule. For example, in some embodiments of the instant disclosure, the amino acid sequence of the marker may comprise a conservative amino acid substitution. Amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. A form of conservative substitution includes an amino acid of one class being replaced with another amino acid of the same type (such that the substitution does not materially alter the biological activity and/or three-dimensional configuration of the marker molecule). Table 1 provides an exemplary listing of examples of amino acids belonging to each class.

Table 1. Exemplary listing of examples of amino acids belonging to each class.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu

According to some embodiments of the present disclosure, kits, including some or all of the components necessary to practicing one or more of the methods disclosed herein, are provided.

A kit, according to the instant disclosure, may be made of any suitable material. Non-limiting examples of kit materials are card-board or other paper product, plastic, glass, wood, metal, and any alloy thereof.

In some embodiments, a kit disclosed herein includes at least one component or a packaged combination of components for practicing a disclosed method. By “packaged combination” it is meant that the kits provide a single package that contains a combination of one or more components, such as probes (for example, an antibody), controls, buffers, reagents (for example, conjugate and/or substrate) instructions, and the like, as disclosed herein. A kit containing a single container is also included within the definition of “packaged combi-

nation.” In some embodiments, the kits include at least one probe, for example an antibody (having specific affinity for an epitope of a biomarker as disclosed herein. For example, the kits may include an antibody that is labelled with a fluorophore or an antibody that is a member of a fusion protein. In the kit, the probe may be immobilized, and may be
5 immobilised in a specific conformation. For example, an immobilized probe may be provided in a kit to specifically bind target protein, to detect target protein in a sample, and/or to remove target protein from a sample.

According to some embodiments, kits include at least one probe, which may be immobilized, in at least one container. Kits may also include multiple probes, optionally immobilized, in one or more containers. For example, the multiple probes may be present in a
10 single container or in separate containers, for example, wherein each container contains a single probe.

In some embodiments, a kit may include one or more non-immobilized probe and one or more solid support that does or does not include an immobilized probe. Some such embodiments may comprise some or all of the reagents and supplies needed for immobilizing
15 one or more probes to the solid support, or some or all of the reagents and supplies needed for binding of immobilized probes to specific proteins within a sample.

In certain embodiments, a single probe (including multiple copies of the same probe) may be immobilized on a single solid support and provided in a single container. In other embodiments, two or more probes, each specific for a different target protein or a different form of a single target protein (such as a specific epitope), are provided in a single container.
20 In some such embodiments, an immobilized probe may be provided in multiple different containers (e.g., in single-use form), or multiple immobilized probes may be provided in multiple different containers. In further embodiments, the probes may be immobilized on multiple different type of solid supports. Any combination of immobilized probe(s) and
25 container(s) is contemplated for the kits disclosed herein, and any combination thereof may be selected to achieve a suitable kit for a desired use.

A container of the kits may be any container that is suitable for packaging and/or containing one or more components disclosed herein, including for example probes (for example, an antibody), controls, buffers, and reagents (for example, conjugate and/or substrate).
30 Suitable materials include, but are not limited to, glass, plastic, cardboard or other paper

product, wood, metal, and any alloy thereof. In some embodiments, the container may completely encase an immobilized probe(s) or may simply cover the probe to minimize contamination by dust, oils, etc., and expose to light. In some further embodiments, the kits may comprise a single container or multiple containers, and where multiple containers are present, each container may be the same as all other containers, different than others, or different than some but not all other containers.

Moreover, it has been shown in the context of the method of the present invention that also GDF-15 is a good marker for the diagnosis of AKI associated with major surgery of the risk thereof. The combined determination of GDF-15 and sFlt-1 further increases the reliability of the diagnosis carried out in the context of the method of the present invention.

Accordingly, the aforementioned method, preferably, further comprises the steps of

- a') determining the amount of GDF-15 in the first sample obtained prior the major surgery
- b') determining the amount of GDF-15 in another sample obtained during or after said first sample, and
- c') comparing the amount of GDF-15 in said first sample to the amount of GDF-15 in said another sample.

Preferably, the "first sample" as set forth in a') is the first sample in which the amount of sFlt-1 is determined (see above). Preferably, said "another sample" as set forth in b') is the "second sample" in which the amount of sFlt-1 is determined (see also above). It is, however, also contemplated that said another sample has been obtained later than the second sample for the determination of sFlt-1. Thus, said another sample may be a third sample. It is preferred that said another sample has been obtained not earlier than 3 hours, but not later than 3 days after surgery. It is even more preferred that said another sample has been obtained not earlier than 3 hours, but not later than 48 hours after surgery. It is most preferred that said another sample has been obtained not earlier than 3 hours, but not later than 36 hours after surgery, or not earlier than 3 hours, but not later than 24 hours after surgery.

Preferably, an increased amount and, more preferably, a significantly increased amount, of sFlt-1 in the second sample as compared to the amount of sFlt-1 in said first sample con-

comitant with an increased amount and, more preferably, a significantly increased amount of GDF-15 in the another sample as compared to the amount of GDF-15 in said first sample is indicative for the diagnosis of AKI (or risk thereof) associated with major surgery in the subject.

5

Preferred increases or sFlt-1 are described herein elsewhere.

Preferably, an increased amount, more preferably, a significantly increased, and most preferably, a statistically significant increase of the amount of GDF-15 in the second sample as compared to the amount of GDF-15 in said first sample is indicative for the diagnosis of AKI (or risk thereof) associated with major surgery in the subject.

10

Moreover, an increase of the amount in the second sample as compared to the amount in the first sample which is considered to be significant and which is, thus, indicative for the diagnosis of AKI associated with major surgery, is an increase which is equal or larger than an increase of the amount in the second sample as compared to the first sample in a control subject or a group of control subjects known to have suffered from AKI associated with major surgery. Preferably, said increase is a significant increase.

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Preferably, the period of time between obtaining the first and the second sample as well as the time points for obtaining said samples in the test subject corresponds to the period of time between obtaining the first and the second sample and to the time points for obtaining said samples in the control subject(s). Of course, the control subject shall have undergone major surgery.

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Preferred significant increases of the amount of GDF-15 which have been found in the course of the invention which are indicative for the diagnosis of AKI associated with major surgery.

30

According to the invention, an increase of the amount of GDF-15 in the second sample compared to the amount in the first sample, preferably, of at least 100 %, more preferably of at least 200 % and, even more preferably, of at least 300 %, and most preferably of at

least 400 % or 500% is considered to be significant and, thus, to be indicative for the diagnosis of AKI (or risk thereof) associated with major surgery.

Moreover, an increase of the amount of GDF-15 in the second sample compared to the amount in the first sample, preferably, of at least 1000 pg/ml, more preferably of at least 2000 pg/ml and even, more preferably, of at least 4000 pg/ml or 6000 pg/ml, and most preferably of at least 8000 pg/ml is considered to be significant and, thus, to be indicative for the diagnosis of AKI (or risk thereof) associated with major surgery.

The increase of GDF-15 being indicative for AKI may depend on the time point at which the second sample is obtained. Thus, if the second sample is obtained at the end of surgery, an increase of at least 1000 or at least 2000 pg/ml shall be indicative for the diagnosis of AKI. If the second sample is obtained at 6 hours or 12 hours after surgery, an increase of at least 6000 or at least 8000 pg/ml shall be indicative for the diagnosis of AKI. If the second sample is obtained at 24 hours after surgery, an increase of at least 2000 pg/ml shall be indicative for the diagnosis of AKI (or risk thereof). Which increased is indicative for the diagnosis of AKI can be determined by the skilled person based on the Examples provide herein without further ado.

