(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2014 (12.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/135701 A1

(51) International Patent Classification:
G01N 33/74 (2006.01)

(21) International Application Number:
PCT/EP2014/054508

(22) International Filing Date:
7 March 2014 (07.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13158401.3 8 March 2013 (08.03.2013) EP

(71) Applicant: **IMMUNDIAGNOSTIK AG** [DE/DE]; Stubenwald-Allee 8a, 64625 Bensheim (DE).

(72) Inventors: **ARMBRUSTER, Franz Paul**; c/o Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim (DE). **HOCHER, Berthold**; Ernst-Thaelmannstrasse 151a, 14532 Kleinmachnow (DE). **ROTH, Heinz Jürgen**; Im Breitspiel 15, 69126 Heidelberg (DE).

(74) Agent: **BENEDUM, Ulrich**; Haseltine Lake LLP, Theatinerstrasse 3, 80333 München (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

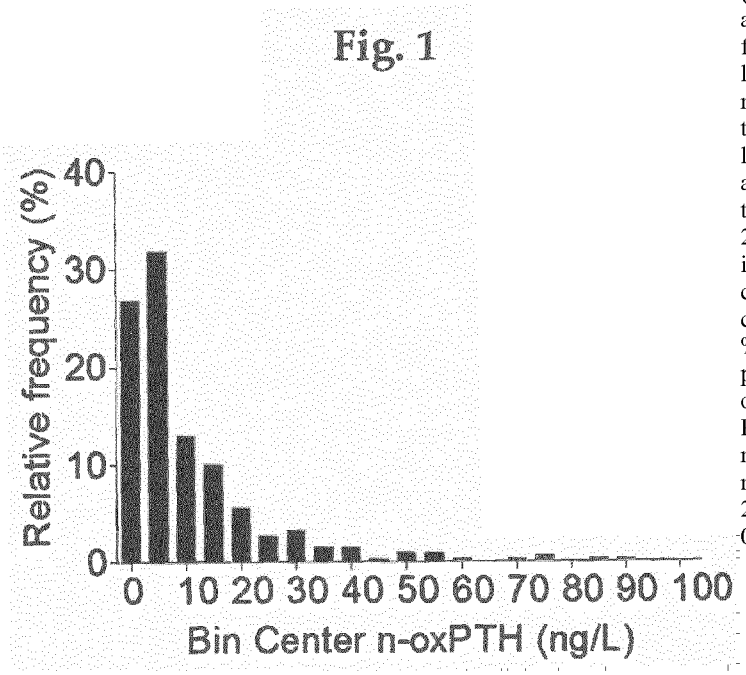
Published:

— *with international search report (Art. 21(3))*

WO 2014/135701 A1

(54) Title: NON-OXIDIZED, BIOLOGICAL ACTIVE PARATHYROID HORMONE DETERMINES MORTALITY IN HEMODIALYSIS PATIENTS

Fig. 1



(57) Abstract: A new method of in vitro monitoring and assessing the need of a medication which interferes with the regulation of the parathyroid hormone level in a kidney patient subject to oxidative stress, notably hemodialysis patients. Figure 1 shows the distribution of n-oxPTH concentrations in 340 hemodialysis patients (224 men and 116 women) with a median age of 66 years (IQR, 56 to 75 years), a median time since initiation of dialysis (dialysis vintage) of 266 days (IQR, 31 to 1209 days), and a median dialysis dose (kt/V) of 1.2 (IQR, 1.1 to 1.3). The cause of chronic kidney disease was nephrosclerosis in 113 cases (33%), diabetic nephropathy in 107 cases (31 %), chronic glomerular nephritis in 29 cases (9%), polycystic kidney disease in 9 cases (3%) and other/unknown in 82 cases (24%). The median n-oxPTH concentration was 5.9 ng/L (IQR, 2.4 to 14.0 ng/L). n-oxPTH concentrations were not different in men and women (5.9 ng/L; IQR, 2.4 to 14.2 ng/L; n = 224; vs. 5.5 ng/L; IQR, 2.4 to 14.0 ng/L; n = 1 16; p = 0.915).

NON-OXIDIZED, BIOLOGICAL ACTIVE PARATHYROID HORMONE DETERMINES MORTALITY IN HEMODIALYSIS PATIENTS

FIELD OF THE INVENTION

[001] The invention relates to means and methods of monitoring parathyroid hormone concentration in plasma samples of patients with chronic kidney disease (CKD).

BACKGROUND OF THE INVENTION

[002] Excess mortality in patients with chronic kidney disease stage 5 is an important unsolved problem. Annual mortality of patients with chronic kidney disease stage 5 is about 10 to 20%. The parathyroid hormone (PTH) seems being a factor responsible for the excess mortality in patients requiring hemodialysis as a recent huge study demonstrated a J-shaped association between PTH and mortality. Consequently, parathyroid hormone (PTH) has been described as a uremic toxin with multiple systemic effects including bone disorders (renal osteodystrophy), myopathy, neurologic abnormalities, anemia, pruritus, and cardiomyopathy. Hyperparathyroidism is common in CKD and results in significant morbidity and mortality if left untreated. Low as well as high PTH levels measured by current PTH assays are associated with a progression of cardiovascular diseases and substantially increased all-cause mortality in patients on hemodialysis (Floege J. et al, *ARO Investigators. Serum iPTH, calcium and phosphate, and the risk of mortality in a European haemodialysis population.* Nephrol Dial Transplant.2011;26:1948-1955; Torres PA et al, *Calcium-sensing receptor, calcimimetics, and cardiovascular calcifications in chronic kidney disease.* Kidney Int. 2012;82:19-25; Souberbielle JC et al. in *Parathyroid hormone measurement in CKD.* Kidney Int. 2010;77:93-100)

[003] Thus, guidelines have been established aiming to keep PTH in concentrations associated with the lowest morbidity and highest survival. The Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines recommend measuring regularly PTH concentrations of patients with chronic kidney disease (CKD) and adjusting the patients' medication (e.g. vitamin D, phosphate binders, calcimimetics) such that plasma PTH levels are kept within a target range in accordance with the stage of CKD (e.g., 150 to 300 ng/L in patients with CKD stage 5). If pharmacological approaches do not work adequately, parathyreodectomy may be considered.

[004] Secondary hyper-parathyroidism may also occur as an adaptive response to deteriorating renal function when circulating 1,25-dihydroxy vitamin D decreases as early as in stage 2 of CKD and continues to fall as the glomerular filtration rate (GFR) decreases. Chronic kidney disease is associated with a progressive loss of 1α-hydroxylase activity, because of functional reasons such as enzyme inhibition by hyperphosphataemia, hyperuricaemia, metabolic acidosis and sometimes also 25-hydroxyvitamin D deficiency. More important is , however, simply the loss of healthy renal tissue – and hense 1α-hydroxylase – explaining functional reduction of 1α-hydroxylase activity in CKD. As GFR decreases below 60 mL/min/1·73·m^2 phosphate is retained which stimulates directly or via the klotho/FGF23 system secretion of PTH. Additionally the 1,25-dihydroxy vitamin D deficiency contributes in this situation to an increased secretion of PTH, since PTH secretion/gene expression in the parathyroid glad is negatively controlled by 1,25-dihydroxy vitamin D.

[005] Hypocalcaemia develops as the GFR decreases below 50 mL/min/1·73·m^2, further stimulating a secretion of parathyroid hormone (PTH) from cells of the parathyroid gland into the blood circulation. In the intact form human parathyroid hormone (hPTH) consists of a single polypeptide chain having 84 amino acids and a molecular weight of ca. 9500 Dalton (see SWISS-PROT: P01270, PTHY-HUMAN). With disease progression, intact hPTH(aa 1–84) half-life increases and immunoreactive C-terminal fragments of the hormone tend to accumulate in serum. A chronic elevation of parathyroid activity then results in bone loss, fractures, vascular calcification, cardiovascular disease, and hence an increased cardiovascular mortality (cf Fraser WD, *Hyperparathyroidism.* Lancet. 2009; 374:145f).

