Comparison of second and third generation parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic Hospital – Are they fit for purpose?

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Chemical Pathology.

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DECLARATION

I, Nokuthula Nhlapo, student number 305014, declare that this research report is my work. I have contributed adequately to the findings issued in this report. It is being submitted for the Master of Medicine in Chemical Pathology under the Department of Chemical Pathology at the University of the Witwatersrand. This report has not been submitted for examination towards a degree at any other University.

Candidate signature:  Date: 13 November 2023

DEDICATION

This research report is dedicated to my family who have supported and encouraged me throughout this journey.

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I would like to acknowledge and give my warmest thanks to my research supervisors; for their immense and invaluable guidance, encouragement and time, without which this research report would not have been possible.

I would also like to give a special mention to all my NHLS laboratory colleagues for the technical assistance they provided, which has aided in the completion of this research report.

STATEMENT

The research report consists of a submissible article (including literature review), a study protocol, and appendices. Before undertaking this research project, a research protocol detailing the study's methodology was written, submitted for assessment, and approved by a panel of assessors from the Health Science Faculty at the University of the Witwatersrand School of Pathology. A copy of the research protocol for background reference and the ethics clearance certificate are included under the appendices of this research report.

The formatting of this report complies with the Health Sciences Faculty of the University of the Witwatersrand style outlined for Theses, Dissertations, and Research Reports. The format of the submissible article may differ from the rest of the research report to comply with the author's guidelines of the PLOS ONE Journal, to which it is intended to be submitted. As recommended by the journal, figure captions have been inserted immediately after the first paragraph in which the figure was cited; with figure files uploaded separately. The tables have been inserted immediately after the first paragraph in which they have been cited.

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ABSTRACT

Background

Parathyroid hormone (PTH) measurement is crucial in the investigation of calcium and phosphate disorders and in the management of chronic kidney disease (CKD). Available PTH assays include second (intact PTH) and third generation (PTH 1-84) assays. Intact PTH assays are widely available and used for clinical guidelines but overestimate PTH in CKD. PTH 1-84 assays are more specific, but lack of standardisation has complicated clinical interpretation.

The study aimed to compare the second and third generation assays to determine the difference in analytical performance and the effect on clinical interpretation.

Methods

A method comparison was done on 481 patient samples with PTH requested at Charlotte Maxeke Johannesburg Academic Hospital. PTH was measured in each sample using both intact PTH and PTH 1-84 assays. Passing Bablok regression and Bland Altman plots were performed to determine method agreement and bias. Analytical performance was assessed using the European Federation of Clinical Chemistry and Laboratory Medicine biological variation specifications. Clinical performance was compared in the diagnosis of hypo- and hyperparathyroidism, and in predialysis and dialysis CKD based on current Kidney Disease Improving Global Outcomes guidelines.

Results

Intact PTH had a higher median concentration than PTH 1-84 (9.93 vs 8.60 pmol/L, $p<0.0001$) but showed good correlation ($r = 0.994$ and $p<0.0001$). Regression analysis revealed significant systematic and proportional differences, with increased deviations at higher concentrations.

The average bias was above allowable bias of 7.1%. Clinical interpretation of hypo- and hyperparathyroidism and predialysis and dialysis groups was unchanged.

Conclusions

There was significant bias observed between the two PTH assays thus, they should not be used interchangeably. However, no significant changes in clinical interpretation were found when one assay was used over the other. The decision to use third over second generation PTH assay should consider the impact on clinical interpretation in the population**.**

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SUBMISSIBLE ARTICLE

Comparison of second and third generation parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic Hospital – Are they fit for purpose?

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INTRODUCTION

Chronic kidney disease (CKD) is a global health problem with a prevalence of 5 - 10% [1]. In Africa, CKD presents a major public health concern particularly against a background of limited resources [2]. Such patients have an increased risk of CKD-mineral and bone disorder (CKD-MBD) which is associated with high morbidity and mortality [1]. Bone histomorphometry is the gold standard in the assessment of renal osteodystrophy, which reflects the skeletal facet of CKD-MBD. However, it is invasive, expensive and requires expertise not readily available, thus measurement of circulating biomarkers including parathyroid hormone (PTH) offer an attractive non-invasive alternative [1, 3]. Changes in PTH concentrations contribute to the bone abnormalities observed in CKD–MBD [4]. This makes its measurement essential in the management of CKD–MBD.

PTH is a key regulator of calcium and phosphate homeostasis and bone remodelling [4]. PTH determination is crucial in the diagnostic workup of hypocalcaemia, hypercalcaemia and in the perioperative assessment in thyroid or parathyroid surgery [1, 4]. Therefore, accurate and reliable PTH assays are needed for proper patient management. PTH is composed of a single polypeptide produced exclusively by the chief cells of parathyroid glands, with a molecular weight of ~9.5 kDa [5]. It is sequentially cleaved at the amino terminal end to yield the mature

biologically active 84 amino acid molecule PTH (1-84), which consists of two main parts; the amino (N) terminus made up of the first 34 amino acids and the carboxyl (C) terminus that contains the remaining 50 amino acids. As a result of rapid proteolytic cleavage in circulation, PTH exists as intact PTH (1-84) and various fragments i.e., N-terminal, mid region and C-terminal PTH, the most abundant being 7-84 PTH [6]. Although the plasma half-life of PTH (1-84) is between 2 and 4 minutes, C-terminal PTH fragments have a longer half-life of several hours due to renal clearance [6, 7]. The latter may account for more than 20% of circulating PTH in normal individuals and up to 45% in patients with renal failure [6]. This heterogeneity of PTH in circulation has made its measurement challenging.

There are numerous pitfalls in the laboratory measurement of PTH. Currently, no reference method exists, and although the use of a single recognised international standard e.g., World Health Organisation (WHO) PTH 95/646 has been proposed, it has not been fully implemented and PTH remains unstandardised [8, 9]. The radioimmunoassay (RIA) was a first generation competitive PTH assay that utilised a single polyclonal antibody generated against epitopes in the mid or C-terminus of the PTH peptide [7]. The RIAs had several limitations, including cross reactivity with the biologically inactive C-fragments, which was more evident in patients with CKD; where decreased renal clearance of the C-fragments led to their plasma accumulation and thus overestimation of PTH levels [10, 11]. To overcome this issue, the immunometric second and third generation PTH assays were developed. The second generation PTH assay is a non-competitive sandwich immunoassay that makes use of two antibodies, generated against the mid region N terminal and C terminal amino acids. It was assumed that it detected the biologically active PTH (1-84) and avoided cross reactivity with C-fragments and thus was referred to as the "intact" PTH assay [11, 12]. Subsequent studies revealed these assays overestimated values in patients with renal failure compared to PTH values in normal subjects due to the accumulation of N-truncated PTH fragments, the most abundant of which

is 7-84 PTH [13, 14]. The third generation PTH assays use a capture antibody, similarly to second generation assays. However, the detection antibody is directed against epitopes in the first 4 amino acids of the N-terminus, which avoids cross reactivity with 7-84 PTH fragments [7, 11]. The third generation assays were termed bio-intact (bio-PTH) or whole PTH assays as they were more specific for PTH (1-84) [12].

The second generation assays have provided the basis for the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines on management of CKD-MBD [12, 15]. Significant inter-method variability among second generation assays has been shown, thus affecting comparability of results [3]. Taking this into consideration, the recent KDIGO guidelines proposed utilisation of multiples of 2 – 9-fold of the upper limit of normal for a given PTH assay instead of absolute values in CKD patients on dialysis. In patients not on dialysis, the KDIGO guidelines state that the target PTH concentration is unknown and thus recommend monitoring trends rather than a single value [15].