The term “Growth-Differentiation Factor-15” or “GDF-15” relates to a polypeptide being a member of the transforming growth factor (TGF)- β cytokine superfamily. The terms polypeptide, peptide and protein are used interchangeable throughout this specification. GDF-15 was originally cloned as macrophage-inhibitory cytokine-1 and later also identified as placental transforming growth factor- β , placental bone morphogenetic protein, non-steroidal anti-inflammatory drug-activated gene-1, and prostate-derived factor (Bootcov loc cit; Hromas, 1997 *Biochim Biophys Acta* 1354:40-44; Lawton 1997, *Gene* 203:17-26; Yokoyama-Kobayashi 1997, *J Biochem (Tokyo)*, 122:622-626; Paralkar 1998, *J Biol Chem* 273:13760-13767). Similar to other TGF- β -related cytokines, GDF-15 is synthesized as an inactive precursor protein, which undergoes disulfide-linked homodimerization. Upon proteolytic cleavage of the N-terminal pro-peptide, GDF-15 is secreted as a ~28 kDa dimeric protein (Bauskin 2000, *Embo J* 19:2212-2220). Amino acid sequences and biological activities for GDF-15 are disclosed in WO99/06445, WO00/70051, WO2005/113585, Bottner 1999, *Gene* 237: 105-111, Bootcov loc. cit, Tan loc. cit., Baek 2001, *Mol Pharma-*

col 59: 901-908, Hromas loc cit, Paralkar loc cit, Morrish 1996, Placenta 17:431-441 or Yokoyama-Kobayashi loc cit.. As used herein, the term “GDF-15”, preferably, encompasses variants of the specific GDF-15 polypeptide. Variants of a polypeptide have at least the same essential biological and immunological properties as said polypeptide. In particular, they share the same essential biological and immunological properties if they are detectable by the same specific assays referred to in this specification, e.g., by ELISA assays using polyclonal or monoclonal antibodies specifically recognizing the said polypeptides. Moreover, it is to be understood that a variant as referred to in accordance with the present invention shall have an amino acid sequence which differs due to at least one amino acid substitution, deletion and/or addition wherein the amino acid sequence of the variant is still, preferably, at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identical with the amino sequence of the specific GDF-15 polypeptide, preferably over the entire length of the human GDF-15, respectively. How to calculate the degree of sequence identity is described elsewhere herein.

Preferably, the amounts of sFlt-1 and GDF-15 are determined in the same sample. It is, however, also preferred that the amount of sFlt-1 is determined in different samples obtained at different time points.

Preferred time points and time periods for obtaining a sample for the determination of sFlt-1 are disclosed above.

With respect to the determination of GDF-15, it is, particularly, preferred that the sample has been obtained after the major surgery (to be more precisely, after the completion of the surgery).

It is particularly contemplated that the sample has been obtained after the surgical procedure has been completed, preferably, within a time period of not later than 1, 2 or 3 days after surgery. Preferably, the sample has been obtained immediately after surgery. The term “immediately after surgery”, preferably, to a sample, that has been obtained not later than about 0.5, not later than about 1, not later than about 2, not later than about 3 or not later than about 6 hours after surgery, most preferably not later than 0.5 hours after surgery. Accordingly, the sample is preferably obtained within the period of 0 to 6 hours, 0 to

3 hours, 0 to 2 hours, or 0 to 1 hour after the surgery (or more precisely after completion of the surgery). It is more preferred that the sample has been obtained not earlier than 3 hours, but not later than 3 days after surgery. It is even more preferred that the sample is obtained not earlier than 3 hours, but not later than 48 hours after surgery. It is also preferred that
5 the sample has been obtained not earlier than 3 hours, but not later than 36 hours after surgery, or not earlier than 3 hours, but not later than 24 hours after surgery. It is most preferred that the sample has been obtained at the end of surgery.

In a preferred embodiment of the method of the present invention, the method further
10 comprises the step of recommending a therapy.

The term "recommending" as used herein means establishing a proposal for a therapy which could be applied to the subject. However, it is to be understood that applying the actual therapy whatsoever is preferably not comprised by the term. The therapy to be recommended depends on the outcome of the diagnosis provided by the method of the present invention. Preferably, nephrotoxic medication shall be avoided once the diagnosis of AKI
15 has been made. In particular, it is also envisaged to monitor the blood pressure once the diagnosis of AKI has been made. In case of increased blood pressure, blood pressure lowering medicaments shall be administered.

In an aspect of the invention, a method for establishing an aid for diagnosing acute kidney injury associated with major surgery in a subject is contemplated, said method comprising the steps of

- a) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a first
25 sample of said subject obtained prior to major surgery; said determining comprises (i) bringing the sample into contact with a detection agent that specifically binds to sFlt-1 for a time sufficient to allow for the formation of a complex of the said detection agent and the sFlt-1 from the sample, (ii) measuring the amount of the formed complex, wherein the said amount of the formed complex is proportional to the amount of sFlt-1 present in the sample, and (iii) transforming the
30 amount of the formed complex into an amount of sFlt-1 reflecting the amount of sFlt-1

b) determining the amount of soluble fms-like thyrosine kinase-1 (sFlt-1) in a second sample of said subject, wherein said second sample has been obtained during or after said major surgery, said determining the steps (i) to (iii) as set forth under a),

5 c) comparing the amount of sFlt-1 in said second sample to the amount of sFlt-1 in said first sample,

d) and establishing an aid for diagnosing acute kidney injury associated with major surgery in said subject based on the comparison made in step c).

10 A suitable detection agent may be, in an aspect, an antibody which is specifically binds to sFlt-1 in a sample of a subject to be investigated by the method of the invention. Another detection agent that can be applied, in an aspect, may be an aptamere which specifically binds to sFlt-1 in the sample. In yet an aspect the, sample is removed from the complex formed between the detection agent and the sFlt-1 prior to the measurement of the amount
15 of formed complex. Accordingly, in an aspect, the detection agent may be immobilized on a solid support. In yet an aspect, the sample can be removed from the formed complex on the solid support by applying a washing solution. The formed complex shall be proportional to the amount of the sFlt-1 present in the sample. It will be understood that the specificity and/or sensitivity of the detection agent to be applied defines the degree of proportion of
20 sFlt-1 comprised in the sample which is capable of being specifically bound. Further details on how the determination can be carried out are also found elsewhere herein. The amount of formed complex shall be transformed into an amount of sFlt-1 reflecting the amount indeed present in the sample. Such an amount, in an aspect, may be essentially the amount present in the sample or may be, in another aspect, an amount which is a certain
25 proportion thereof due to the relationship between the formed complex and the amount present in the original sample.

In yet another aspect of the aforementioned method, steps a) and b) may be carried out by an analyzing unit, in an aspect, an analyzing unit as defined elsewhere herein.

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In an aspect of the method of the invention, the amount determined in step a) is compared to the amount determined in step b). Based on an increase of the amount as determined in step b) as compared to the amount determined in step a) as set forth elsewhere herein, AKI

can be diagnosed.. In an aspect, the comparison is carried out automatically, e.g., assisted by a computer system or the like.

The aid for diagnosing is established based on the comparison carried out in step c) by allocating the subject either into a group of subjects suffering from AKI with certain likelihood or a group of subjects not suffering therefrom. As discussed elsewhere herein already, the allocation of the investigated subject must not be correct in 100% of the investigated cases. Moreover, the groups of subjects into which the investigated subject is allocated are artificial groups in that they are established based on statistical considerations, i.e. a certain preselected degree of likelihood based on which the method of the invention shall operate. Thus, the method may establish an aid of diagnosis which may, in an aspect, require further strengthening of the diagnosis by other techniques. In an aspect of the invention, the aid for diagnosing is established automatically, e.g., assisted by a computer system or the like.

In an aspect of the method of the invention, said method further comprises a step of recommending and/or managing the subject according to the result of the aid of diagnosis established in step d). Such a recommendation may, in an aspect, be the application of therapeutic measures as set forth elsewhere herein in detail.

In an aspect of the aforementioned method, steps c) and/or d) are carried out by an evaluation unit as set forth elsewhere herein.

The same applies, if the biomarker GDF-15 is determined.

It is to be understood that the definitions and explanations of the terms made above and below apply mutatis mutandis for all embodiments described in this specification and the accompanying claims (except stated otherwise).

Moreover, the present invention relates to a method for diagnosing acute kidney injury associated with major surgery in a subject, said method comprising the steps of

- a) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a sample of the subject obtained during or after a major surgery; and

- b) comparing the determined amount of sFlt-1 with a reference amount, whereby acute kidney injury is diagnosed.

Preferably, acute kidney injury is diagnosed by carrying out the further step of c) diagnosing acute kidney injury based on the result of the comparison carried out in step b).

The method of the present invention, preferably, is an ex vivo or in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate to sample pre-treatments or evaluation of the results obtained by the method. The method may be carried out manually or assisted by automation. Preferably, step (a) and/or (b) may in total or in part be assisted by automation, e.g., by a suitable robotic and sensory equipment for the determination in step (a) or a computer-implemented comparison and/or diagnosis based on said comparison in step (b).

As set forth above, the subject according to the present invention, preferably, has undergone or shall undergo a major surgery at the time at which the sample as referred to in the context of the aforementioned method is obtained.