[006] Part of the problem with the use of PTH measurements has been confusion concerning the interpretation of the assays utilized. The measurement of PTH in blood has evolved since the early 1960s when RIAs were first developed for measurement of PTH (Berson SA et al, Proc Natl Acad Sci U S A. 1963; 49:613-617). However, these first-generation assays proved not to be reliable owing to different characteristics of the antisera used and the realization that PTH circulates not only in the form of the intact 84-amino-acid peptide but also as multiple fragments of the hormone, particularly from the mid and carboxy (C)-terminal regions of the PTH molecule. The PTH peptide following secretion is degraded within minutes in the kidney in active and inactive fragments and the respective fragments have further varying half-lifes. A second generation of PTH immunoassays was developed using two antibodies one binding in the aminoterminal portion of the PTH peptide with the biologic activity and the other in its carboxyterminal portion (John MR et al. (1999), J. Clin. Endocrinol.

Metab., 84. 4287-4290; Gao P et al. 2000, Poster M455, ASBMR 22nd Annual Meeting; Roth HJ et al. (2000), Poster P1288; 11th International Congress of Endocrinology, Sydney). However, there was still a discrepancy between measured immunoreactive PTH concentration in serum and clinical findings (Goltzman D et al, in *Discordant disappearance of bioactive and immunoreactive parathyroid hormone after parathyroidectomy,* J Clin Endocrinol Metab 1984, 58(1):70-75. Thus, a third generation of intact PTH assay has been developed which however fails to improve the diagnosis of bone diseases or other clinical signs of secondary hyperparathyroidism in uraemic patients (Brossard JH et al., *Influence of glomerular filtration rate on non-(1-84) parathyroid hormone (PTH) detected by intact PTH assays,* Clin Chem. 2000; 46:697-703). It seems meanwhile accepted that some immunoreactive PTH fragments have a biological activity comparable with intact PTH peptides whereas others such as hPTH(3-34) seem to inhibit the effects of parathyroid hormone (see EP-A 0 349 545; Schmidt-Gayk et al. (1999) Osteologie forum, 5, 48-58), Suva et al. (1987) Science, 237, 893ff; EP 0 451 867). It has further been postulated that large inactive but immunoreactive non-(1-84) PTH fragments lead to erroneous determinations (LePage R. et al. (1998) Clin. Chem., 44, 805-809). Additionally, dipeptidyl peptidase-4 (DPP4) is expressed on the surface of many cell types and a rather indiscriminate serine exopeptidase. This led to the hypothesis of PTH being a substrate of DPP4 or a similar exoproteinase while the utmost two N-terminal amino acids are necessary for cAMP-cyclase activity and binding of the PTH peptide to its receptor. Consequently, a also two-site immunoassays have been developed employing antibodies that can distinguish between aminoterminally „intact" PTH peptide chains and PTH peptides that are missing one or two amino acids at the utmost aminoterminus (see WO 2001/44818 (Armbruster et al), WO 96/10041 (Mägerlein et al); WO 2003/03986 (Hutchison JS)).

[007] The discovery of oxidized PTH peptide chains in serum samples of uraemic patients has further led to the development of an immunoassay for determination of non-oxidized PTH (1-84) and biologically active fragments thereof (WO 2002/082092). Thus, there are plethora of immunoassays available for measuring parathyroid hormone in plasma and concentrations of various "bioactive" PTH peptide embodiments which are in some patient groups similar and other patient groups noticeably different. It would therefore be desirable to obtain reliable information on the patients' PTH status which allows an adaptation of the medication of kidney patients to reduce morbidity and mortality (see also Sprague SM et al, *The Case for Routine Parathyroid Hormone Monitoring,* Clin J Am Soc Nephrol, Oct 2012, as doi: 10.2215/CJN.04650512e; Goldsmith DJA, ebuttal: *The Case for Routine Parathyroid Hormone Monitoring* Clin J Am Soc Nephrol 8: 319–320, 2013. doi:

10.2215/CJN.10231012). The state of the art PTH measurement therefore represents a problem.

SUMMARY OF THE INVENTION

[008] The problem is solved by a method of PTH monitoring and assessing which gives reliable information on how to adopt the drugs usually prescribed to CKD patients that interfere with PTH secretion. The use of these drugs are also guided by the international guidelines.

[009] Consequently, the instant disclosure relates to a method of *in vitro* monitoring and assessing the need of a medication which interferes with the regulation of the parathyroid hormone level in a kidney patient subject to oxidative stress comprising the steps of purifying a sample of plasma or serum from said kidney patient from human PTH peptides oxidized at either methionine 8 or 18 or both or at tryptophan 22 by contacting said sample with an antibody which recognizes and specifically binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides but which antibody does not bind non-oxidized human PTH (1-84) and fragments thereof; determining the amount of immunoreactive human PTH (iPTH) peptides in said sample by an immunoassay based on antibodies against human PTH(1-84) and fragments thereof that contain at least the domains responsible for receptor binding and activation of the cAMP-cyclase located between amino acids 3 to 34 of the human PTH sequence; and obtaining a PTH status value (n-oxPTH value) for said kidney patient which includes the rate of immunoreactive human PTH (iPTH) peptides secreted by cells of the parathyroid gland into the circulation and the rate by which immunoreactive human PTH (iPTH) peptides are oxidized by the oxidative stress suffered by said patient; and comparing the PTH status value (n-oxPTH) with a reference value at which the morbidity and all-cause mortality is low to determine the need of a medication with respect to a regulation of the PTH status value or for supplementation of the patient with human parathyroid hormone or active fragments thereof or both. The method can be used in particular for a sample from a kidney patient subject to a hemodialysis treatment. Otherwise, the sample may be from a kidney patient afflicted of chronic kidney disease (CKD) or uremia or hyperparathyroidism.

[010] The method preferably comprises the step of contacting said sample of plasma or serum with a solid phase having bound an antibody which recognizes and specifically binds a three-dimensional epitope located between amino acids 3 to 34 of

oxidized human PTH peptides but which antibody does not bind non-oxidized human PTH (1-84) and fragments thereof.

[011] The method comprises a determination of immunoreactive potent human PTH (iPTH) peptides which may encompass the use of a two-site immunoassay wherein one antibody is a monoclonal antibody that binds to a domain involved in the binding of the PTH peptide to PTH receptors 1 and 2. Alternatively, the determination may comprise the use of a two-site immunoassay wherein one antibody binds an antigenic determinant comprising the utmost aminoterminal amino acids valine and serine of the human PTH and the other antibody binds in the region between amino acids 14 to 34 of the human PTH sequence. In another embodiment, the determination of immunoreactive potent human PTH (iPTH) may comprise the use of a two-site immunoassay wherein one antibody binds an antigenic determinant comprising amino acids 1 to the 13 of the human PTH sequence.

[012] The employed antibody against oxidized human PTH peptides selectively binds a three-dimensional epitope between amino acids 3 to 34 of the human PTH sequence which does not comprise the oxidized methionines at position 8 and/or 18. In other words, for analysis showed that the employed monoclonal antibody specific for oxidized human binds to a conformational epitope between amino acids 3 to 34 of the human PTH sequence which does comprise any one of the methionine sulfoxides or methionine sulfones.

[013] An aspect of the disclosure relates to a determination of the ratio of immunoreactive human PTH (iPTH) and the amount of PTH peptides bound by said antibody which recognizes and selectively binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides.

[014] A further aspect relates to a kit comprising a solid phase with an antibody that recognizes and selectively binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides, and a combination of antibodies for determining the immunoreactive intact human PTH (iPTH) in plasma or serum as disclosed above.