Research has shown that third generation assays produce results that are 50-60% and 15% lower in CKD and non-CKD patients, respectively, when compared to second generation assays [10, 12]. Third generation assays are more specific to bioactive PTH than second generation and should in theory be more accurate in the evaluation of PTH status. Yet, there is still dispute as to which assay is preferable and whether third generation assays offer improved clinical value [5]. Second generation assays are readily available, extensively researched and form part of current clinical guidelines [12]. In contrast; there is insufficient research for the third generation to be universally adopted in clinical practice [12].

In our setting, the National Health Laboratory Service (NHLS) supplies two major academic hospitals i.e., Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) which utilise the second and third generation PTH

assays respectively, from the same manufacturer with similar reference intervals. There is considerable movement of patients and blood samples between the two hospitals which may affect PTH interpretation and clinical decision making depending on the assay used. Thus, this study aimed to compare the analytical performance and the effect on clinical interpretation between second and third generation PTH assays.

The primary objectives were to determine both the analytical performance and agreement between the two PTH assays using patient samples according to CLSI 09 protocol.

The secondary objectives were; firstly, to determine the clinical interpretation of PTH results in various clinical scenarios including hypo- and hyperparathyroidism, when second and third generation assays are used. Secondly, to determine whether misclassification of patients with CKD based on the KDIGO guidelines occurs, when one PTH assay is used over the other.

MATERIALS AND METHODS

SAMPLES

A total of 505 residual patient samples on which a PTH was requested at CMJAH NHLS laboratory, were obtained from both in- and outpatients. The study was conducted between 06 April 2022 and 21 September 2022. The following equation was used to derive the sample size: $= \frac{z^2 p(1-p)}{d^2}$ [16]. It utilised a prevalence of 6.4% in South Africa for CKD; where n is the sample size, Z is the statistic corresponding to level of confidence of 95%, P is expected prevalence (6.4 %) and d is precision which reflects the effect size and was estimated to be 5% [16, 17]. A minimum of 92 samples from CKD patients were required to demonstrate a difference between the two assays.

Samples were collected in BD Vacutainer® EDTA tubes™ (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Samples of sufficient volume and within recommended stability period of 48 hours when stored at 2 - 8 °C were included. The samples were initially analysed using the routine third generation, Roche PTH 1-84 assay and then immediately re-analysed using the newly validated second generation, Roche intact PTH assay. The instrument middleware was set up, to allow analysis of second generation PTH immediately after the requested third generation PTH was analysed to minimise time delays between analyses.

To aid with clinical interpretation of PTH results; data was collected from the NHLS TrakCare™ (InterSystems, Cambridge, MA, USA) laboratory information system (LIS) and included: age, gender, calcium, phosphate and creatinine and 25 (OH) vitamin D. The glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) 2009 equation without the race factor [18].

ETHICAL APPROVAL

This study has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificate No. M210475) and performed in line with the Declaration of Helsinki.

LABORATORY ANALYSIS

A verification study for second generation PTH assay was performed according to CLSI EP15-A3 guideline [19]. This was done because the second generation PTH assay was not validated on the Roche Cobas e602 module at CMJAH. Assay performance was within the acceptable limits of the manufacturer's claims with intra-assay and inter-assay variability of 0.9% and 2.2 % respectively.

The PTH assays' analytical performance was compared as per the CLSI EP 09 - A3 guideline [20]. Analyses of samples for PTH were performed using Roche intact PTH (second generation) and PTH 1-84 (third generation) reagents and calibrators on an e602 module of a Cobas 8000© system from Roche Diagnostics (Mannheim, Germany). The analytical characteristics of the PTH assays used are depicted in table 1 [21, 22]. Biochemical parameters including creatinine, total calcium and phosphate were measured using standard chemistry methods on the Roche Cobas 8000 analyser 702 module.

Table 1: Analytical Characteristics of Roche PTH Assays used

	Elecsys Intact PTH (Second generation)	**Elecsys PTH 1-84 (Third generation)**
Methodology	Electrochemluminescent (ECLIA) C terminal epitope: ruthenium labelled monoclonal mouse antibodies reactive with epitopes in the amino acid region 38 - 84. N terminal epitope: biotinylated monoclonal mouse antibodies reactive with epitopes in the amino acid regions 26 - 32 and 37 - 42.	ECLIA Monoclonal mouse antibodies used. A biotinylated monoclonal antibody reacts with the N-terminus of the PTH molecule and a ruthenium labelled monoclonal antibody reacts with the C-terminal fragment PTH. Epitopes not explicitly stated.
Limit of Detection (pmol/L)	0.127	0.583

Measuring Range (pmol/L)	0.127 – 530	0.583 – 244
Calibration Standard	Traceable to a commercial PTH (RIA) assay.	Traceable to the WHO International Standard 95/646.
Reference Interval (pmol/L)	1.6 – 6.9	1.58 – 6.03
Analytical coefficient of variation (CV) (%)	6.24 %	5.62 %

STATISTICAL ANALYSIS

The data obtained was captured onto Microsoft Excel® (Microsoft, Seattle, WA, USA) and analysed using MedCalc®, version 200218 (MedCalc Software, Ostend, Belgium). The Shapiro-Wilks test was used to assess for normality. Data were demonstrated to be non-parametric and thus expressed as median values with interquartile range (IQR). Categorical data were expressed as proportion and percentage. The Wilcoxon test was used to compare PTH concentrations between the two assays.

Intact PTH was used as the comparative method (X) and PTH 1-84 as the test method (Y). Passing-Bablok regression was used to assess method agreement and the Bland-Altman difference plot was used to determine the magnitude of bias between the two methods. The desirable analytical performance specifications for PTH were derived from the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation database [23]. A percentage bias of ±7.1 % was used as the allowable limit of acceptable analytical performance.

The McNemar test was used to determine if there were significant differences in the classification of patients and if the clinical interpretation changes when one assay is used over the other. A p-value of <0.05 was considered statistically significant.

CKD patients on dialysis were classified as above, below, or within the PTH target range of 2 – 9 times the upper limit of normal (ULN) as recommended by the KDIGO guidelines, using the two PTH assays. A kappa statistic was used to assess the performance of the classification using both assays with a value of ≥ 0.60 representing acceptable agreement [24].

RESULTS

METHOD COMPARISON

A total of 505 residual patient samples were identified for the study. However, 24 samples were excluded; 17 had results exceeding the measuring range of the Elecsys PTH 1-84 assay and seven had exceeded the stability period. Thus, 481 samples were analysed and utilised for the method comparison study.

The median PTH concentration for third generation was significantly lower at 8.60 pmol/L compared to the second generation median of 9.93 pmol/L, $p <0.0001$. The regression analysis equation was calculated as $y = 0.713x + 0.887$ (Fig 1). There was good correlation between the two methods with a coefficient correlation (r) = 0.994 and $p <0.0001$. The intercept was calculated at 0.887 pmol/L (95% confidence interval 0.788 – 1.005) and the slope at 0.713 pmol/L (95% confidence interval 0.703 - 0.723); indicating that there were systematic and proportional differences between the two methods, with increased deviations observed at higher PTH concentrations.

Fig 1: Scatter plot and regression analysis comparing Intact PTH and PTH 1-84 for all samples.

The Bland-Altman analysis showed an average overall difference between the PTH assays of 9.00 pmol/L (18.5%, $p < 0.0001$) among all samples analysed (Fig 2 and Fig 3). The bias in the entire CKD group and predialysis CKD group was 27.2% and 25.3% ($p<0.0001$) respectively (Fig 4 and Fig 5). The dialysis group, however, demonstrated the highest bias of 35.2% ($p<0.0001$) (Fig 6). This is in stark contrast to the difference observed within the non-CKD group with eGFR >60 ml/min/1.73m^2, which was demonstrated to be 5.7% ($p<0.0001$) (Fig 7).