The sample as referred to in the context of the aforementioned method is, preferably, obtained during or after the major surgery. It is particularly contemplated that the sample has been obtained after the surgical procedure has been completed, preferably, within a time period of not later than 1, 2 or 3 days after surgery. It is more preferred that the sample has been obtained immediately after or not later than 1 day after completion of the intervention. It is most preferred that the sample has been obtained immediately after surgery. The term “immediately after surgery”, preferably, to a sample, that has been obtained not later than about 0.5 hours, not later than about 1 hour, not later than about 2 hour, not later than about 3 hours or not later than about 6 hours after surgery. Accordingly, the sample is preferably obtained within the period of 0 to 6 hours, 0 to 3 hours, 0 to 2 hours, or 0 to 1 hour, or 0 to 0.5 hours after the surgery (or more precisely: after completion of the surgery).

The term “comparing” as used herein, preferably, encompasses comparing the amount of the peptide or polypeptide comprised by the sample to be analyzed with an amount of a

suitable reference source specified elsewhere in this description. It is to be understood that comparing as used herein refers to a comparison of corresponding parameters or values, e.g., an absolute amount is compared to an absolute reference amount while a concentration is compared to a reference concentration or an intensity signal obtained from a test sample is compared to the same type of intensity signal of a reference sample. The comparison referred to in step (b) of the method of the present invention may be carried out manually or computer assisted. For a computer assisted comparison, the value of the determined amount may be compared to values corresponding to suitable references which are stored in a database by a computer program. The computer program may further evaluate the result of the comparison, i.e. automatically provide the desired assessment in a suitable output format. Based on the comparison of the amount determined in step a) and the reference amount, it is possible to assess whether a cardiac disease is an acute inflammation-associated cardiac disease or an acute inflammation-independent cardiac disease. Therefore, the reference amount is to be chosen so that either a difference or a similarity in the compared amounts allows for diagnosing AKI, and, thus, for identifying those test subjects which suffer from AKI associated with major surgery (rule-in) or not (rule out).

Accordingly, the term "reference amount" as used herein, preferably, refers to an amount which allows assessing whether a subject as referred to herein suffers from AKI associated with major surgery or not. Accordingly, the reference amount for sFlt-1 may, in principle, from a sample from:

- (i) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to suffer from AKI associated with major surgery, and/or
- (ii) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known not to suffer from AKI associated with major surgery.

Preferably, the said reference subject undergoes or has undergone major surgery. Preferably, said sample from said reference subject or group of reference subjects has been obtained during or after a major surgery as specified elsewhere herein. Preferably, the sample from the reference subject (or group of reference subjects) from which the reference amount is derived is obtained within the same time period or, more preferably, at essentially the same time point with respect to the major surgery as the test sample from the test

subject. Preferred time periods for obtaining the sample from the test subject are disclosed elsewhere herein. The person skilled in the art understands what is meant by the expression “essentially the same time point”. E.g., if the sample from the test subject has been obtained after 3 hours after a major surgery, the sample from which the reference amount is
5 derived, is preferably, obtained from a reference subject within 2 to 4 hours, and, more preferably, after 3 hours after the major surgery in the reference subject.

The reference subject, i.e. the subject from the the reference sample(s) is (are) derived, in the context of method of the present invention may exhibit a pre-existing disorder of the
10 renal tubules before major surgery. However, it is preferred that the reference subject does not exhibit a pre-existing disorder of the renal tubules (particularly, if the test subject does also not exhibit a pre-existing disorder of the renal tubules).

Preferably the following is applied as a diagnostic algorithm:

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Preferably, an essentially identical amount of sFlt-1 or an increased amount of sFlt-1 in the sample of the test subject as compared to the reference amount is indicative for the diagnosis of acute kidney injury associated with major surgery (or the risk thereof), if the reference amount is derived from a subject or a group of subjects known to suffer from acute
20 kidney injury associated with a major surgery.

Preferably, an essentially identical amount of sFlt-1 or a decreased amount of sFlt-1 in the sample of the test subject as compared to the reference amount indicates that the subject does not suffer from acute kidney injury associated with major surgery (and/or is not at
25 risk thereof), if the reference amount is derived from a subject or a group of subjects known not to suffer from acute kidney injury associated with a major surgery.

The reference amount applicable for an individual subject may vary depending on various physiological parameters such as age, gender, or subpopulation, as well as on the means used for the determination of the polypeptide or peptide referred to herein. A suitable reference
30 amount may be determined from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the test sample.

Reference amounts can be calculated for a cohort of subjects as specified above based on the average or mean values for a given biomarker by applying standard statistically methods. In particular, accuracy of a test such as a method aiming to assess a condition, or not, is best described by its receiver-operating characteristics (ROC) (see especially Zweig
5 1993, Clin. Chem. 39:561-577). The ROC graph is a plot of all of the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of data observed. The clinical performance of a diagnostic method depends on its accuracy, i.e. its ability to correctly allocate subjects to a certain prognosis or diagnosis. The ROC plot indicates the overlap between the two distributions by plotting the sensitivity versus 1-
10 specificity for the complete range of thresholds suitable for making a distinction. On the y-axis is sensitivity, or the true-positive fraction, which is defined as the ratio of number of true-positive test results to the product of number of true-positive and number of false-negative test results. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-
15 positive fraction, or 1-specificity, which is defined as the ratio of number of false-positive results to the product of number of true-negative and number of false-positive results. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of the event
20 in the cohort. Each point on the ROC plot represents a sensitivity/-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the
25 lower left corner to the upper right corner. Most plots fall in between these two extremes. If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa. Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the
30 test. Dependent on a desired confidence interval, a threshold can be derived from the ROC curve allowing for the diagnosis or prediction for a given event with a proper balance of sensitivity and specificity, respectively.

Accordingly, the reference to be used for the aforementioned method of the present invention, i.e. a threshold which allows for discriminating between a subject suffering from AKI associated with major surgery and a subject not suffering from AKI associated with major surgery, preferably, by establishing a ROC for said cohort as described above and deriving
5 a threshold amount therefrom. Dependent on a desired sensitivity and specificity for a diagnostic method, the ROC plot allows deriving suitable thresholds. It will be understood that an optimal sensitivity is desired for excluding a subject not suffering from AKI associated with major surgery (i.e. a rule out) whereas an optimal specificity is envisaged for a subject suffering from AKI associated with major surgery (i.e. a rule in).

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Preferred reference amounts for sFlt1 derived from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to suffer from AKI associated with major surgery are, in increasing order of preference 300 pg/ml, 400 pg/ml or, more preferably, 500 pg/ml.

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Preferred reference amounts for sFlt1 derived from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to suffer from AKI associated with major surgery are, in increasing order of preference 250 pg/ml, 100 pg/ml or, more preferably, 150 pg/ml.

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In a preferred embodiment, the method of the present invention further comprises the steps of determining the amount of Growth Differentiation Factor-15 (GDF-15) in a sample from said subject obtained during or after a major surgery, and comparing the determined amount to a reference amount.

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The term "reference amount" is described elsewhere herein. The reference amount for GDF-15 may be derived, in principle, from a sample from:

(i) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to suffer from AKI associated with major surgery, and/or

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(ii) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known not to suffer from AKI associated with major surgery.

Preferably, the said reference subject undergoes or has undergone major surgery. Preferably, said sample from said reference subject or group of reference subjects has been obtained during or after a major surgery as specified elsewhere herein. Preferably, the sample from the reference subject (or group of reference subjects) from which the reference amount is derived is obtained within the same time period or, more preferably, at essentially the same time point with respect to the major surgery as the test sample from the test subject. Preferred time periods or time points for obtaining the sample from the test subject are disclosed above.

10 Preferably, the following is applied as a diagnostic algorithm:

Preferably, an essentially identical amount of sFlt-1 and GDF-15 or an increased amount of sFlt-1 and GDF-15 in the sample from the test subject (or in the samples from the test subject in case sFlt-1 and GDF-15 are determined in two different samples) as compared to the reference amount for sFlt-1 and GDF-15 is indicative for the diagnosis (or for the diagnosis of the risk) of acute kidney injury associated with major surgery, if the reference amount for GDF-15 and sFlt-1 is derived from a sample (from samples) of a subject or a group of subjects known to suffer from acute kidney injury associated with a major surgery.

20 Preferably, an essentially identical amount of sFlt-1 and GDF-15 or a decreased amount of sFlt-1 and GDF-15 in the sample from the test subject (or in the samples from the test subject in case sFlt-1 and GDF-15 are determined in two different samples) as compared to the reference amount for sFlt-1 and GDF-15 is indicative for the diagnosis that no acute kidney injury associated with major surgery occurred and/or that the patient is not at risk of suffering from acute kidney injury associated with major surgery, if the reference amount for GDF-15 and sFlt-1 is derived from a sample (from samples) of a subject or a group of subjects known not to suffer from acute kidney injury associated with a major surgery.