[015] The disclosed method usually comprises a purifying of a sample of plasma or serum from a patient from PTH peptides chains subject to oxidative stress and oxidized at either methionines 8 or 18 or both or at tryptophan 22 by contacting said sample with an antibody that binds an oxidized antigenic determinant of the human parathyroid hormone. PTH oxidation and hence inactivation is an issue in CKD patients with hemodialysis since these patients suffer from oxidative stress which

interfere with conventional PTH measurements. The method further comprises a determination of the secreted parathyroid hormone status in said purified sample by an immunoassay that measures the concentration of those peptides of the human parathyroid hormone that contain at least the domains responsible for receptor binding and activation of the cAMP-cyclase so that a PTH status is obtained, hereinafter "n-oxPTH" status, that corresponds to the equilibrium of the rate of PTH peptides secreted by cells of the parathyroid gland into circulation and clearance of PTH peptides from circulation that have been oxidized by the oxidative stress through the hemodialysis treatment. The disclosed method then compares the measured n-oxPTH status with a n-oxPTH status at which the all-cause mortality hemodialysis patients is low to determine the need of a medication with respect to a regulation of the secreted parathyroid hormone or by a direct supplementation of parathyroid hormone.

[016] The method comprises a contacting of said plasma sample with a solid phase having bound antibodies which bind an oxidized antigenic determinant of the human parathyroid hormone. The binding material may be in the form of a slurry.

[017] According to a preferred aspect, the method comprises a use of a two-site immunoassay wherein one antibody is a monoclonal antibody that binds to a domain involved in the binding of the PTH peptide to PTH receptors 1 and 2. The antibody may be one which recognizes and selectively binds an epitope between amino acids 1 to 13 of the human parathyroid hormone as disclosed in WO 2003/003986 (Hutchison JS) or as described in WO 01/44818 (Armbruster FP et al) or in US 6,030,790 (Adermann et al). Further suitable antibodies for determining the concentration of secreted potent human PTH peptides are disclosed in WO 00/42437.

[018] In another embodiment the method comprises the use of a two-site immunoassay wherein one antibody binds an antigenic determinant comprising the utmost aminoterminal amino acids valine and serine of the human PTH and the other antibody binds in the region between amino acids 14 to 34 of the human PTH sequence.

[019] In a preferred embodiment the antibody for purifying the immunoreactive potent PTH peptides recognizes a three-dimensional antigenic determinant located between amino acids 3 to 34 of the human parathyroid hormone which is formed by a change of peptide conformation following the oxidation of the native parathyroid hormone at either methionine in position 8 and/or 18 or at tryptophan in position 22. While no NMR data are available a skilled person will appreciate that the same change

of PTH peptide conformation is brought about by a proteolytic removal of the utmost aminoterminal amino acids serine and valine so that such a conformation antibody will also remove such misfolded peptides. Whether the change of conformation leads synergistically to an oxidation of the peptide following oxidative stress remains to be elucidated. However, oxidation and change of conformation are indicative for the clearance rate of PTH from plasma.

[020] An aspect of the invention relates to the testing of serum or plasma samples from a patient afflicted of chronic kidney disease. A further aspect relates to the testing of samples from uraemic patients or patients with hyperthyroidism.

BRIEF DESCRIPTION OF DRAWINGS

[021] The present invention is best understood when read in conjunction with the accompanying tables and figures, which serve to illustrate the preferred embodiments. It is understood, however, that the invention is not limited to the specific embodiments disclosed in the figures.

Fig. 1 is a diagram showing the relative frequency (%) distribution of measured serum values for a measured non-oxidized parathyroid hormone status (n-oxPTH) in 340 hemodialysis patients.

Fig. 2 is a diagram showing Kaplan-Meier survival curves for death in 340 hemodialysis patients; the patients were stratified into tertiles of measured non-oxidized parathyroid hormone (n-oxPTH) status concentrations (Log rank test, chi square = 14.30; p = 0.0008).

Fig. 3 are diagrams showing Kaplan-Meier survival curves for death in hemodialysis patients having a measured iPTH level higher than the normal iPTH range in healthy subjects (70 ng/L). (A) When the patients were stratified according to the median of measurable non-oxidized parathyroid hormone (n-oxPTH) status (Log rank test, chi square = 0.046; p = 0.80) the n-oxPTH status does not correlate with the patients' outcomes. (B) When the patients were stratified according to the median of iPTH concentrations (Log rank test, chi square = 3.852; p = 0.049) the oxidized PTH levels measured using the iPTH assay is predictive for the patients' outcome.

Fig. 4 is a diagram with Kaplan-Meier survival curves for death in 340 hemodialysis patients. According to international guidelines patients were stratified into five iPTH categories representing very low (<20 ng/L), low (20 to 65 ng/L), medium (65 to 150 ng/L), target (150 to 300 ng/L), and high (>300 ng/L) levels (Chi square 16.35; P = 0.0026 by log-rank test). The J-shaped pattern between iPTH levels and outcome is characteristic for hemodialysis patients and thus indicates that the findings seen in the current study are of general applicability.

DETAILED DESCRIPTION OF THE INVENTION

[022] Animal studies have shown that oxidation of PTH at methionine residues results in a loss of biological activity. Any oxidative stress or a oxidative deactivation of the parathyroid hormone may be compensated or even over-compensated by an the increased secretion of parathyroid hormone into the circulation. Here, we disclose an oxidative stress parameter and its impact on the mortality in hemodialysis patients which gives rise to a therapeutic decision and specific medication. More precisely, the effects of non-oxidized intact immunoreactive PTH (n-oxPTH) and intact immunoreactive PTH (iPTH) in serum are compared and associated with the survival of hemodialysis patients.

[023] "Intact PTH" is defined as the amount or concentration of immunoreactive PTH peptides when determined by a one or two-site immunoassay detecting the PTH peptide that contain at least the domains responsible for receptor binding and activation of the cAMP-cyclase. Those are located in the aminoterminal part of the PTH protein (hPTH(1-37)) The cAMP receptor-binding domain comprises the region from His^{14} to Phe^{34} and the DNA synthesis stimulating domain comprises Asp^{30} to Phe^{34}. The complete aminoterminal peptide hPTH(1-34) is required for correct folding and stimulation of the cAMP-dependent signal pathway. The stimulatory potential is lost on deletion of Ser^{1} and Val^{2} but the cAMP-receptor binding capacity is not influenced by this deletion, indicating that the activation and receptor-binding sites are located in different domains.

[024] The immunoreactive "intact PTH" as defined above is not synonymous with "bioactive PTH" since PTH bioactivity is affected by the correct folding of the PTH peptide chains and the absence of an oxidation of one or more methionines at positions 8 and/or 18 or at tryptophan 22. Despite numerous studies the routes for the bioactivity of the parathyroid hormone and its deactivation remain unclear and can hardly be correlated to a PTH peptide entity which is said to comprise the "effective

bioactivity". The discordant disappearance of PTH bioactivity and plasma immunoreactivity in patients point to a dynamic PTH equilibrium so that a medication with PTH requires a determination of the momentary secretion rate of "intact PTH" activity and a momentary clearance rate of "oxPTH peptides" so that the disclosed n-oxPTH status measurement takes account of both PTH rates and can give directions for a suitable PTH medication required by the patient.

N-oxPTH measurements can be performed using test systems for the determination of „bioactive" PTH which have no clinically relevant cross-reactivity with the many C-terminal PTH degradation products, say hPTH(35-84) and fragments thereof. These are for example the Elecsys™2010 PTH immunoassay of Roche Diagnostics GmbH, Mannheim and the CAP-PTH-IRMA of the company Scantibodies. The electrochemiluminescence immunoassay employs a monoclonal captor antibody against an aminoterminal conformation epitope comprising amino acids 26 to 32 of hPTH and a ruthenium complex marked tracer antibody against a C-terminal hPTH epitope corresponding to the amino acids 55 to 64. The monoclonal captor antibody recognizes the inactive „large hPTH(7-84) fragment" but not bioactive hPTH-fragments such as hPTH(1-34), hPTH(1-35) or hPTH(1-37) (Gao P et al. 2000, Poster M455, ASBMR 22nd Annual Meeting, Roth H J et al. (2000), Poster PI 288; 11th International Congress of Endocrinology, Sydney). The CAP-PTH-IRMA of the company Scantibodies (Santee, CA, US) employs polyclonal antibodies against the N-terminal region of hPTH(1-6) which are described as not binding to the large PTH (7-84) fragments. Moreover, Quest Diagnostics Inc. Introduced a "Bio-Intact PTH test" and obtained FDA-clearance for it as it said to recognize the entire parathyroid hormone molecule, rather than fragments of the molecule, which has a tendency to break up. Currently, PTH measurement is often complicated by the presence of inactive PTH fragments in blood, which impacts the clinical utility of such testing. In recognizing the entire PTH molecule, which consists of an 84-amino acid chain, the Bio-Intact PTH assay has specificity for the N-terminal region of PTH, which is considered essential for the biological effect of PTH. The Bio-Intact PTH test uses proprietary antibodies which are described binding a three-dimensional epitope comprising amino acids 1-13. Whatever immunoassay is used for determining the secretion rate of hPTH into circulation the resulting hPTH status does not reflect the true PTH bioactivity because these immunoassay cannot take account of all relevant factors such as the routes of degradation, the multitude of active, partly active and inactive PTH fragments, nor of their differing half-lifes in serum or plasma.

