UNIVERSITY OF LIEGE

FACULTY OF MEDICINE

DEPARTMENT OF CLINICAL CHEMISTRY

Professor J.P. Chapelle

PARATHORMONE DETERMINATION IN THE CLINICAL LABORATORY: BIOCHEMICAL, ANALYTICAL AND CLINICAL ASPECTS.

Thesis submitted by

Etienne CAVALIER

for the degree of

« Docteur en Sciences Biomédicales et Pharmaceutiques »

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Department of Clinical Chemistry.

Parathormone determination in the Clinical Laboratory: biochemical, analytical and clinical aspects.

Abstract.

The aim of our work was to provide comprehensive data to the study of the Parathyroid hormone (Parathormone, or PTH). The first part of the manuscript concerns the "laboratory" aspects of PTH determination. We thus studied the stability of the peptide when stored at different temperatures (pre-analytical phase). From an analytical point of view, we compared different methods for PTH determination and the impact of any change in PTH determination for the follow-up of the hemodialyzed patients. We also studied some analytical interferences and provided a validation strategy that takes into consideration these interferences. Finally, for the post-analytical phase, we established the reference range of PTH with two different methods. We provided guidelines which could help laboratories to establish their own reference range for PTH and studied the clinical impact of applying this newly established reference range in the daily routine.

The second part of the manuscript is more dedicated to the "clinical" aspects of PTH determination. We focused on the problems encountered when determining PTH in CKD and hemodialyzed patients. We also studied different bone markers in these patients to see if one of them could, in the future, replace PTH for the follow-up of these patients. Finally, we studied the "amino-PTH", a newly discovered form of PTH, in a large population of patients suffering from parathyroid cancer. We thus presented the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio as a potential marker of parathyroid carcinoma.

Université de Liège – Faculté de Médicine
Service de Chimie Médicale

Détermination de la PTH au Laboratoire de Chimie clinique : aspects biochimiques, analytiques et cliniques.

Résumé.

Notre travail est consacré à l'étude de l'hormone parathyroïdienne (parathormone, PTH). La première partie de l'ouvrage concernera plus particulièrement les aspects "laboratoire" du dosage de la PTH. Ainsi, d'un point de vue pré-analytique, nous avons étudié la stabilité du peptide lorsqu'il était conservé sous différentes conditions de température. D'un point de vue analytique, nous avons comparé différentes méthodes de dosage de la PTH et étudié l'impact d'un changement de méthode sur le suivi longitudinal des patients dialysés. Ensuite, nous avons étudié différentes interférences analytiques et nous avons proposé une stratégie qui permet de prendre en compte ces interférences lors de la validation biologique des résultats. Enfin, d'un point de vue post-analytique, nous nous sommes interrogés sur la meilleure façon de définir la « population de référence » pour établir les valeurs de référence de la PTH. Nous avons ainsi établi les valeurs de référence pour deux méthodes analytiques différentes et étudié l'impact de l'utilisation de nos propres valeurs de référence par rapport à celles fournies par le fabricant des kits de dosage.

La deuxième partie de l'ouvrage est consacrée aux aspects plus « cliniques » du dosage de la PTH. Nous nous sommes ainsi consacrés aux difficultés rencontrées lors du dosage de la PTH chez les patients insuffisants rénaux et dialysés. Nous avons également étudié la possibilité d'utiliser d'autres marqueurs osseux chez ces patients afin de voir si l'un d'entre eux pourrait, éventuellement, remplacer la PTH pour le suivi de ces patients. Enfin, nous avons étudié l' « amino-PTH », une forme de PTH récemment découverte dans une importante population de patients souffrant de carcinome parathyroïdien sévère. Nous proposons ainsi d'utiliser le rapport $\frac{\text{"}\boldsymbol{PTH\ de.3ème.génération}\text{"}}{\text{"}\boldsymbol{PTH\ de.2ème.génération}\text{"}}$ comme marqueur tumoral du cancer parathyroïdien.

Arrivé au terme à cet ouvrage, il m'est agréable de repenser à toutes les personnes qui, de près ou de loin, m'ont aidé à son aboutissement. Je voudrais ainsi profiter de ce moment pour les en remercier.

Mes premières pensées vont au Professeur Gielen qui m'a confié la responsabilité du Laboratoire d'Endocrinologie « 2 », premier jalon de ce travail.

Je voudrais sincèrement remercier le Professeur Chapelle, Chef du Service de Chimie médicale pour la confiance qu'il m'a toujours témoignée et pour m'avoir aidé à trouver ce fameux « fil rouge » qui m'a permis de donner à cet ouvrage la structure qu'il a aujourd'hui. Je le remercie également pour sa rigueur scientifique et pour m'avoir initié aux multiples facettes de l'Assurance qualité.

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Mille mercis à Agnès Carlisi, Responsable technique du Laboratoire d'Endocrinologie « 2 » qui m'a tant aidé pour que je puisse en arriver là. Agnès, je crois qu'aucune parole ne me permettra jamais de t'exprimer toute ma gratitude…

Merci aussi à tous les membres du labo d'Endocrinologie « 2 », Anne-Catherine, Claudette, Renée, Georges, Nunzio et Olivier pour leur professionnalisme, leur bonne humeur (parfois un peu bruyante…) et pour toujours accepter d'un sourire toutes les lubies qui me passent par la tête.

Je voudrais également remercier les super « datas » de Néphro, Marie-Antoinette et Michèle, pour leur indéfectible disponibilité et leur aide précieuse, ainsi que le personnel du Dispatching, dont le rôle souvent méconnu est pourtant primordial. Merci aussi à tout le

personnel de Chimie médicale, et particulièrement à l'équipe du Laboratoire des Urgences et aux secrétaires de Monsieur Chapelle pour leur aide quotidienne.

Tout ce travail n'aurait probablement jamais vu le jour sans une personne qui est devenue, au fil du temps, un vrai ami. Le Docteur Pierre Delanaye m'a initié à sa discipline sans jamais cacher son savoir. Il m'a aidé à faire mes premiers pas dans le monde de la littérature scientifique. Il a partagé mes joies et m'a toujours soutenu lors des moments plus difficiles ou lorsque le moral était en baisse. Ce travail, fruit de notre collaboration, est aussi le sien. Pierre, le mot « merci » me paraît un peu vide de sens pour t'exprimer vraiment ce que je ressens…

Ce travail est certainement un aboutissement aussi pour les personnes qui m'ont aidé et soutenu dès mes premiers jours…Maman, Papa, je ne mesurerai jamais assez l'importance que vous avez eue pour, au cours des ans, faire de moi l'homme que je suis devenu. Vous avez toujours su stimuler ma curiosité – ou me stimuler tout court – et m'avez toujours guidé pour me permettre de me développer tant intellectuellement qu'humainement. Je crois qu'à force de petites gouttes régulières (et non de seaux d'eau de temps en temps), on arrive enfin à la dernière ligne droite et que le bout du tunnel n'est plus vraiment loin…

Enfin mes derniers remerciements iront vers celles qui partagent ma vie au quotidien. Merci mes deux chéries de me pardonner le temps que je ne vous consacre pas. Je vous aime.

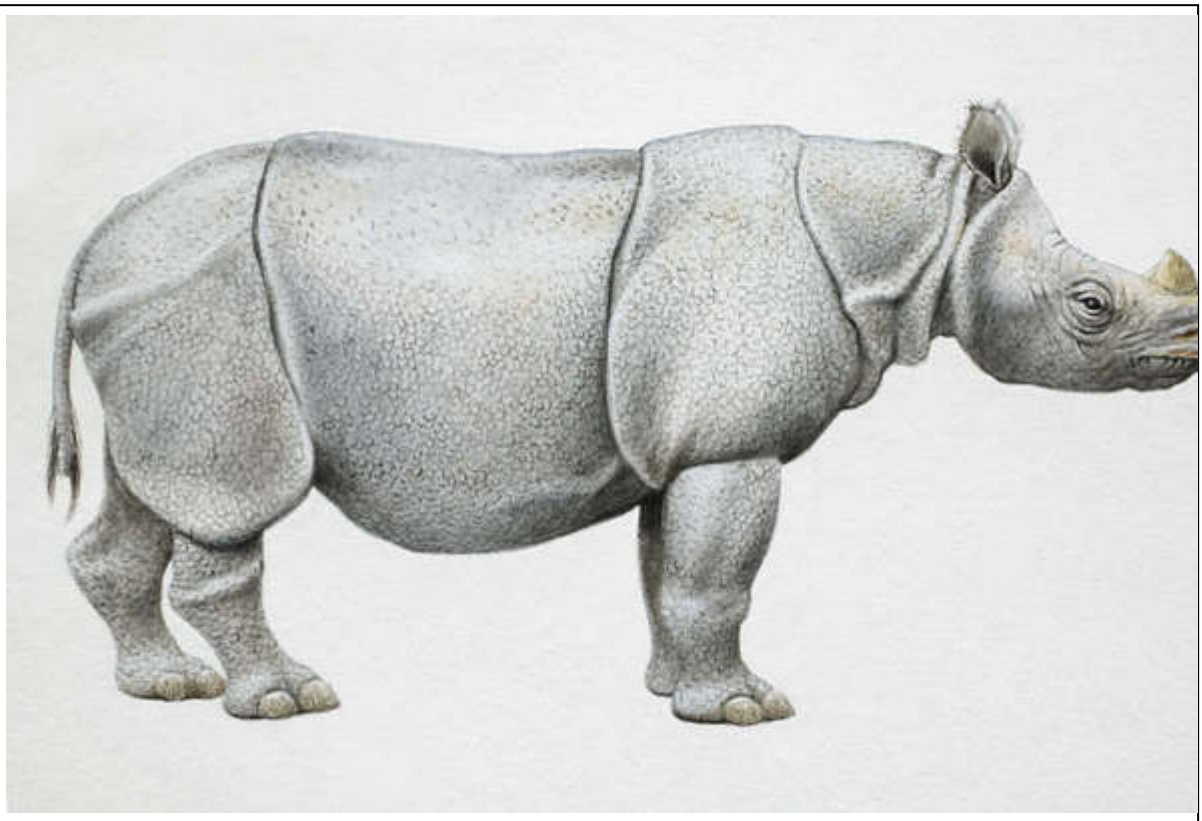
INDEX

I. GENERAL INTRODUCTION

"The first telescope opened the heavens; the first microscope opened the world of the microbes; radio-isotopic methodology, as exemplified by RIA, has shown the potential for opening new vistas in science and medicine"

Rosalyn S Yalow,
Nobel Prize Lecture,
8th December 1977

A. PARATHORMONE AND THE PARATHYROID GLANDS



RHINOCEROS UNICORNIS

1. Discovery of the parathyroid glands

In May 1834, the Zoological Society of London purchased a Great Indian Rhinoceros (*Rhinoceros unicornis*). The animal died in November 1849 and its carcass was offered to Sir Richard Owen (1804–1892), Professor and Conservator of the Museum in the Royal College of Surgeons of England [1]. The dissection of the 2 tons animal took place in the winter months of 1849 to 1850. The notes of the dissection, published in 1862, include the rhinoceros's last days as recorded in the Head-Keeper's minute book [2].

In his detailed description of the anatomy, Owen observed '***a small compact yellow glandular body attached to the thyroid at the point where the vein emerged'***: this structure is now known as the parathyroid gland.

Even if Owen is the first one to have described the parathyroid glands, Ivar Viktor Sandström from Sweden (1852–1889) is credited for the discovery and the name of the glands. This medical student was employed in the Department of anatomy in the University of Uppsala when he observed interesting structures in a dog's neck. In 1880, he wrote:

'I encountered on the thyroid of a dog a small hardly hemp-seed sized structure which was included in the same capsule as the thyroid but distinguished itself from it by a brighter colour. A superficial examination revealed an organ of a structure entirely different from that of the thyroid and particularly amply vascularised, because of which I considered it probable that here a vascular gland had been encountered, analogous to the carotid glands'[3]**.**

He then identified the organ in other animals before proceeding to human anatomy and to find it in the first individual examined, '*found on both sides of the inferior border of the thyroid an organ of the size of a small pea which judging from its exterior, did not appear to be a lymph gland, or an accessory thyroid gland and which upon histological examination*

showed a rather peculiar structure'. He named these new structures ***glandulae parathyroidae***.

The relevance of the Sandström's discovery remained unappreciated until Eugene Gley (1857–1930), Professor of Physiology and Endocrinology at the *Collège de France* in Paris, understood the endocrine function of the parathyroid glands. In 1891, he observed that tetany and death caused by experimental thyroidectomy in dogs occurred only if the excised material included the glands described by Sandström [4]. It was also in 1891 that the famous German pathologist Friedrich Daniel Von Recklinghausen (1833-1910) described *osteitis fibrosa cystica*, a disease associated with recurrent fractures of several bones with negligible trauma and 'bending' of the long bones with extensive fibrosis, cysts and brown tumours [5]. However, he was not aware that this disease was associated with an excessive secretion of parathormone.

In 1907, Jakob Erdheim (1874-1937) a young pathologist working in Vienna noted the relation between the function of the parathyroid glands and the structure of bone and teeth [6].

However, the central role played by the parathyroid glands in calcium metabolism was not yet appreciated until 1908. At that time, the correlation between parathyroidectomy or parathyroid insufficiency and convulsive symptoms, spasmodic states or eclampsy had been established [7]. In 1901, Jacques Loeb (1859-1924), a German biologist working in Chicago, noticed that "*the muscle itself contains calcium salts, and we consider it likely that these calcium salts might help in preventing contractions*" [8]. This led two famous scientists, the Canadian William G. MacCallum (1874-1944), and the Swiss Carl Voegtlin (1879-1960), working together at the Johns Hopkins Hospital as respectively Professor of Pathological Physiology and Professor of Pharmacology to set that the function of the parathyroid glands was connected with calcium metabolism [9, 10].

In 1915, Friedrich Schlagenhaufer (1866–1930), Professor of pathology in Vienna, suggested that an enlarged parathyroid might be the cause of bone disease and not the result of it. He presented two patients with osteomalacia each of whom was found to have a single parathyroid tumour at necropsy [11].

Ten years later, Felix Mandl (1892–1957), from the University of Vienna was the first one to remove a parathyroid tumour [12]. Under local anaesthesia, he removed a '*yellowish-brown almond shaped tumour'* measuring 25×15×12 mm from the left inferior area behind the thyroid gland of a patient suffering from severe von Recklinghausen's disease, with immediate improvement. Indeed, this impotent patient (a 1914-1918 soldier that had been invalidated by tuberculosis in the trenches) recovered rapidly and within a few days, his blood and urine calcium content lowered considerably and his bones recalcified.

2. Discovery of the parathyroid hormone

Adolph Melanchton Hanson (1888-1959), an American surgeon from Faribault (Minnesota), a little city located in the south of Minneapolis, was trying to extract the active substance from parathyroid glands. Using hydrochloric acid, he extracted from bovine parathyroid glands a substance that could treat experimental tetany, raise the calcium levels after parathyroidectomy in dogs, and cause osteoporosis if given over a period of time. He published these observations in 1923 and in 1924 [13, 14].

However, he was not the only one to work on this field and had a serious competitor. James Bertram Collip (1892-1965) was Professor of Biochemistry at Edmonton University, in Canada. He had already been involved in the discovery of insulin and awarded the Nobel Prize for this discovery (together with Macleod, Banting and Best) in October 1923. Based on his "insulin" experience, he postulated that the extracts of parathyroid glands, which caused tetany, produced a hormone. With hot hydrochloric acid, he extracted a crude substance from parathyroid glands, and by injecting this extract to parathyroidectomized dogs, he was able to maintain them alive for several months. Thus, he proved that his extract could serve as replacement therapy, but he went further and treated patients suffering from tetany with success in a clinical trial of his preparation. Collip had kept good contacts with Eli Lilly since their collaboration in the commercialization of insulin for *diabetes mellitus* treatment. Even if Hanson had been the first to isolate parathormone and to contact Eli Lilly, the director of research of the company urged Collip to publish his results and to make a mention to Hanson's papers [15].

B. DETERMINATION OF THE PARATHYROID HORMONE

In 1963, Solomon Berson and Rosalyn Yalow from the Bronx Veterans Administration Hospital of New-York published the description of the first immuno-assay for parathyroid hormone [16]. The PTH standard had been obtained from bovine parathyroid glands and labelled with ^{131}I. They had obtained their anti-PTH antibodies by injecting the bovine parathyroid glands extracts in guinea pigs and rabbits at intervals of 2-4 weeks. With their technique, they were able to detect endogenous PTH in plasma samples obtained from 2 subjects with hyperparathyroidism, but not in plasma obtained from 9 subjects with hypoparathyroidism. They also detected PTH in random samples obtained from subjects without known parathyroid disease. Rosalyn Yalow (Solomon Berson had passed over at that time) was awarded the Nobel prize in 1977 for her contribution to the development of RIA for peptide hormones.

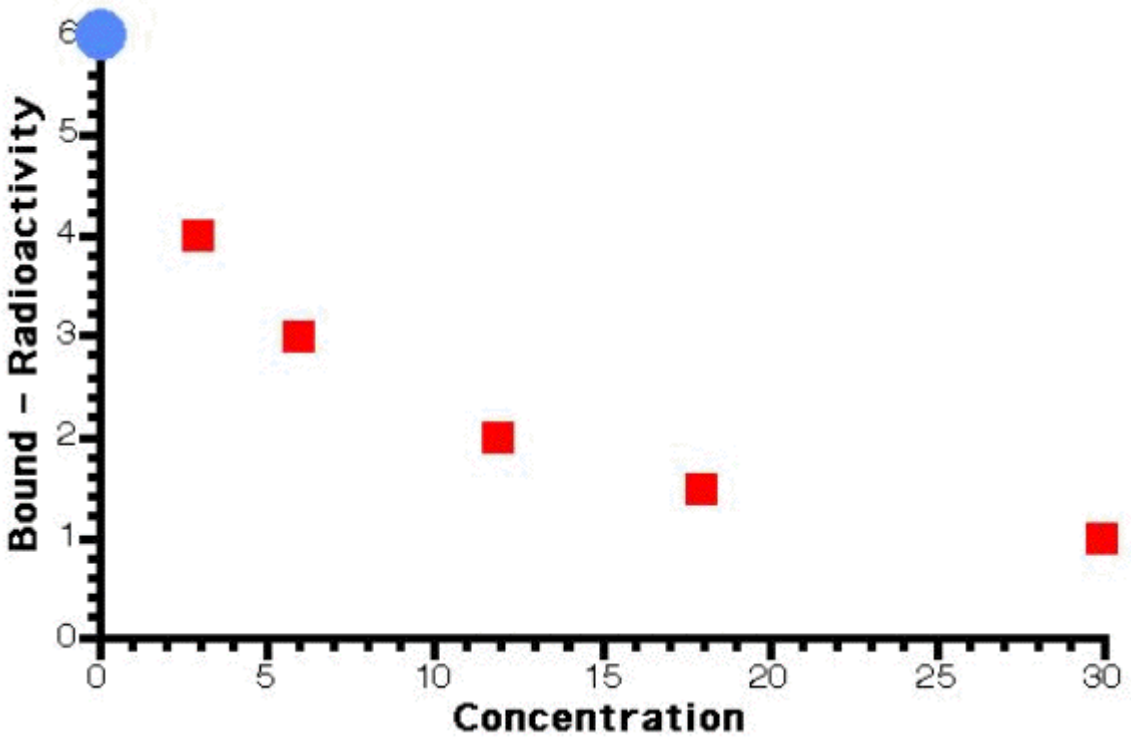
In 1968, Reiss and Canterbury developed an assay using anti-PTH antibodies obtained by injecting bovine PTH to chickens [17]. This assay presented a high affinity and a strong cross-reactivity with human PTH. They used an arbitrarily selected hyperparathyroid serum derived from a uremic subject with severe secondary hyperparathyroidism as a standard, which was assigned a concentration of 1000 picograms equivalents per millilitre. With this kit, they showed that in a "normal" population, the PTH ranged between 2 and 54 and was not measurable in hypoparathyroid patients. In the hyperparathyroid group, the lowest assay value of PTH was 100 whereas it reached 2000 in a patient with hypercalcemic crisis resulting from carcinoma of a parathyroid gland [18].

In the early seventies, many laboratories used to perform PTH determination with what we call now the "first generation" assays. These radio-immunoassays (RIA assays) used a single anti-PTH antibody that was either produced by the analytical laboratory himself (by

immunization of an animal - generally a rabbit - with the peptide) or a commercially available antibody.

The peptide (from bovine origin) was bought in specialized societies and labelled with ^{125}I. The samples were incubated with the anti-PTH antibody for 4 days at 4°C. Then the ^{125}I marked PTH was added and a second incubation of 3 days at 4°C occurred. This second incubation resulted in a competition for the anti-PTH antibody between the marked PTH and the patient's PTH. Then a second antibody (a sheep anti-rabbit IgG) precipitated the complex "PTH of the patient or marked PTH-anti-PTH antibody". This precipitate was washed and counted with a gamma-counter. The "counts" were inversely proportional to the PTH concentration of the patient (Figure 1).

Figure 1: a typical RIA curve.



At that time, in Liège, PTH determination was performed by the *Laboratoire de Radio-immunologie de la Faculté de Médecine* (Dr Heynen and Prof. Franchimont). The antibody used was the antiserum 211/32 bought from Wellcome Laboratories at the final dilution of $\frac{1}{400.000}$. This antibody was able to recognize the native (1-84) molecule and the fragments that contained the amino-terminal sequence (1-34). In 1977, our predecessors published a non-equivocal paper entitled "*La Parathormone sérique: problèmes récents*" (sic) [19]. Indeed, these "first generation" assays were important for a better comprehension of the phosphocalcic metabolism and helped for the diagnosis of primary and secondary hyperparathyroidism. However, they suffered from important interferences due to the "carboxy-terminal fragments" (COOH-fragments), which were PTH fragments of approximately 7000 kD. This was a consequence of the lack of a total specificity of the antiserums used for the aminoterminal sequence. These COOH-fragments were already known at that time to have a longer half-live than the native (1-84) peptide and the cross-reactivity of the antiserum and the COOH-fragments explained partially the discrepancies in PTH results observed between different laboratories.