In an aspect, the aforementioned method does not encompass the determination of PlGF in the sample from the subject. In another aspect, the aforementioned method does not encompass the calculation of a ratio of sFlt-1 to PlGF and vice versa.

In an aspect of the invention, a method for establishing an aid for diagnosing acute kidney injury associated with major surgery in a subject is contemplated, said method comprising the steps of

- a) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in sample of said subject obtained prior to major surgery; said determining comprises (i) bringing the sample into contact with a detection agent that specifically binds to sFlt-1 for a time sufficient to allow for the formation of a complex of the said detection agent and the sFlt-1 from the sample, (ii) measuring the amount of the formed complex, wherein the said amount of the formed complex is proportional to the amount of sFlt-1 present in the sample, and (iii) transforming the amount of the formed complex into an amount of sFlt-1 reflecting the amount of sFlt-1
- b) comparing the amount of sFlt-1 to a reference amount,
- c) and establishing an aid for diagnosing acute kidney injury associated with major surgery in said subject based on the comparison made in step b).

Moreover, the present invention relates to the use of the biomarker sFlt-1 and/or of a detection agent which specifically binds thereto in a first and preferably also in a second sample from a subject for diagnosing acute kidney injury associated with a major surgery. In addition, the invention relates to the use of the biomarker sFlt-1 and/or of a detection agent which specifically binds thereto in a first and preferably also in a second sample from a subject for predicting the risk of the subject to suffer from acute kidney injury associated with a major surgery, preferably the risk to suffer from AKI after the surgery.

As set forth above, AKI associated with major surgery can be diagnosed or predicted by determining the amount of sFlt-1 in sample of a subject obtained during or after a major surgery. However, no correlation was found between the amount of sFlt-1 in samples obtained prior to surgery and the outcome AKI.

In contrast, it has been shown in the context of the present invention that the amount of L-FABP in a urine sample obtained prior to a major surgery is indicative for an increased risk of acute kidney injury after said major surgery. Interestingly, it was observed that the majority of subjects with the outcome AKI had either

- i) increased urinary L-FABP levels prior to surgery,

- ii) increased sFlt-1 levels after surgery, or
- iii) increased urinary L-FABP levels prior to surgery and increased sFlt-1 levels after surgery (see Example 2).

5 These data suggest the following (for subjects with the outcome AKI):

- i) increased urinary L-FABP levels prior to surgery indicate that pre-existing disorders of the renal tubules are the predominant cause of AKI (which of course may be triggered by minor ischemic complications during major surgery)
- 10 ii) increased sFlt-1 levels after surgery indicate that ischemic complications caused by the major surgery are the predominant cause of AKI, and
- iii) increased urinary L-FABP levels prior to surgery and increased sFlt-1 levels after surgery indicate that the AKI is caused by a combination of both ischemic complications caused by major surgery and pre-existing disorders of the renal tubules.

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Accordingly, the present invention relates to a method for differentiating in a subject suffering from acute kidney injury (AKI) associated a major surgery between (i) a pre-existing disorder of the renal tubules (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent disorder of the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant cause for said AKI, comprising the steps of

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- a) determining the amount of L-FABP (liver fatty acid binding protein) in a urine sample of said subject obtained prior to the major surgery, and comparing the, thus, determined amount of L-FABP to a reference amount for L-FABP,
- 25 b) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a sample of a subject obtained during or after said major surgery, and comparing the, thus, determined amount of sFlt-1 to a reference amount for sFlt-1,

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whereby it is differentiated between (i) a pre-existing underlying disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent underlying disorder of the renal tubules and ischemic complications in the kidney caused by said major surgery, as the predominant cause for said acute kidney injury.

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The definitions and explanations given elsewhere herein in the context of the other methods of the present invention apply mutatis mutandis to the aforementioned method.

Preferably, it is differentiated between (i), (ii) and (iii) by carrying out the further step c) of
5 differentiating between i) a pre-existing disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent underlying disorder of the renal tubules and ischemic complications in the kidney caused by said major surgery, as the predominant cause of AKI.

10 The term “differentiating” as used herein means to distinguish between i) a pre-existing underlying disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent underlying disorder of the renal tubules and ischemic complications in the kidney caused by said major surgery, as the predominant cause of acute kidney injury. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for 100% of the subjects to be tested. The
15 term, however, requires that the assessment is correct for a statistically significant portion of the subjects (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student’s t-test, Mann-Whitney test etc.. Details are found in Dowdy and
20 Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001.

25 The term “ischemic complications in the kidney” as used herein, preferably, refers to a condition of the kidney characterized by a reduced delivery of oxygen (undersupply) to kidney tissue, preferably, the renal tubules. Preferably, tissue is affected by ischemia, if the amount of oxygen that is supplied to said tissue is not sufficient in order to cover the need of the cells comprised by said tissue, and, thus, to meet the rate of mitochondrial oxidation
30 in said cells. As a consequence AKI may occur. In the context of the aforementioned method, the ischemic complications shall be caused by the major surgery.

The “disorder of the renal tubules” is well understood by the skilled person. As used herein, the preferably refers to renal tubular damage, and, thus, to epithelial injury in renal tubule cells. The disorder of the renal tubules may be the consequence of a cardiac dysfunction or a cardiovascular disease, including coronary artery disease and heart failure. The test subject, preferably, exhibits the disorder of the renal tubules before initiating the major surgery. Thus, said disorder shall be a pre-existing disorder. Moreover, it is preferred that said pre-existing disorder is a chronic condition. In case the cause for is a pre-existing disorder of the renal tubules, minor ischemic complications may be the trigger for AKI. However, the pre-existing disorder shall be the predominant cause.

A subject exhibiting a pre-existing disorder of the renal tubules, preferably, shall not exhibit impaired renal function prior to major surgery (for an explanation of this term see above). How to assess whether a subject exhibits impaired renal function is well known in the art. Renal disorders can be diagnosed by any means known and deemed appropriate. Particularly, renal function can be assessed by means of the glomerular filtration rate (GFR). For example, the GFR may be calculated by the Cockcroft-Gault or the MDRD formula (Levey 1999, Annals of Internal Medicine, 461-470). GFR is the volume of fluid filtered from the renal glomerular capillaries into the Bowman's capsule per unit time. Clinically, this is often used to determine renal function. The GFR was originally estimated (the GFR can never be determined, all calculations derived from formulas such as the Cockcroft Gault formula of the MDRD formula deliver only estimates and not the “real” GFR) by injecting inulin into the plasma. Since inulin is not reabsorbed by the kidney after glomerular filtration, its rate of excretion is directly proportional to the rate of filtration of water and solutes across the glomerular filter. In clinical practice however, creatinine clearance is used to measure GFR. Creatinine is an endogenous molecule, synthesized in the body, which is freely filtered by the glomerulus (but also secreted by the renal tubules in very small amounts). Creatinine clearance (CrCl) is therefore a close approximation of the GFR. The GFR is typically recorded in milliliters per minute (mL/min). The normal range of GFR for males is 97 to 137 mL/min, the normal range of GFR for females is 88 to 128 mL/min. Thus, it is particularly contemplated that the GFR of a subject who does not exhibit impaired renal function is within this range. Moreover, said subject preferably, has a blood creatinine level (in particular a serum creatinine level) of lower than 0.9 mg/dl, more preferably of lower than 1.1 mg/dl and most preferably of lower than 1.3 mg/dl.

The urine sample for the determination of the amount of L-FABP as set forth in step a) of the aforementioned method shall have been obtained before the major surgery. More preferably, the sample has been obtained not more than two weeks, not more than one week, not more than 3 days, and more preferably, not more than one day before said surgery. It is further contemplated that the sample has been obtained not more than 6 hours, not more than 3 hours, not more than 2 hours, and, more preferably, 1 hour before the major surgery.

The sample for the determination of the amount of s-Flt-1 as set forth in step a) of the aforementioned method shall have been obtained during or after a major surgery. A definition for the term "sample" has been given elsewhere herein. It is particularly contemplated that the sample has been obtained after the surgical procedure has been completed, preferably, within a time period of not later than 1, 2 or 3 days after surgery. It is more preferred that the sample has been obtained immediately or not later than 1 day after completion of the surgery. It is most preferred that the sample has been obtained immediately after surgery. The term "immediately after surgery", preferably, to a sample, that has been obtained not later than about 0.5, not later than about 1, not later than about 2, not later than about 3 or not later than about 6 hours after surgery, most preferably not later than 0.5 hours after surgery. Accordingly, the sample is preferably obtained within a period of 0 to 6 hours, 0 to 3 hours, 0 to 2 hours, or 0 to 1 hour after the surgery (or more precisely after completion of the surgery). It is most preferred that the sample has been obtained at the end of surgery. Preferably, the time "X hours after surgery" is calculated from the time point when the respiration support (e.g. heart-lung machine) is disconnected, when the patient leaves the operation room or when the subject arrives at the intensive-care unit.