Fig 2: The absolute difference plot of Intact PTH and PTH 1-84 assays

Fig 3: The percentage difference plot of Intact PTH and PTH 1-84 assays

Figure 4: The percentage difference plot of Intact PTH and PTH 1-84 assays in entire CKD group

Figure 5: The percentage difference plot of Intact PTH and PTH 1-84 assays in predialysis CKD group

Figure 6: The percentage difference plot of Intact PTH and PTH 1-84 assays in the dialysis group

Figure 7: The percentage difference plot of Intact PTH and PTH 1-84 assays in the non-CKD group with eGFR >60 ml/min/1.73 m^2

CLINICAL INTERPRETATION

The clinical performance of the two assays was assessed in the diagnosis of hypoparathyroidism and hyperparathyroidism and in CKD patients, including those on dialysis. A total of 276 patients were used for this purpose as depicted in the flow diagram (Figure 8). The median age was 52 years with majority being female (62%) (Table 2). Vitamin D results were not included as very few patients had results available on LIS.

Figure 8: Flow diagram of PTH samples used for clinical interpretation

Table 2: Demographics and biochemical data for the patient samples used in clinical interpretation.

Parameters	**Overall**	**CKD**	**Non-CKD**
Age (years)	52.50 (49.00 – 54.00)	46.90 (44.61 – 49.19)	55.00 (54.00 – 59.00)
Sex (n) (%) Female n (%) Male n (%)	276 170 (62%) 106 (38%)	146 80 (55%) 66 (45%)	130 90 (69%) 40 (31%)
Parathyroid Hormone (PTH) pmol/L Intact PTH PTH 1-84	 12.33 (10.52 – 16.96) 10.36 (9.10 – 12.81)	 26.28 (20.84 – 36.28) 19.95 (15.50 – 27.29)	 6.75 (5.68 – 8.10) 6.05 (5.00 – 7.04)
Serum Calcium (mmol/L)	2.30 (2.27 – 2.32)	2.26 (2.20 – 2.30)	2.36 (2.31 – 2.41)
Phosphate (mmol/L)	1.26 (1.17 – 1.36)	1.30 (1.19 – 1.42)	1.16 (1.06 – 1.33)
Creatinine (µmol/L)	306.00 (2264.55 – 417.04)	599.00 (428.03 - 697.86)	90.00 (82.08 – 107.93)
eGFR (ml/min/1.73m^2) CKD Stage 1 (3) CKD Stage 2 (9)	16.00 (12.00 – 22.45)	8.00 (6.00 – 10.00) 93 * 65.00 (58.41 – 81.03)	66.00 (52.23 – 74.92)

CKD Stage 3a (9)		52.00 (45.28 – 56.00)	
CKD Stage 3b (9)		36.00 (33.14 – 37.86)	
CKD Stage 4 (18)		19.00 (16.00 – 21.22)	
CKD Stage 5 (70)		5.50 (5.00 – 6.00)	
CKD Stage 5D (28)		5.50 (4.36 – 7.00)	

CKD - chronic kidney disease diagnosed with > 3-month history of eGFR<60 ml/min/1.73 m^2. **Non-CKD** - Patients without confirmed history of CKD and with estimated glomerular filtration rate (eGFR). Values are expressed as median (25^{th} and 75^{th} interquartile) or number of participants (percentage). * Sample size insufficient to calculate confidence interval.

The McNemar test did not demonstrate any statistically significant differences in clinical interpretation as shown by p values above 0.05 in the detection of low, normal and high PTH using both assays. In both the CKD and dialysis group, no significant differences in interpretation were found using McNemar as shown in table 3.

Table 3: Differences in clinical interpretation using manufacturer reference ranges.

	Category	**Intact PTH (n, %)**	**PTH 1-84 (n, %)**	**p value**
Overall	PHPT	15 (5.4%)	16 (5.8%)	1.0000
	SHPT	185 (66.5%)	184 (66.2%)	1.0000
	Normal	57 (20.5%)	58 (20.9%)	1.0000
	Hypoparathyroidism	20 (7.2%)	21 (7.6%)	1.0000
CKD	SHPT	133 (91.1%)	135 (92.5%)	0.500
	Normal	11 (7.5%)	9 (6.2%)	0.500

	Hypoparathyroidism	2 (1.4%)	2 (1.4%)	UTC
Dialysis	SHPT	28 (100%)	28(100%)	UTC

PHPT – primary hyperparathyroidism; **SHPT** – secondary hyperparathyroidism; **UTC** – unable to calculate and perform test.

The clinical impact of using either second or third generation in the classification of CKD patients on dialysis, in terms of the KDIGO guidelines was assessed. Patients were classified as below, above or within the recommended PTH target range of 2 to 9 times the upper limit of normal. For the kappa analysis, 29 confirmed patients on dialysis were included. Classification according to KDIGO revealed minimal discordance between the two generations, with a κ coefficient of 0.79 (Table 4).

Table 4: Classification According to KDIGO guidelines for Intact PTH and PTH 1-84 Assays

Manufacturer ULN (pmol/L)	**< 2 x ULN**	**2 – 9 x ULN**	**>9 x ULN**
Intact PTH (1.6 – 6.9)	0 (0.0%)	15 (53.6%)	13 (46.4%)
PTH 1-84 (1.6 – 6.0)	1 (3.6%)	16 (57.1)	11 (39.3%)

ULN – upper limit of normal.; Weighted Kappa = 0.797 (0.594 – 1.000)

DISCUSSION

Currently two different generations of PTH assays exist in the market, with significant inter-assay variability [3]. The lack of standardisation between these assays has created some confusion for clinicians especially in the management of CKD patients.

We found that there was a significant inter-assay bias between the second and third generation PTH assays. However, there was no significant change in the clinical classification of hypoparathyroidism, PHPT, and secondary hyperparathyroidism in predialysis and dialysis CKD patients when one assay was used over the other.

With regards to analytical performance, the methods were strongly correlated. However, as the percentage mean bias surpassed the EFLM biological variation goal, this implies that these assays are not interchangeable nor comparable. Our study showed that for the entire patient group, the third generation PTH assay produced results that were on average approximately 20% lower than the second generation assay, and even lower at 27% and 35% in CKD and dialysis patients, respectively. This is in keeping with other reported research [3, 14, 15, 25-27]. Studies by Hashim et al and O'Flaherty et al, both using Roche PTH assays found that third generation assays were lower by 30% and 40% compared to second generation assays [28, 29]. In contrast, in the non-CKD group with eGFR above 60 ml/min/1.73 m^2, the observed bias was 5.7%. This indicates that in patients with normal GFR, there are minimal differences between second and third generation assays, which is keeping with other reported studies [26, 28].

Although the observed differences between the two PTH assays were significantly higher in both the predialysis and dialysis CKD groups, there was no change in interpretation of secondary hyperparathyroidism with either assay. The KDIGO guidelines do not state the target

PTH range in predialysis CKD patients [15]. Furthermore, we did not find studies that assessed the impact on clinical interpretation in this group.

There is a paucity of studies that have compared second and third generation PTH assays in dialysis patients using the KDIGO classification of 2 – 9 fold of the ULN [26, 30]. In the dialysis group, using the manufacturer's ULN, classification using KDIGO did not demonstrate statistically significant discordance between the two assays. This study was limited by a small sample size of confirmed dialysis patients which reflects the limited dialysis resources in our country. However, our finding was in contrast to a few published studies; including that by Dupuy et al, which demonstrated a high percentage of discordant results, with a κ coefficient of <0.20 [26]. In addition, a study by Beko et al, found that using the manufacturer's ULN, the clinical classification was altered in up to 23% of dialysis patients because of the switch from second to third generation PTH assay [30]. Presence of discordance is important as PTH concentrations are used in the management of CKD-MBD. Hence, the use of second over third generation PTH assays may result in misclassification leading to inadequate management [18].