The interference of the COOH-fragments was especially important in hemodialyzed patients. In these patients, PTH concentrations measured with these assays were always greatly increased. This was even the case in certain patients clearly identified by bone biopsy as suffering from low turnover bone disease, a condition associated with a defect in PTH action. Furthermore, these assays had a poor analytical sensitivity in the low concentrations of the peptide, which did not really allowed the discrimination between low- and normal-levels [20].

In 1974, at its 26th meeting, the Expert Committee on Biological Standardization of the World Health Organization (ECBS, WHO), remarked that the different PTH immunoassays presented a growing interest for different clinical purpose. They thus asked the National Institute for Biological Standards and Control (London), to obtain material of human origin

which could serve as an international reference preparation (IRP). In December 1978, a nominal amount of 150 μg of hPTH, stated to be more than 95% pure, was donated to WHO as a proposed IRP. Ampoules, labelled 79/500 were prepared in 1981, evaluated in a multicentric study and became the International Reference Preparation (IRP) of Parathyroid Hormone, Human, for Immunoassay [21]. The results of this study emphasized the heterogeneity of the results obtained in the different home-made or commercially available immunoassay systems used at that time. Due to mainly lack of stability and reconstitution problems, the WHO standard has really never been used.

In 1987, Nichols launched an Immunoradiometric (IRMA) kit called "***Allegro***" [22]. This immunoradiometric assay (IRMA) used a pair of different antibodies: a capture antibody coated to a plastic bead was directed against the (39–84) portion of the PTH molecule, and a ^{125}I labelled antibody recognized the (13–24) portion of the peptide.

This "sandwich" assay was thus not influenced any more by the COOH-terminal or mid-fragments which were measured with the first-generation assays. This "second-generation" assay kit and the ones that followed were globally called ***''intact'' PTH*** assays as they were thought to measure only the full-length (1–84) PTH.

During the following years, several similar assays, either IRMA or fully automated chemiluminescent assays became available on the market. In Liège, the Allegro assay replaced the "first-generation" PTH. Then, we used an "intact" PTH from Incstar, a company bought later by DiaSorin. The Incstar/DiaSorin IRMA was used until 2004, as we decided to move to the Liaison (DiaSorin), a chemiluminescent automate.

Some commercially available "intact PTH" assays use an anti-N-terminal antibody directed, like the Allegro assay, towards the proximal (13–24) portion of the hormone, whereas others, like Roche Elecsys intact PTH assay, recognize a more distal epitope, in the (26–32) portion.

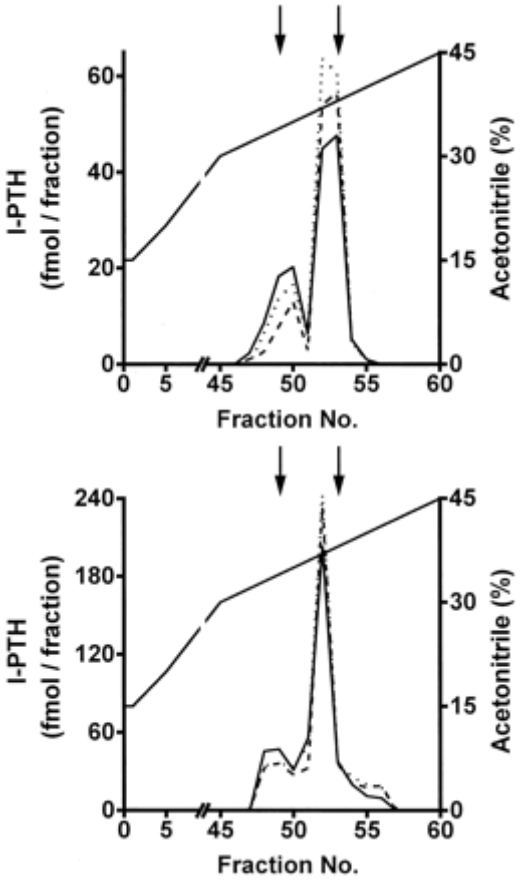
Even if these second generation kits gave results much more consistent with the clinic, they were rapidly shown to present some limitations. Indeed, several reports suggested that they overestimated the degree of secondary hyperparathyroidism in CRF patients [23, 24]. Some of these patients presented elevated "intact" PTH concentrations whereas they presented histological features of low turnover bone disease…

These apparent discrepancies were explained in 1998, after the demonstration that several ''intact'' PTH assays recognized, with various cross-reactivities (from approximately 50% to 100%), a PTH molecule different from the (1–84) PTH, which co-eluted in HPLC with a synthetic (7–84) PTH fragment [25]. This fraction was then called the "***non-(1-84) PTH***" or, more generally, ***"(7-84) PTH"*** (Figure 2).

Figure 2: HPLC profiles of circulating I-PTH in two pools of uremic samples (top, ~60 pmol/L; bottom, ~100 pmol/L).

Assays: Nichols (—), Incstar (... .), and DSL (- - -). Left arrow, hPTH(7–84) standard; right arrow, hPTH(1–84) standard. From Lepage et al [25].

We can see from this graph that, when the substrate caught by the antibodies used in the "intact" PTH kits is processed in chromatography, two different moieties are observed, (1-84) PTH and a fraction that migrates with (7-84) PTH.

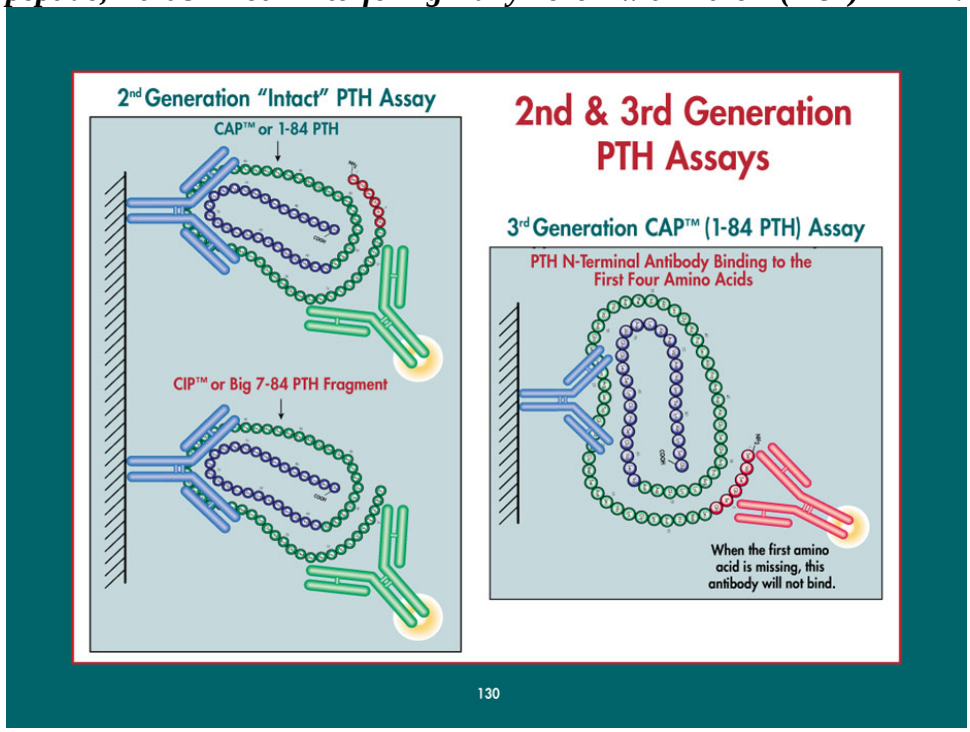


In 1999, the first "third-generation" PTH assay was developed by Scantibodies Laboratories [26]. This IRMA, called ***"Whole PTH assay"*** or "***Bio-intact PTH"***, uses an anti-COOH-terminal antibody similar to those of the ''intact'' PTH assays, but an anti-N-terminal antibody directed against the very first amino-acids (1 to 4) (Figure 3). Thus, this IRMA does not measure the ''non-1–84'' PTH fragments anymore. This kit does neither cross-react with

the N-truncated fragments. With the help of this kit, it became possible to evaluate the amount of the non-(1-84) PTH. Indeed, by removing the value of the "whole" PTH from the "intact" PTH (if this intact PTH cross-reacts at 100% with the non-(1-84)PTH), we obtain the amount of the non-(1-84) PTH present in the serum of the patient.

$$non-(1-84)PTH = "\text{int}\,actPTH - "whole"\,PTH$$

Figure 3: on the left side of the picture, we see that, in the 2nd Generation of PTH kits, the 2nd antibody catches (1-84) PTH and (7-84) PTH, whereas on the right side of the picture, the 2nd antibody of the 3rd generation kits is specific for the first four aminoacids of the peptide, thus not interfering anymore with the (7-84) PTH. © Scantibodies



Recently, it has been shown that a N-terminal molecular form of parathyroid hormone (amino-PTH), a PTH possibly phosphorylated on the serine in position 17, was overproduced in parathyroid carcinoma [27, 28] and in rare cases of severe primary hyperparathyroidism [29], leading to an inversion of the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio.

Indeed, amino-PTH cross reacts with the antibodies used in the 3rd generation PTH ("whole" or "complete' PTH), which are directed against the first four amino-acids of the peptide, but

not with the antibodies used in the 2nd generation kits ("intact" PTH). It should also be noted that the Roche Elecsys kit (a second generation PTH assay that uses an antibody directed against a more distal part of the peptide) also presents an interference with amino-PTH [20]. In normal individuals, amino-PTH represents approximately 10% of the circulating PTH [30]. Thus, the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio should always be <1 because the large non-(1-84) fragments detected with the 2nd generation (and not the 3rd) kits represent a larger proportion of the circulating PTH than amino-PTH does. We will see later the potential interests of amino-PTH in the diagnosis of parathyroid carcinoma.

The different PTH moieties recognized by the different PTH generation kits are summarized in Table 1.

Table 1: main PTH circulating fragments and whether they are measured by the various PTH assay-generations

	1st generation assays	2nd generation assays	3rd generation assays
Most common identifications	C-PTH assays, Mid-PTH assays	"Intact" PTH assays	Whole PTH assay, Ca-PTH assay, BioIntact PTH assay
Methodology	RIA (competition assays)	IRMA, Chemiluminescence, (sandwich assays)	IRMA (sandwich assays)
(1-84) PTH recognition?	Yes	Yes	Yes
Non-(1-84) recognition?	Yes	Yes (with various cross-reactivity)	No
C-terminal fragments recognition?	Yes	No	No
Amino-PTH recognition?	Yes	Depends on the epitope of the N-terminal antibody. No if the epitope is proximal (13-24), Yes if the epitope is distal (26-32) like Roche Elecsys PTH	Yes

C. ROLE, STRUCTURE AND METABOLIZATION OF PARATHORMONE

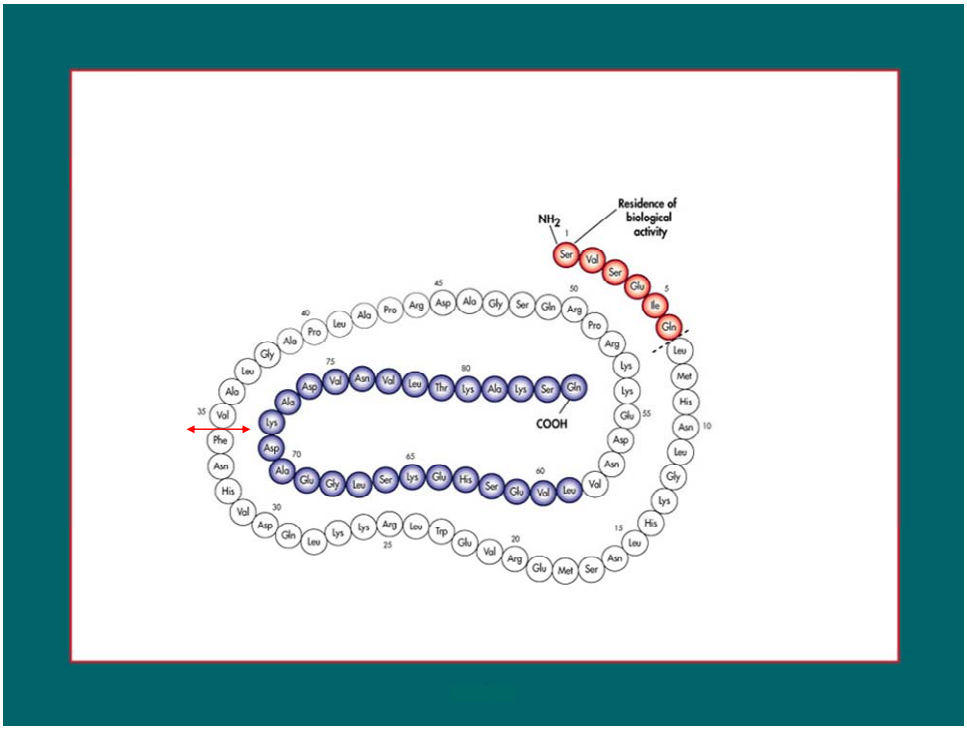


FIGURE 4: THE PARATHORMONE

Parathormone, together with 1,25(OH)2 vitamin D, plays a key role in the phosphocalcic regulation. Even if the study of the phosphocalcic metabolism is not the main purpose of this work, some essential information on the physiology of calcium and phosphate, as well as their regulation, is necessarily before going any further.

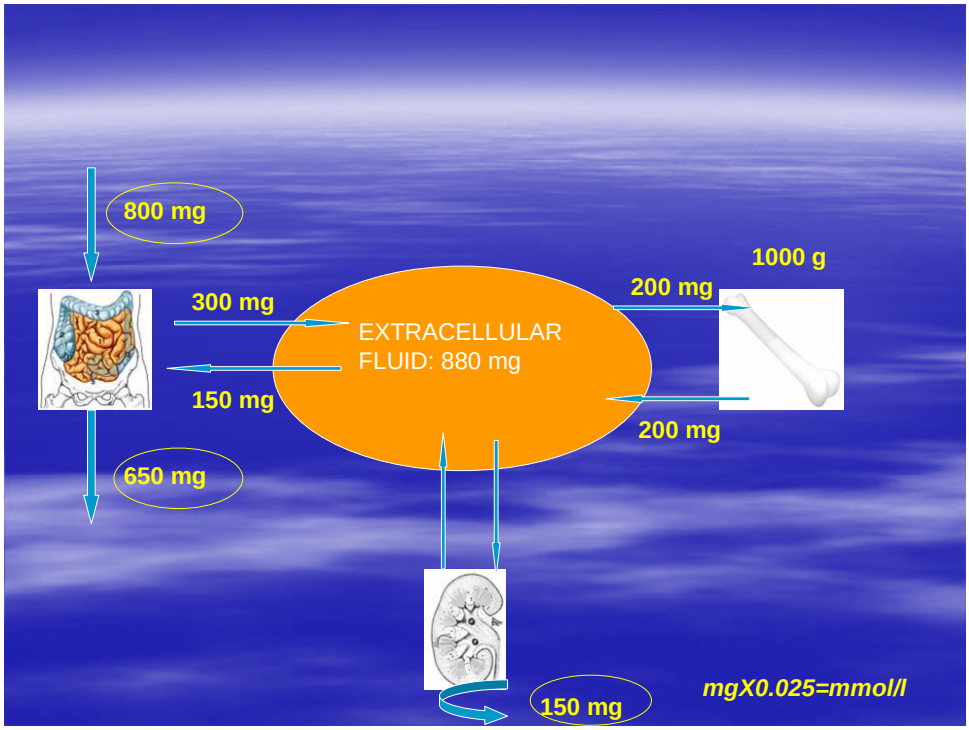
1. Calcium.

Our organism contains approximately 25000 mmol (1000 g) of calcium. The majority of calcium is contained in the bones (more than 99%) whereas less than 1% can be found in the extracellular fluids.

Calcium is normally present in our alimentation and the needs are approximately of 1 gram per day. Thirty to 35% of this alimentary calcium is absorbed through the small intestine, under the influence of 1,25(OH)2 vitamin D or passively. In normal situation, the sum of the absorbed calcium corresponds to the amount of calcium eliminated in the urine through the kidneys and the total balance for calcium is null. However, this kidney elimination has some limits. Indeed, under physiological normal conditions, it is quite difficult with a Western diet to eliminate less than 100 mg/day of calcium in the urine [31]. Thus, in the situations where the alimentation contains less than 600 mg/day (which is quite frequent in our lifestyle) or when there is an alteration of the intestinal absorption of calcium, the neat calcium balance becomes negative.

Bone remodelling, in young adults, is responsible for the release of approximately 200 mg of calcium in the circulation. On the other hand, the bone formation process will also need approximately 200 mg in order to mineralize the newly formed bone matrix (Figure 5).

Figure 5: Calcium flux in a normal adult having a diet containing the recommended amount of calcium. Personal illustration.



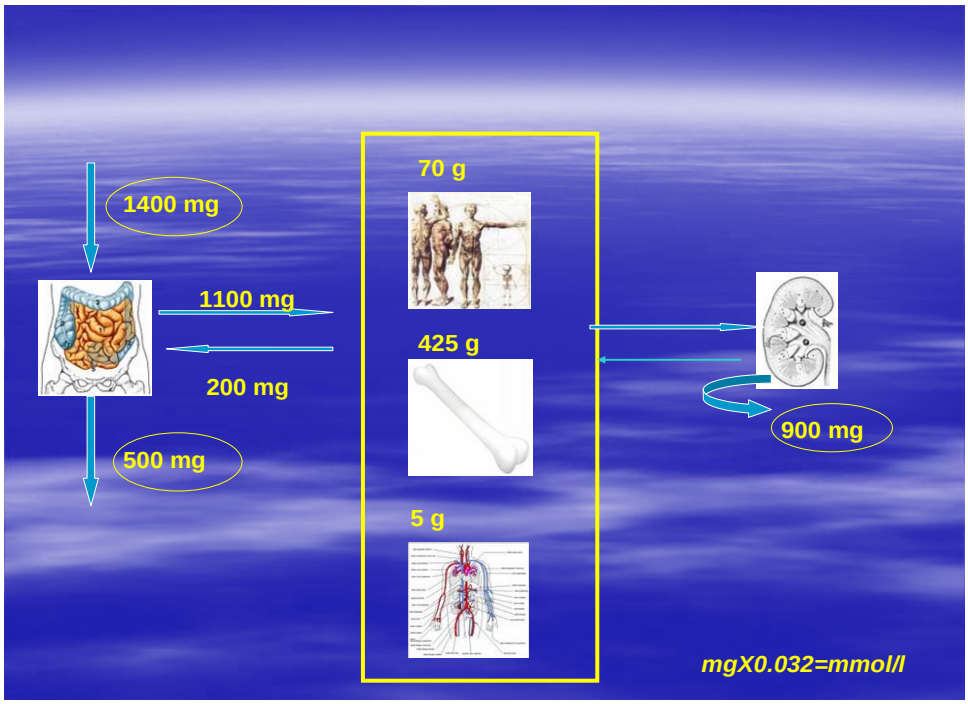
The only "active" and regulated form of calcium is the ionized calcium (Ca^{++}), which represents 50 to 55% of the total circulating calcium. However, due to analytical and preanalytical problems, total calcium, and not Ca^{++}, is generally measured. One should then keep in mind that in some conditions, where pH or albumin variations are observed, the total calcium will not reflect the true value of Ca^{++}. Some formulas exist to "correct" the total calcium, but with imperfections (particularly in hemodialyzed patients), as we and others have shown [32, 33].

2. Phosphate

Our organism contains approximately 500 grams of phosphate, mainly found in bones (85%). Fourteen percent of phosphate is present in the intracellular fluid and 1% in the extracellular fluid (ECF). In the ECF, one third of the phosphate circulates as a free form, weakly linked to proteins, calcium or magnesium (Figure 6). The total balance of phosphate is null in normal adults, positive in children and negative in the elderly. The total amount of phosphate found in the diet ranges approximately from 800 to 2000 mg/day. An important part of this phosphate (approximately 70%) is absorbed in the duodenum and in the jejunum, via two mechanisms. The first one is a passive, unregulated and non-saturable mechanism, and depends only on the phosphate gradient between the intestinal lumen and the interstitial liquid. The other (saturable and regulated) mechanism relies on the sodium/phosphate cotransporter Npt2b. In situations where high levels of phosphate are present in the intestine, the passive mechanism is predominant whereas if the quantity of phosphate is low in the diet, the active mechanism will be preponderant.

In normal situations, the quantity of phosphate coming from the intestine corresponds to the one that is eliminated by the kidneys. Kidneys play the central role in the phosphate regulation, as the intestinal absorption of phosphate from diet is poorly regulated and bones do not significantly participate to its regulation [34].

Figure 6: Phosphorus flux in a normal adult receiving a diet containing the recommended amount of phosphate. Personal illustration.



3. The phosphocalcic regulation.

In normal individuals, the calcemia is remarkably stable (intra-individual variation <2%, according to Westgard and Ricos). This is due to the different fluxes of calcium between bone, kidneys and the extracellular fluids. Briefly, in fasting conditions, the sum of calcium eliminated by the kidneys is compensated by an equal quantity of calcium liberated by the bone. This process is independent from the bone-remodelling process, which is a slow phenomenon, not adapted to the correction of a quick calcium variation. Rapid release of calcium from bone implicates another cellular mechanism (via the osteocytes, probably) and affects only the newly mineralized bone. After the meal, and in normal situations, the total amount of calcium that had been lost by the bones to maintain the calcemia is completely compensated.

As already said, calcium regulation is under the control of 2 hormones, PTH and 1,25(OH)2 vitamin D. Calcium levels are also maintained by the calcium itself, via the Calcium Sensing Receptor (CaSR). This (CaSR) is a G-coupled receptor of 1078 amino acids which consists of a large N-terminal extracellular domain, seven transmembrane helical domains and an intracellular C-tail segment (Figure 7) [35].

Figure 7: the Calcium Sensing Receptor.