Preferably, the amount of the L-FABP in said urine sample is normalized to the amount of creatinine in said urine sample. The normalization, preferably, allows to account for creatinine clearance and urine flow. How to carry out this normalization step is well known in the art. Particularly, the ratio of the amount of L-FABP to the amount of creatinine shall be formed.

Preferably, the reference amount for L-FABP as set forth in step a) of the aforementioned method has been derived from a reference urine sample from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to have

a pre-existing disorder of the renal tubules as the predominant cause for AKI associated with major surgery. Preferably, said reference urine sample has been obtained before major surgery in said subject. Preferred time points or time periods for obtaining a sample before a surgery are described above in the context of step a) of the aforementioned method.

- 5 Moreover, is it preferred that the reference urine sample has been obtained at the same time, or the same time period at which the test sample, i.e. the sample obtained from the subject to be tested, has been obtained. Also the reference amount is, preferably, normalized to the amount of creatinine in said urine sample.
- 10 Preferred reference amounts for L-FABP, in particular, for normalized L-FABP, derived from a subject or groups of subjects as set forth above are, preferably, 10,8 $\mu\text{g/g}$ creatinine, more preferably, 29.5 $1\mu\text{g/g}$ creatinine and most preferably 32.3 10,8 $\mu\text{g/g}$ creatinine.

- Preferably, the reference amount for sFlt-1 as set forth in step a) of the aforementioned method has been derived from a reference sample from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to have ischemic complications in the kidney caused by the major surgery as the predominant cause for AKI associated with major surgery. Preferably, said reference sample has been obtained during or after a major surgery. Preferred time points or time periods for obtaining a sample during or after surgery are described above in the context of step b) of the aforementioned method. Moreover, is it preferred that the reference sample has been obtained at the same time, or the same time period at which the test sample, i.e. the sample obtained from the subject to be tested, has been obtained.
- 15
- 20

- 25 Preferred reference amounts for sFlt1 derived from a subject or groups of subjects as set forth above are, in increasing order of preference 300 pg/ml, 400 pg/ml and 500 pg/ml.

Preferably the following is applied as a diagnostic algorithm:

- 30 i. preferably, an essentially identical amount of L-FABP or an increased amount of L-FABP in the urine sample from the test subject as compared to the reference amount for L-FABP indicates that a pre-existing disorder of the renal tubules is the predominant cause for said AKI.

- ii. preferably, an essentially identical amount of sFlt-1 or an increased amount of sFlt-1 in the sample from the test subject as compared to the reference amount for sFlt-1 indicates that ischemic complications of the kidney caused by a major surgery are the predominant cause of AKI, and
- 5 iii. preferably, an essentially identical amount of sFlt-1 or an increased amount of sFlt-1 in the sample from the test subject as compared to the reference amount for sFlt-1, in combination with an essentially identical amount of L-FABP or an increased amount of L-FABP in the urine sample from the test subject as compared to the reference amount for L-FABP, indicates that the
- 10 AKI is caused by both ischemic complications in the kidney caused by major surgery and a pre-existent disorder of the renal tubules.

In case of i), the amount of sFlt-1 in the sample (as set forth step b)) is, preferably, lower than the reference amount for sFlt-1. More preferably, the amount of sFlt-1 is lower than

15 the amount of sFlt-1 in a reference sample that has been derived from a sample from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known not to have the major surgery as the predominant cause for AKI associated with major surgery. Preferably, said reference sample has been obtained during or after a major surgery. Preferred time points or time periods for obtaining a sample during or

20 after surgery are described above in the context of step b) of the aforementioned method. Moreover, is it preferred that the reference sample has been obtained at the same time, or the same time period at which the test sample, i.e. the sample obtained from the subject to be tested, has been obtained.

25 In case of ii), the amount of L-FABP in the urine sample (see step a)) is, preferably, lower than the reference amount for L-FABP. More preferably, the amount of L-FABP is lower than the amount of L-FABP in a reference urine sample that has been derived from a sample from a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known not to have a pre-existing disorder of the renal tubules

30 as the predominant cause for AKI associated with major surgery. Preferably, said reference urine sample has been obtained before major surgery in said subject. Preferred time points or time periods for obtaining a sample before a surgery are described above in the context of step a) of the aforementioned method. Moreover, is it preferred that the reference urine

sample has been obtained at the same time, or the same time period at which the test sample, i.e. the sample obtained from the subject to be tested, has been obtained.

The term “liver-type fatty acid binding protein” (L-FABP, frequently also referred to as FABP-1) relates to a polypeptide that is encoded by the FABP1 gene. The term, preferably, relates to the human L-FABP polypeptide. L-FABP belongs to a family of small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. Liver-type fatty acid binding protein is an intracellular carrier protein of free fatty acids that is, e.g., expressed in the proximal tubules of the human kidney. Kamijo et al. (Urinary liver-type fatty acid binding protein as a useful biomarker in chronic kidney disease. *Mol. Cell Biochem.* 2006; 284) reported that urinary excretion of L-FABP may reflect various kind of stresses that cause tubular interstitial damage and may be a useful clinical marker of the progression of chronic renal disease. The sequence of human L-FABP is e.g. disclosed by Chan et al. (Human liver fatty acid binding protein cDNA and amino acid sequence. Functional and evolutionary implications *J. Biol. Chem.* 260 (5), 2629-2632 (1985)), or GenBank Acc. Number M10617.1 (DNA sequence) and AAA52419.1 (protein sequence). Genbank is available from the NCBI, USA. Further, the term “L-FABP” encompasses variants of said specific human L-FABP. Such variants have at least the same essential biological and immunological properties as the specific L-FABP polypeptide. Variants are deemed to share the same essential biological and immunological properties if they are detectable by the same specific assays referred to in this specification, e.g., by ELISA assays using polyclonal or monoclonal antibodies specifically recognizing the said L-FABP polypeptides. A preferred assay is described in the accompanying Examples. Moreover, it is to be understood that a variant as referred to in accordance with the present invention shall have an amino acid sequence which differs due to at least one amino acid substitution, deletion and/or addition wherein the amino acid sequence of the variant is still, preferably, at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identical with the amino sequence of the specific L-FABP polypeptides. How to assess degree of identity is described elsewhere herein.

In an aspect of the invention, a method for establishing an aid for differentiating in a subject suffering from acute kidney injury (AKI) associated a major surgery between (i) a pre-existing disorder of the renal tubules (ii) ischemic complications in the kidney caused by

major surgery, or (iii) both a pre-existent disorder of the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant cause for said AKI is established, comprising the steps of

- 5 a) determining the amount of L-FABP (liver fatty acid binding protein) in a urine sample of said subject obtained prior to the major surgery, and comparing the, thus, determined amount of L-FABP to a reference amount for L-FABP, said determining comprises (i) bringing the sample into contact with a detection agent that specifically binds to L-FABP for a time sufficient to allow for the formation of a complex of the said detection agent and the L-FABP from the sample, (ii)
10 measuring the amount of the formed complex, wherein the said amount of the formed complex is proportional to the amount of L-FABP present in the sample, and (iii) transforming the amount of the formed complex into an amount of L-FABP reflecting the amount of L-FABP
- 15 b) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a sample of a subject obtained during or after said major surgery, and comparing the, thus, determined amount of sFlt-1 to a reference amount for sFlt-1, said determining comprises (i) bringing the sample into contact with a detection agent that specifically binds to sFlt-1 for a time sufficient to allow for the formation of a complex of the said detection agent and the sFlt-1 from the sample, (ii) measuring the
20 amount of the formed complex, wherein the said amount of the formed complex is proportional to the amount of sFlt-1 present in the sample, and (iii) transforming the amount of the formed complex into an amount of sFlt-1 reflecting the amount of sFlt-1,
- 25 c) comparing the amount of L-FABP determined in step a) to a reference amount for L-FABP, and comparing the amount of sFlt-1 determined in step b) to a reference amount for sFlt-1, and
- 30 d) establishing an aid for differentiating between (i) a pre-existing underlying disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent underlying disorder of the renal tubules and ischemic complications in the kidney caused by said major surgery, as the predominant cause for said acute kidney injury based on the comparison made in step c).