There have been various attempts to reduce the discrepancies between the second and third generation PTH assays in dialysis patients; by applying a correction factor or deriving an equation to allow interconversion between the two assays [31]. Some authors have suggested the use of 1–84/7–84 PTH ratio to report PTH results to minimise potential misdiagnosis of CKD-BMD in CKD patients [31]. However, further validation studies are needed, and these efforts are not currently adopted by clinical guidelines.

Overestimation of PTH by second generation assays because of PTH fragment cross-reactivity is of negligible consequence in patients with primary hyperparathyroidism (PHPT). Nonetheless, the question whether third generation PTH assays offer superior diagnostic efficacy remains. In our study, there were no differences in interpretation between the two

assays with regards to diagnosis of PHPT. This is consistent with other published studies [32, 33]. Taking into consideration published evidence, the recent international guideline on evaluation and management of PHPT, recommend the use of either second or third generation PTH assays [34].

The IFCC Committee for Bone Metabolism has established a working group for the purpose of creating PTH standardisation to make the results comparable among different assays [35]. In the absence of a reference measurement method, the IFCC has recommended the use of the WHO standard, but this has not been completely implemented due to lack of commutability [9, 10]. Even though second generation PTH assays are the most utilised assays, third generation PTH assays offer the best hope for harmonisation of PTH because majority are traceable to the WHO standard [35].

To the best of our knowledge, this is the first study in Africa to evaluate the differences in analytical performance and impact on clinical interpretation of second and third generation PTH assays. However, our study had a few limitations. Firstly, we had a small sample size of patients on dialysis. Secondly, the study was based on information available on LIS with limited clinical information.

CONCLUSION

The second and third generation PTH assays correlate very well, though there was significant bias observed between the two methods which increases with rising PTH concentrations that prevail in CKD patients. However, using the manufacturer's assay specific reference intervals and KDIGO guideline on PTH classification, there were no major changes in clinical interpretation when either assay was used. Thus, the decision to use third over second

generation PTH assays should take all this into account. We recommend that assays not be used interchangeably. Therefore, as stated by the manufacturer and in accordance with the KDIGO recommendations, clinical laboratories should state the generation of PTH assay employed and inform clinicians of any change in clinical methods to facilitate appropriate interpretation of PTH results.

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I would also like to give a special mention to all my NHLS laboratory colleagues for the technical assistance they provided, that has aided in the completion of this research report.

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APPENDIX 1: FIGURES

Figure 1: Scatter plot and regression analysis comparing Intact PTH and PTH 1-84 for all samples.

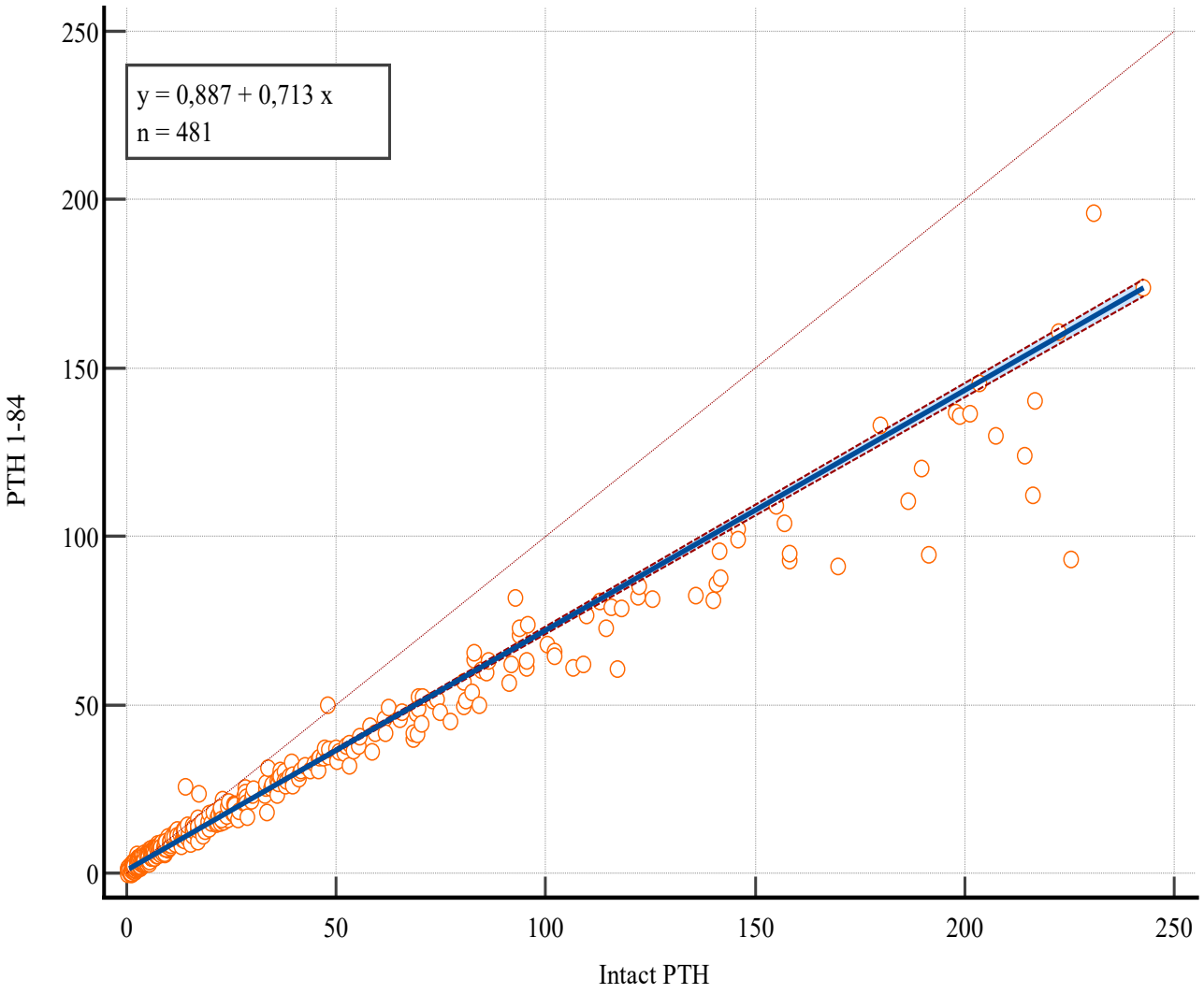


Figure 2: The absolute difference plot of Intact PTH and PTH 1-84 assays.