[025] The present application discloses that hemodialysis patients having n-oxPTH levels in the upper n-oxPTH tertile show an increased survival compared to

patients of the lower n-oxPTH tertile. After multivariable adjustment higher n-oxPTH tertile reduced whereas higher age increased the odds for death in hemodialysis patients. The validity of the present disclosure is strengthened by the fact that stratification of iPTH data from our cohort into five categories according to international guidelines reveal that hemodialysis patients with target iPTH levels according to the guidelines have longer median survival compared to the other groups. The J-shaped survival pattern confirms results derived from iPTH-data from a large mortality meta-analysis. Current PTH-assays do not distinguish between secretion and clearance of PTH forms, although it is well-known that oxidation of PTH results in loss of its biological activity. Our analysis in a subgroup of hemodialysis patients showing iPTH above the upper normal range (70 ng/L) clearly separated the true effects of the hormone from the disastrous effects of increased clearance and oxidation. Increased clearance of immunoreactive PTH would be noted by most current immunoassays but not increased oxidation and deactivation. We observed that increased mortality in this subgroup depended on protein oxidation of iPTH as a surrogate of overall protein oxidation and oxidation associated impairment of protein function and structure but not on biologically active n-oxPTH.

[026] Human PTH is secreted by the chief cells of the parathyroid glands as a polypeptide having 84 amino acids. After secretion into the circulation, the bioactive PTH peptides comprising the essential domains increase blood calcium by an activation of parathyroid hormone 1 receptor, present in high levels in bone and kidney, and the parathyroid hormone 2 receptor, present in high levels in the central nervous system, pancreas, testis, and placenta. The half-life of those bioactive PTH peptides is approximately 4 minutes only. It was known that also the oxidation of those PTH peptides may result in a loss of biological activity (Galceran T et al, in *Absence of biological effects of oxidized parathyroid hormone-(1-34) in dogs and rats.* Endocrinology. 1984;115:2375-2378; Horiuchi N. *Effects of oxidation of human parathyroid hormone on its biological activity in continuously infused, thyroparathyroidectomized rats.* J Bone Miner Res. 1988;3:353-358; Zull JE et al, in *Effect of methionine oxidation and deletion of amino-terminal residues on the conformation of parathyroid hormone.* Circular dichroism studies. J Biol Chem. 1990;265:5671-5676). Indeed, many publications have been concerned with the effect of oxidation stress in the case of chronically kidney insufficient patients (Martin-Mateo MC et al (1999), Ren Fail 21:55-167; Hasselwander O et al (1998) Free Radic Res 29:1-11; Zoccali C et al.(2000) Nephrol Dial Transplant 15: Suppl. 2; Canaud B et al (1999) Blood Purif 17:99-106). Various working groups have investigated oxidized parathyroid hormone and its biological activity (Alexiewicz L M et al. (1990), J Am Soc

Nephrol 1:236-244; Zull J E et al (1990) J Biol Chem 265:5671-5676; Pitts T O et al. (1988) Miner Electrolyte Metab 15: 267-275; Horiuchi N (1988) J Bone Miner Res 3:353-358; Frelinger A L et al. (1986) Arch Biochem Biophys 244: 641-649; GalceranT et al. (1984) Endocrinology 115,2375-2378; Frelinger A L et al. (1984) J Biol Chem 259:5507-5513; O'Riordan JLH et al. (1974) J Endocr 63:117-124; Logue FC et al (1991) Ann Clin Biochem 28:160-166; Logue F C (1991b) J Immun Meth 39:159). The oxidation stress in dialysis patients and its consequence for morbidity and all-cause mortality have, however, not so far been investigated and recognized.

[027] The so-called intact PTH (iPTH) and bio-intact sandwich assays do not differentiate between non-oxidized PTH peptide chains (n-oxPTH) and oxidized PTH peptide chains (oxPTH). Using mass spectroscopy we recently demonstrated that oxidative stress in hemodialysis patient may lead to an oxidation of human PTH *in vivo* and to a variety of inactive PTH products with oxidized methionine residues at positions 8 and/or 18 (Hocher B et al in *Measuring parathyroid hormone (PTH) in patients with oxidative stress--do we need a fourth generation parathyroid hormone assay?* PLoS One. 2012;7:e40242). This discovery and the immunological distinction between non-oxidized and oxidized "intact" PTH peptide chains in plasma or serum which gives rise to a n-oxPTH status which allows a new medication of haemodialysis patients. Hereinafter, the term n-oxPTH is used for an immunoreactive "intact" PTH (iPTH) concentration as defined above in serum or plasma after taking account of the PTH clearance in serum or plasma by the oxidation of PTH peptide chains. Whether or not the measured n-oxPTH concentration represents the „true" PTH bioactivity present in serum is not relevant since we discovered that the all-cause mortality of hemodialysis patients is linked to the equilibrium of immunoreactive secreted intact PTH and PTH peptide chains which are immunoreactive for oxidation in the aminoterminal portion of PTH. Only after removal of those oxidized PTH chains, the resulting rate of secreted iPTH into the serum gives an *in vitro* parameter which allows a therapeutic decision as whether a therapy with vitamin D, phosphorus binders or calcimimetics needs adjustment to achieve treatment goals provided by international guidelines. The discovery of a dynamic PTH parameter which correlates with the all-cause mortality in hemodialysis patients then allows a reasonable medication and therapy. In the examples described below, the PTH concentration in serum was measured by means of a third generation intact-PTH immunoassay system (Elecsys™2010 PTH Roche), both directly (total intact parathyroid hormone, iPTH) and after removal of oxidized PTH molecules from the samples using a monoclonal antibody which binds oxidized human PTH peptide chains.

[028] The hemodialysis patients (224 men/116 women) in our study had a median age of 66 years. 170 patients (50%) died during the follow up time of 5 years. Median n-oxPTH levels were higher in survivors (7.2 ng/L) compared to deceased patients (5.0 ng/L; p=0.002). Survival analysis showed an increased survival in the highest n-ox-PTH tertile compared to the lowest n-oxPTH tertile (Chi square 14.3; p=0.0008). Median survival was 1702 days in the highest n-oxPTH tertile, whereas it was only 453 days in the lowest n-oxPTH tertile. Multivariable-adjusted Cox regression showed that higher age increased odds for death, whereas higher n-oxPTH reduced the odds for death. Another model analyzing a subgroup of patients with secreted iPTH concentrations at baseline above the upper normal range of the iPTH assay (70 ng/L) revealed that mortality in this subgroup was associated with PTH oxidation but not with n-oxPTH levels. The huge numerical difference between the target PTH levels according to International Guidelines (PTH target = 150 to 300 ng/L) and the median n-oxPTH value in survivors (7.2 ng/L) will not escape attention of the skilled reader and could not have been anticipated. In conclusion, the predictive powers of n-oxPTH and iPTH levels on mortality of hemodialysis patients differ substantially. Measurements of n-oxPTH therefore reflect the dynamic PTH hormone status more precisely. The iPTH associated mortality especially when iPTH levels are high reflects mainly mortality associated with PTH protein oxidation and oxidative stress. This gives rise to a new medication program and therapy. The skilled person will also appreciate that the degree of PTH oxidation and of a measurement of the inherent oxidative stress leads to new therapies and medication programs. Thus, the quantitative amount of oxidized PTH peptides within serum or plasma represents an important medical parameter which requires regulation and monitoring.