Detection agents have been disclosed elsewhere herein. In yet an aspect of the aforementioned method, step a) and b) may be carried out by an analyzing unit, in an aspect, an analyzing unit as defined elsewhere herein. In an aspect of the method of the invention, the amounts determined in steps a) and b) are compared to a reference. In an aspect, the references for sFlt-1 and L-FABP are references as defined elsewhere herein. In yet another aspect, the reference takes into account the proportional relationship between the measured amount of complex and the amount present in the original sample. Thus, the references applied in an aspect of the method of the invention are artificial references which are adopted to reflect the limitations of the detection agent that has been used. In another aspect, said relationship can be also taken into account when carrying out the comparison, e.g., by including a normalization and/or correction calculation step for the determined amount prior to comparing the value of the determined amount and the reference. Again, the normalization and/or correction calculation step for the determined amount adopts the comparison step such that the limitations of the detection agent that has been used is reflected properly. In an aspect, the comparison is carried out automatically, e.g., assisted by a computer system or the like.

The aid for differentiation is established based on the comparisons carried out in step c) by allocating the subject either into a group of subjects suffering from acute kidney injury (AKI) associated a major surgery between (i) a pre-existing disorder of the renal tubules (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent disorder of the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant. As discussed elsewhere herein already, the allocation of the investigated subject must not be correct in 100% of the investigated cases. Moreover, the groups of subjects into which the investigated subject is allocated are artificial groups in that they are established based on statistical considerations, i.e. a certain preselected degree of likelihood based on which the method of the invention shall operate. Thus, the method may establish an aid of diagnosis which may, in an aspect, require further strengthening of the diagnosis by other techniques. In an aspect of the invention, the aid for differentiating is established automatically, e.g., assisted by a computer system or the like.

Moreover, the present invention relates to the use of the biomarker sFlt-1 and/or of a detection agent which specifically binds thereto in a sample from a subject for diagnosing acute kidney injury associated with major surgery.

- 5 Also, the present invention relates to the use of i) the biomarker sFlt-1 and GDF-15 or ii) to the use of a detection agent which specifically binds to sFlt-1 and of a detection agent which specifically binds to GDF-15 in a first and second sample from a subject for diagnosing acute kidney injury associated with major surgery.
- 10 Also, the present invention relates to the use of i) the biomarker sFlt-1 and GDF-15 or ii) to the use of a detection agent which specifically binds to sFlt-1 and of a detection agent which specifically binds to GDF-15 in a sample from a subject for diagnosing acute kidney injury associated with major surgery.
- 15 Also, the present invention relates to the use of i) the biomarker sFlt-1 (in a sample of a subject obtain during or after major surgery) and L-FABP in a urine sample obtained before major surgery, or ii) to the use of a detection agent which specifically binds to sFlt-1 (in a sample of a subject obtain during or after major surgery) and of a detection agent which specifically binds to L-FABP in a urine sample obtained before major surgery for
- 20 differentiating between (i) a pre-existing disorder of the renal tubules (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent disorder or the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant cause for said AKI. Preferably, said subject shall suffer from acute kidney injury (AKI) associated a major surgery

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The term “detection agent” as used herein refers to an agent which is capable of specifically recognizing and binding to a biomarker present in a sample, preferably to sFlt1, to L-FABP or to GDF-15. The term is interchangeably used with the term “ligand” herein. Mo

The agent shall allow for direct or indirect detection of the complex formed by the said

30 agent and the biomarker. Direct detection can be achieved by including into the agent a detectable label. Indirect labelling may be achieved by a further agent which specifically binds to the complex comprising the biomarker and the detection agent wherein the said further agent is than capable of generating a detectable signal. Suitable compounds which

can be used as detection agents are well known in the art. Preferably, the detection agent is an antibody or aptamere which specifically binds to the biomarker protein or a nucleic acid encoding the biomarker. The term “antibody” as used herein includes both polyclonal and monoclonal antibodies, as well as any modifications or fragments thereof, such as Fv, Fab and F(ab)₂ fragments. The antibody shall be capable of specifically binding to the polypeptide referred to herein (i.e. to sFlt-1, to GDF-15, or to L-FABP).

Also encompassed is a device adapted for carrying out the method for diagnosing AKI associated with major surgery comprising

- a. an analyzing unit comprising a detection agent which specifically binds to sFlt1 (and/or, preferably, a detection agent which specifically binds to GDF-15), said unit being adapted for determining the amount of sFlt1 (and/or preferably, for determining the amount of GDF-15) in a first and second sample from a subject; and
- b. an evaluation unit for comparing the determined amount in said first sample with the amount in said second sample whereby AKI associated with major surgery can be diagnosed, said unit comprising a database with increases of the amount of sFlt1 (and/or preferably, of increases of the amount of GDF-15) in the second sample as compared to the first sample, said increases being, preferably, derived from a subject or a group of subjects known to have developed AKI associated with major surgery and a computer-implemented algorithm for carrying out a comparison step.

Said increases of the amount are, preferably, increase which are indicative for the diagnosis of AKI (and/or risk thereof).

Moreover, the present invention relates to a device adapted for carrying out the method of the present invention for diagnosing AKI associated with major surgery, comprising

- a. an analyzing unit comprising a detection agent which specifically binds to sFlt1 (and/or, preferably, a detection agent which specifically binds to GDF-15), said unit being adapted for determining the amount of sFlt1 (and/or preferably, for determining the amount of GDF-15) in a sample of a subject; and
- b. an evaluation unit for comparing the determined amount with a reference amount whereby AKI can be diagnosed, said unit comprising a database with reference

amount values for sFLT1 (and/or, preferably, with reference amount values GDF-15) and a computer-implemented algorithm for carrying out a comparison step.

In the context of the aforementioned device, said reference amounts are preferably derived from a sample from:

(i) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known to suffer from AKI associated with major surgery, and/or

(ii) a subject (herein also referred to as reference subject) or a group of subjects (group of reference subjects) known not to suffer from AKI associated with major surgery (see also above)

Moreover, the present invention relates to a device adapted for carrying out the method of the present invention for differentiating in a subject suffering from acute kidney injury (AKI) associated a major surgery between (i) a pre-existing disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent disorder of the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant cause for said AKI, comprising

- a. an analyzing unit comprising a detection agent which specifically binds to sFlt1, said unit being adapted for determining the amount of sFlt1 in a sample of a subject;
- b. an analyzing unit comprising a detection agent which specifically binds to L-FABP, said unit being adapted for determining the amount of L-FABP in a urine sample of a subject;
- c. an evaluation unit for comparing the determined amounts with reference amounts whereby it can be differentiated between (i), (ii) and (iii) as set forth above a the predominant cause of AKI, said unit comprising a database with a reference amount (reference amounts) for sFlt1 and L-FABP and a computer-implemented algorithm for carrying out a comparison step.

Preferred reference amounts are the reference amounts as set forth in the context of the corresponding method (see above).

The term “device” as used herein relates to a system comprising the aforementioned units operatively linked to each other as to allow the diagnosis or monitoring according to the methods of the invention. Preferred detection agents which can be used for the analyzing unit are disclosed elsewhere herein. The analyzing unit, preferably, comprises said detection agents in immobilized form on a solid support which is to be contacted to the sample comprising the biomarkers the amount of which is to be determined. Moreover, the analyzing unit can also comprise a detector which determines the amount of detection agent which is specifically bound to the biomarker(s). The determined amount can be transmitted to the evaluation unit. Said evaluation unit comprises a data processing element, such as a computer, with an implemented algorithm for carrying out a comparison between the determined amount and a suitable reference (e.g. a reference amount, or the amount of the marker in a first or second sample from the subject). Suitable references can be derived from samples of subjects to be used for the generation of reference amounts as described elsewhere herein above. The results may be given as output of parametric diagnostic raw data, preferably, as absolute or relative amounts. It is to be understood that these data will need interpretation by the clinician. However, also envisaged are expert system devices wherein the output comprises processed diagnostic raw data the interpretation of which does not require a specialized clinician.

Moreover, the present invention envisages a kit adapted for carrying out the method of the present invention for diagnosing AKI associated with major surgery, said device comprising a detection agent for the biomarker sFlt1. Preferably, the kit further comprises a detection agent for GDF-15. Moreover, the kit further may comprise instructions for carrying out the said method.