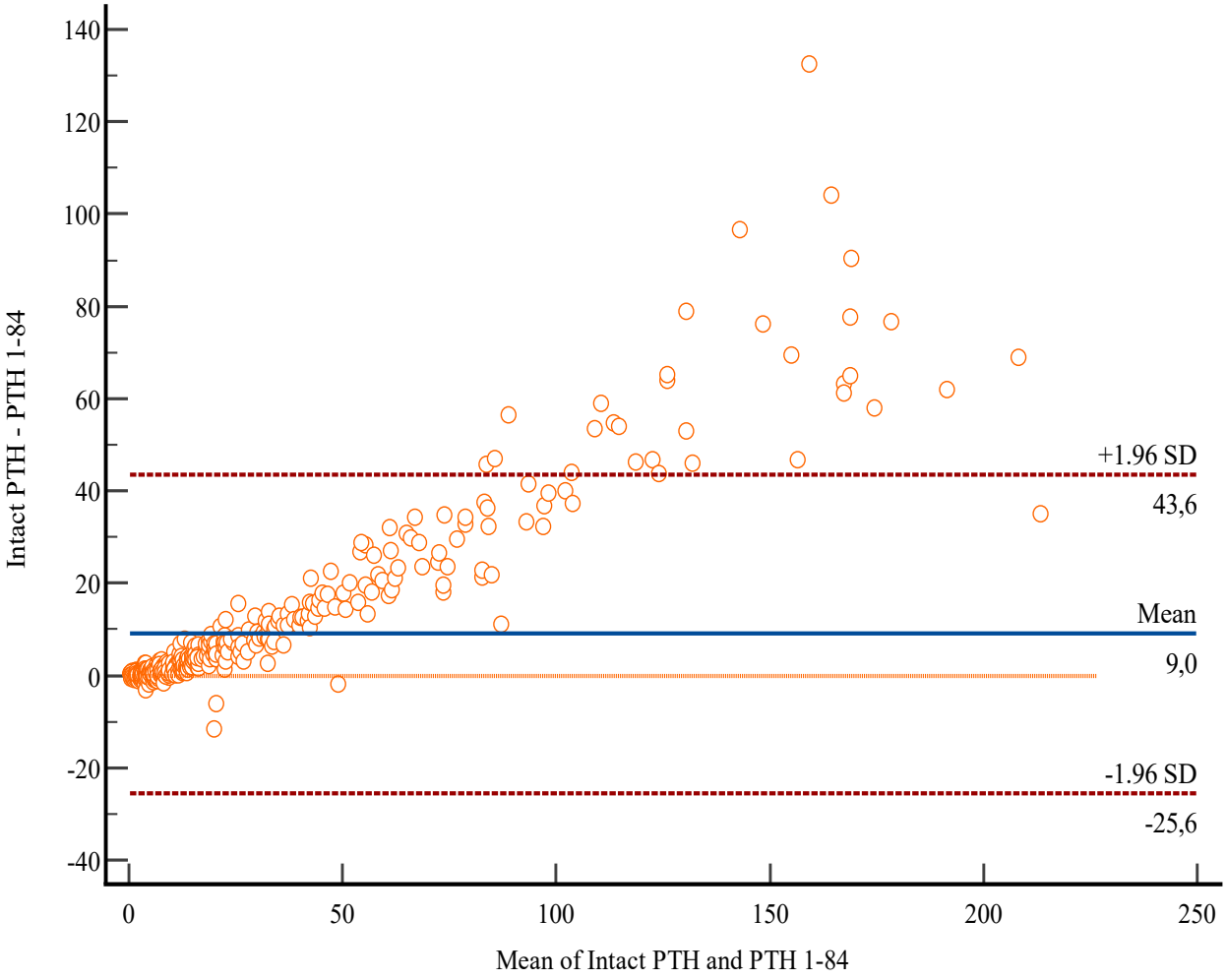


Figure 3: The percentage difference plot of Intact PTH and PTH 1-84 assays.

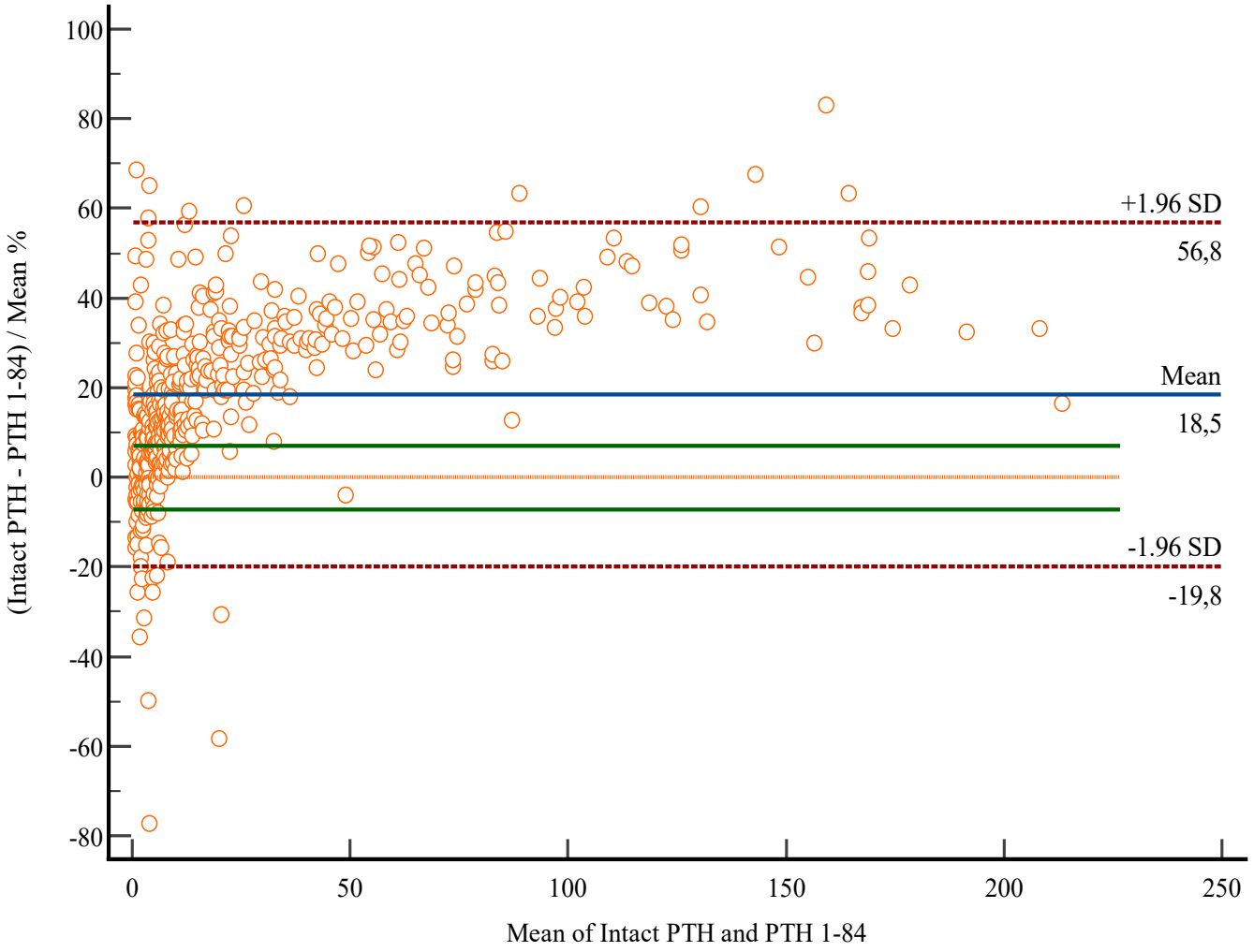


Fig 4: The percentage difference plot of Intact PTH and PTH 1-84 assays in entire CKD group.