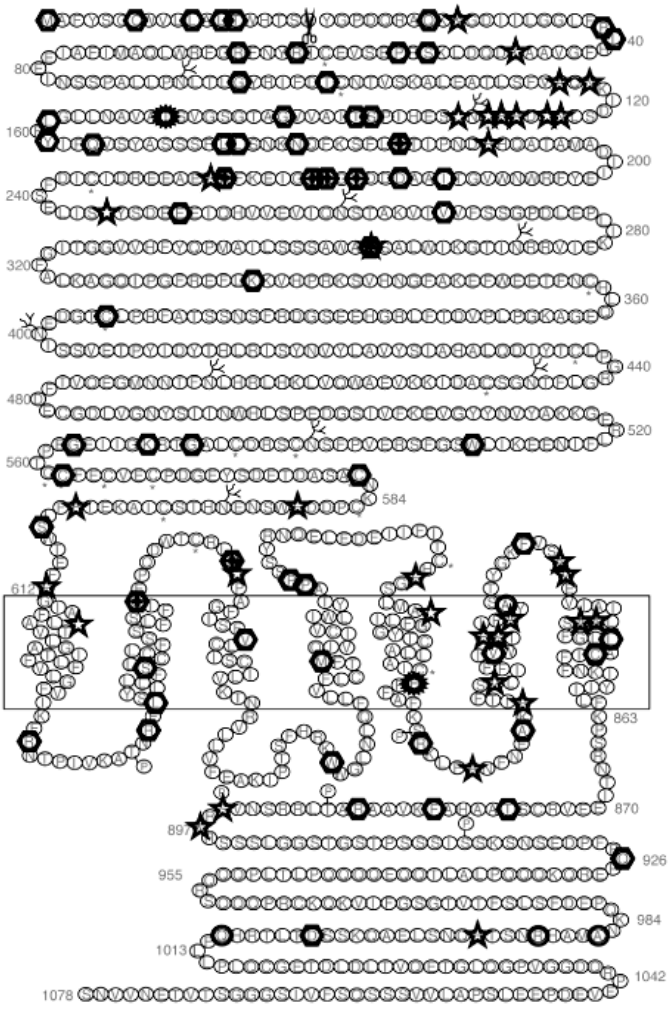
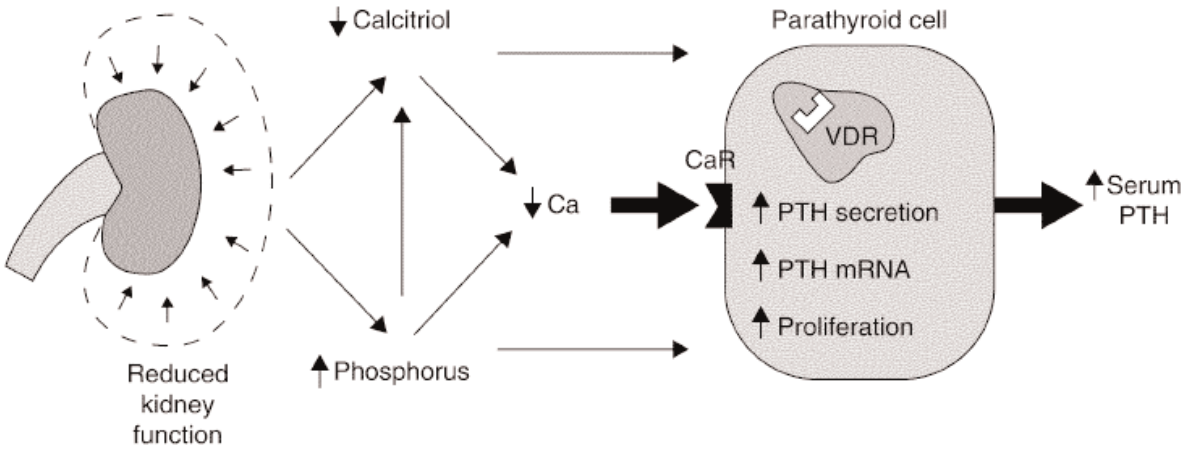


Figure 1. Topography and positions of mutations in the CASR. Symbols ○ indicate positions with one inactivating mutation, ⊛ positions with two different inactivating mutations, ★ positions with one activating mutation, ✪ positions with two different activating mutations and ⊙ indicate positions with polymorphisms. Adapted from D'Souza-Li in CASR mutation database, 1999. Reproduced with permission.

This receptor is present in the organs that secrete calcium-regulating hormones (the parathyroid glands and the C-cells of the thyroid) and in the target tissues of these hormones (renal tubules of the kidney, bone and intestine). It plays a key role in the control, by the ionized calcium levels, of PTH secretion and in the renal reabsorption of calcium.

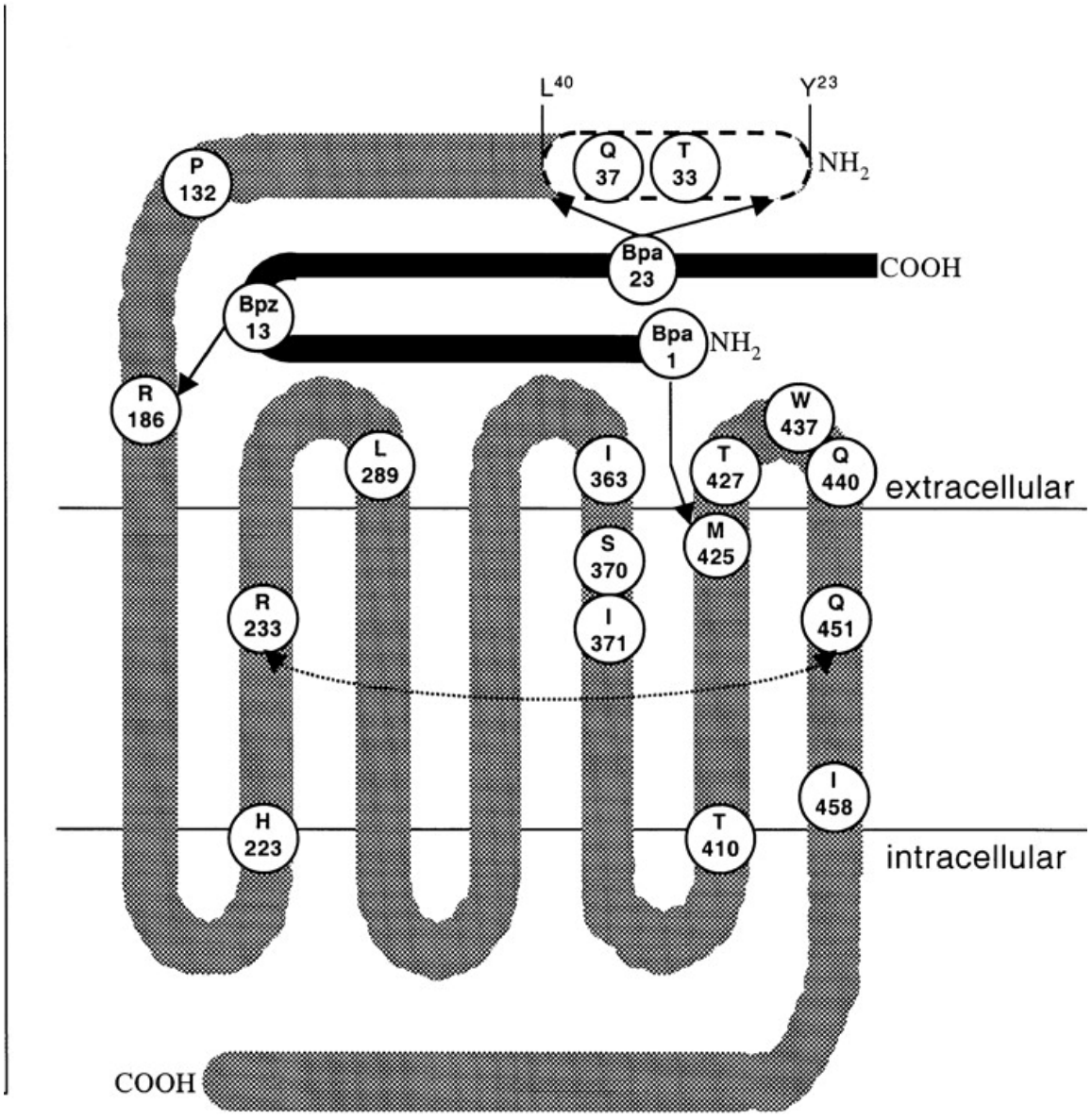
Figure 8: central role of the calcium-sensing receptor in the development of secondary hyperparathyroidism (from de Francisco AL, Expert Opin Pharmacother 2008).



PTH acts primarily on kidney and bone where it binds to cells expressing the type 1 PTH/PTHrP receptor (PTH1R). This G-protein coupled receptor is characterized by seven transmembrane domains (Figure 9). When it binds PTH, the PTH/PTHrP receptor transduces its signal through activation of protein kinases A and C pathways [36].

The very first N-terminal amino-acids of the PTH molecule are indispensable for this interaction [37].

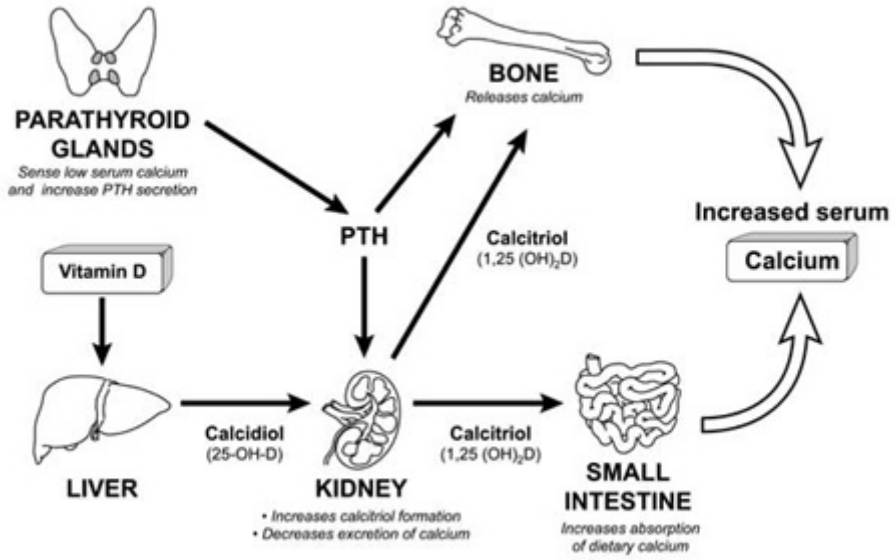
Figure 9: The PTHR1 receptor, from Mannstadt et al, Am J Physiol Renal Physiol [38]
Schematic shows some of the sites in the PTH-1receptor (thick gray-shaded line) and PTH (or PTHrP) ligand (narrower solid line) that have been found by mutagenesis or photochemical cross-linking methods to have potentially important roles in ligand binding or receptor function. Receptor amino acids and position number correspond to the human PTH-1 receptor sequence.



In the kidney, PTH stimulates the reabsorption of calcium in the distal tubule and the activity of the 1-alpha hydroxylase in the renal proximal tubule, thus enhancing the synthesis of 1,25 dihydroxy-vitamin D. This active metabolite of vitamin D increases the intestinal absorption of calcium (and phosphorus) and exerts an endocrine feed-back on the secretion of the peptide. PTH also decreases the renal reabsorption of phosphate in the proximal tubule (which decreases serum phosphate) through the endocytosis of the apical sodium dependant cotransporter Npt2a [39].

In bone, as explained above, PTH can induce a rapid release of calcium from the matrix.

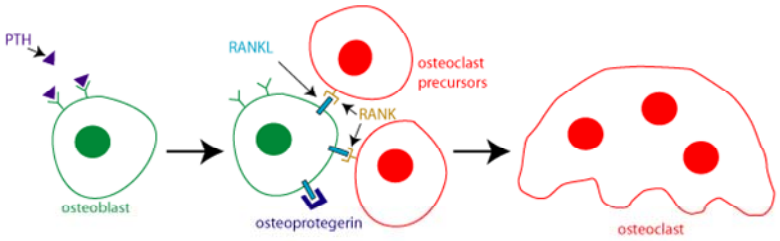
Figure 10: Calcium regulation



At longer term, PTH induces changes in Ca metabolism by its action on osteoblasts and indirectly on osteoclasts. Briefly, a continuous production of PTH will stimulate bone resorption by enhancing the transformation of pre-osteoclasts into active osteoclasts, through

the osteoblasts. Indeed, PTH stimulates the osteoblastic production of the Receptor Activator for Nuclear Factor κ B Ligand (RANK-L) together with Interleukin-1 and 1,25 $(OH)_2$-vitamin D and inhibits the expression of osteoprotegerin, an inhibitor of the RANK receptor. Precisely, this receptor is present on the surface of the pre-osteoclasts and its activation by the RANK-L will induce the transformation of pre-osteoclasts into active osteoclasts (Figure 11).

Figure 11: Schematic action of PTH on osteoclasts via the osteoblasts through the RANK/RANKL system.



On the other hand, one should notice that PTH stimulates bone formation, as it helps for the recruiting, proliferation and differentiation of the osteoblasts as well as reducing their apoptosis. This property is now used in clinical practice for the treatment of osteoporosis [40] (PTH given on one daily injection).

Calcitonin, a polypeptide secreted by the C-cells of the thyroid gland also inhibits the bone resorption by regulating the number and the activity of the osteoclasts. It is secreted in response to serum hypercalcemia and may prevent large oscillations in serum calcium levels. However, in comparison to PTH and 1,25(OH)2 vitamin D, the role of calcitonin is minor and

measurements of serum calcitonin levels is therefore not useful in the diagnosis of Ca homeostasis.

4. Synthesis and metabolization of parathormone

The human PTH gene is located on the short arm of chromosome 11 [41].

The peptide is first formed as a large polypeptide that then undergoes two successive proteolytic cleavages to yield PTH, the principal form of the hormone stored in and secreted from the glands. The peptide is synthesized as a pre-proparathyroid hormone (pre-proPTH) containing 115 amino acids (Figure 12). Within 1 min of synthesis, pre-proPTH is cleaved in its amino-terminal part and looses 25 amino acids to give the proparathyroid hormone (proPTH), an intermediate precursor of 90 amino acids. Pro-PTH is transported to the Golgi apparatus in 15 minutes, where a second cleavage of 6 AA in the terminal part occurs, resulting in the formation of the 84 amino acids PTH. The purpose and mechanism of the successive cleavages are unknown [42], but mutations preventing cleavage in pre-proPTH can cause hypoparathyroidism [43], indicating the importance of the signal sequences for a proper activity of the peptide.

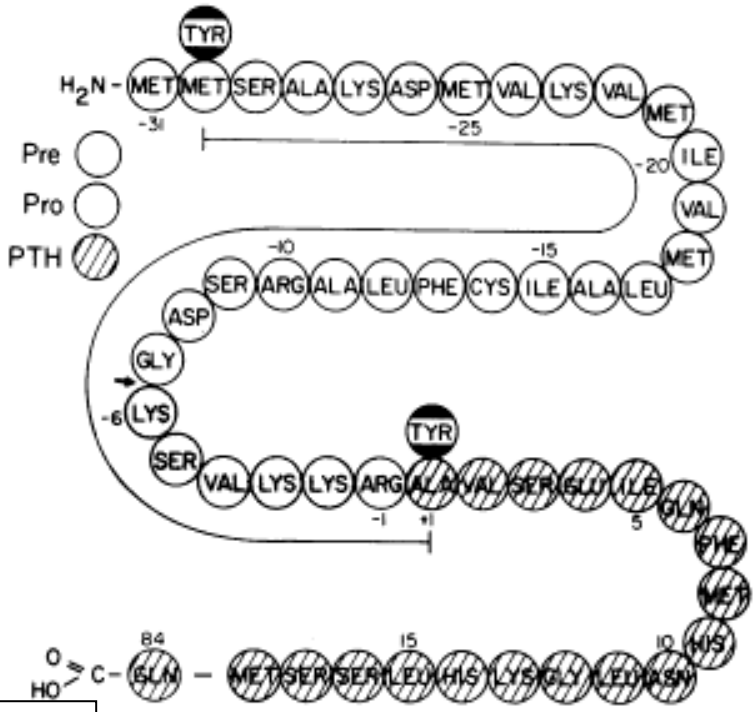


Figure12 **Amino acid sequence of the NH_2-terminal extension of bovine pre-proPTH determined by radiomicrosequencing of the major product of translation of parathyroid mRNA in the wheat-germ cell-free system. Open, stippled, and hatched residues indicate pre sequence, pro sequence, and PTH sequence, respectively. Residues 19–83 in the sequence of PTH are omitted for clarity. Continuous line: region of sequence synthesized by chemical means; barred tyrosine residues: substitutions in the synthetic peptides.**

The metabolization of the PTH is performed in the liver by the Kupffer cells. This releases different amino-truncated PTH fragments in the circulation. These fragments are generally called COOH-terminal fragments because they have kept the carboxyl-terminal part of the peptide. [44].

These fragments:

- are cleared from the circulation through the kidneys, as PTH itself [45], thus accumulating in the blood of patients suffering from chronical renal failure (CRF) [46].
- were thought to be inactive (we will see in a next chapter that this is not the case).
- have a higher half-live than the (1-84) PTH.

- will have an impact on the activity and the analytical determination of the peptide (especially in CRF patients).
- are not only a degradation product of PTH. Indeed, they are also secreted by the parathyroid glands.
- in normal individuals, account for 15-30% of the total PTH, but in CRF patients, this percentage can be as high as 70-80%.

II. AIMS OF THE WORK

In the last years, the role of the Clinical Biologist has changed. Indeed, if he remains the "analytical partner" of the Clinician, helping in the diagnosis with results of valuable quality, he has now to give much more detailed information on different topics, which are now considered to be of crucial importance to obtain reliable results. These points concern notably the pre-analytical phase [47], the reference range of the analytes [48], the analytical interferences [49] and the extensive validation of the methods. He also has to warn the Clinicians against some pitfalls which could lead to erroneous diagnostics. All these requirements are clearly requested by the ISO 15189 [50], the most recent international quality Guideline dedicated to biomedical laboratories.

Based on this evolution, our aims were to provide comprehensive data to the study of Parathormone. The first part of the results will concern the analytical aspects of PTH determination. The structure of this part will follow the classical laboratory flow and will be divided in "pre-analytical", "analytical" and post-analytical" aspects.

For the pre-analytical phase, we studied the stability of the peptide when stored at different temperatures and under different conditions. The analytical phase will mainly concern the evaluation of different methods for PTH testing and the clinical impact of any change in the method for the follow-up of the hemodialyzed patients. We also studied different analytical interferences and provided a strategy for the validation of the PTH results that takes into account these possible analytical interferences. We will illustrate by clinical cases that these interferences can lead to erroneous diagnostics. Finally, for the post-analytical phase, we focused on the establishment of the "reference" range of PTH.

The second part of the results will deal with the clinical aspects of PTH determination.

The first chapter will concern the hemodialyzed patients and in the second one, we will study the amino-PTH in patients suffering from severe parathyroid carcinoma.

Our work is based on these 9 following publications:

1- ***Cavalier E***, Delanaye P, Krzesinski JM, Chapelle JP. Comparison of Liaison N-tact PTH (DiaSorin) and N-tact PTH SP IRMA (DiaSorin) in hemodialyzed patients. Clin Chem Lab Med 2005; 43:890-1

2- ***Cavalier E***, Delanaye P, Carlisi A, Krzesinski JM, Chapelle JP. Analytical validation of the new version of the Liaison N-Tact PTH assay. Clin Chem Lab Med 2007; 45:105-7.

3- ***Cavalier E***, Delanaye P, Collette J, Krzesinski JM, Chapelle JP. Evaluation of different bone markers in hemodialyzed patients. Clin Chim Acta 2006; 371:107-11.

4- ***Cavalier E***, Delanaye P, Carlisi A, Krzesinski JM, Chapelle JP. Stability of intact Parathyroid hormone in samples from hemodialysis patients. Kidney Int 2007; 72:370-2.

5- ***Cavalier E***, Delanaye P, Hubert P, Krzesinski JM, Chapelle JP, Rozet E. Estimation of the stability of Parathyroid Hormone when stored at -80°C for a long period. CJASN, in press.

6- ***Cavalier E***, Carlisi A, Chapelle JP, Delanaye P. False positive PTH results: an easy strategy to test and detect analytical interferences in routine practice. Clin Chim Acta 2008; 387:150-2.

7- ***Cavalier E***, Delanaye P, Carlisi A, Chapelle JP, Collette J. An unusual interference in parathormone assay caused by anti-goat IgG: a case report. Clin Chem Lab Med 2009;47:118.

8- ***Cavalier E***, Carlisi A, Chapelle JP, Orfanos P, Uzan M, Falque V, Delanaye P, Hachicha M. Human anti-mouse antibodies interferences in Elecsys PTH assay after OKT3 treatment. Transplantation 2009; 87:451-2.

9- ***Cavalier E***, Delanaye P. Defining a “Reference Population”: No Easy Task. J. Bone Miner Res, 24(9) 2009, 1638.

III. RESULTS:

III - 1: ANALYTICAL ASPECTS

A. STABILITY OF PARATHORMONE IN SAMPLES FROM HEMODIALYZED PATIENTS.

Stability of intact parathyroid hormone in samples from hemodyalisis patients.

Cavalier E, Delanaye P, Carlisi A, Krzesinski JM, Chapelle JP

Kidney Int 2007; 72:370-2.

Estimation of the stability of Parathormone when conserved at -80°C for a long period: influence of the patient, of the method and of the sample type.

Etienne Cavalier, Pierre Delanaye, Philippe Hubert, Jean-Marie Krzesinski, Jean-Paul Chapelle, Eric Rozet. CJASN; in press.