The term “kit” as used herein refers to a collection of the aforementioned components, preferably, provided in separately or within a single container. The container also comprises instructions for carrying out the method of the present invention. These instructions may be in the form of a manual or may be provided by a computer program code which is capable of carrying out the comparisons referred to in the methods of the present invention and to establish a diagnosis accordingly when implemented on a computer or a data processing device. The computer program code may be provided on a data storage medium or device such as an optical storage medium (e.g., a Compact Disc) or directly on a computer or data

processing device. Moreover, the kit may, preferably, comprise standards for reference amounts as described elsewhere herein in detail.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

The following examples are only intended to illustrate the present invention. They shall not limit the scope of the invention in any way.

Example 1

Patients, Materials and methods

A total of 126 consecutive patients undergoing elective coronary artery bypass graft (CABG) surgery were included into the study. The patients were clinically stable, and, thus did not exhibit symptoms of ACS at the time of surgery and within one week prior to surgery. There were 68 males and 58 females, median age 68 (52-81) years. Serum creatinine levels were normal in all patients. All patients had two or more vessel disease as indicated by at least one stenosis exceeding 50 % of the lumen. Patients were followed for 30 days with respect to mortality and development of acute kidney injury. Endpoints of the study were: acute kidney injury (AKI, creatinine increase at least 0.3 mg/dl within 3 days after surgery), new requirement for haemodialysis or mortality (within 30 days after surgery). Blood was taken before surgery and immediately thereafter as well as 1, 2 and 3 days after surgery. Samples were centrifuged for 30 minutes and stored at -20° Celsius until analyzed.

sFlt-1, Troponin T (hsTNT), GDF-15 and NT-proBNP were determined with sandwich immunoassays using analyzers from Elecsys or COBAS e-series. The assays comprise two monoclonal antibodies specific for the respective peptide. The first of these is biotinylated and the second one is labelled with a Tris(2,2'-bipyridyl)ruthenium (II)-complex. In a first incubation step both antibodies are incubated with the sample. A sandwich complex comprising the peptide to be determined and the two different antibodies is formed. In a next

incubation step streptavidin-coated beads are added to this complex. The beads bind the sandwich complexes. The reaction mixture is then aspirated into a measuring cell where the beads are magnetically captured on the surface of the electrode. The application of a voltage then induces a chemiluminescent emission from the ruthenium complex which is measured by a photomultiplier. The emitted amount of light is dependent on the amount of sandwich complexes on the electrode. sFlt-1 amounts between 10 to 85,000 pg/ml, GDF-15 amounts between 300 pg to 20,000 pg/ml, and NT-proBNP amounts between 2 pg/ml and 35,000 pg/ml can be measured. The high sensitivity Troponin T test used in this study has a sensitivity of 2 pg/ml. The amounts of sFlt-1, Troponin T and NT-proBNP in Examples are given in "pg/ml".

L-FABP was determined by using the L-FABP ELISA-Kit from CMIC Co., Ltd, Japan. The test was based on an ELISA 2-step assay. L-FABP standard or urine samples were firstly treated with pretreatment solution as provided with the test, and transferred into a L-FABP antibody coated microplate containing assay buffer and incubated. During this incubation, L-FABP in the reaction solution bound to the immobilized antibody. After washing, the 2nd Antibody-POD conjugate was added as the secondary antibody and incubated, thereby forming sandwich of the L-FABP antigen between the immobilized antibody and conjugate antibody. After incubation, the plate was washed and substrate for enzyme reaction was added, color develops according to the L-FABP antigen quantity. The L-FABP concentration was determined based on the optical density.

Creatinine was determined using a modification of the Jaffe method (Foster-Swanson A et al., 1994, Clinical Chemistry, Abstract #361; Seelig HP and Wüst H, 1969, Ärztliches Labor, 15: 34-39; Bartels H et al., 1972, Clinical Chimica Acta, 37: 193-197). Briefly, picric acid reacts with creatinine in alkaline solution to form a yellow-orange complex. Said complex was detected photometrically with Roche/Hitachi analyzers.

sFlt-1 levels (pg/ml) before surgery are shown in Table 1.

Table 1: sFlt-1 levels (pg/ml) before surgery

Percentile	All patients	No AKI	AKI
	N = 126	N = 89	N = 37
25	97	99	97
50	157	163	131
5 75	221	222	207

As can be seen sFlt-1 levels were higher in patients not developing AKI than in those who developed AKI, however sFlt-1 levels were in general higher in CABG patients than in a control group of 149 apparently healthy individuals who had sFlt-1 levels of 71 pg/ml (median) and 64.8 and 78 pg/ml (25 % and 75 % percentile respectively). This is due to the fact that not all patients were clinically stable or had undergone invasive diagnostic procedures. In any case, for these reasons sFlt-1 before intervention can not be used as a predictor of AKI developing after intervention.

In contrast, sFlt-1 is useful in the early recognition of AKI as demonstrated by sFlt-1 levels taken immediately after surgery. This is shown in Table 2 when tertiles of sFlt-1 were formed and compared to the outcome AKI

Table 2: Post-surgery sFlt-1 levels in patients with outcome AKI taken immediately after surgery.

sFlt-1 tertiles	1.	2.	3.
pg/ml	N = 39	N = 39	N = 39
N = AKI	8	8	18
sFlt-1 median pg/ml	185	287	647
25	(103 – 222)	(223 – 405)	(408-2632)

The data obtained clearly indicate that the level of sflt-1 reflecting the extent of ischemia during the intervention clearly correlates with probability of the outcome AKI.

Table 3 shows an overview on the sFlt-1 levels in patients with and without AKI at various time points (given are the median levels in pg/ml). Whereas an increase of only 75% was observed (with respect to the median level of sFlt-1) in patients without AKI, patients who developed AKI showed an increase of more than 200 % at the end of surgery. The largest increase was obtained at the end of surgery or immediately after surgery. However, significantly increased sFlt-1 levels were also observed 24 hours after surgery.

Table 3: sFlt-1 levels in patients with and without outcome AKI at various time points

sFlt-1	start of surgery	end of surgery	24 hours after surgery
no AKI	163	268	212
AKI	131	405	322

As it can be seen from table 3, patients with the outcome AKI had significantly increased sFlt-1 levels at the end of surgery as compared to patients without AKI. sFlt-1 levels are still increased even 24 hours after surgery, although to a lower extend as immediately after surgery. Therefore, sFlt-1 is a reliable marker for differentiation between AKI and non AKI even after 24 hours after surgery. However, it is particularly preferred to obtain the test sample immediately after surgery.

Moreover determination of GDF 15 after surgery indicates that patients developing AKI have significantly higher GDF 15 levels than those not developing AKI. This is reflected in Table 4. Whereas an increase of only 60% was observed (with respect to the median level of GDF-15) in patients without AKI, patients who developed AKI showed an increase of approximately more than 150% at the end of surgery. 24 hours after surgery, difference regarding the GDF-15 levels in patients with AKI and patients without AKI is even more significant as compared to the difference at the end of surgery. sFlt-1 is therefore considered as a very early marker for acute kidney injury, whereas GDF- 15 is an early to late marker. In contrast, creatinine as a later marker which allows for the diagnosis of AKI only after two days after the onset of the acute event.

Table 4: GDF15 levels in patients with and without AKI at various time points

GDF15	start of surgery	end of surgery	24 hours after surgery
no AKI	1105	1807	1903
AKI	1347	3389	4675

Case studies

- 5 A patient exhibiting multivessel disease was subjected to coronary bypass surgery. In a sample obtained before the surgery, the levels of sFlt-1 and GDF-15 were determined (sFlt-1: 120 pg/ml, GDF-15 913 pg/ml). The levels of sFlt-1 and GDF-15 were also determined in a sample obtained immediately after surgery (sFlt-1: 163 pg/ml, GDF-15: 1330 pg/ml). Thus, GDF-15 and sFlt-1 were only slightly increased relative to the respective
- 10 reference levels. The patient did not develop AKI within 30 days after surgery.

- A further patient exhibiting multivessel disease was also subjected to coronary bypass surgery. In a sample obtained before the surgery, the levels of sFlt-1 and GDF-15 were determined (sFlt-1: 161 pg/ml, GDF-15 1682 pg/ml). The levels of sFlt-1 and GDF-15 were
- 15 also determined in a sample obtained immediately after surgery (sFlt-1: 570 pg/ml, GDF-15: 3401 pg/ml). Thus, sFlt-1 and GDF-15 were significantly increased at the end of surgery. The patient did develop AKI within 30 days after surgery.