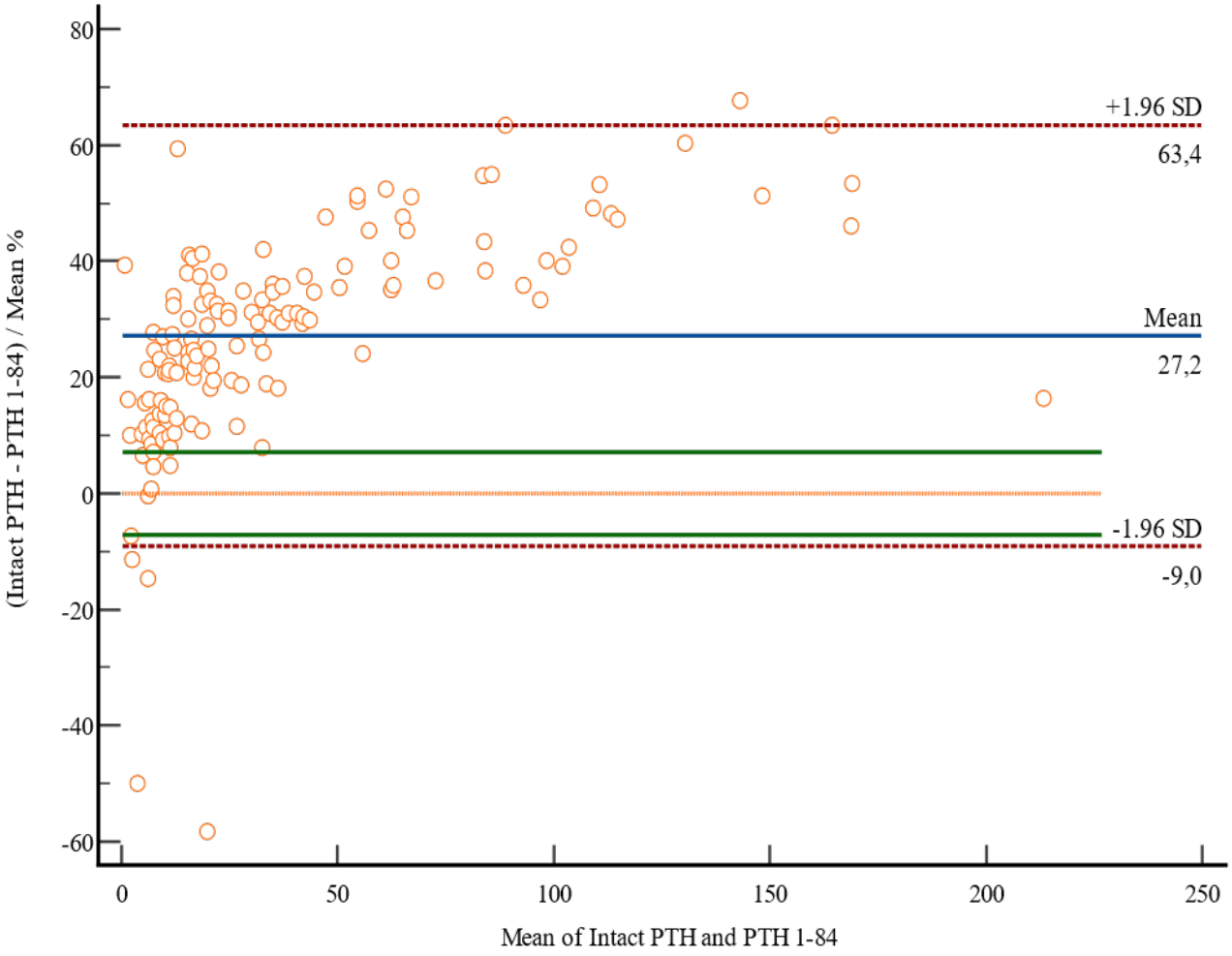


Fig 5: The percentage difference plot of Intact PTH and PTH 1-84 assays in predialysis CKD group.

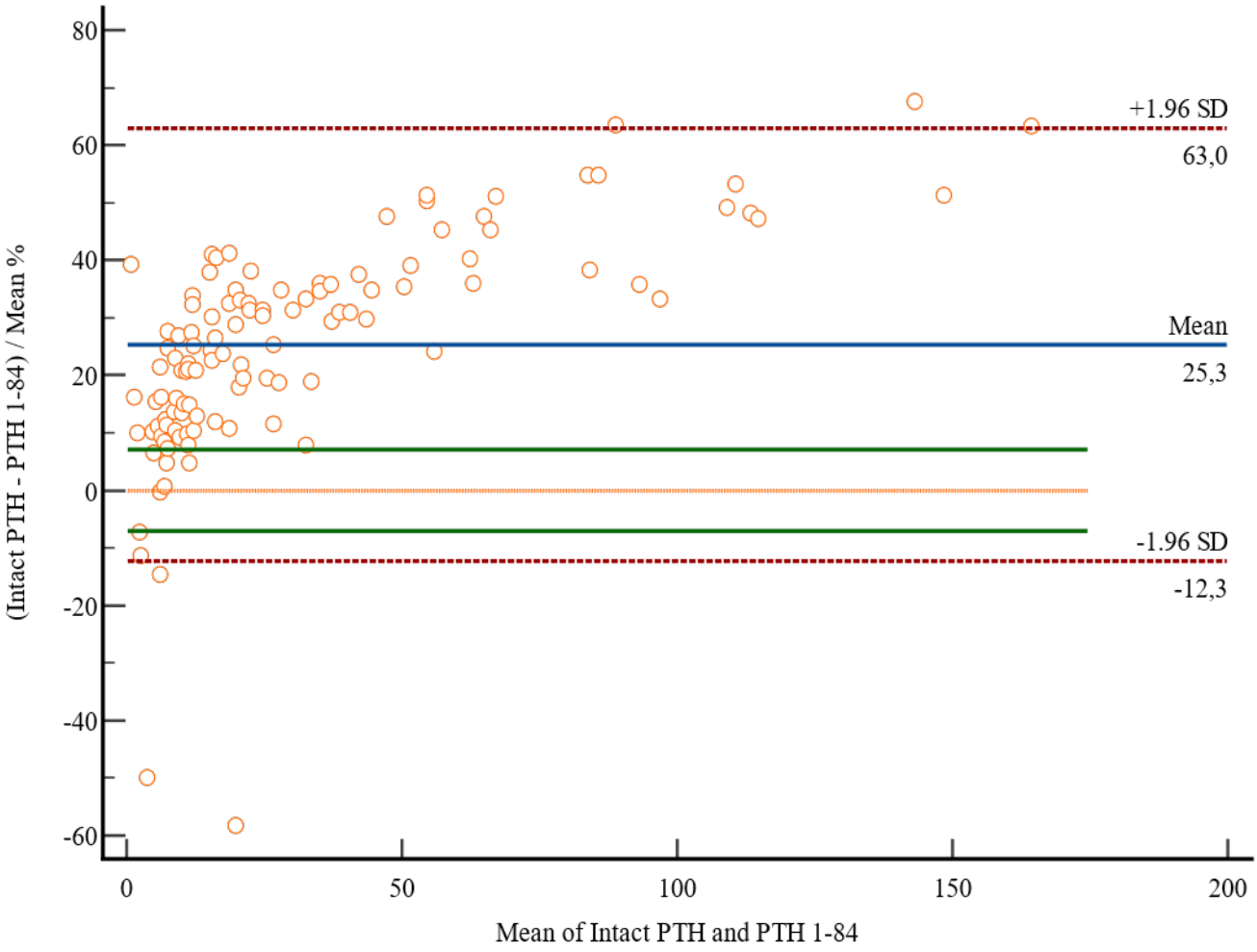


Fig 6: The percentage difference plot of Intact PTH and PTH 1-84 assays in the dialysis group.