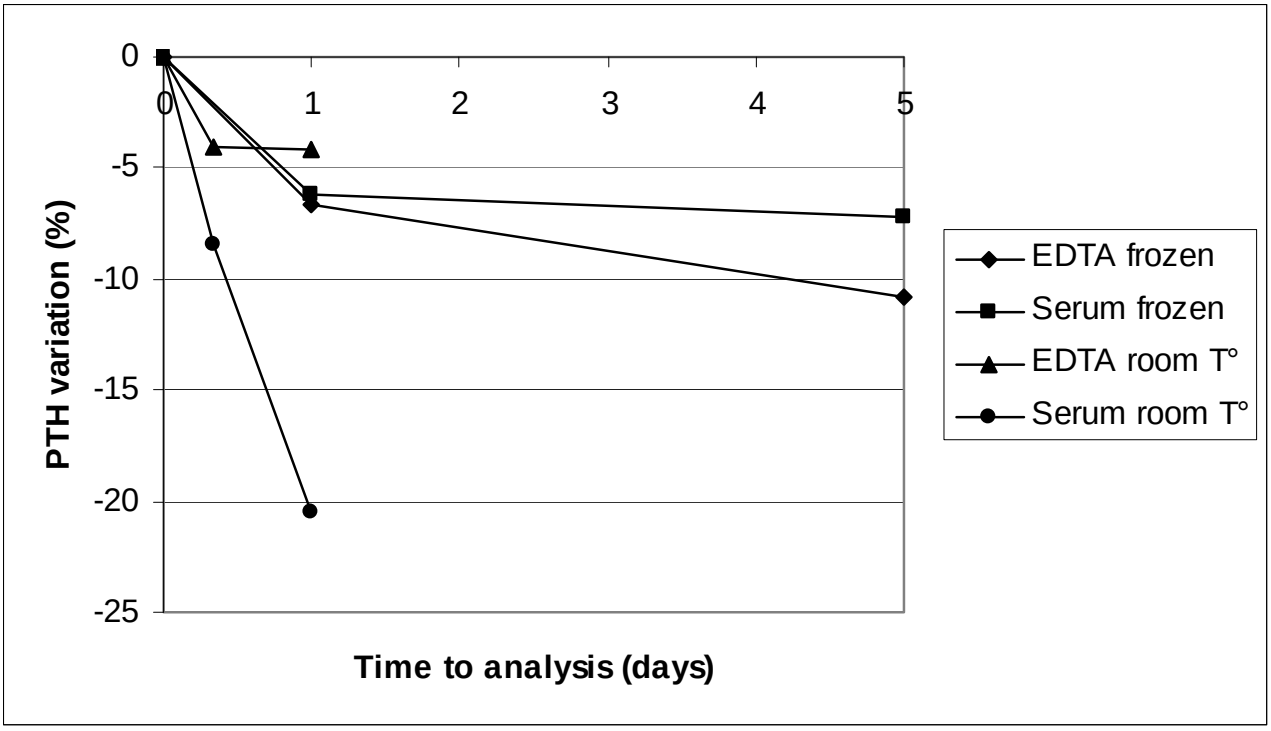
The ISO 15189 Norm requires that laboratories should perform stability studies of the biological parameters. Indeed, this factor is of importance for a good pre-analytical handling of the samples. In long-term studies, samples are sometimes stored for long periods at -80°C, allowing the determination of the analytes in the same batch. Unfortunately, the question of stability is poorly addressed and as we will see, this can have serious consequences on clinical decisions. In this chapter, we want to discuss the stability of PTH at different temperatures and in different conditions. It will be divided in two parts: the first one will deal with the stability of PTH when conserved as serum, EDTA plasma or total whole blood at room temperature and -20°C; the second will study the long-term stability of the peptide when conserved as plasma EDTA or serum at -80°C. In this last study, we will also show the impact of the method (Elecsys and Liaison), and of the patient on the maximum storage time.

1. Stability of PTH at Room temperature and -20°C.

Analytical and preanalytical conditions are of importance for a good follow-up of the CKD patients. So,we evaluated analytical and preanalytical influences on Roche Elecsys PTH determination [51]. Our approach was designed to be as close as possible to the hospital real life. Two series of blood samples were collected in an interval of a month from 16 hemodialyzed patients immediately before starting renal dialysis. All the samples were drawn into two tubes, one 5-ml EDTA tube and one gel separator with clot activator tube purchased from Becton-Dickinson. In all cases, the tubes were filled completely and brought to the laboratory within 30 minutes. On the first set of samples, drawn in October, we evaluated the stability of PTH when stored frozen as serum or EDTA plasma at -20°C. The stability of the 'frozen' PTH was evaluated after 24 h and 5 days. On the second set of samples, drawn in December, we investigated the stability of PTH when conserved in the sample tube as EDTA whole blood or clotted blood at room temperature (21°C). The stability of 'temperate' PTH was evaluated at 0, 8, and 24 h. For both series, the reference was the level of the corresponding tube assayed directly after centrifugation at 4°C.

Considering the two groups of patients as independent samples, the results obtained on the tubes assayed directly after sampling (zero time) did differ neither between October and December nor between EDTA and serum sample tubes. The mean percentages of PTH degradation under our experimental conditions are illustrated in Figure 13.

Figure 13: Mean PTH variations (%) when samples are conserved at room temperature as whole EDTA blood and clotted serum for 8 and 24 hours, or frozen aliquots of EDTA plasma and serum for 1 and 5 days.



Our results showed that there was no significant difference in PTH degradation between serum and EDTA after 1-day conservation at -20°C (-6.2 and -6.6%, respectively).

Nevertheless, when samples were kept frozen for 5 days, PTH degradation was more important in EDTA aliquots than in serum aliquots (-10.8±2.4 *vs.* -7.2±2.2%, p=0.0004). On the other hand, when samples were conserved at room temperature, PTH in EDTA whole blood remained relatively stable (mean loss of activity: -4.1%), whereas its degradation in clotted serum was already important after 8 h (-8.4±4.8%) and even more after 24h (-20.5±10.1%).

This difference in stability between serum and EDTA tubes was very significant ($p<0.0001$).

Clinically, by applying the criterion of the Royal Australasian College of Pathologists Quality Assurance Program, 33% of serum samples stored at room temperature failed assurance

criteria (<25% difference between the sample and the target) and would have thus lead to a diagnostic misclassification. In frozen series and EDTA kept at room temperature, no transgression of this quality criterion was observed.

As previously said, our approach in this first part of the work was designed to be as close as possible as the 'real life' of a sample tube in a hospital. The samples drawn in October mimicked the different options usually faced by a laboratory: immediate assay after reception or freezing of the samples for a further determination, usually 24 h to 5 days after reception. The goal of the study on the samples drawn in December was to see what happened if the tubes were left at room temperature from 8 to 24 h before arriving in the laboratory. This kind of 'pragmatic' methodology is original. Indeed, reports on PTH stability in serum and plasma are sometimes contradictory, due to the great differences in the methodologies used. For instance, the definition of the 'zeropoint' is, for some authors, a freshly frozen aliquot, whereas others consider the sample centrifuged and assayed within 3 h. Moreover, storage time at -20°C before analysis is not mentioned, which could let suppose that this parameter is of minor or no importance.

The definition of the term 'stability' is not also well defined, as most of the authors let degrade PTH in serum or plasma EDTA and not in whole EDTA blood or clotted serum. The conclusions of these studies are then only applicable to sample management in laboratories and cannot be extrapolated to pre-analytical handling of the tubes in the wards.

From a strict point of view, it may be difficult to draw conclusions from these studies.

Nevertheless, we agree that EDTA tubes are preferable in situations where rapid delivery of blood to the laboratory cannot be achieved. We have also observed that if PTH was considerably more stable in EDTA than serum when left at room temperature, it was not completely stable. On the other hand, some authors have shown that, when using the Siemens Immulite automate, PTH kept in EDTA tubes was higher (8%) after 3 days at room

temperature[52]. We have also observed such behaviour with the DiaSorin Liaison (data not shown).

The in vitro observation that PTH decline is observed in serum and not in EDTA plasma suggests that the increased protease activity may be due to the clotting process. Some authors showed that the addition of aprotinin significantly reduced the decline in PTH at 24 h – even if there was still a difference with EDTA samples. Others found that an addition of two protease inhibitors (aprotinin and leupeptin) eliminated completely the decline of PTH [53].

We did not find any difference between serum and EDTA plasma at baseline, whereas some authors reported significant higher values for EDTA plasma. Teal et al.[54] did not find differences when they compared PTH stability in serum and EDTA plasma with a third-generation PTH kit. They made the hypothesis that amino-PTH could be more stable than PTH itself. As Roche Elecsys PTH antibodies also recognize amino-PTH, our findings reinforce this hypothesis.

When we compared the stability of PTH after 24 h in the 'frozen' EDTA tube with the 'temperate' EDTA tube, we observed that PTH was more stable in EDTA whole blood than in EDTA plasma conserved at -20°C. This astonishing observation might be attributable to a greater PTH sensitivity to freezing/unfreezing than to PTH degradation by plasmatic metalloproteases. However, when we validated the method, we observed that PTH stability after four cycles of freezing/unfreezing did not support this hypothesis.

We have shown here that PTH is not always more stable in EDTA than in serum. Indeed, EDTA is preferable when samples are left in a ward or in a practitioner's office at room temperature, but once the sample is treated in the laboratory and kept frozen, PTH is more stable in serum than EDTA, compared with a 'fresh' determination.

Our findings are experimental. We do not know why we observed this difference. On the other hand, the greater degradation of PTH observed in serum compared with EDTA plasma still remains unexplained.

In conclusion of this first part, the type of samples used for PTH determination should depend on the way laboratories work. On one hand, if a lab has a practice of general practitioners or works for a multisite hospital, EDTA tubes conserved at room temperature are better. EDTA tubes are also preferable if a lab works in continuous flow. On the other hand, if a lab works in batches with automates, as it used to be with immunoradiometric assay series, and keep samples frozen before determination, serum tubes are the best choice.

When taking a decision on the best tube for PTH, one must also take into consideration that calcium – which should systematically be assayed together with PTH – can not be performed on EDTA tubes...

2. Estimation of the stability of Parathormone when conserved at -80°C for a long period: influence of the patient, of the method and of the sample type.

To our knowledge, the stability of PTH at -80°C for a long storage period (1 year or more) has never been studied. To know if there is, or not, a significant degradation of PTH when samples are stored for a long period, like in studies where a serum or plasma EDTA bank have been constituted (*for instance* in [55-57]), might thus be of importance for the conclusions of these studies. The aim of this study was to evaluate the stability of PTH when conserved for a long period as serum or plasma EDTA samples at -80°C, and to see if there was an influence of the method (Roche Elecsys or DiaSorin Liaison) on the results obtained.

Material and Methods.

Samples were collected in 16 hemodialyzed patients at 08:00h, immediately before commencing renal dialysis. All the samples were drawn into 5-ml EDTA (EDTA) and gel separator with clot activator tubes (DRY) purchased from Terumo (Haasrode, Belgium). In all cases, the tubes were filled completely and brought to the laboratory within 30 minutes. They were centrifuged at +4°C and plasma and serum were immediately aliquoted in cap-closed tubes and stored at -80°C. An aliquot was directly assayed on Liaison (DiaSorin, Saluggia, Italy) and Elecsys (Roche Diagnostics, Mannheim, Germany) to determine the "baseline" values for serum and EDTA plasma.

For each patient, one aliquot of serum and plasma EDTA was thawed after respectively 1, 3, 6 and 12 months, vortexed, centrifuged and assayed on the 2 automates.

In our hands, the coefficients of variation obtained with the Roche Elecsys PTH and the DiaSorin Liaison are <10% [58].

Data treatment

All the results were reported as recovery values, that is the ratios of the analyte concentration at each storage time to the analyte concentration at time 0. The factors "method" (Elecsys or Liaison), "tubes" (Dry or EDTA), "subjects" (n=16) and "time" (in months) were included in a linear mixed model to evaluate their effects on PTH concentration. Statistical significance of those factors were evaluated using α=0.05. Bootstrap prediction intervals for the linear mixed model were also computed when relevant [59]. JMP v7 (SAS Institute, Cary, USA) and R v2.8.1 (CRAN, http://cran.r-project.org) were used for model fitting and evaluation and to compute prediction intervals.

Results

The concentration of PTH was modeled as a linear mixed model of factors "methods", "tubes", "subjects" and "time" using the following model:

$$Y = \gamma_{0,j} + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \gamma_{4,j} x_4 + \varepsilon \quad \textbf{(Eq. 1)}$$

where *Y* is the measured PTH concentration (pg/mL), $\gamma_{0,j}$ is the random intercept, $\gamma_{4,j}$ is the random slope for time and the β_i's (*i*=1 to 3) are the coefficients of the factors "methods", "tubes" and "subjects, respectively. $\varepsilon \propto N(0;\sigma_\varepsilon^2)$ is the residual error assumed normally distributed, $\gamma_{0,j} \propto N(0;\sigma_{\gamma_1}^2)$ and $\gamma_{4,j} \propto N(0;\sigma_{\gamma_4}^2)$ are also assumed normally distributed and account for the variability of all the *j*=1 to 16 subjects.

The model adjusted R^2 equaled 0.9 suggesting a good adequacy of the model fitted: 90% of the observed variability of the PTH concentration is explained by the retained factors.

Statistical evaluation of the coefficient of the factors showed that the type of the method used to analyze the samples is highly significant (p-value<0.0001) as shown in Figure 14. Similarly, the type of tube used to collect the sample is also significant (p-value=0.041; Figure 14). The time of conservation showed a statistically (p-value=0.002) decreasing slope of -2.06 as indicated in Figure 14.

Due to the significant effect of the factors "method" and "tube", separate analysis for each pair of ["method";"tube"] was performed to further assess the stability of PTH concentration over time using a linear mixed model. For each pair of ["method";"tube"], time of conservation has a significant effect over the concentration of PTH (p-value<0.05). About 60% of the predicted slopes for each subject were found negative. This finding asserts a non negligible degradation of PTH over time at -80°C, although not systematic for all biological sources of samples.

However, in practice, it is not the average of samples coming from different subjects that is given as a clinically relevant result, but it is rather the PTH concentration of a single patient that is of interest, whoever the patient is. Moreover, the model given by Eq.1, as well as the smallest models used to evaluate each pair of ["method";"tube"] showed that the slope of the degradation was subject-dependant.

Although the average degradation slope is statistically significant and knowing that it is variable from one patient to another, this degradation may have a practical implication. The question is what impact these slopes do have on the future results that will be obtained when thawing a single sample of any subject and analyzing it after 6, 12 or even 24 months of storage ? This answer can be effectively answered through a graphical representation and using prediction interval methodology [59, 60]. Prediction intervals allow to predict where a future result can be obtained with a defined probability. Figures 15 a-d show the recovery of PTH concentration of each subject obtained during the storage of 16 patients samples for each

pair ["method";"tube"]. Added to these figures are drawn their 95% lower prediction interval (bold continuous line). Thus, these prediction limits show where a single result of any possible future patient can be obtained in the future at each storage time with 95% probability. It is thus evident from these figures, that stable storage of these samples at -80°C is not infinite. There is indeed a Maximum Storage Time that should be evaluated. Furthermore, this is a requirement for laboratories aiming accreditation such as ISO 17025 [61] or ISO 15189 [50]. One effective way to determine this storage time is to add to the graph acceptance limits as shown in Figures 15 a-d. These acceptance limits represent the maximum degradation that is acceptable. The Maximum Storage Time is defined when this acceptance limit crosses the lower prediction interval. Those acceptance limits could be for example the limits chosen for assessing the validity of the method, or 2 or 3 times the intermediate precision standard deviation of the method. The acceptance limits shown in Figures 15 a-d are settled by allowing a degradation of maximum -30%, *ie*. a minimum recovery of 70% (dashed line) [62]. The maximum storage times when using the Liaison method with either Dry tubes or EDTA ones depicted in Figures 15a and 15b are the shortest and are estimated of 9 and 2 months, respectively. So samples collected with EDTA tubes should not be stored more than 2 months using this type of automate as the degradation will exceed the maximum acceptable level of degradation of 30%. By opposite when using the Elecsys method, the maximum storage times are of at least 27 months whatever the type of tube used, as shown in Figure 15c and 15d. When using the Elecsys automate samples could be stored up to 2 years, still with acceptable stability of the PTH. Indeed, the degradation will not exceed the maximum acceptable level of -30%. Furthermore, the statistical difference between the two methods has shown here the practical implication of such a lack of comparability on the stability of PTH.

Discussion.

We presented here the first study on the conservation of PTH at -80°C for a long period. We introduced an original method that allowed us to take into account different parameters, like the method used to determine PTH, the kind of tube used for sampling, the length of conservation and the patient variability. We were thus able to build a model that explained 90% of the variability of PTH concentration. We also established a Maximum Storage Time for the conservation of the peptide using an original methodology. This Maximum Storage Time is an important parameter that should be taken into account in studies where samples are kept for a long period at -80°C before processing. Indeed, we have shown that there was a systematic degradation of PTH and, moreover, that this degradation was patient-dependant. In other words, we will observe degradation in some patient samples, but not in others; it means that we will be obliged to be restrictive and use the lowest Maximum Storage Time for the limit of conservation. In this way, the patient will be protected against inadequate decisions made using the analytical results of the stored samples.

Another striking point is the impact of the assay method used on the Maximum Storage Time. Indeed, we observed a more important degradation with Liaison than with Elecsys. This can be explained in two points: firstly, Elecsys recognize, next to the PTH molecule itself, another peptide called "amino-PTH" which is not recognized by the Liaison antibodies [28]. We and others have already hypothesized that this amino-PTH was more stable than PTH itself [51, 54], explaining why we observe higher levels with Elecsys. Secondly, it is now well known that C-terminal fragments accumulate in the serum of hemodialyzed patients [25]. These fragments are more stable than PTH itself [46]. The Liaison is one of the methods that cross-reacts at the lesser extent with PTH 7-84, the most representative fragment of the C-terminal fragments. This is particularly true, when compared to the Elecsys (see Table IV in [63]), which could let us think

that the decrease observe with the Liaison, and not the Elecsys, could be due to a degradation of the PTH molecule itself.

Conclusion

Conservation of PTH at -80°C is not infinite, but method and patient-dependant. An evaluation of the Maximum Storage Time should be performed to ascertain that the samples will present the same reactive profile after conservation for a long period. Only in such a way will consistent decisions be made with the results obtained from the analysis of the stored samples.

Figure 14: ***Effect of the factors "method", "tube" and "time" on PTH concentration***.