EXAMPLES

EXAMPLE 1

PTH-measurements

[029] PTH was measured by means of a third-generation electrochemiluminescence PTH immunoassay system both directly (iPTH) and after prior removal of misfolded or oxidized PTH molecules from the samples using monoclonal antibodies raised against the oxidized human PTH (n-oxPTH). Removal of oxidized PTH was performed using an anti-human oxidized PTH monoclonal antibody as described below. The anti-human oxidized PTH monoclonal antibody was immobilized on CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences, Uppsala, Sweden). Hundred µl aliquot of the slurry was filled in a column (MobiSpinColumn, MoBiTec, Göttingen, Germany) and equilibrated with PBS buffer, pH 7.4. Then, 500 µl of plasma samples were applied on the columns which were incubated mixing end-over-end for 2 h at room temperature, washed with 250 µl of 0.1 M ammonium acetate buffer pH 7.0, followed by a wash with 250 µl of 0.1 M ammonium acetate buffer pH 7.0, containing 20% acetonitrile, and then eluted 2 times with 200 µl of elution buffer (0.05 M formic acid, pH 3.5). Flow-through was collected separately and lyophilized.

[030] Then the samples were reconstituted in 500 µl of PBS buffer, pH 7.4 and aliquots analyzed for iPTH. The employed iPTH immunoassay (ECLIA Elecsys 2010; Roche Diagnostics, Mannheim, Germany) is based on a biotinylated monoclonal antibody, which reacts with amino acids 26–32, and a capture ruthenium-complexed monoclonal antibody, which reacts with amino acids 55–64. The determinations were performed on a Roche Modular E 170. The intra-assay CV was 4.1% and the inter-assay CV was 5.8% at concentrations of 35.0 and 180.0 ng/L, respectively.

EXAMPLE 2

Monoclonal antibodies against a conformation epitope of oxidized PTH(aa 1-38)

[031] Monoclonal antibodies were raised in BALB/c-mice. The mice were immunized with the oxPTH(aa 1-38) thyreoglobulin conjugate at 200 µg for both primary and secondary immunizations with incomplete Freund's (mineral oil only) in the intraperitoneal cavity. Each of the antisera was tested for binding to non-oxidized biotin-hPTH(1-38). To detect antibodies specifically recognizing oxPTH(aa 1-38) peptides, we used the double antibody separation technique and as tracer biotin-

oxPTH(aa1-38) labelled with ^{125}I-streptavidin. After cell fusion and HAT selection, selected hybridomas were screened in the same way, namely for binding to human oxidized PTH(aa 1-84) but not to human PTH(1-84).

[032] For ultimate characterization of the specificity of the monoclonal antibodies (MAB) and for identification of a monoclonal antibody recognizing a conformation epitope common to oxidized hPTH(1-38) peptides, say common to all forms of oxidized hPTH(aa 1-38) independently from oxidation status and chirality (Met-R-O, Met-S-O, and $MetO_2$ at positions 8, 18 and both), the antibody was immobilized on CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences, Uppsala, Sweden). Hundred µl aliquot of the slurry was filled in a column (MobiSpinColumn, MoBiTec, Göttingen, Germany) and equilibrated with PBS buffer, pH 7.4. Then 2.5 µg of lyophilized oxidized hPTH(1-84) were dissolved in 300 µl of equilibrating buffer and applied on the column. The column was incubated end-over-end for 1 h at room temperature, washed with 300 µl of equilibrating buffer, followed by 3 washes with 300 µl of distilled water, and then eluted 2 times with 200 µl of elution buffer (0.1% TFA). Flow-through, wash fractions (equilibrating buffer and water) as well as eluate of the column were collected separately, lyophilized and analyzed by nanoLC-ESI-FT-MS. A monoclonal antibody ("oxPTH-ConforMAB") recognizing a conformation epitope present on all forms of oxidized hPTH(aa 1-84) and fragments thereof was selected for further analysis and characterization. The selected oxPTH-ConforMAB specifically recognized with high affinity all forms of oxidized and misfolded hPTH fragments, but not non-oxidized PTH (aa 1-84).

EXAMPLE 3

Patients

[033] A prospective cohort study in 340 hemodialysis patients was followed up for 5 years. Our eligibility criteria included all adult prevalent patients on hemodialysis treatment due to end-stage chronic kidney disease stage 5 and presence of informed consent. Informed consent from each patient and ethical approval by the local ethics committee were obtained. Data on dialysis vintage at inclusion and duration of hemodialysis treatment per session were obtained. All of the patients were routinely dialyzed for 4 to 5 hours three times weekly using biocompatible membranes with no dialyser re-use. Blood flow rates were 250 to 300 mL/min, dialysate flow rates were 500 mL/min, dialysate conductivity was 135 mS. Blood pressure was measured before

dialysis. Predialysis blood samples were taken at study entry. Blood was collected immediately before the start of the hemodialysis session.

[034] Clinical and laboratory data included age, gender, medications (use of angiotensin-converting-enzyme inhibitors, ß-blockers, calcium channel blockers, and erythropoietin), body mass index (calculated as body weight divided by height squared), systolic and diastolic blood pressure, serum albumin, serum cholesterol, serum triglyceride, serum urea, serum creatinine, serum calcium, serum potassium, and serum phosphate.

[035] 170 patients (50%) died during the follow up time of 5 years. The causes of death were classified as cardiovascular including sudden death, infection, or cancer.

EXAMPLE 4

Statistical analysis of n-oxPTH measurements

[036] Figure 1 shows the distribution of n-oxPTH concentrations in 340 hemodialysis patients (224 men and 116 women) with a median age of 66 years (IQR, 56 to 75 years), a median time since initiation of dialysis (dialysis vintage) of 266 days (IQR, 31 to 1209 days), and a median dialysis dose (kt/V) of 1.2 (IQR, 1.1 to 1.3). The cause of chronic kidney disease was nephrosclerosis in 113 cases (33%), diabetic nephropathy in 107 cases (31%), chronic glomerular nephritis in 29 cases (9%), polycystic kidney disease in 9 cases (3%) and other/unknown in 82 cases (24%). The median n-oxPTH concentration was 5.9 ng/L (IQR, 2.4 to 14.0 ng/L). n-oxPTH concentrations were not different in men and women (5.9 ng/L; IQR, 2.4 to 14.2 ng/L; n = 224; vs. 5.5 ng/L; IQR, 2.4 to 14.0 ng/L; n = 116; p = 0.915).

[037] Table 1 summarizes the distribution of cases and laboratory variables stratified by tertiles of n-oxPTH. Tertile limits were n-oxPTH concentrations of 3.3 ng/L and 10.3 ng/L, respectively. Hemodialysis patients of the upper n-oxPTH tertile had higher weight, body mass index, and higher urea, a proxy for dietary protein intake, higher creatinine, a proxy for muscle mass, and typical signs of impaired mineral metabolism, i.e. lower serum calcium and higher serum phosphorous concentrations. Furthermore, serum phosphorus concentrations were directly (Spearman r = 0.245; p < 0.001) and serum calcium concentrations were inversely (Spearman r = -0.160; p = 0.004) correlated with n-oxPTH concentrations. On the other hand, age (Spearman r = -0.099) and dialyses vintage (Spearman r = 0.098) were not significantly correlated with n-oxPTH.

Table 1.

Baseline clinical and biochemical characteristics of hemodialysis patients by tertiles of non-oxidized intact parathyroid hormone (n-oxPTH).