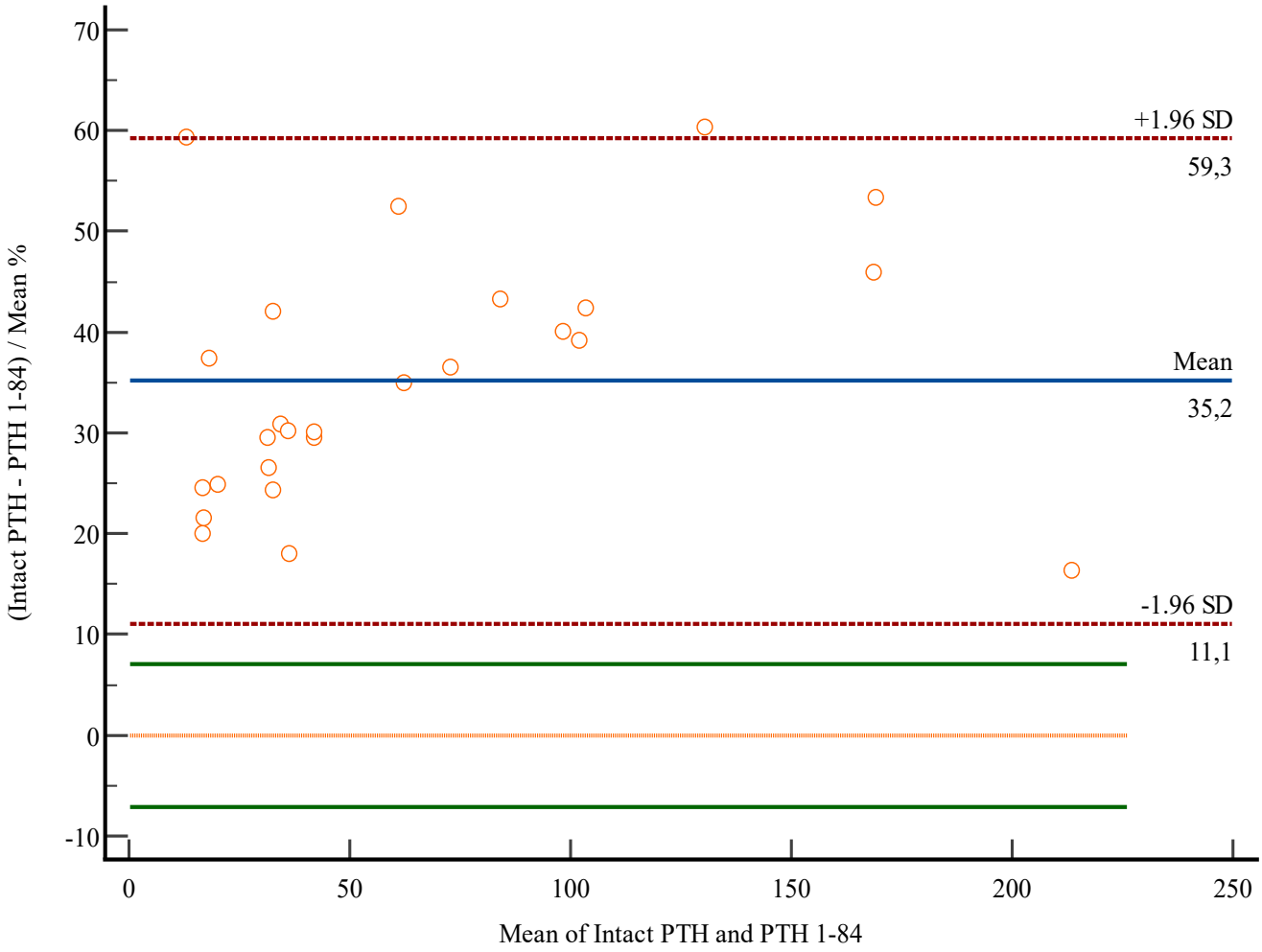


Fig 7: The percentage difference plot of Intact PTH and PTH 1-84 assays in the non-CKD group with eGFR >60 ml/min/1.73m^2.

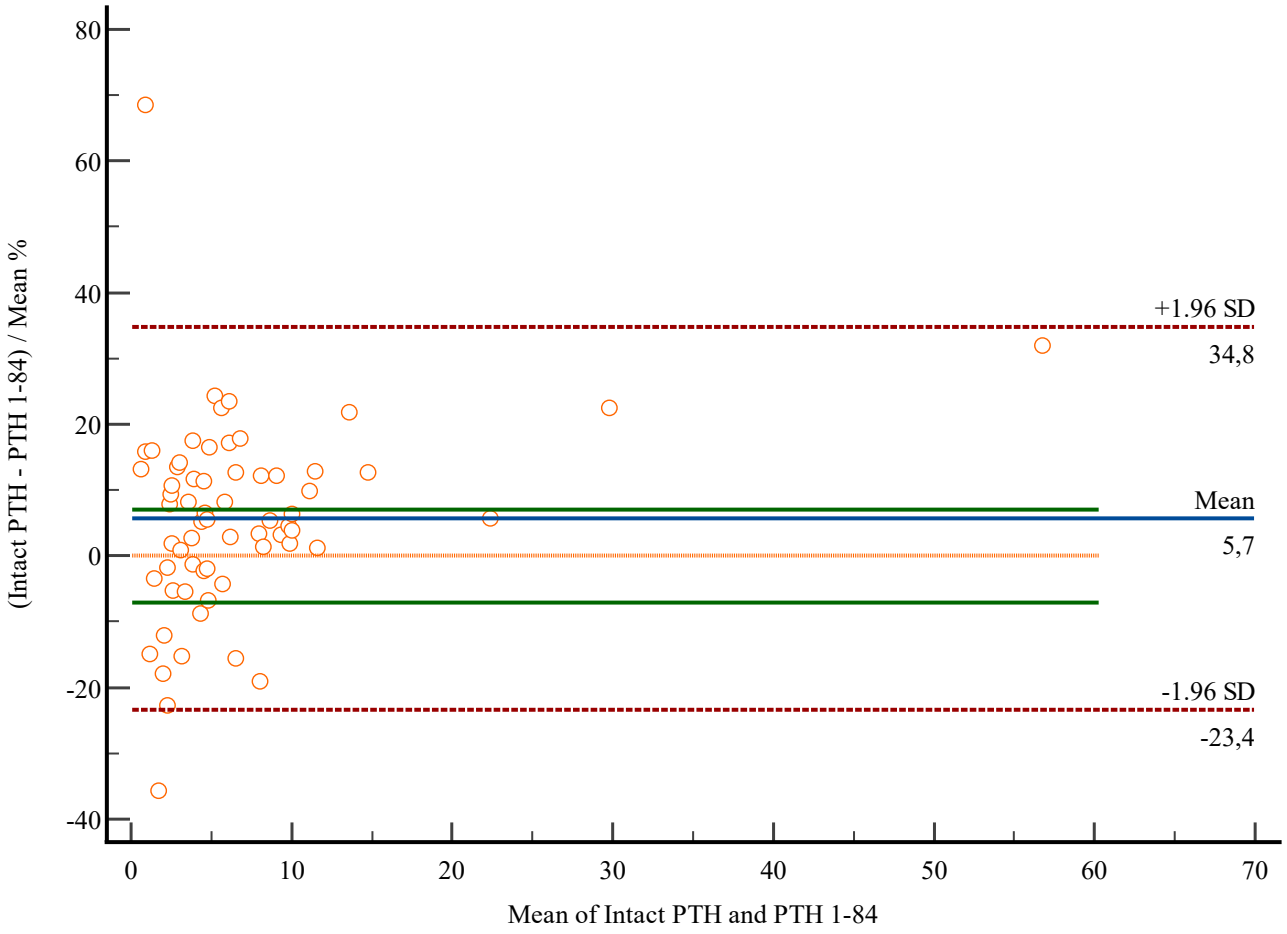
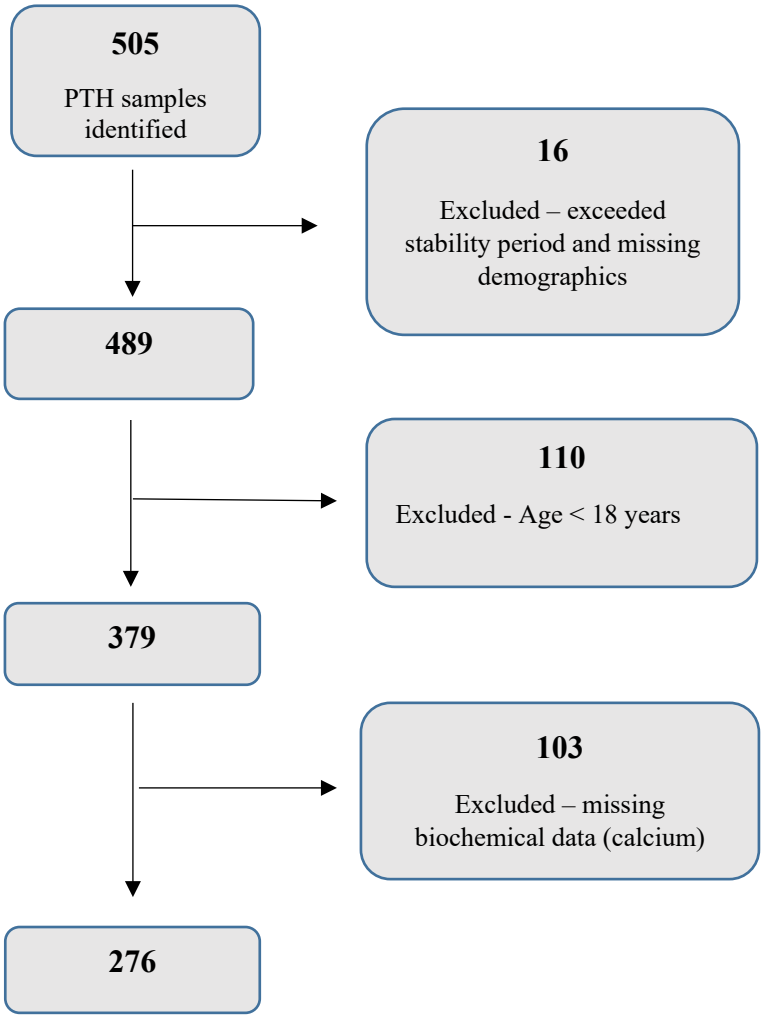