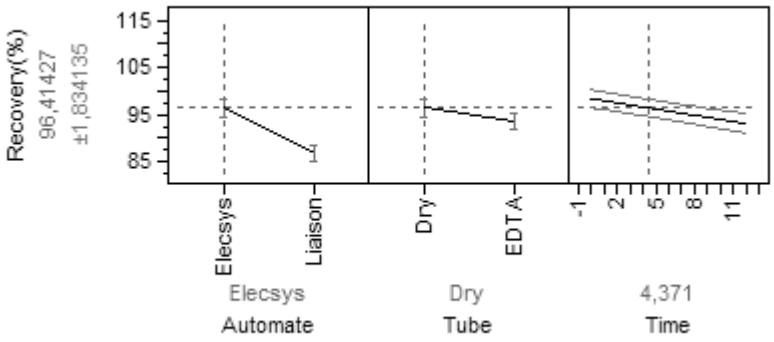
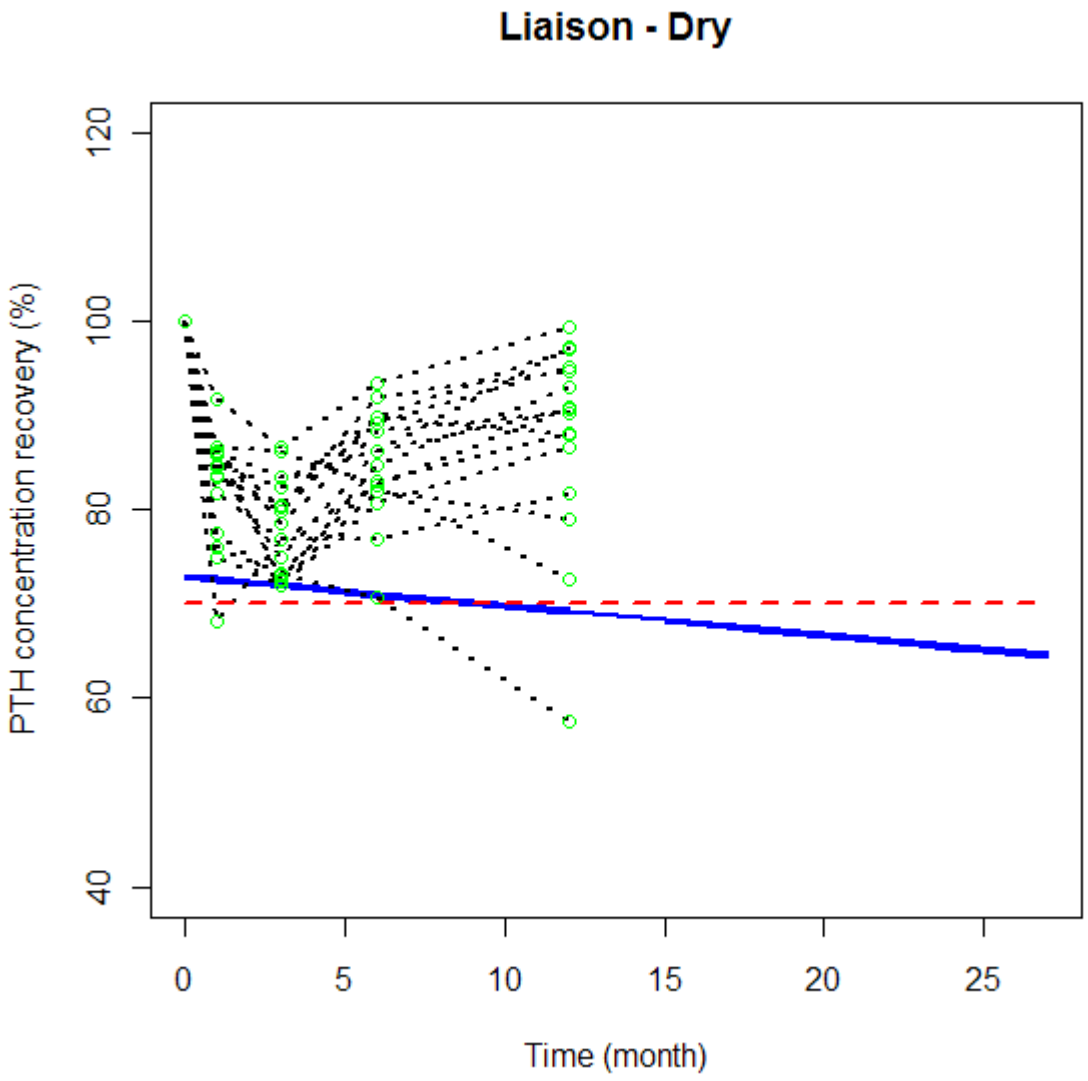
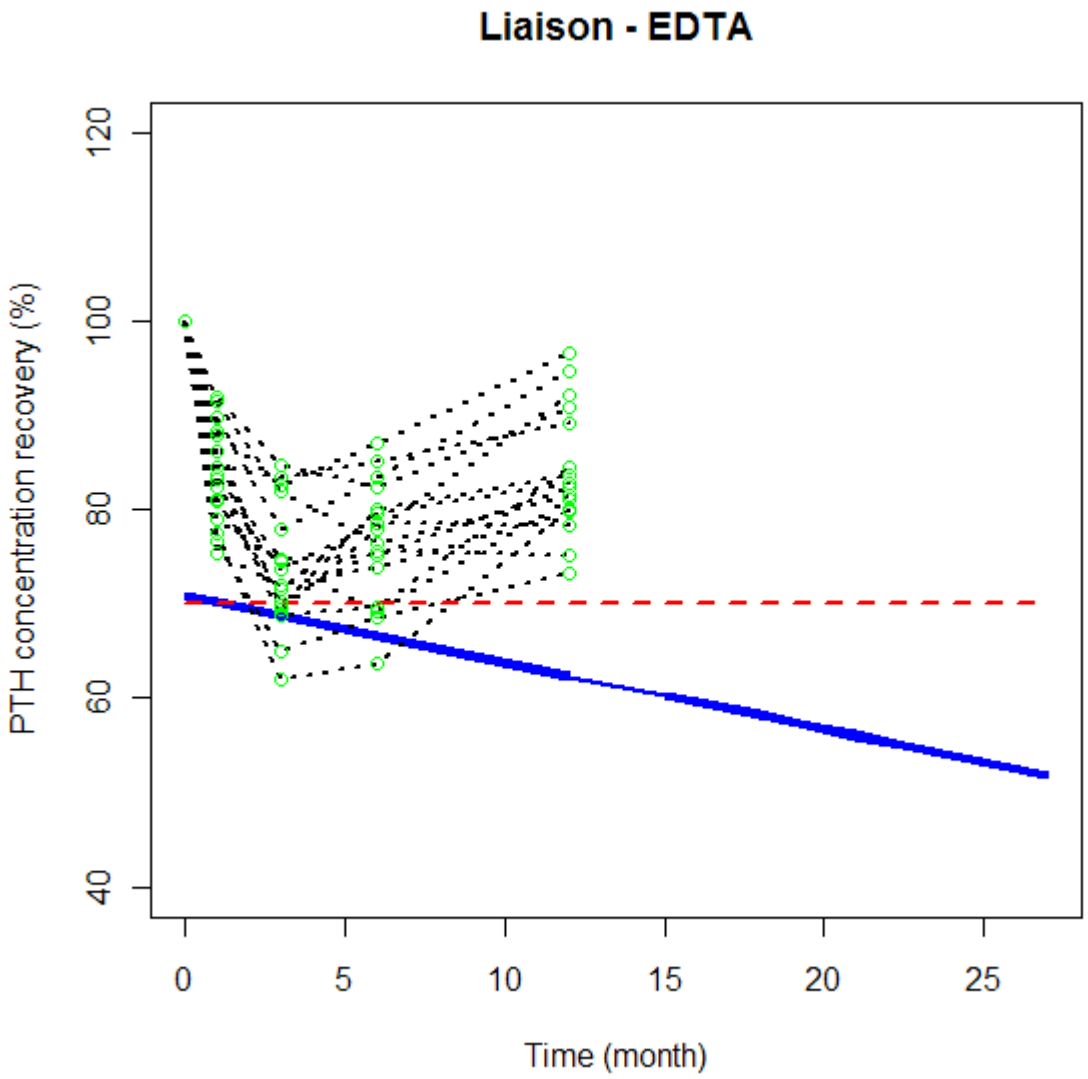


Figure 15 : ***Prediction of the linear degradation of PTH concentration overt time for each pair of [“method”;”tube”]:, The circles and the dotted lines show the evolution of the PTH recovery with respect to time point “0 month”. The bold continuous lines are the lower 95% prediction limits and the dashed lines are the lower acceptance limits settled at 70% of recovery.***

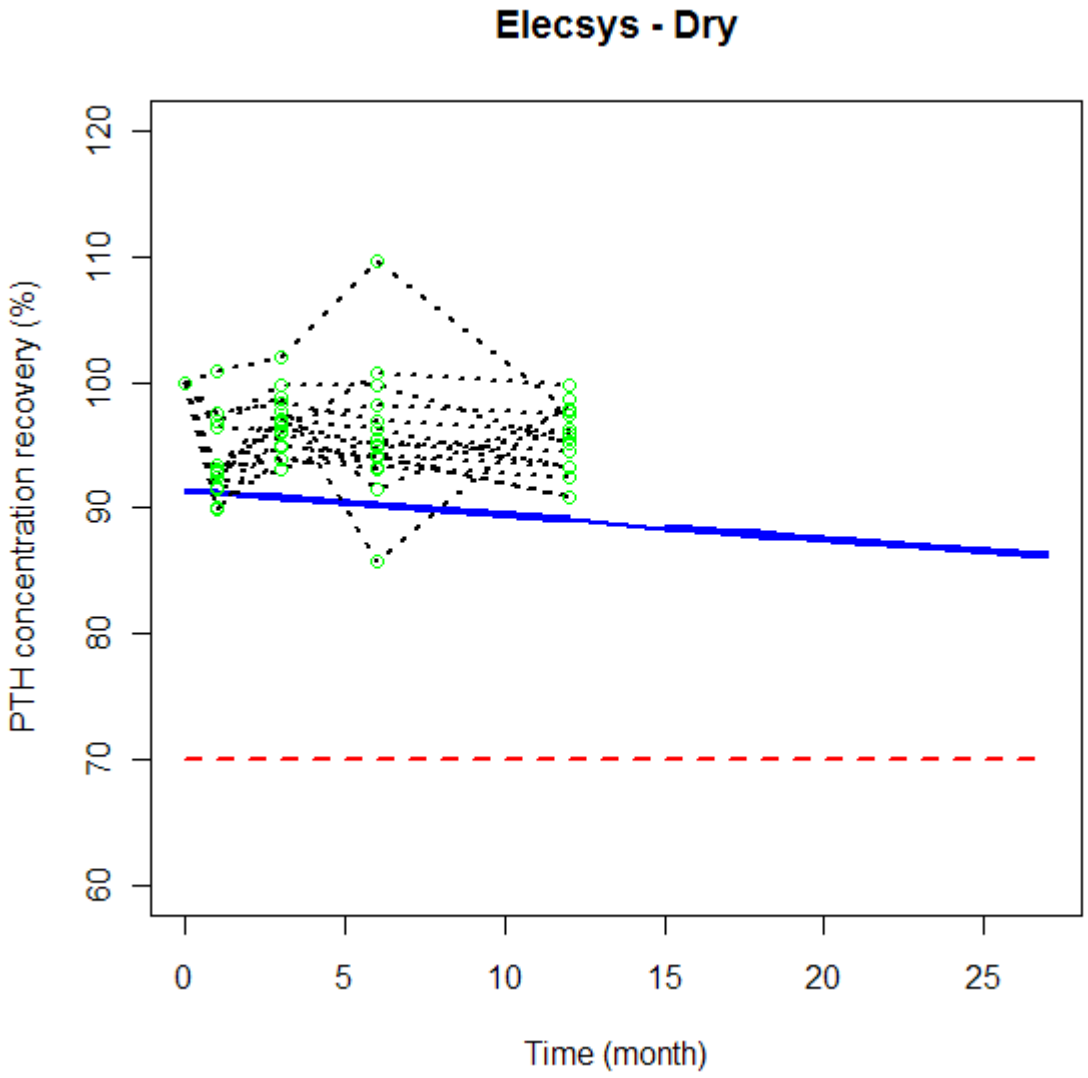
(a) [Liaison; Dry]



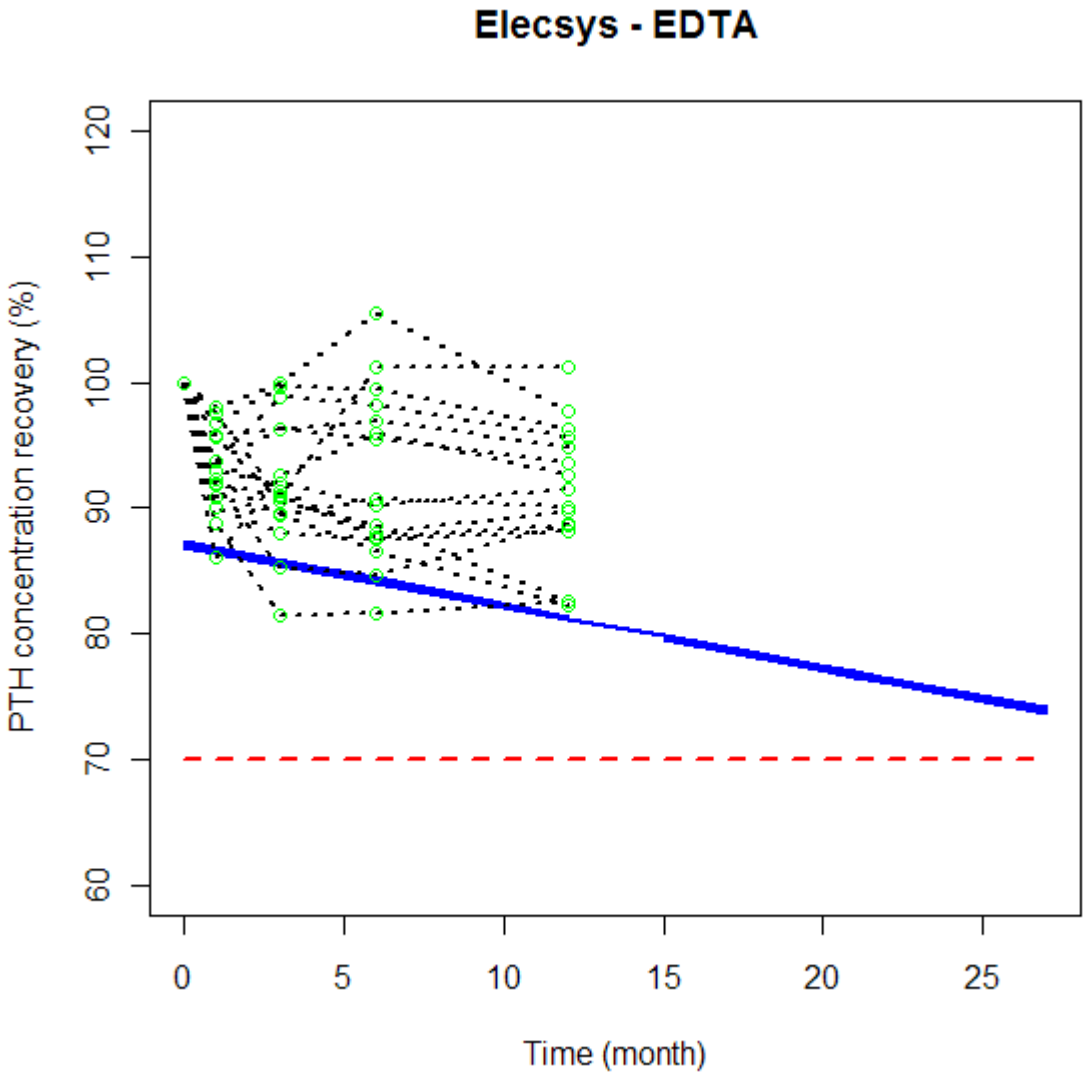
(b) [Liaison; EDTA],



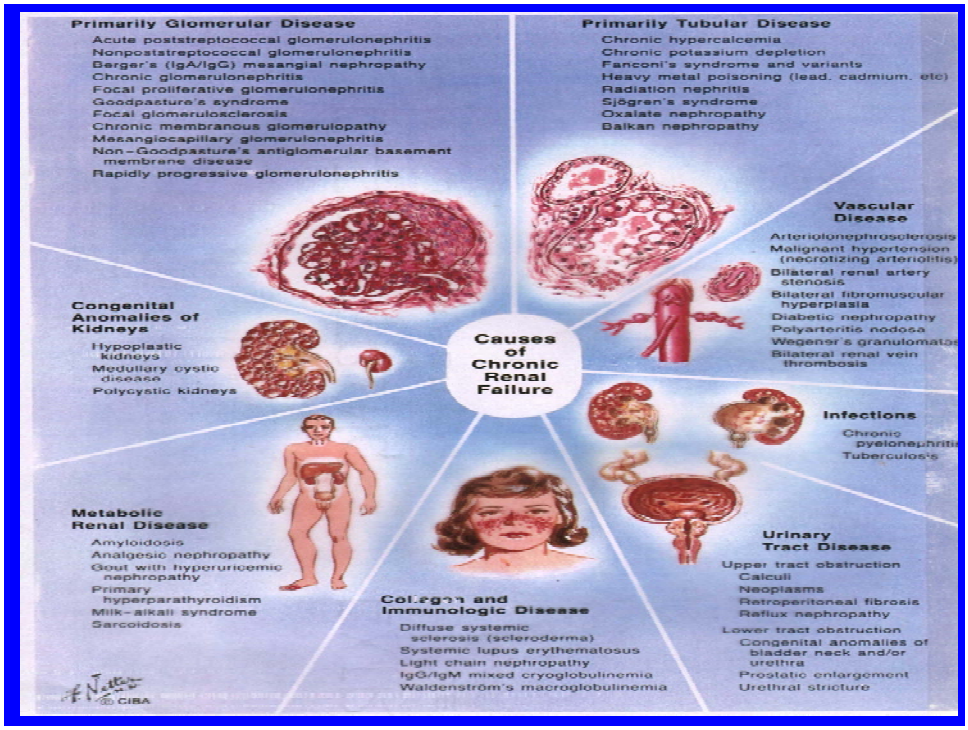
(c) [Elecsys; Dry],



(d) [Elecsys; EDTA]



B. PTH DETERMINATION IN CHRONIC RENAL FAILURE PATIENTS: NOT AN EASY TASK



Comparison of Liaison N-tact PTH (DiaSorin) and N-tact PTH SP IRMA (DiaSorin) in hemodialyzed patients.

Cavalier E, Delanaye P, Krzesinski JM, Chapelle JP.

Clin Chem Lab Med 2005; 43:890-1.

Analytical validation of the new version of the Liaison N-Tact PTH assay.

Cavalier E, Delanaye P, Carlisi A, Krzesinski JM, Chapelle JP.

Clin Chem Lab Med 2007; 45:105-7.

1. The K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease: impact of the PTH determination on the follow-up of the patients.

Routinely, Nephrologists rely on the generally accepted K/DOQI guidelines for the management of the CKD patients [64].

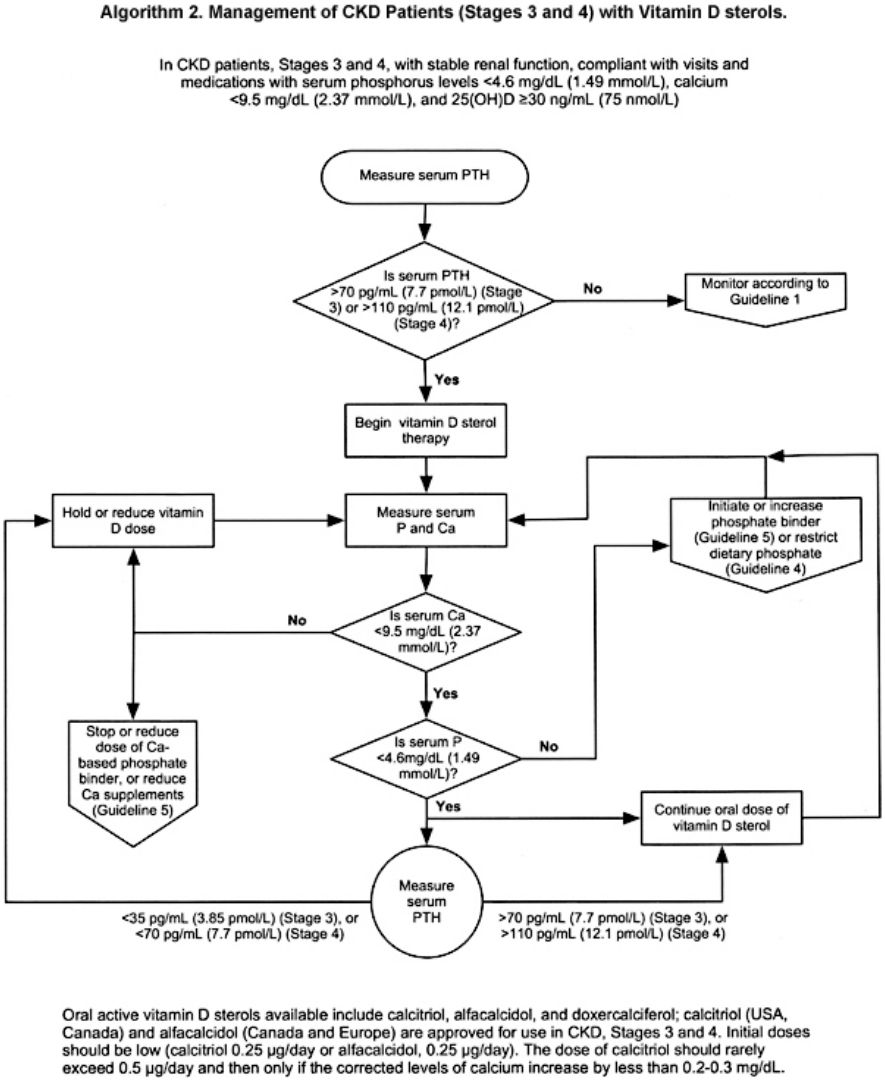
Guideline #13 concerns how to treat bone disease in CKD patients.

Paragraph A of this Guideline concerns "Hyperparathyroid (high-turnover) and mixed (high turnover with mineralization defect) bone disease". In this paragraph, we can find information on the therapeutic and dietary instructions on the treatment of CKD patients (stages 3 and 4) ***"with intact PTH> 70 pg/ml (stage 3) or >110 pg/ml (stage 4) on more than 2 consecutive measurements"*** and for stage 5 CKD patients ***"with intact PTH> 300 pg/ml"***.

Paragraph C of the Guideline deals with adynamic bone disease in stage 5 CKD patients (determined by bone biopsy or ***intact PTH< 100 pg/ml***).

Guideline #14 recommends parathyroidectomy in patients with severe hyperparathyroidism (defined as persistent ***serum levels of intact PTH> 800 pg/ml***, associated with hypercalcemia and/or hyperphosphatemia that are refractory to medical therapy).

Figure 16: Management of CKD patients (stages 3 and 4) regarding the PTH levels of the patient



It should be noted that these intact PTH "cut-offs" have been obtained with the ***Nichols Allegro kit*** – which is no longer available on the market. This point is not specified in the guidelines.

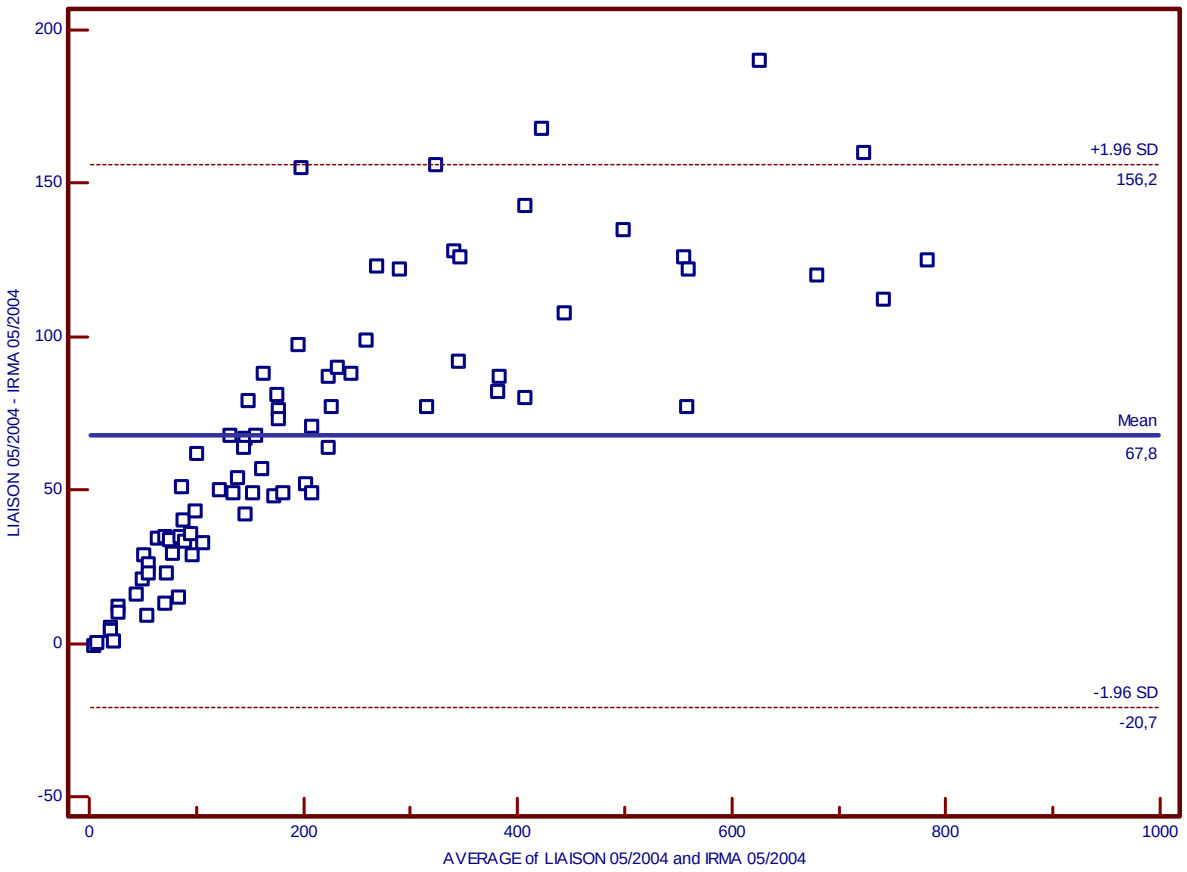
Given the various cross-reaction degrees of the antibodies used in the intact PTH kits and the non-(1-84) PTH, this has two serious consequences:

- the intact PTH goals described in the Guidelines are only applicable *sensu stricto* to the Allegro kit.
- any change in the PTH determination method can affect the longitudinal follow-up of the patients

We emphasized this point in 2005 [65]. Indeed, as many other laboratories, we planned to move from an IRMA method (DiaSorin) to a chemiluminescent automate (Liaison, DiaSorin) in order to have the possibility to perform both PTH and 25-OH vitamin D at the same time on the machine.