CHARACTERISTIC	1 TERTILE	2. TERTILE	3. TERTILE	P-VALUE
Age (years)	68 (57-76)	67 (56-77)	65 (54-72)	0.125
Gender (% Female)	35%	35%	35%	0.928
Vintage (days)	241 (31-1233)	263 (58-913)	425 (31-1507)	0.429
Diabetes mellitus (%)	31%	46%	38%	0.076
Smoker (%)	31%	30%	35%	0.732
Weight (kg)	70 (60.0-80)	70 (60-78)	75 (67-85)	0.003
Body mass index (kg/m^2)[b]	24.3 (21.1-26.5)	24.1 (21.9-26.3)	25.6 (22.9-29.4)	0.003
Systolic blood pressure (mmHg)	134 (110-146)	133 (112-149)	138 (118-153)	0.326
Diastolic blood pressure(mmHg)	69 (58-80)	67 (57-80)	72 (60-83)	0.185
Hemoglobin (mg/dL)	10.0 (9.1-11.4)	10.2 (9.3-11.2)	10.5 (8.9-11.8)	0.589
Leukocytes (10^9/L)	8.0 (5.9-10.1)	8.4 (6.0-10.8)	8.1 (6.3-11.3)	0.895
Platelets (10^9/L	218 (178-272)	221 (167-275)	230 (174-296)	0.846
Serum albumin (g/L)	3.3 (2.9-3.7)	3.3 (2.8-3.6)	3.4 (2.9-3.8)	0.371
Serum cholesterol (mg/dL)	152 (129-186)	151 (125-190)	150 (126-189)	0.963
Urea (mg/dL)	69 (47-93)	57 (48-89)	77 (57-102)	0.026
Serum creatinine (mg/dL)	5.5 (3.8-7.9)	5.6 (3.7-7.9)	7.1 (5.8-9.3)	0.001
Serum potassium (mmol/L)	4.8 (4.0-5.3)	4.5 (4.1-5.2)	4.9 (4.2-5.3)	0.238
Serum calcium (mmol/L)	2.30 (2.19-2.50)	2.19 (2.06-2.37)	2.22 (2.06-2.40)	0.001
Serum phosphorus (mg/dL)	1.55 (1.07-1.91)	1.43 (1.21-1.90)	1.90 (1.36-2.30)	0.001
Dialysis dose (ktV)	1.2 (1.1-1.3)	1.2 (1.1-1.4)	1.2 (1.0-1.4)	0.227
Angiotensin converting enzyme Inhibitors (%)	26%	26%	26%	0.992
ß-Blockers (%)	60%	60%	60%	0.996
Calcium channel blockers (%)	28%	34%	29%	0.583
Erythropoietin therapy (%)	57%	53%	42%	0.064

a) Continuous variables are given as medians and interquartile range. Between groups, comparisons were made using non-parametric Kruskal-Wallis test for continuous variables and using Chi square test for categorical variables.

b) Body mass index was calculated as weight in kilograms divided by height in meters squared.

[038] 170 patients (50%) died during the follow up time of 5 years. Death occurred at a median of 217 days (IQR, 67 to 564 days) after study entry. The causes of death were cardiovascular diseases in 102 patients (60%), infections in 39 patients (23%), cancer in 19 patients (11%), and other/unknown reasons in 10 (6%). Median n-oxPTH levels were higher in survivors (7.2 ng/L; IQR 3.1 to 16.5 ng/L) compared to deceased patients (5.0 ng/L; IQR, 1.9 to 11.1 ng/L; p = 0.002 by Mann Whitney test). Survival analysis showed an increased survival in the upper n-oxPTH tertile compared to the lower n-oxPTH tertile (Chi square 14.30; p = 0.0008 by log-rank test). Median survival was 1702 days in the upper n-oxPTH tertile, whereas it was only 453 days in the lower n-oxPTH tertile (Figure 2).

[039] Multivariable-adjusted survival analyses were performed using the proportional hazards regression model with backward variable selection, using $p < 0.05$ for variable retention. Variables tested were plasma n-oxPTH, iPTH category, dialysis dose, dialysis vintage, age, haemoglobin, and serum phosphorus. iPTH category, dialysis dose, dialysis vintage, and serum phosphorus did not show significant effects. This multivariable-adjusted Cox regression showed that that higher age increased odds for death, whereas higher n-oxPTH reduced the odds for death (Table 2).

Table 2.

Multivariable-adjusted Cox regression showing the odds for death in hemodialysis patients.

VARIABLE	B (SE)	ODDS RATIO	(95% CI)
n-oxPTH Tertile	-0.276 (0.103)	0.759	(0.620 to 0.929)
Age	0.068 (0.008)	1.070	(1.053 to 1.087)
Hemoglobin	-0.169 (0.055)	0.844	(0.756 to 0.940)

Multivariable-adjusted survival analyses were performed using the proportional hazards regression model with backward variable selection, using P < 0.05 for variable retention. Variables tested were plasma n-oxPTH, iPTH category, dialysis dose, dialysis vintage, age, hemoglobin, serum phosphorus. iPTH category, dialysis dose, dialysis vintage, and serum phosphorus did not show a significant effects.

[040] Furthermore, a model analyzing only patients with iPTH above the upper normal range (70 ng/L) revealed that mortality in this subgroup depended on protein oxidation of iPTH but not on biologically active n-oxPTH. In other words, n-oxPTH had no impact on mortality in patients with iPTH levels above the upper normal range, whereas in these patients iPTH was associated with all-cause mortality (Figure 3).

[041] Using another model, we *a priori* stratified iPTH levels into five categories according to international guidelines, representing very low (<20 ng/L), low (20 to 65 ng/L), medium (65 to 150 ng/L), target (150 to 300 ng/L), and high (>300 ng/L). Survival analysis showed a J-shaped pattern, i.e. patients with target iPTH levels had longer median survival compared to the other categories (Chi square 16.35; P = 0.0026 by log-rank test). This J-shaped pattern between iPTH levels and outcome is characteristic for hemodialysis patients, thereby confirming that our data were obtained from a typical hemodialysis cohort (Figure 4).

EXAMPLE 5

Statistical Analysis

[042] Continuous variables were expressed as median with interquartile range (IQR) and compared with nonparametric Mann-Whitney test or non-parametric Kruskal-Wallis test and Dunn's multiple comparison post-hoc test, where appropriate. Associations between variables were tested using non-parametric Spearman correlation. Time-to-event analyses were performed using the Kaplan-Meier method. Comparison of survival curves were performed using the log-rank (Mantel-Cox) test. Categorical variables were expressed as proportions and compared with the Chi-square test. Multivariable-adjusted survival analyses were performed using the proportional hazards regression model. Multivariable models were constructed with backward variable selection, using $p < 0.05$ for variable retention. 45 patients (13%) underwent kidney transplantation during the follow up. These patients were censored on the day of transplantation. All hypothesis tests were 2-sided, with statistical significance defined as having a p value of less than 0.05. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) or SPSS for windows (version 15; SPSS, Chicago, IL).

SUMMARY

[043] The present application examines the dynamic equilibrium in plasma or serum between secreted intact PTH (iPTH), clearance of PTH activity by oxidation the and resulting parameter corresponding to non-oxidized intact PTH (n-oxPTH) on the survival of hemodialysis patients. N-oxPTH measurements were performed with intact-PTH immunoassay after purifying the sample from oxidized PTH peptide chains. The present study indicates that hemodialysis patients in the upper n-oxPTH tertile enjoy increased survival compared to the patients in the lower n-oxPTH tertile. After multivariable adjustment higher n-oxPTH tertile reduced whereas higher age increased

the odds for death in hemodialysis patients. The validity of the present disclosure is strengthened by the fact that stratification of iPTH data from our cohort into five categories according to international guidelines reveal that hemodialysis patients with target iPTH levels according to the guidelines had longer median survival compared to the other categories. This J-shaped survival pattern confirms results derived from iPTH-data from a large mortality meta-analysis. The analysis of a subgroup of hemodialysis patients showing iPTH above the upper normal range (70 ng/L) clearly separated the positive effects of secreted iPTH peptides from the negative effect of PTH peptides subjected to oxidation. The increased mortality in this subgroup depended on protein oxidation of iPTH.

[044] A nephrologist familiar with this topic will appreciate that this analysis should be extended to dialysis patients with very high iPTH concentrations, which means patients who are considered to have secondary hyperparathyroidism according to classical diagnostic standards. The number of patients with such high iPTH concentrations in our study cohort was too low to allow clear statements. Analyses from cohorts of dialysis patients with secondary hyper-parathyroidism as investigated in the EVOLVE study cohort may help to address this important clinical question.