Example 2

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Correlation between L-FABP before surgery and sFlt-1 after surgery in patients with the outcome AKI

Urinary L-FABP is a marker for tubular damage and is a good predictor for AKI in patients undergoing cardiac surgery (European patent application 10165964.7, filed on 15.06.2010 unpublished). In particular, increased levels of L-FABP in a sample obtained prior to surgery are indicative for an increased risk of AKI. However, it is a less reliable marker after surgery.

In contrast, no correlation was found between the amount of sFlt-1 in samples obtained prior to surgery and the outcome AKI. However, sFlt-1 is a reliable marker for AKI if measured in samples after surgery.

Because of the differences between these two markers, it was assessed whether both markers identify the same subgroup of patients. In particular, it was assessed, whether patients with AKI with increased urinary levels of L-FABP also had increased levels of sFlt-1 after surgery.

A total of 27 patients developing AKI after cardiac surgery were analyzed. A level of urinary L-FABP in a sample obtained prior to surgery of larger than 10,8 µg/ g creatinine was considered as being indicative for an increased risk of AKI. A level of sFlt-1 in a sample obtained at the end of surgery of larger than 500 pg/ml was indicative for the diagnosis of AKI.

A total of 8 patients out of 27 patients had L-FABP levels larger than the cut-off value indicating an increased risk of AKI.

A total of 11 patients had increased sFlt-1 levels after surgery, i.e. levels above the cut-off, indicating AKI (the number of positives would be larger, if the cut-off value would be lowered).

Interestingly, only two patients had increased levels of L-FABP before the surgery, and increased sFlt-1 levels after the surgery. Thus, the determination of L-FABP before surgery and of sFlt-1 after surgery allows for identifying two subgroups of patients with AKI. Whereas patients which have an increased risk of AKI due pre-existing renal disorders such as tubular damage can be identified by measuring the amount of L-FABP before surgery, patients which develop AKI caused by major surgery can be identified by measuring sFlt-1 in a sample obtained after surgery. Interestingly, the two groups do not comprise the same patients. Therefore, L-FABP and sFlt-1 are independent markers for the risk or diag-

nosis of AKI. Moreover, by determining the amounts of both markers as described above, the predominant cause of AKI can be assessed.

This illustrates that development of AKI is complex and composed of pre-existing illness of the kidney which predisposes to AKI and events occurring during surgery associated with ischemia.

The value of diagnosing pre-existing kidney damage in spite of normal kidney function lies in the fact that specific measures can be taken to avoid AKI, the diagnostic value of sFIT1 is the early diagnosis of ischemia associated with surgery which allows a careful monitoring of these patients and the avoidance of factors that might cause further damage to the kidney and ultimately leading to AKI.

Summary

Acute kidney injury is a frequent and serious complication of coronary bypass surgery and other major surgery. While there are indicators for increased risk for acute kidney injury already before surgery such as older age, impaired kidney function or pre-existing heart failure there was currently no method to identify risk factors resulting from the intervention itself. Thanks to the current invention sFIT1 concentration after surgery and its increase from baseline (before surgery) turned out to be a good indicator for acute kidney injury. This has implications for monitoring, time necessary on intensive care unit, and application of drugs to be administered (avoidance of nephrotoxic drugs and careful fluid balance and maintenance of blood pressure).

Claims

1. A method for diagnosing acute kidney injury associated with a major surgery in a subject, said method comprising the steps of
 - 5 a) determining the amount of soluble fms-like thyrosine kinase-1 (sFlt-1) in a first sample of said subject obtained prior to the major surgery;
 - b) determining the amount of soluble fms-like thyrosine kinase-1 (sFlt-1) in a second sample of said subject, wherein said second sample has been obtained during or after said major surgery, and
 - 10 c) comparing the amount of sFlt-1 in said second sample to the amount of sFlt-1 in said first sample, whereby acute kidney injury is diagnosed, wherein an increase of the amount of sFlt-1 in the second sample as compared to the first sample is indicative for the diagnosis of AKI.
- 15 2. The method of claim 1, wherein an increase of more than 200 % is indicative for the diagnosis of AKI.
3. The method of claims 1 and 2, wherein said second sample has been obtained at or after completion of said major surgery.
- 20 4. The method of any one of claims 1 to 3, wherein the method does not encompass the determination of PlGF (Placental Growth Factor)
5. The method of claim 1 to 4, wherein said subject does not suffer from a pre-existing disorder of the renal tubules.
- 25 6. The method of any one of claims 1 to 5, wherein said major surgery is coronary artery bypass surgery (CABG).

7. The method of any one of claims 1 to 6, wherein the method further comprises the steps of
- a') determining the amount of GDF-15 in a first sample obtained before the major surgery
 - 5 b') determining the amount of GDF-15 in another sample obtained during or after said first sample, and
 - c') comparing the amount of GDF-15 in said first sample to the amount of GDF-15 in said another sample.
- 10 8. The method of any one of claims 1 to 7, further comprising the step of recommending a therapy.
9. A method for diagnosing acute kidney injury associated with a major surgery in a subject, said method comprising the steps of
- 15 a) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a sample of the subject obtained during or after the major surgery; and
 - b) comparing the determined amount of sFlt-1 with a reference amount, whereby acute kidney injury is diagnosed.
- 20 10. The method of claim 9, wherein the reference amount is derived from a subject or a group of subjects known to suffer from acute kidney injury associated with a major surgery, wherein an essentially identical amount of sFlt-1 or an increased amount of sFlt-1 in the sample of the subject as compared to the reference amount is indicative for the diagnosis of acute kidney injury associated with major surgery.
- 25 11. The method of claims 9, wherein the reference amount is derived from a subject or a group of subjects known not to suffer from acute kidney injury associated with a major surgery, and wherein an essentially identical amount of sFlt-1 or decreased amount of sFlt-1 in the sample of the subject as compared to the reference amount
- 30 indicates that the subject does not suffer from acute kidney injury associated with major surgery.

12. The method of any one of claims 9 to 11, wherein said sample has been obtained within 24 hours after said major surgery.
13. A method for differentiating in a subject suffering from acute kidney injury (AKI) associated a major surgery between (i) a pre-existing disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent disorder of the renal tubules and ischemic complications in the kidney caused by major surgery, as the predominant cause for said AKI, comprising the steps of
- a) determining the amount of L-FABP (liver fatty acid binding protein) in a urine sample of said subject obtained prior to the major surgery, and comparing the, thus, determined amount of L-FABP to a reference amount for L-FABP, and
 - b) determining the amount of soluble fms-like tyrosine kinase-1 (sFlt-1) in a sample of a subject obtained during or after said major surgery, and comparing the, thus, determined amount of sFlt-1 to a reference amount for sFlt-1,
- whereby it is differentiated between (i) a pre-existing underlying disorder of the renal tubules, (ii) ischemic complications in the kidney caused by major surgery, or (iii) both a pre-existent underlying disorder of the renal tubules and ischemic complications in the kidney caused by said major surgery, as the predominant cause for said acute kidney injury.
14. Use of the biomarker sFlt-1 and/or of a detection agent which specifically binds thereto in a first and second sample from a subject for diagnosing acute kidney injury associated with a major surgery.
15. A device adapted for carrying out the method of claims 1 to 8, comprising
- a. an analyzing unit comprising a detection agent which specifically binds to sFlt1, said unit being adapted for determining the amount of sFlt1 in a first and second sample from a subject; and
 - b. an evaluation unit for comparing the determined amount in said first sample with the amount in said second sample whereby AKI associated with major surgery can be diagnosed, said unit comprising a database with increases of the amount of sFlt1 (and/or preferably, of increases of the amount of GDF-15) in the second

sample as compared to the first sample, said increases being, preferably, derived from a subject or a group of subjects known to have developed AKI associated with major surgery and a computer-implemented algorithm for carrying out a comparison step.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/057619

A. CLASSIFICATION OF SUBJECT MATTER
 INV. G01N33/68 G01N33/74 C12Q1/48
 ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C12Q

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Jan Runte ET AL: "Soluble fms-like tyrosine kinase 1 and Human placental growth factor: New "troponins" for cardiac-surgery-associated acute kidney injury?", 24. Herbsttreffen des wissenschaftlichen Arbeitskreises Kardioanästhesie, 13 November 2010 (2010-11-13), XP055004335, Retrieved from the Internet: URL: http://www.ak-kardioanaesthesie.de/informationen-und-links/downloads/doc_view/95-posterpreis3-new-troponine.raw?tmpl=component [retrieved on 2011-08-08]	1-6, 8-12,14, 15
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Rosin, Oliver

INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

PCT/EP2012/057619

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