Even if the antibodies used in the two methods were the same, this change has had an important impact on the results of the CKD patients. We showed that, in 78 hemodialyzed patients, we had a mean difference of 68 pg/ml on the Bland-Altman plot, which is far from negligible (Figure 17).

Figure 17: Comparison of the values obtained in 78 hemodialyzed patients with the DiaSorin IRMA and Liaison with the Bland and Altman plot.



Moreover, all the results obtained with the Liaison were higher than those that we used to have with the IRMA (mean difference of 33.8%). In the study, 22 patients were within the 150-300 pg/ml range with the DiaSorin IRMA. As mentioned above in the Guidelines, this range was supposed to be the target range to avoid secondary hyperparathyroidism or adynamic bone disease in the hemodialyzed patients. In this subgroup, the Bland-Altman plot showed a mean difference of 37%, and the 90% confidence interval range found by the Liaison assay for this population was 204-409 pg/ml, with some of these patients classified as suffering from secondary hyperparathyroidism.

The discrepancies linked to the different assays used were nicely described one year later by Jean-Claude Souberbielle [63]. In this multicentric study, he correlated all the kits available on the market with the famous 150, 300 and 1000 pg/ml cut-offs determined on Allegro. It was striking to see that, with some kits, patients could either be considered as suffering from adynamic bone disease or secondary hyperparathyroidism!

Figure 18: Results obtained with different PTH assays for "Allegro" values of 150, 300 and 1000 ng/L (Souberbielle et al. Kidney Int 2006). The highlighted techniques are the "standard" Allegro intact PTH, the N-tact PTH IRMA and the Liaison from DiaSorin and the kits that present the highest and the lowest values for each pool.

Assay	PTH (ng/l)	PTH (ng/l)	PTH (ng/l)	Median bias (%)
Allegro intact PTH	150	300	1000	0
N-tact PTH IRMA	83	160	517	−44.9 (−68.0; −26.2)
PTH IRMA Immunotech	188	369	1216	23.9 (−6.1; 108.3)
ELISA-PTH	149	290	948	−1.6 (−24.3; 47.2)
Total intact PTH IRMA	134	262	857	−14.5 (−41.5; 23.5)
DSL PTH IRMA	323	638	2108	123.0 (53.1; 188.9)
DSL PTH ELISA	264	523	1734	79.6 (−8.0; 180.9)
Elecsys PTH	161	311	1011	7.3 (−13.8; 80.3)
Immulite 2000 intact PTH	212	410	1334	37.8 (3.8; 130.8)
PTH-ACS 180	185	374	1256	18.8 (−9.9; 69.4)
PTH AdviaCentaur	168	342	1154	9.5 (27.6; 55.6)
Intact PTH advantage	174	339	1109	14.6 (−10.4; 72.2)
LIAISON N-tact PTH	111	223	748	−23.4 (−68.2; −1.9)
Ca-PTH IRMA	84	165	543	−44.8 (−65.6; −22.8)
BioIntact PTH advantage	109	214	704	−27.6 (−53.0; 12.5)

However, when we went back to our population described one year before with the IRMA and the Liaison, we saw that what we considered as patients "under control" (between 150 and 300 pg/ml with the DiaSorin IRMA) were clearly suffering from hyperparathyroidism as the values of our kit were roughly the half of the Allegro kit…(Table 2). Moving to Liaison reduced this gap, as our values raised by 37%, which allowed us to slightly better classify the patients.

Table 2: Classification of 78 patients from the Hemodyalisis Department of our Hospital, according to DiaSorin IRMA (the currently used method I the Laboratory), Liaison (the new method) and the "equivalent Allegro" calculated according to Souberbielle.

	IRMA	Liaison	Equivalent Allegro
ABD	46	32	24
Normal	17	23	22
Hyperparathyroidism	15	23	32

Thus, with the DiaSorin IRMA, any classification of the patients according to the K/DOQI guidelines was impossible. Indeed, the prevalence of the patients suffering from adynamic bone disease was clearly overestimated, and the number of patients suffering from hyperparathyroidism was underestimated. Finally, "normal" patients with the DiaSorin IRMA suffered, in fact, from secondary hyperparathyroidism!

Two years later, DiaSorin launched a second version of the Liaison intact PTH. We validated this new version according to our quality procedures and published the results of this validation [58]. For this validation, we compared, in hemodialyzed patients, the results obtained with the new version and another chemiluminescent method, Roche Elecsys PTH. We had also the data obtained with the former version of the Liaison PTH and the DiaSorin IRMA.

When we go back to these data, some points are striking:

- the regression equation between Elecsys (which is probably calibrated against the Allegro, as we can see from the already cited paper of Jean-Claude Souberbielle) and the DiaSorin IRMA is DiaSorin IRMA=0,5738XElecsys+3,3 (R^2=0,9499, p<0,001). When we used this equation to evaluate what are the "DiaSorin IRMA equivalents" for Elecsys values of 150 and 300 pg/ml, we found data that are closed to those

published by Souberbielle for this method (89 and 175 vs. 83 and 160 pg/ml for Souberbielle).

- Souberbielle confirms in his paper the 37% difference that we observed between the IRMA and the 1st version of the Liaison PTH.
- In 2007, when we published the validation of the 2nd version of the Liaison PTH, our results showed that the DiaSorin IRMA was still underestimating the Elecsys by 50%. However, the 2nd and even the 1st version of the PTH Liaison were much more in accordance with the Roche Elecsys PTH. Indeed, the results obtained with the regression equations were, for Elecsys values of 150 and 300 pg/ml: 162 and 296 for the 1st, and 158 and 288 pg/ml for the 2nd version of the Liaison. This indicates that, during a two-year period, DiaSorin modified the calibration of the kits on Liaison in order to give values that are closer to the Elecsys (and Allegro). This implies, *de facto*, that the results of our patients changed from 2005 to 2007, with possible clinical consequences on the follow-up of the CKD patients.

2. The new KDIGO guidelines

Very recently (August 2009), the KDIGO (Kidney Disease Improving Global Outcomes) stated, in Chapter 4.2 "*Treatment of abnormal PTH levels in CKD–MBD*" that "In patients with CKD stage 5D, we suggest maintaining iPTH levels in the range of approximately two to nine times the upper normal limit for the assay". As we will see later, the problem of defining a "reference range" for PTH is quite difficult and the discrepancies between the different kits and the different laboratories will probably remain.

C. ANALYTICAL INTERFERENCES IN PTH TESTING.

False positive PTH results: an easy strategy to test and detect analytical interferences in routine practice

Cavalier E, Carlisi A, Chapelle JP, Delanaye P..
Clin Chim Acta 2008; 387:150-2

An unusual interference in parathormone assay caused by anti-goat IgG: a case report.

Cavalier E, Delanaye P, Carlisi A, Chapelle JP, Collette J.
Clin Chem Lab Med 2009; 47:118

Human anti-mouse antibodies interferences in Elecsys PTH assay after OKT3 treatment

Cavalier E, Carlisi A, Chapelle JP, Orfanos P, Uzan M, Falque V, Delanaye P, Hachicha M..
Transplantation 2009; 87:451-2

Immuno-assays are largely used throughout the world for the diagnostic or the follow-up of many diseases. Clinicians rely on those tests and are not necessarily aware of interferences, preanalytical [66] and analytical [67-69] problems that can lead to misdiagnosis or expensive complementary explorations [70]. Natural antibodies are the major source of heterophile antibody interference (HAMA) [71]. Many authors have also shown that rheumatoid factors (RF), which are autoantibodies mainly of the IgM class binding preferably to IgG antibodies, can cause interference in immunoassays [72-74]

As other immunoassays, PTH determination is not free from these interferences, which can induce falsely elevated results, leading to misdiagnosis and expensive unnecessary explorations. However, in routine practice, these interferences are not always obvious to detect. We thus established a validation protocol which could allow us to detect these interferences in our routine PTH determinations [75].

We have reported two cases of HAMA interference: one with the Liaison and one with Elecsys. The interference observed with Liaison has been detected in our laboratory by our validation protocol and has had no impact. The sample on which we observed an interference with Elecsys was sent to us for an expertise. Unfortunately, in this case, the interference has led to unnecessary and cost-effective extra investigations.

1. False positive PTH results: an easy strategy to test and detect analytical interferences in routine practice.

We aimed to evaluate the occurrence of analytical interferences on PTH determination with DiaSorin Liaison and to present a protocol for PTH validation, easily applicable in practice and which takes these interferences in consideration.

PTH results have to be interpreted together with serum 25-hydroxyvitamin D (25VTD), ionized calcium (Ca++) results and with the estimation of glomerular filtration (eGFR) rate by the MDRD equation [76]. We considered an elevated PTH (>60 pg/ml, 6.30 pmol/l) result with normal 25VTD (>32 ng/ml, 80 nmol/l), normal Ca++ (comprised between 1.15 and 1.30 mmol/l) and eGFR >60 ml/min/1.73 m² as suspicious. Suspicious samples were treated, according to the manufacturer's instructions, with Heterophile Blocking Tubes (Scantibodies, Shantee, CA) to evaluate a possible interference with HAMA. As controls, 118 HAMA-negative patients were treated. We observed a recovery of 91.7%, a valuable result to consider HAMA treatment as acceptable, according to Ellis and Livesey [77].

Then, RF determination was performed on HAMA negative samples on BNII (Siemens Diagnostics, ex-Dade Behring, Marburg, Germany). A sample was considered as RF-positive when the RF level detected was > 11 UI/ml.

RF-positive samples were then treated with RF-Absorbent® (IBL, Hamburg, Germany), a reagent containing anti-human IgG antibodies to precipitate RF as follows: 400 µl of serum were added with 50 µl of RF-Absorbent, incubated 1 hour at room temperature and analyzed in parallel with the non-treated serum.

Ten RF-negative samples containing a suspicious level of PTH were treated as controls.

A sample presenting a drop in PTH level of 20% (twice the inter-assay of the test) after any treatment was considered as significantly influenced.

We started to apply our validation criteria in May 2007. In the end of June, we had determined PTH for 2084 patients. Amongst them, 743 presented an elevated level of PTH (36%). We found a plausible reason for this result in 91% of the cases (54% of them were renal insufficient, 34% had a suboptimal level of 25VTD and 3% an abnormal calcium level). However, there were 63 patients for which the level of PTH could be considered as suspicious. After treatment of these sera with HBT tubes to search for HAMA interference, we observed a decrease of PTH in 25 of them (mean fall: 29%) and the level of 13 samples returned into normal range (Figure 19).

Amongst the 38 remaining patients with suspicious PTH which did not present HAMA interference, 21 were found to be positive for rheumatoid factor. PTH results obtained after RF-Absorbent treatment on these 21 samples, as well as RF values, are presented in Table 3. We observed a fall of more than 20% in PTH in 9 samples (43%) and the mean decrease in PTH levels observed in these samples was 46%. For 6 of them, PTH even returned into the normal laboratory ranges.

Table 3: Results of PTH obtained in 21 RF-positive samples before and after treatment with RF-Absorbent®. In bold, samples in which a decrease of more than 20% has been observed.

RF (UI/ml)	PTH before treatment (pg/ml)	PTH after treatment (pg/ml)	Difference (%)
20	114	107	-6
21	80	71	-11
21	63	59	-6
35	**63**	**40**	**-37**
35	63	63	0
48	79	75	-5
58	**134**	**104**	**-22**
59	101	90	-11
71	62	60	-3
83	79	67	-15
95	**74**	**56**	**-24**
116	114	96	-16
161	**65**	**50**	**-23**
360	68	56	-18
402	**186**	**60**	**-68**
418	**62**	**44**	**-29**
438	210	204	-3
439	**139**	**14**	**-90**
500	**66**	**35**	**-47**
503	**169**	**62**	**-63**
527	63	62	-2

There was no correlation between RF level and the difference in PTH observed after treatment.

In the control population, the mean PTH decrease was 5% and none of the 10 samples tested presented a fall of more than 20%.

Immuno-assays are very largely used throughout the world for the diagnostic or the follow-up of many diseases. Clinicians rely on those tests and are not necessarily aware of interferences that can lead to misdiagnosis or expensive complementary explorations [69, 70]. Natural antibodies are the major source of heterophile antibody interference [71] and many authors have shown rheumatoid factor interference in immunoassays [72-74].

PTH determination on Liaison, as well as many other immunoassays, is not free from these interferences. In this study, we have shown that the serum of 34 of the 743 patients with an elevated PTH level presented in fact interference due to HAMA or RF (interference rate: 4.5%). Moreover, PTH of 18 of these patients returned into normal range after treatment. Nevertheless, one should keep in mind that even if the result observed after treatment was not back into the normal range, this does not mean that the patient has a pathologic PTH, as the interference hides the "true value". However, as it is not economically viable to test any positive PTH sample with HBT or RF-absorbent, a strategy should be applied to validate PTH.

We have presented a simple three steps protocol, easy to apply in routine with new generation of laboratory information systems, which allowed us to screen for suspect PTH results. Firstly, we took into consideration 3 parameters, essential for a correct PTH clinical interpretation and that should be available at the time of validation: 25 OH-vitamin D, calcium levels and an estimation of the renal function. With these criteria, we found a plausible explanation for an elevation of PTH levels in 91% of our patients.

Then, suspect PTH values were confirmed by HBT treatment to remove the possible HAMA interference. We found that 40% of the 63 suspect samples were positive for HAMA.

Amongst the 38 HAMA-negative samples, we performed a rheumatoid factor determination. We found it to be positive in 55% of the cases. Finally, these 21 RF-positive samples were treated with RF-Absorbent®.

This strategy, tested on Liaison, could basically be applied to any PTH method. However, it should be noticed that RF-Absorbent treatment has to be validated in order to avoid matrix effects. Moreover, we can not presume the percentages of interferences that might be observed with these other methods. Indeed, lack of standardisation and complexity of PTH determination [63] are closely related elements explaining, at least partially, these assays

problems. Once again, one should also keep in mind that the validity of any treatment used to overcome interferences should be checked for each PTH determination kit [77].

In conclusion, by this method, we managed to avoid spuriously elevated PTH results which could have cause medical errors as well as unnecessary cost-effective extra-investigations.

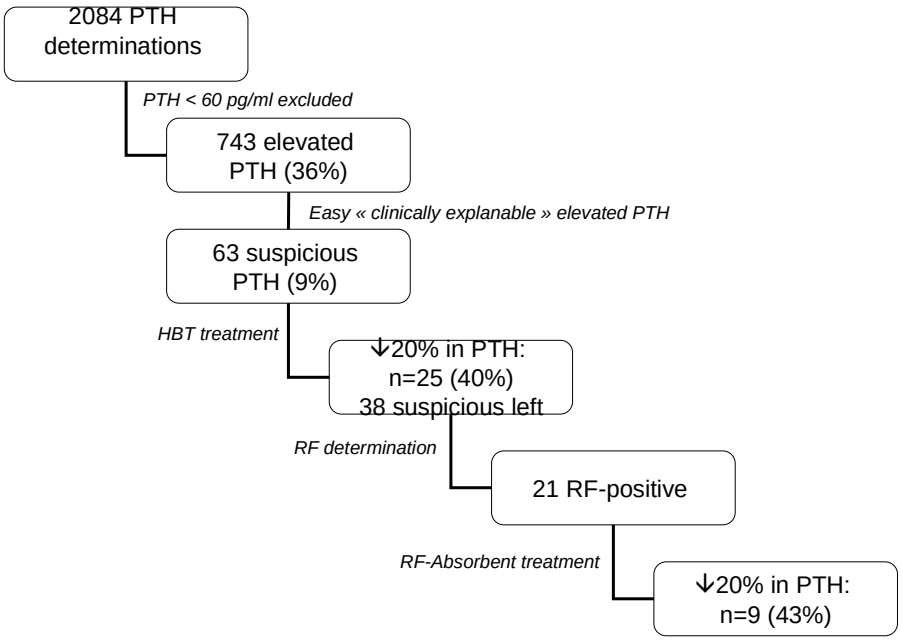


Figure 19: Results of the strategy used in the Laboratory to validate the PTH results

2. An unusual interference in PTH assay caused by anti-goat IgG: a case report.

We reported the case of a 29 yo woman, working as a nurse, who underwent a blood analysis to monitor hypothyroidism. In addition to the "classical" parameters, her physician ordered a parathormone (PTH) determination. The result of the PTH was found to be >2000 pg/ml (normal range: 12-58 pg/ml) with Liaison. As we just discussed [75] ,we considered this result as "spurious" and we treated the sample with HBT (Heterophilic Blocking Tubes, Scantibodies, Santee, CA) and RF-Absorbent (IBL, Hamburg, Germany) to remove a possible interference due to human anti-animal antibodies (HAAA) and rheumatoid factor (RF), respectively. We also determined PTH with Roche Elecsys. We observed that the result remained unchanged after HBT treatment whereas PTH dramatically felt at a value of 40 pg/ml after anti-RF treatment. On Elecsys, the PTH was also normal (24 pg/ml). We thus suspected an interference due to RF, but the patient neither suffered from rheumatoid arthritis (nor any other autoimmune disease), neither was positive when we tested her for RF (BN2, Siemens, Marburg, Germany). To explore the reason for this interference on Liaison we then performed a chromatographic separation with a Superdex 200 HR 10/30 column (Amersham Biosciences, Piscataway, NJ) and we concluded that there was PTH reactivity in the fraction that corresponded to the molecular weight of the IgG's. This confirmed the results of the RF-Absorbent treatment, which, indeed, is a treatment of the sample with anti-IgG antibody.

As the antibodies used for PTH Liaison are polyclonal goat antibodies (whereas Roche Elecsys use monoclonal mouse antibodies), we suspected that there was anti-goat IgG present in the serum of the patient that could interfere with the assay. To confirm this hypothesis, we incubated 400 µl of the patient's serum with 40 µl of goat serum. In this condition, we observed that PTH dropped from >2000 to 41 pg/ml.

Human anti-animal antibodies are a known cause of interferences in immunoassays [78]. This patient had no obvious reason to present anti-goat antibodies (any animal contact, professional exposition, celiac disease, drug, vaccination or blood transfusion). She did not eat goat cheese nor drink milk. A possible cause could be a pregnancy three years before, even if she was primiparous [79]. Even if the manufacturers write in the inserts of the kits that this kind of interference remains possible, one should be careful when interpreting the results of any immunoassay. We must keep in mind the tragic outcomes that can be observed with these interferences [80]. Indeed, these antibodies can give spurious results, leading to unnecessary cost-effective and stressful extra-investigations.

3. Human anti-mouse antibodies interferences in Elecsys PTH assay after OKT3 treatment

We also reported the case of a 61- year old white man with end stage renal disease secondary to an autosomal dominant polycystic kidney disease (ADPKD). Dialysis therapy was commenced in 2003 and he received a renal transplant from a deceased donor in 2004. His circulating PTH concentration before transplantation was 287 pg/ml as measured by the Roche Elecsys immunoassay analyser, in the target of the K/DOQI recommendations [64]. The transplanted kidney functioned until 2007 and then failed due to acute cellular rejection. He received standard anti-rejection medication (i.v. steroids and OKT3) but did not respond, lost function of his renal graft and returned to hemodialysis in February 2007. Four months later, the renal graft was removed because of systemic manifestations of rejection. From March to December 2007, plasma PTH concentrations were measured every 3 months again by Roche Elecsys. The results showed a rise of PTH after 7 months on hemodialysis, and this was even more dramatic after 10 months. No parathyroid mass was identified either by ^{99}Tc-Sestamibi or ultrasound scans. An interference in the PTH assay was thus suspected and the sample was sent to our Laboratory where extra-investigations were performed, as described in the previous chapter. The treatment of the sample with HBT (Heterophilic Blocking Tubes, Scantibodies, Shantee, CA), which removes human anti-animal antibodies (HAAA), resulted in an important decrease of PTH, from 3748 to 552 pg/ml. Treatment with anti-rheumatoid factor (IBL, Hamburg, Germany) did not result in a significant change in PTH. PTH was then measured by a different second-generation chemiluminescent immunoassay (Liaison) which uses polyclonal anti-goat antibodies and showed a result at 605 pg/ml, not altered by inclusion of HAAA or RF. By contrast the suspect Roche Elecys intact PTH assay uses two murine monoclonal antibodies. Thus these two assays use antibodies derived from different animal species which bind to different epitopes on the PTH molecule. This led us to conclude that

there was an analytical interference in the PTH determination by Elecsys, and that this interference was due to a human anti-mouse IgM.

Treatment with OKT3, a murine monoclonal antibody directed against the CD23 of human T-cell antibodies, is well known to induce the production of human anti-mouse antibodies [81]. These antibodies can reduce the efficiency of the drug by blocking the interaction with the target cells, but can also interfere with the immunoassays that use murine antibodies [82]. Such interferences are not always obvious to detect, particularly in the case of hemodialyzed or transplanted patients, where high circulating concentrations of PTH can be expected.