[045] Current guidelines recommend to measure PTH levels routinely and to obtain target PTH levels (i.e. from 150 to 300 ng/L), because several studies observed worse outcome with PTH levels above 300 ng/L. In contrast we found that hemodialysis patients in the upper tertile, i.e. having n-oxPTH levels above 10.3 ng/L enjoying increased survival. This finding is surprising but while not wishing to be bound by any theory, there may be an explanation. The studied dialysis cohort comprised only a few patients with iPTH concentrations above 300 ng/L so that answers for this sub-cohort require a larger study group. Clearance of iPTH from plasma or serum occurs mainly by the liver and the kidney but there is evidence that the half-life of oxidized iPTH exceeds that of non-oxidized iPTH. In essence, the metabolic clearance rate of non-oxidized iPTH is the range of about 21.6 mL/min per kg body weight, whereas the metabolic clearance rate of oxidized iPTH is 8.8 mL/min per kg body weight only (Neuman WF et al in *The metabolism of labeled parathyroid hormone. V. Collected biological studies.* Calcif Tissue Res. 1975;18:271-287; Hruska KA et al in *Peripheral metabolism of intact parathyroid hormone. Role of liver and kidney and the effect of chronic renal failure*, J Clin Invest. 1981;67:885-892). Thus, high iPTH levels in the prior art literature may merely represent large amounts of oxidized PTH peptide chains and that patients were suffering from increased oxidative stress. Moreover, wasting may have an impact on the measurable immunoreactive intact PTH. Wasting is further associated with inflammation and oxidative stress. Thus the impact by wasting on the

prior art intact PTH measurements is likely related to the presence of oxidized PTH peptides. However, this needs to be proven in future studies.

[046] Removal of the parathyreoid glands in animal models of uremia as well as in patients suffering from hyperparathyroidism proves that high concentrations of PTH contributes to vascular calcification, hence cardiovascular morbidity and mortality in uremia. At the same time, it is also true that oxidative stress is related to cardiovascular mortality in end-stage-renal disease patients as well. Our data indicate that the J-shaped survival curve for iPTH represents a overlay of two different biological processes. Conventional immunoreactive iPTH assays do either not differentiate between those PTH forms nor was the size of the oxidation stress on the PTH measurement appreciated. There was no knowledge how to use the information on the n-oxPTH status with respect to therapy and medication for CKD patients. In conclusion, the predictive power of immunoreactive n-oxPTH and iPTH for all cause mortality differ substantially. Thus, clinical decisions based on any immunoreactive PTH peptide concentration in plasma or serum may be misleading in patients with end-stage renal disease if the oxidative PTH clearance is not taken into account.

CLAIMS

1. Method of *in vitro* monitoring and assessing the need of a medication which interferes with the regulation of the parathyroid hormone level in a kidney patient subject to oxidative stress comprising the steps of

purifying a sample of plasma or serum from said kidney patient from human PTH peptides oxidized at either methionine 8 or 18 or both or at tryptophan 22 by contacting said sample with an antibody which recognizes and specifically binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides but which antibody does not bind non-oxidized human PTH (1-84) and fragments thereof;

determining the amount of immunoreactive human PTH (iPTH) peptides in said sample by an immunoassay based on antibodies against human PTH(1-84) and fragments thereof that contain at least the domains responsible for receptor binding and activation of the cAMP-cyclase located between amino acids 3 to 34 of the human PTH sequence; and

obtaining a PTH status value (n-oxPTH value) for said kidney patient which includes the rate of immunoreactive human PTH (iPTH) peptides secreted by cells of the parathyroid gland into the circulation and the rate by which immunoreactive human PTH (iPTH) peptides are oxidized by the oxidative stress suffered by said patient; and

comparing the PTH status value (n-oxPTH) with a reference value at which the morbidity and all-cause mortality is low to determine the need of a medication with respect to a regulation of the PTH status value or for supplementation of the patient with human parathyroid hormone or active fragments thereof or both.

2. The method of claim 1, wherein the sample is from a kidney patient subject to a hemodialysis treatment.

3. The method of claim 1 or claim 2, wherein the sample is from a kidney patient afflicted of chronic kidney disease (CKD) or uremia or hyperparathyroidism.

4. The method of any claim 1 to 3, comprising the step of contacting said sample of plasma or serum with a solid phase having bound an antibody which recognizes and specifically binds a three-dimensional epitope located between

amino acids 3 to 34 of oxidized human PTH peptides but which antibody does not bind non-oxidized human PTH (1-84) and fragments thereof.

5. The method of any claim 1 to 4, further comprising a quantitative determination of said peptides bound by said antibody which recognizes and specifically binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides.

6. The method of any claim 1 to 5, wherein the determination of immunoreactive human PTH (iPTH) peptides comprises the use of a two-site immunoassay wherein one antibody is a monoclonal antibody that binds to a domain involved in the binding of the PTH peptide to PTH receptors 1 and 2.

7. The method of any claim 1 to 5, wherein the determination of immunoreactive human PTH (iPTH) peptides comprises the use of a two-site immunoassay wherein one antibody binds an antigenic determinant comprising the utmost aminoterminal amino acids valine and serine of the human PTH and the other antibody binds in the region between amino acids 14 to 34 of the human PTH sequence.

8. The method of any claim 1 to 5, wherein the determination of immunoreactive human PTH (iPTH) comprises the use of a two-site immunoassay wherein one antibody binds an antigenic determinant comprising amino acids 1 to the 13 of the human PTH sequence.

9. The method of any claim 1 to 8, wherein said antibody specific for oxidized human PTH peptides binds a three-dimensional epitope between amino acids 3 to 34 of the human PTH sequence which does not comprise the oxidized methionines at position 8 and/or 18.

10. The method of any claim 1 to 9, further comprising a determination of the ratio of immunoreactive human PTH (iPTH) and the amount of PTH peptides bound by said antibody which recognizes and selectively binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides.

11. A kit comprising a solid phase with an antibody that recognizes and selectively binds a three-dimensional epitope located between amino acids 3 to 34 of oxidized human PTH peptides, and a combination of antibodies for determining

the immunoreactive intact human PTH (iPTH) in plasma or serum as disclosed in any claim 4 to 10.

5

Fig. 1

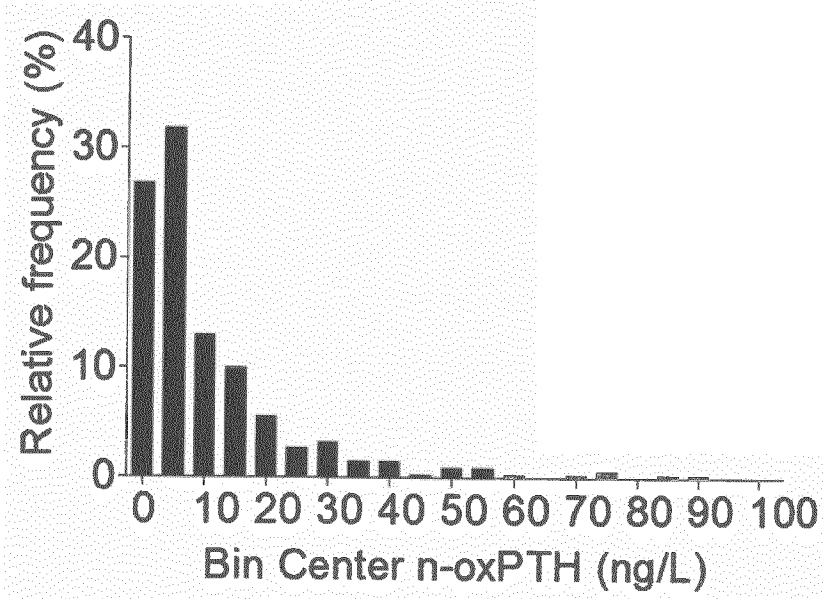

Relative frequency (%)
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Bin Center n-oxPTH (ng/L)