D. ESTABLISHMENT OF A "REFERENCE RANGE FOR PTH": IMPACT ON THE DIAGNOSIS OF PRIMARY AND SECONDARY HYPERPARATHYROIDISM.

Analytical validation of the new version of the Liaison N-Tact PTH assay.

Cavalier E, Delanaye P, Carlisi A, Krzesinski JM, Chapelle JP

Clin Chem Lab Med 2007; 45:105-7

Defining a "Reference Population": No Easy Task

Cavalier E, Delanaye P

Journal of Bone and Mineral Research, 2009 Sep;24(9):1638

The diagnostic of primary and secondary hyperparathyroidism is essentially biological. Thus, determination of a strong reference range for PTH is of importance. This has recently been emphasized by Eastell *et al* when they published the new guidelines for the management of the asymptomatic primary hyperparathyroidism [83]. Indeed, they insisted on the necessity to determine optimal reference ranges for PTH – which are lacking for the moment. On the other hand, ISO 15189 guidelines ask laboratories to check periodically the reference range proposed by the manufacturers. However, these guidelines do not explain ***how*** to check for these reference ranges. Generally, the best way to establish them is to select a determined number of samples from a healthy population and to assay the parameter concerned. Then, if the distribution is "normal" (this can be evaluated by statistical means, *ie* with the Kolmogorov-Smirnov test) the mean +/- 2 standard deviations represent 95% of the population. So if we take these values for the upper and lower limits, it means that 2.5% of healthy people will be classified as having "too high" and "too low" values for the parameter studied. For some parameters, where a "cut-off" is needed, the 5% can be one-sided, right or left.

If the population is not statistically "normal", there are different means, as logarithmic transformation or the use of non-parametric tests, to define the reference ranges.

Nevertheless, one question remains: how do we define a "normal population"?

Generally, blood donors were used to be considered as a "normal healthy population" and a lot of reference values have been established in this population. However, on one hand, most of laboratories do not have access to these patients and on the other hand, it may be subjective to think that these people are always "healthy" for every parameter tested: these patients are generally young, declare that they consider their selves as "healthy" and have a "conventional" sexual behaviour. This does not mean that they suffer from a deficiency that

can not modify some of their parameters, nor that their diet or alcohol consumption is "normal"….

From our point of view, we think that the best way to define a reference population is to select the samples on which the parameter will be assayed with the help of pre-determined biological (or clinical) criteria [48]. So, if we apply this concept to the determination of the reference ranges for PTH, we should test samples that do not present obvious biological signs of primary or secondary hyperparathyroidism. These samples should also be free from any obvious interference, like rheumatoid factor.

We thus generated fasting reference intervals for the Liaison PTH and Roche Elecsys PTH assays using 60 patients (11 male, 49 female) aged 23–85 years [58].

Our criteria for inclusion were:

- 25-OH vitamin D levels >50 nmol/L,
- glomerular filtration rate evaluated by the MDRD formula >60 mL/min/1.73 m²
- negative rheumatoid factor (RF)
- total calcium level within the laboratory reference range (2.20-2.60 mmol/L).
- total phosphorus level within the laboratory reference range (0.74-1.51 mmol/L).

The reference interval was determined to be 12–54 pg/mL for the DiaSorin Liaison and 14–52 pg/mL for the Roche Elecsys assays. We found no age- or gender related differences.

These upper values of our established reference interval were much lower than the ones proposed by the Manufacturers (-19 pg/mL for DiaSorin, -13 pg/mL for Roche). Indeed, these societies did not select the patients as we did, including patients that could have presented secondary or primary hyperparathyroidism in their reference population.

The impact of lowering the reference range was clinically important: indeed, in 2008, we have performed 15243 PTH determinations with the DiaSorin Liaison. Among them, 2732 (17.9%)

patients were comprised between 54 (our calculated upper value) and 73 pg/mL (the upper value proposed by DiaSorin). We found that the majority of these "elevated" PTH were due to vitamin D insufficiency that had led to secondary hyperparathyroidism.

Once again, as the diagnostic of primary and secondary hyperparathyroidism is mainly biological, the patients with a mildly elevated PTH will be missed by using the manufacturers' reference range.

Therapeutic actions (ie, supplementation with native vitamin D) will thus be delayed.

On the other hand, as we already mentioned, the KDIGO recently published new guidelines, stating that "*in patients with CKD stage 5D, we suggest maintaining iPTH levels in the range of approximately two to nine times the upper normal limit of the assay*".

We already explained the problems encountered when Nephrologists wanted to follow the K/DOQI guidelines and transposed the "150-300 pg/mL" to the other techniques. The good point with the KDIGO guidelines is that they do not deal anymore with absolute values, but rather refer to the "normal range" of the assay used in the laboratory. Unfortunately, we are facing another problem: indeed, most of the labs use, as reference range, the data provided by the manufacturers in the inserts of the kits whereas all manufacturers implicitly request in these folders that the laboratories have to establish their own reference range…

If we multiply by 2 and 9 the upper reference range published by DiaSorin for the Liaison (73 pg/mL), we obtain a range of ***143 to 657*** pg/mL; for the Elecsys (65 pg/mL), it will be ***130 – 585*** pg/mL. However, if we use the PTH reference range established with our criterion (cf supra), the patients should be maintained between ***108 and 486*** pg/mL if they are followed with Liaison and ***104 – 468*** pg/mL if they are followed with Elecsys. These discrepancies are far from negligible.

III-2: CLINICAL ASPECTS

A. NON-(1-84) PTH, PTH RESISTANCE AND CKD MINERAL BONE DISORDERS.

Evaluation of different bone markers in hemodialyzed patients.

Cavalier E, Delanaye P, Collette J, Krzesinski JM, Chapelle JP.

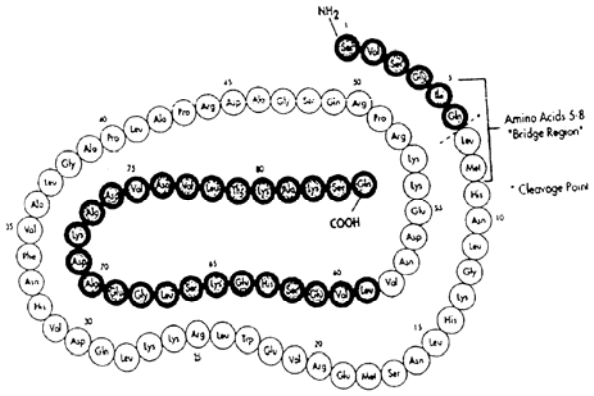
Clin Chim Acta. 2006 Sep;371(1-2):107-11

1. The (non-1-84) PTH.

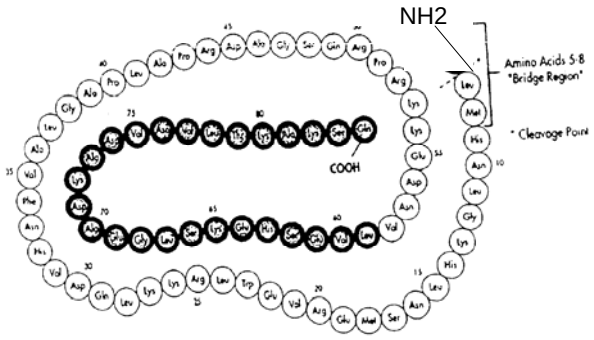
As we already mentioned, PTH circulates in the blood in its "complete" (1-84) form, next to different amino- or carboxyl-truncated fragments. These fragments can come from the hepatic degradation of the peptide or can be secreted by the parathyroid gland itself. We already saw that these fragments, and particularly the big C-terminal fragments, interfere with the PTH assays. This was true for the 1st generation PTH RIA kits, but the 2nd generation kits presented also an interference with the "non-(1-84) PTH".

This "non-(1-84) PTH" is composed of a family of fragments, the longest and the shortest fragment starting at position 4 and at position 15, respectively. The major component of the non-(1-84) PTH is a peptide starting at position 7. This is the reason why the non-(1-84)PTH is generally called PTH (7-84) [84].

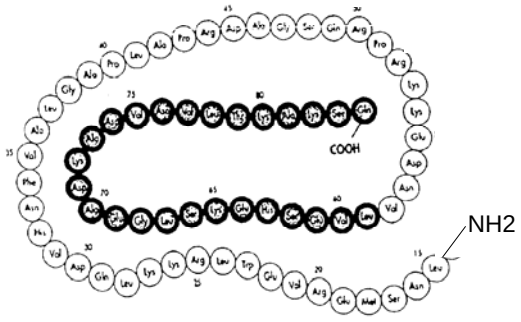
(1-84) PTH



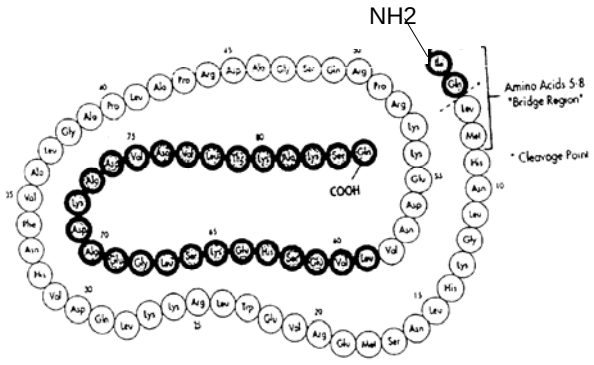
(7-84) PTH: the most important fragment of the non-(1-84) PTH



(15-84) PTH: the shortest fragment of the non-(1-84) PTH



(4-84) PTH: the longuest fragment of the non-(1-84) PTH



Non-(1-84) PTH represents about 20% of intact PTH in normal individuals, but up to 50% in patients with terminal CRF. The fragments that compose the non-(1-84) PTH are cleared by the kidneys and accumulate in CKD patients, proportionally to the importance of their renal disease. They are also secreted by the parathyroid glands and generated when PTH is metabolized. In fact, two forms of secretory granules have been identified in the parathyroid cells [85]. The first one contains, of course, the (1-84) PTH in its complete form, but the second type also contains different cleavage enzymes (cathepsins B and H) [86] and (1-84) PTH. Low extracellular calcium promotes the release of PTH (1-84) in the circulation. However, when the CaSR is activated by circulating elevated Ca^{++} levels, it causes the release of intracellular Ca in the parathyroid cells, which in turn inhibits the secretion of PTH (1-84) in the circulation. This inhibition is accompanied by an enhanced proteolysis of the NH2-terminal part of the PTH peptide by the cathepsins. The result of this proteolysis thus becomes the non-(1-84) PTH.

2. Activity of the non-(1-84) PTH: the PTH resistance

Traditionally, the non-(1-84) fragments have been supposed to be inactive, as they lacked the amino-terminal form of the (1-84) PTH.

However the dynamic balance between the secretion of these fragments and of (1-84) PTH, controlled by free calcium, raises some questions [87]. Moreover, as the peripheral metabolism of PTH is unaffected by changes in calcium [88], one could think that the secreted ratio between the different PTH moieties is of clinical importance.

As already said in the previous chapter, PTH1R signaling requires the first four amino-acids of the peptide. However, it has been shown that the non-(1-84) PTH down-regulated the biological activity of 1-84 PTH and caused internalization of the PTH1R receptors, without activating them [89]. This is of importance in uremic patients, where high levels of these fragments can be found, and could probably explain part of the tissue "resistance" to PTH that characterizes chronic renal failure (this "resistance" can be defined as the difference in biological response of target organs to a same excessive concentration of PTH).

Indeed, if PTH resistance is not completely understood, two theories have emerged to explain it. The first one, developed by Slatopolsky [90], is based on the fact that PTH (7-84) antagonizes the calcemic actions when given to a molar ratio 1:1 with PTH (1-84) in parathyroidectomized rats. These authors think that PTH (7-84) could thus act as a competitive inhibitor of PTH (1-84) for PTH1R.

There are some limitations to this theory: indeed, as pointed by Friedman [85], downregulation of PTH1R is generally observed in CRF [91-93], independently of any resistance. Secondly, it was proven in rats, not in humans - and these rats had not renal insufficiency.

At that point of the discussion, we should introduce the Na+/K+/Exchanger Regulatory Factor 1 (NHERF1) and its role on PTH1R regulation. NHERF1 is a cytoplasmic protein that regulates trafficking of several G protein-coupled receptors – including PTH1R. NHERF1 promotes the membrane retention of PTH1R, stabilizes it at the cell membrane and increases the fraction of receptor at the cell surface [94]. NHERF1 thus inhibits endocytosis without affecting PTH1R recycling and such an effect protects against PTH resistance or PTH1R downregulation.

In kidneys, PTH1R is expressed in proximal tubules, cortical ascending limbs and distal convoluted tubules [95]. Distal tubular cells lack sufficient amounts of NHERF1 and thus, in these cells, the PTH1R receptors are more prone to be downregulated by PTH (7-84) [85]. This downregulation will be associated in these cells with a reduction in PTH mediated calcium transport.

In the other cells that contain both PTH1R and sufficient amounts of NHERF1 (proximal tubules and cortical ascending limbs cells), there is no internalization of the PTH1R after exposure to PTH (7-84). As these cells contribute to the PTH-regulated phosphorus transport, the inhibitory effect of PTH on phosphorus transport is not affected.

The consequences of the effects of PTH (7-84) would thus be a resistance to the hypercalcemic effect of PTH, but a preservation of its phosphaturic effect.

The second theory is based on the studies published by Divieti and Bringhurst [96-98] which introduce evidence for a new PTH receptor, which who would interact with the carboxyl-terminal part of the peptide (C-PTHR). Indeed, PTH (7-34) analogues do not lower calcium in hypercalcemic mice [99], suggesting that the end of the peptide is important for the inhibitory

action. On the other hand, amino-truncated fragments stimulate bone alkaline phosphatase (bAP) activity and induce the expression of mRNAs for bAP and calcitonin [100, 101].

This hypothesis is elegant, but the C-PTHR receptor has still to be found. We have an indirect proof of its existence as we know, from experiments, that PTH (19-84) can bind osteoblastic and osteolytic cell lines from mice KO for the PTH1R [96]. Moreover, the hypothetic role played by this receptor in the PTH resistance has still to be clarified.

Further work is thus needed to completely understand the PTH resistance in uremia.

3. CKD Mineral Bone Disorders

CKD Mineral Bone Disorders represents the various forms of skeletal disease of the CKD patients, and may appear as a high bone turnover due to hyperparathyroidism, a mixed form (hyperparathyroidism with defect of mineralization) or a low bone turnover (adynamic bone). In all cases, the patients suffering from this disease are at high risk of fractures, vascular and extra-vascular calcifications.

The gold standard for the diagnosis of the CKD Mineral Bone Disorders is the histomorphometric and the histochemical examination of a bone biopsy specimen obtained after double labelling with tetracycline [102]. However, this invasive procedure is only performed in specialized centres and is not easily accepted by the patients. Bone mineral densitometry [103] has been evaluated in the diagnosis and/or monitoring of renal bone disease, but data are clearly insufficient. Routinely, nephrologists rely on measurements of different serum biochemical markers to diagnose which type of renal bone disease is present, initiate appropriate treatment and make the follow-up of the patients. The most frequently used is PTH (in conjunction with calcium and phosphorus), but PTH measurement has clear limitations, especially in CKD patients.

In 2001, however, the scientific community paid a particular attention to a paper published by Monier-Faugere et al in Kidney International [104]. Indeed, these authors reported that all their patients with low bone turnover (LBT) presented a $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio <1.

The $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio was thus presented by these authors as a new tool for the diagnosis of LBT. This study had been performed in 51 hemodialyzed patients vs. bone biopsy. The patients enrolled had not received active vitamin D or any other drug that could

have had an effect on bone metabolism for 6 months. The mean vintage time on dialysis of these young (47±3 y.) patients was 25.6±3 months.

In the literature, an important scientific debate immediately followed this publication.

Indeed, one year later, Coen et al [105] did not find similar data. For these authors, there was no correlation between the ratio and bone alkaline phosphatase (bAP), bone formation rate or osteoblast surface (which are histomorphic and histodynamic measurements). Coen at al based their results on a study involving 35 patients against bone biopsy. Among these patients, 9 (26%) suffered from LBT and the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio observed in these patients was not different from that in the other histological groups. One should notice that Coen's population was a little older (57±12 y.) and had spent longer times on hemodialysis (61±66 months) than the one of Monier-Faugère. The majority of Coen's patients had discontinued calcitriol therapy between 1 and 4 months before biopsy (*vs.* over >6 months for Monier-Faugère). However, the mean 25-OH vitamin D levels of these patients was quite elevated, at 25±13 ng/mL (data not presented by Monier-Faugère).

One year later, in a study involving 33 paediatric patients (13±4 yo) treated with continuous cycling peritoneal dialysis for 13±9 months and who underwent bone biopsy, Salusky et al [106] showed that PTH concentrations (2nd or 3rd generation) were a better predictor factor than the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio. In these patients, calcitriol therapy had been held for 4 weeks before the bone biopsy. The 25-OH vitamin D data were not provided. Ten over 33 (30%) of the patients were considered to have normal/reduced bone turnover (the sample size of patients with adynamic bone was very small). The bAP values of the patients suffering from secondary hyperparathyroidism was significantly higher than those of the patients in the

normal/low bone turnover group (469 vs. 264 IU/L, p<0.02), but the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio did not differ between the two groups, in contrast to the study of Monier-Faugère.

The same year, Reichel et al, in a study involving 141 patients, but no bone biopsy [57] indirectly showed that the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio could not be a predictor of LBT, because in most of the studies, the proportion of patients that suffer from this disease is around 30% and in his population, only 4% of the patients presented a $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio <1. The patients enrolled in Reichel's study were monthly treated with 20 000 IU vitamin D3 (mean serum 25-OH vitamin D levels: 50±27 ng/mL) and most of them were under calcitriol or alfacalcidol therapy.

When we look at these different and conflicting results, we must admit that it is not obvious to make one's opinion on the interest of using the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio in routine practice.

However, an important point is the methodology used in these studies – and particularly the time elapsed between the biopsy and the last active vitamin D (or calcimimetics) injection. Indeed, we are dealing here with some patients presenting a low or a high bone turnover on the bone biopsy. In patients having stopped recently (say one month) an active vitamin D treatment, bone biopsy may reflect a low turnover (as it can take many months to achieve a remodelling cycle in this case) whereas PTH may be high reflecting the current status of the calcium balance. In those presenting a high bone turnover, the PTH or bone turnover markers could rapidly decrease after the injection of calcitriol whereas the biopsies will still present signs of high bone turnover. When we analyse the different studies, we must admit that it was Monier-Faugère's protocol that could better explain the correlation of the markers and the results of the biopsy, since the patients enrolled had not received active vitamin D or any other drug that could have had an effect on bone metabolism since 6 months.

In 2005, we performed a transversal study in 73 hemodialyzed patients. The aim of this study was to determine PTH with the 2nd and 3rd PTH generation kits and to estimate the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio in our patients.

The results of the laboratory tests observed in our population are presented in Table 4.

Table 4: Results of the Laboratory tests performed in 73 hemodialyzed patients.

Analyte	Normal range	Mean ± SD	Median	Range
Intact PTH Scantibodies (pg/ml)	16-66	244±247	185	5-1237
bAP (ng/ml)	3-23	21.5±42.5	11.2	5.2-355
CTXS (pg/ml)	<35-680	1913±1322	1624	131-6000
P1NP (ng/ml)	20-76	596±1340	287	45-11380
TRAP5b (U/l)	1.4-4.7	4.1±1.9	3.6	1.2-9.5
Whole PTH (pg/ml)	5-39	148±164	89	4-820
Non (1-84) PTH (pg/ml)		96±88	83	1-444
$\frac{(1-84)PTH}{non-(1-84)PTH}$ (%)		1.5±0.7	1.5	0.6-4.0
PTH Liaison version 1 (pg/ml)	7-82	262±231	217	5-1287
Ca^{++} (mmol/l)	1.15-1.30	1.10±0.09	1.09	0.91-1.36
Serum calcium (mmol/l)	2.15-2.60	2.35±0.14	2.36	2.02-2.74
Serum phosphate (mg/l)	23-47	52±13	50	23-87
Serum creatinine (mg/l)	3.8-12.1	89±30	88	25-145
25(OH)D (ng/ml)	> 32	17 ± 8	16	7 – 54
$1,25(OH)_2$ D (pg/ml)	19-79	5.6 ± 6.2	2.95	<2.1 - 42.2

Our results, published in 2006 [107], showed that there was a strong correlation between bone markers concentrations, particularly between bAP and P1NP (r=0.953).

The presumed non-(1–84) PTH represented 41.7% of the "intact PTH" in our patients group. Intact, whole and non-(1-84) PTH moieties correlated very well together, as well as with the other bone markers.

The $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio correlated positively with whole PTH and TRAP 5b and negatively with total calcium. No correlation was found between this ratio and the usual bone markers.

There was no correlation between 25VTD and the other analytes.

We then separated our population into three groups according to the intact PTH levels observed with our routine method (group 1: <100pg/ml; group 2: ≥100 to <300pg/ml and group 3: ≥300pg/ml).

Bone formation markers were significantly ($p<0.01$) higher in group 3 than in the other groups.

Bone resorption markers were significantly ($p<0.05$) in group 1 than in group 2, and also lower ($p<0.01$) in group 2 than in group 3.

The $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio was significantly ($p<0.05$) lower in group 2 than in the two other groups, demonstrating similar levels.

Table 5: Comparison of biomarkers (mean and 95% confidence interval) in subgroups of patients separated according to their Liaison® iPTH levels (pg/ml)

Biomarker	Group 1: iPTH <100 pg/ml (n=21)	Group 2: 100 ≤ iPTH < 300 pg/ml (n=27)	Group 3: PTH≥300 pg/ml (n=25)
bAP (ng/ml)	10 (7-12)	11 (9-14)	14 (10-25) a
CTXS (pg/ml)	1114 (676-1457) b	1709 (933-2150) c	2338 (1587-2752)
P1NP (ng/ml)	247 (175-417)	280 (164-568)	419 (233-805)
TRAP5b (U/l)	3.2 (2.7-4.3)	3.5 (2.3-4.1)	4.3 (3.5-5.4) a
$\frac{(1-84)PTH}{non-(1-84)PTH}$ (%)	1.63 (1.04-1.99)	1.13 (1.31-2.2) d	1.73 (1.31-2.2)
25VTD (ng/l)	16 (11-22)	16 (13-19)	16 (12-19)
Homocystein (µmol/l)	27 (20-49)	37 (25-43)	30 (25-36)

a Analyte from group 3 significantly higher ($p<0.01$) than in group 1 or group 2.
b Analyte significantly lower ($p<0.05$) in group 1 than in group 2.
c Analyte significantly lower ($p<0.01$) in group 2 than in group 3.
d Result significantly lower ($p<0.05$) in group 2 than in the two others groups

In this non-native vitamin D supplemented population, 95% of the patients had a suboptimal level of 25VTD (<32 ng/ml). Twenty-four patients (36%) had even severe vitamin D insufficiency (arbitrarily defined as 25(OH)-vitamin D levels ≤13ng/ml). For all the studied

biological markers, there was no difference between this group and the group of the patients with vitamin D levels >14ng/ml. The only exception was for homocystein, which showed significantly higher concentrations in the very low 25VTD group (41 vs. 28µmol/l, $p<0.05$).

In our patients, the mean $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio was 1.5±0.7%. Twenty percent of them presented a $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio <1.

We did not find any differences in the biomarkers studied between the low ratio group and the other patients, excepted that they were significantly younger (58 years vs. 74, $p<0.02$). They also presented higher homocystein levels (48 vs.27µmol/l, $p<0.02$), not influenced by folate intake or folate level. This might be interesting as homocystein is well known to be associated with hip fractures and osteoporosis in healthy elderly people [108-110].

However, when we separated the population in three groups of similar size according to their intact PTH levels, we found that the patients in the lowest intact PTH group presented significantly lower levels of bone markers. It was in the group of patients considered as "in normal ranges" by the K/DOQI guidelines that we found a significantly lower $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio.

The major limit in our study was the lack of bone biopsy, only performed in very specialized centres. Nevertheless, in our non vitamin D3 supplemented population, our data showed a weak, but positive correlation between homocystein and 25VTD in the subgroup of patients with severe vitamin D deficiency. This correlation could be accidental, as this population is more at high cardiovascular risk, but it needs to be tested on larger populations.

4. bAP: the new marker of choice?

The debate is still ongoing to see if the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio is interesting in clinical practice or not. Our results are not really in favour of this ratio. However, we paid much more attention to the Bone alkaline Phosphatase for the follow-up of the hemodialyzed patients.

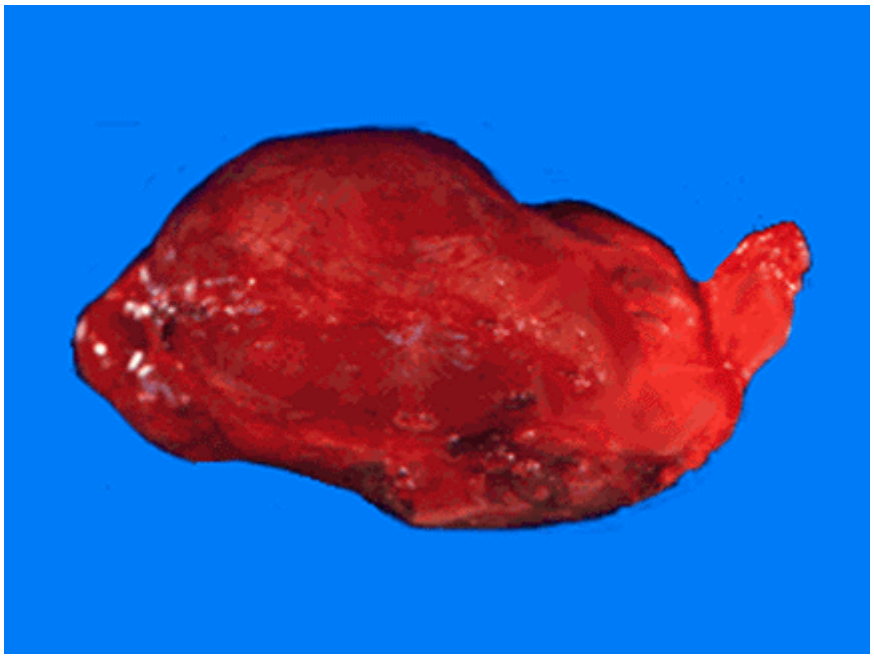
Indeed, Bervoets et al. [111] found that low levels of bAP (≤10µg/l) was a good index for the presence of adynamic bone disease (ABD) in end stage renal failure patients not yet on dialysis. None of these patients were administered vitamin D. They compared the biochemical results with bone biopsy and found that bAP was a useful diagnostic marker to differentiate between ABD/normal bone. More recently, Ueda et al. [112] showed that bAP was a clinically useful bone formation marker for predicting reduction of bone mineral density in hemodialyzed patients and in diabetic hemodialysis patients with low PTH.

In our population, 62% of the patients presented bAP levels >10µg/l, and the patients belonging to this group were younger, had higher levels of P1NP and CTXS (2.0 X higher), TRAP (1.5 X), but the intact PTH concentration, the $\frac{(1-84)PTH}{non-(1-84)PTH}$ ratio and homocystein levels did not differ between the two groups.

The strict correlation between bAP and P1NP is interesting and means that both markers observe a closely related phenomenon. Ueda et al. [113] have already shown the usefulness of P1NP as a marker of osteoblast function in hemodialyzed patients and its usefulness for predicting radius bone loss. We think for our part that P1NP, a specific indicator of type 1 collagen deposition and thus a true bone formation marker might be a new marker of interest.

To summarize, for us, bAP among different markers seems to offer the best clinical and analytical profile for the diagnosis of bone disease. Indeed, this bone mineralization marker is eliminated by the liver – and thus not influenced by the renal insufficiency -, is much more stable than PTH and presents very interesting analytical properties. Unfortunately, we lack guidelines for the follow-up of the patients with bAP. These guidelines could be established, but we need to compare bAP results vs. bone biopsy in a large and well clinically documented population.

B. THE AMINO-PTH AND THE PARATHYROID CARCINOMA



The ratio of PTH as measured by third and second generation assays as a marker for parathyroid carcinoma

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Parathyroid carcinoma (PCa) is a rare disease, with a prevalence of 0.005% of all cancers [114]. Fewer than 400 cases of parathyroid carcinoma were reported in the literature before 1993 [115] and in the US National Cancer Database, only 286 cases have been described over 10 years [116]. PCa accounts for less than 1% of sporadic primary hyperparathyroidisim (PHP) and is associated with more severe clinical features than parathyroid adenomas [117]. The consequences of the severe hypercalcemia linked to unchecked and recurrent PTH hypersecretion are the main cause of morbidity and death in patients with PCa.

Differentiating a rare PCa from a parathyroid adenoma is often challenging, particularly as the histopathology of parathyroid tumors can be equivocal [118]. Indeed, the diagnosis of PCa is often made definitively only when recurrence or metastasis occurs. Surgery remains the only curative treatment for PCa. As better outcomes are associated with complete resection of the tumor at the time of initial surgery [119], it is important to make a correct diagnosis at the time of first occurrence. Hence, a biological marker that could help to reliably distinguish PCa from parathyroid adenoma would be useful.

As already mentioned, Parathyroid hormone (PTH) circulates in the blood in different forms that include full length PTH 1-84, amino truncated PTH fragments of which PTH 7-84 is the most abundant, and an N-terminal form of PTH, "amino-PTH", which may be phosphorylated on the serine at position 17 [30]. Under physiological conditions, PTH 7-84 represents 15-30 % and amino-PTH <10% of total circulating PTH [30]. Since 1987 clinical laboratories have routinely performed determinations of "intact" PTH using various methods. Second-generation PTH assays use a capture antibody directed against the (39–84) portion of the PTH molecule and a second antibody that recognizes the (13–24) portion of the peptide. The term "intact" is misleading as these PTH assays recognize not only PTH 1-84, but also cross-react to varying degrees (50-100%) with a PTH molecule that co-elutes with a synthetic 7–84 PTH fragment in HPLC [25]. As PTH 7-84 accumulates in patients suffering from chronic renal

failure (CRF) this explains in part the overestimation of the secondary hyperparathyroidism in CRF cases [23, 24]. A new generation ("*third generation*") of PTH assays appeared in 1999. The initial third-generation PTH assay used an anti-COOH-terminal antibody like the second-generation assays, but employed an anti-N-terminal antibody directed against the first four amino-acids of PTH [26]. These newer assays, therefore, do not measure PTH 7-84 fragment, Amino-PTH cross-reacts with third generation PTH kit antibodies but not with antibodies used in most second generation assays. The Roche Elecsys assay is an exception and uses an antibody that also recognizes amino-PTH [20].

In healthy individuals, therefore, if the amount of PTH obtained with a third generation assay is divided by the amount of PTH obtained with a second generation assay, this $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio (or $\frac{(1-84)-PTH+A\min o-PTH}{(1-84)-PTH+(7-84)-PTH}$ ratio) will not be greater than 1. Indeed, PTH 7-84 physiologically represents a larger proportion of the circulating PTH than does amino-PTH. Recently, it has been shown that the amino-PTH is overproduced in PCa [27, 28] and in some rare cases of severe primary hyperparathyroidism [29]. In such patients the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio may be inverted (> 1) as compared with normal individuals.

To date, limited patient series support the hypothesis that the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio may have clinical utility as a marker to discriminate PCa from most cases of benign PHP [27].

The aim of this study was to undertake an assessment of the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio in a large population of 23 patients suffering from advanced PCa that were referred for assessment for an experimental cancer immunotherapy treatment [120].

Methods

Patients

Twenty three patients from Europe, the United States and Australia were suffering from advanced PCa. The clinical characteristics of these patients are described in Table 6. All had previously undergone surgery and had been admitted to local hospitals for later administration of an investigational treatment to induce an immunologic (anti-PTH antibody) response to their tumors after repeated injections of modified PTH fragments [120, 121]. All patients underwent assessments of PTH levels before immunological treatment using second and third generation assays to derive the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio.

As a first comparator group we evaluated the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio in a population of 73 chronic renal failure patients undergoing hemodialysis at the Dialysis Unit of the CHU de Liège [107]. The characteristics of these patients are summarized in Table 7. We also evaluated the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio in a population of 60 renal transplanted patients (Table 8) and finally in 82 consecutive healthy elderly patients (mean age 62.0±8.0 years; 53% female), who voluntary underwent blood sampling to screen for renal insufficiency.

PTH assays

PTH was determined with the Duo PTH immuno-radiometric kit from Scantibodies Laboratory (Santee, CA). This kit includes two different assays: 1) a second generation PTH assay that recognizes the PTH 1-84 and the non-PTH 1-84 components, but not the amino-PTH; and 2) a third generation assay that exclusively recognizes (1-84)-PTH and the amino-PTH. In our hands, the coefficients of variation obtained with these two assays are <10%.

Statistics.

Statistical analysis was carried out by the Medcalc software (Mariakerke, Belgium). The differences between groups were calculated by the Student's t-test, a p-value <0.05 indicating a significant difference.

Results

The results of the second generation and third generation PTH assays and the $\frac{3rdgeneration}{2ndgeneration}$ ratios are summarized in Table 9. The mean $\frac{3rdgeneration}{2ndgeneration}$ ratio was 0.58±0.10 in the 73 dialysis patients, 0.54±0.10 in the renal transplant group and 0.54±0.12 in the elderly healthy patients. All 207 of these patients presented a PTH $\frac{3rdgeneration}{2ndgeneration}$ ratio of <1.

In contrast to the control populations, we observed an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio (>1) in 20 PCa patients, whereas only 3 patients had a "normal" ratio <1. The mean $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio was statistically significantly higher in the 20 patients presenting an inverted ratio (mean: 1.50) as compared to those with a ratio <1 (mean: 0.75; p<0.0001).

Discussion

PTH circulates as a mixture of PTH 1-84 itself and different amino-truncated fragments. These fragments are not only products of degradation of PTH 1-84 but are also secreted by the parathyroid gland itself.

With the advent of the third generation PTH assays, a new circulating form of PTH, amino-PTH, was discovered. It is still unknown whether this amino-PTH is biologically active or not, but has previously been shown to be overproduced in PCa [27, 28] and rarely in cases of severe primary hyperparathyroidism [29]. This leads to an inversion of the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio, which is normally <1 in healthy and non PCa populations. In the current study we demonstrate that an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio was seen in nearly 87% of PCa patients, as compared to 0% of a series of three relevant control populations.

The current study of the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio as a marker for PCa is the largest to address this important question and is itself one of the largest series of PCa patients reported to date. Previous work has suggested the potential utility of this PTH ratio in differentiating PCa from the more common benign parathyroid adenoma. Caron et al observed an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio (>1) in a patient with PCa [27], but in only 1/30 osteoporotic PHP patients with PHP and in 0/294 osteoporotic patients without PHP. In the PHP patient, the inverted ratio remained after surgery (1.54) and the patient exhibited marginal hypercalcemia over the course of 3 years follow-up. Blachowicz reported similar results with 0/32 patients with PHP exhibiting an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio [122]. In one of the largest study to date, Rubin et al reported that of eight patients with PCa studied within a larger cinacalcet study, four (50%) presented a high ratio [28]. This study also showed that when cinacalcet

lowered PTH, the inverted ratio remained unchanged. This is not the case when a cure is achieved by resection of the tumor, when the ratio is reported to return to normal [29].

In our study we found the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio to be systematically <1 among a group that included 73 hemodialysis patients, 60 renal transplant patients and 82 healthy elderly patients. Assessing the various published data as a whole, the prevalence of an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio is very low in PHP patients (1.6%; 1/61) and non-PHP controls (0%; 0/530). In stark contrast, the occurrence of an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio in the PCa population is very high at 78.1% or 25/32 PCa patients reported in our study and by others. We observed three PCa patients with no PTH ratio inversion, whereas Rubin and colleagues reported four individuals. Hence, an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio has a sensitivity of 75.8% and a specificity of 98.4% amongst PHP patients as a tumor marker for PCa.

Our results have been observed in patients suffering from advanced PCa. It would be clinically useful to evaluate the PTH ratio in a population of PCa patients presenting with a less advanced stage of malignancy. A useful clinical application would be the ability to identify patients with PCa at an early stage, when the difficulty in distinguishing these challenging cases from benign parathyroid adenoma patients is most pronounced. Also, it is clear that a prospective longitudinal study is necessary to capture the PTH ratio in a large group of PHP patients before they undergo surgery, and to follow up the entire cohort to determine whether those with a persistently inverted PTH ratio or those that later develop an inverted ratio are at a greater risk of evolving into cases of PCa.

The diagnosis of PCa remains very difficult. Our results, based on one of the largest reported cohorts of PCa patients, shows that an inverted $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio may indeed have clinical utility as a future tumor marker for PCa, as suggested in smaller series. Future investigations will be needed to assess if the dual determination of PTH with 2nd and 3rd generation assays could be proposed in treated patients suspected of having PCa or as a pre-operative screening test to detect patients in whom an elevated suspicion for PCa exists. Alternatively, the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio could also be used as a new tool in the follow-up of the operated patients. As the ratio returns to normal when the patient is cured, a non-inverted ratio after surgery, or a ratio that returns to >1 during follow-up could be a sign of disease persistence or relapse of PCa. Finally, in patients undergoing experimental therapies (e.g. immunotherapy), changes in the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio could be evaluated as an efficacy measure.

Patient	Sex	Age	Age at diagnosis	Calcium (mmol/L)	PTH (ng/L)	Creatinine (mg/L)	Metastases (Y/N)	Location of Metastases
1	F	59	42	3.69	736	11.1	Y	Lung
2	M	63	61	3.10	623	15.2	Y	Lung
3*	M	36	31	2.89	860	8.8	Y	Lymph node
4	M	58	55	3.13	205	9.7	Y	Mediastinum, lymph node, diaphragm, bone
5*	F	55	53	2.96	489	8.6	N	
6	F	65	60	4.62	718	9.7	N	
7	M	76	68	3.34	1277	9.2	Y	Lung
8	M	57	52	4.03	785	11.8	Y	Lung
9	M	55	49	3.47	350	11.4	Y	Lung
10	M	53	43	3.72	475	13.4	Y	Mediastinum, bone
11*	M	70	62	3.40	137	11.8	N	
12	M	55	52	4.16	500	15.4	Y	Lung
13	F	66	60	4.30	230	8.3	Y	Tracheostomy site
14	F	58	51	4.10	1092	12.7	N	
15	M	62	55	2.74	223	17.3	Y	N/A
16	F	52	43	2.92	953	8.4	Y	N/A
17	F	42	28	4.56	723	21.2	Y	Esophagus
18	F	50	48	3.57	990	18.0	Y	Left thyroid gland
19	M	68	65	2.98	324	15.6	Y	
20	F	72	69	4.00	2471	4.0	Y	Oesophagus
21	M	77	68	2.92	357	37.4	N	
22	M	58	55	3.10	168	10.1	Y	Liver
23	F	35	34	4.85	1148	12.8	Y	N/A

*Table 6: Clinical characteristics in 23 patients with advanced parathyroid cancer. * patients with a normal* $\frac{3rdgeneration}{2ndgeneration}$ *PTH ratio (<1); N/A: not available*

Characteristics	**Result±standard deviation**
Sex	38% female
Age (years)	65.2±16.5
Mean vintage on hemodialysys (in months)	88±71
Serum creatinine (mg/L)	89±30
2^{nd} generation PTH (pg/mL)	244±247
3^{rd} generation PTH (pg/mL)	148±164
Plasma calcium (mmol/L)	1.10±0.09

Table 7: Characteristics of 73 hemodialysis patients used as comparator group

Characteristics	**Result±standard deviation**
Sex	45% female
Age (years)	50.6±12.2
Time elapsed since renal graft (in months)	56±49
Serum creatinine (mg/L)	15.5±7.4
2^{nd} generation PTH (pg/mL)	90.2±57.6
3^{rd} generation PTH (pg/mL)	49.5±34.5
Plasma calcium (mmol/L)	1.25±0.09

Table 8: Characteristics of 60 renal transplant patients.

Inverted $\frac{3rd generation}{2nd generation}$ PTH **ratio (>1)**			**Normal** $\frac{3rd generation}{2nd generation}$ PTH **ratio (<1)**		
Intact PTH (pg/mL)	**Whole PTH (pg/mL)**	**Ratio**	**Intact PTH (pg/mL)**	**Whole PTH (pg/mL)**	**Ratio**
261	435	1.66	814	668	0.82
464	544	1.17	452	361	0.80
188	333	1.77	695	438	0.63
367	487	1.33			
1098	1363	1.24			
806	1101	1.37			
828	888	1.07			
606	768	1.27			
622	691	1.11			
185	370	2.00			
230	638	2.78			
71	99	1.40			
272	365	1.34			
363	583	1.61			
628	810	1.29			
404	542	1.34			
1063	1989	1.87			
197	358	1.82			
99	102	1.03			
747	1076	1.44			
Mean					
475	***677***	***1.50***	***654***	***489***	***0.75***

Table 9: Individual and mean second generation and third generation PTH assays and the $\frac{3rd generation}{2nd generation}$ *PTH ratio in 23 patients with advanced parathyroid carcinoma*

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IV. CONCLUSIONS AND PERSPECTIVES

The determination of parathormone remains very difficult. Indeed, since the first assay developed by Rosalyn Yalow, our understanding of "what" is PTH and its implication in physiopathology, especially in CKD patients, has considerably evolved.

The aim of our work was to provide a contribution to the study of parathormone, particularly in the light of a better pre-, post- and analytical management, as requested by the ISO 15189.

Our contribution to the preanalytical phase of PTH determination is very important. Indeed, we have shown that the peptide is not very stable and that this stability depends on many factors, *ie*. the conservation temperature, the kind of tube used for the sampling, the automated method used for the determination and the patient himself.

For the post-analytic phase, we have also provided useful information on the way that the reference range for PTH should be established. Based on these considerations, we have provided a methodology that allows a comprehensive interpretation of PTH results and that take into account the possible interferences due to rheumatoid factor or human anti-animal antibodies.

For the analytic phase, and particularly in patients suffering from CKD, the choice of a 2nd vs. 3rd generation kit is not obvious. Clearly, the recommendations of the K/DOQI are based on a 2nd generation kit (Allegro), but are not easily transposable to the other 2nd generation kits.

Recently, the KDIGO guidelines have been published. These guidelines are also based on 2nd generation kits but do not provide 'raw values" anymore. This is a good point, but as they are based on the "laboratory's published reference range", they open the question on "what is a reference range" for PTH…

The 3rd generation assays offer the advantage to be more easily "standardizable" then the 2nd generation ones, but are prone to the interference of the amino-PTH, which is only the case for the Roche Elecsys kit, a 2nd generation assays. Recently, Melamed et al have shown that the 3rd generation kits could be used as predictors of mortality in dialysis patients, but we need

more information and future studies are needed. A solution might perhaps be a 4th generation PTH, which would be free of the amino-PTH interference. Indeed, such a kit could be calibrated against an International standard and we would exactly know (unless we find a new PTH species, which is always possible!) what we assay. Nevertheless, even if this kit appeared on the market, prospective studies against bone biopsies would be needed (as well as clinical studies in patients with normal kidney function and various diseases, like hyperparathyroidism). This is also the main criticism that could be addressed to the use of bAP as a surrogate for PTH.

Another point is that PTH determination is an important routine test. So, this new 4th generation kit should be available in every laboratory. It should not thus be an IRMA method (each lab is not necessarily allowed to manipulate isotopic methods), nor an ELISA method (which would be a regression). Thus, it should be an automated method. But then, what would happen to the labs that do not have this particular automate…

Finally, we think that a prospective multicentric study in consecutive patients suffering from primary hyperparathyroidism that undergo surgery would be interesting to evaluate the $\frac{3rdgeneration}{2ndgeneration}$ PTH ratio.

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VI. APPENDIX