Fig. 2

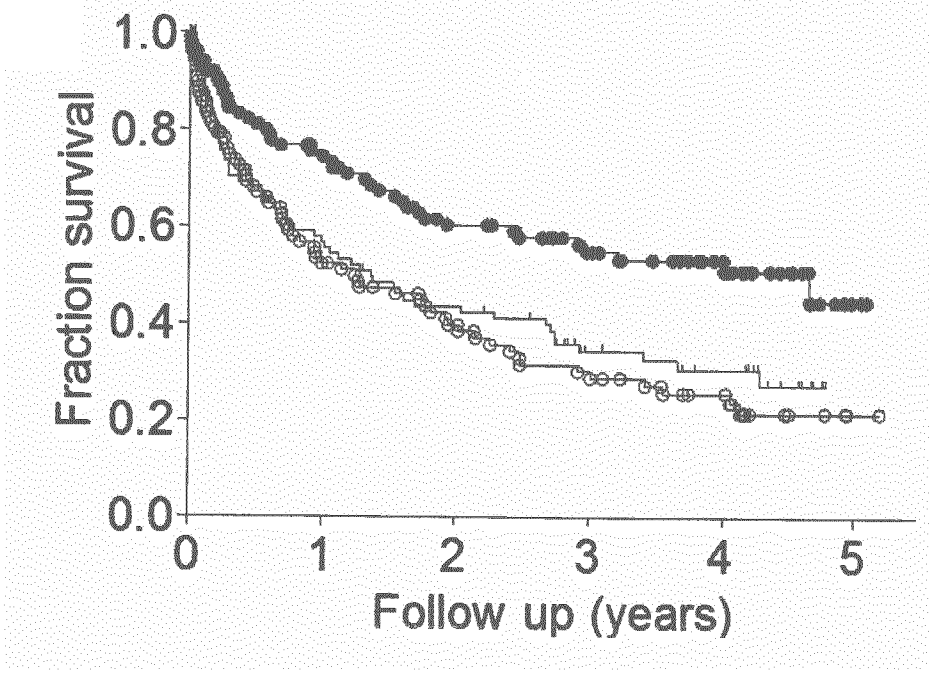

Fraction survival
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5
Follow up (years)

Fig. 3

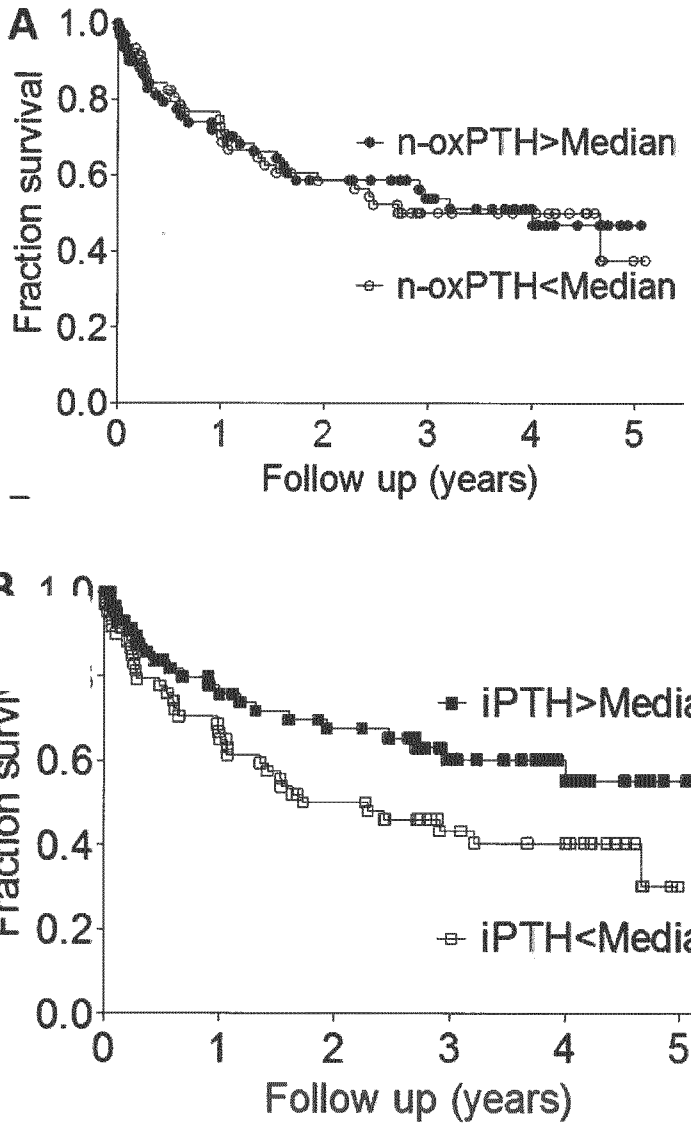
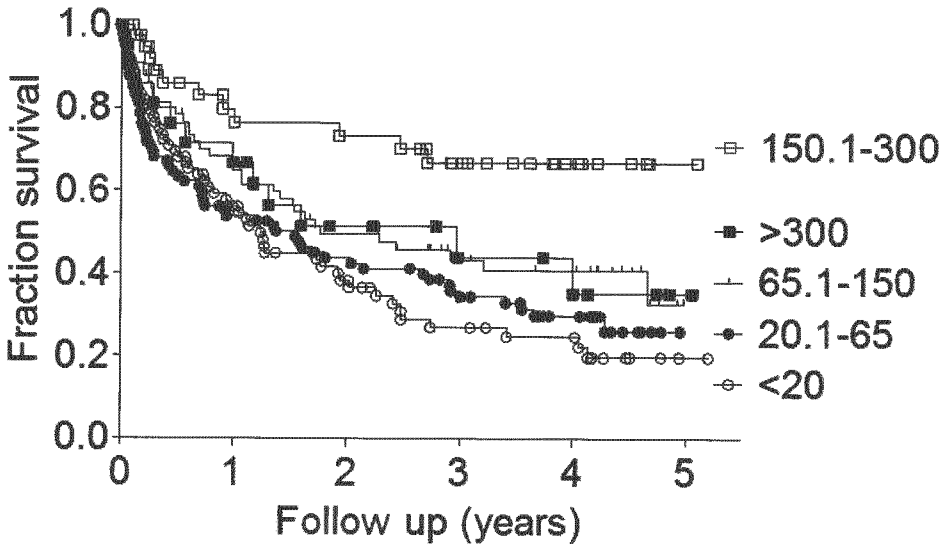

A
1.0
0.8
0.6
0.4
0.2
0.0
Fraction survival
n-oxPTH>Median
n-oxPTH<Median
0
1
2
3
4
5
Follow up (years)
B
Fraction survi
iPTH>Median
iPTH<Median
Follow up (years)

Fig. 4


1.0
0.8
0.6
0.4
0.2
0.0
Fraction survival
0
1
2
3
4
5
Follow up (years)
150.1-300
>300
65.1-150
20.1-65
<20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/054508

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

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOCHER BERTHHOLD ET AL: "Measuring parathyroid hormone (PTH) in patients with oxidative stress - do we need a fourth generation parathyroid hormone assay?", PLOS ONE, PUBLIC LIBRARY OF SCIENCE, vol. 7, no. 7, 6 July 2012 (2012-07-06), pages 1-10, XP002696480, ISSN: 1932-6203 cited in the application	1-11
Y	the whole document -----	1-10
Y	WO 02/082092 A2 (ABC ARMBRUSTER BIOCHEMICALS [DE]; ARMBRUSTER FRANZ PAUL [DE]; ROTH HEI) 17 October 2002 (2002-10-17) cited in the application page 5, line 26 - line 31 -----	1-10

☐ 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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2014	20/06/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gundlach, Björn

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/054508

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02082092 A2	17-10-2002	AT 408842 T	15-10-2008
		DE 10116552 A1	10-10-2002
		DK 1425588 T3	26-01-2009
		EP 1425588 A2	09-06-2004
		EP 2006688 A2	24-12-2008
		JP 4294961 B2	15-07-2009
		JP 2004529342 A	24-09-2004
		US 2006211054 A1	21-09-2006
		WO 02082092 A2	17-10-2002