Figure 8. Flow diagram of PTH samples used for clinical interpretation.



APPENDIX 2: ETHICS CLEARANCE

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Dr N Nhlapo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210475

NAME: **(Principal Investigator)**	Dr N Nhlapo
DEPARTMENT:	School of Pathology Department of Chemical Pathology Medical School University
PROJECT TITLE:	*Comparison of second and third generation parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic Hospopital - are they fit for purpose?*
DATE CONSIDERED:	2021/04/30
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Drs M Maphayi and D Mabuza
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/09/28

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **April** and therefore reports and re-certification will be due in the month of **April** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

10 October 2021
Date

Research Report - 305014-1.docx

by Nokuthula Nhlapo

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SUBMISSIBLE ARTICLE

Comparison of second and third generation parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic Hospital – Are they fit for purpose?

Nokuthula Nhlapo, Siyabonga P Khoza, Doreen Jacob, Mpho R Maphayi

INTRODUCTION

Chronic kidney disease (CKD) is a global health problem with a prevalence of 5 - 10% [1]. In Africa, CKD presents a major public health concern particularly against a background of limited resources [2]. Such patients have an increased risk of CKD-mineral and bone disorder (CKD-MBD) which is associated with high morbidity and mortality [1]. Bone histomorphometry is the gold standard in the assessment of renal osteodystrophy, which reflects the skeletal facet of CKD-MBD. However, it is invasive, expensive and requires expertise not readily available, thus measurement of circulating biomarkers including parathyroid hormone (PTH) offer an attractive non-invasive alternative [1, 3]. Changes in PTH concentrations contribute to the bone abnormalities observed in CKD–MBD [4]. This makes its measurement essential in the management of CKD–MBD.

PTH is a key regulator of calcium and phosphate homeostasis and bone remodelling [4]. PTH determination is crucial in the diagnostic workup of hypocalcaemia, hypercalcaemia and in the perioperative assessment in thyroid or parathyroid surgery [1, 4]. Therefore, accurate and reliable PTH assays are needed for proper patient management.

PTH is composed of a single polypeptide produced exclusively by the chief cells of parathyroid glands, with a molecular weight of ~9.5 kDa [5]. It is sequentially cleaved at the amino terminal

end to yield the mature biologically active 84 amino acid molecule PTH (1-84), which consists of two main parts; the amino (N) terminus made up of the first 34 amino acids and the carboxyl (C) terminus that contains the remaining 50 amino acids. As a result of rapid proteolytic cleavage in circulation, PTH exists as intact PTH (1-84) and various fragments i.e., N-terminal, mid region and C-terminal PTH, the most abundant being 7-84 PTH [6]. Although the plasma half-life of PTH (1-84) is between 2 and 4 minutes, C-terminal PTH fragments have a longer half-life of several hours due to renal clearance [6, 7]. The latter may account for more than 20% of circulating PTH in normal individuals and up to 45% in patients with renal failure [6]. This heterogeneity of PTH in circulation has made its measurement challenging.

There are numerous pitfalls in the laboratory measurement of PTH. Currently, no reference method exists, and although the use of a single recognised international standard e.g., World Health Organisation (WHO) PTH 95/646 has been proposed, it has not been fully implemented and PTH remains unstandardised [8, 9]. The radioimmunoassay (RIA) was a first generation competitive PTH assay that utilised a single polyclonal antibody generated against epitopes in the mid or C-terminus of the PTH peptide [7]. The RIAs had several limitations, including cross reactivity with the biologically inactive C-fragments, which was more evident in patients with CKD; where decreased renal clearance of the C-fragments led to their plasma accumulation and thus overestimation of PTH levels [10, 11]. To overcome this issue, the immunometric second and third generation PTH assays were developed. The second generation PTH assay is a non-competitive sandwich immunoassay that makes use of two antibodies, generated against the mid region N terminal and C terminal amino acids. It was assumed that it detected the biologically active PTH (1-84) and avoided cross reactivity with C-fragments and thus was referred to as the “intact” PTH assay [11, 12]. Subsequent studies revealed these assays overestimated values in patients with renal

failure compared to PTH values in normal subjects due to the accumulation of N-truncated PTH fragments, the most abundant of which is 7-84 PTH [13, 14]. The third generation PTH assays use a capture antibody, similarly to second generation assays. However, the detection antibody is directed against epitopes in the first 4 amino acids of the N-terminus, which avoids cross reactivity with 7-84 PTH fragments [7, 11]. The third generation assays were termed bio-intact (bio-PTH) or whole PTH assays as they were more specific for PTH (1-84) [12].

The second generation assays have provided the basis for the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines on management of CKD-MBD [12, 15]. Significant inter-method variability among second generation assays has been shown, thus affecting comparability of results [3]. Taking this into consideration, the recent KDIGO guidelines proposed utilisation of multiples of 2 – 9-fold of the upper limit of normal for a given PTH assay instead of absolute values in CKD patients on dialysis. In patients not on dialysis, the KDIGO guidelines state that the target PTH concentration is unknown and thus recommend monitoring trends rather than a single value [15].

Research has shown that third generation assays produce results that are 50-60% and 15% lower in CKD and non-CKD patients, respectively, when compared to second generation assays [10, 12]. Third generation assays are more specific to bioactive PTH than second generation and should in theory be more accurate in the evaluation of PTH status. Yet, there is still dispute as to which assay is preferable and whether third generation assays offer improved clinical value [5]. Second generation assays are readily available, extensively researched and form part of current clinical guidelines [12]. In contrast; there is insufficient research for the third generation to be universally adopted in clinical practice [12].

In our setting, the National Health Laboratory Service (NHLS) services two major academic hospitals i.e., Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) which utilise the second and third generation PTH assays respectively, from the same manufacturer with similar reference intervals. There is considerable movement of patients and blood samples between the two hospitals which may affect PTH interpretation and clinical decision making depending on the assay used. Thus, this study aimed to compare the analytical performance and the effect on clinical interpretation between second and third generation PTH assays.

The primary objectives were to determine both the analytical performance and agreement between the two PTH assays using patient samples according to CLSI 09 protocol.

The secondary objectives were; firstly, to determine the clinical interpretation of PTH results in various clinical scenarios including hypo- and hyperparathyroidism, when second and third generation assays are used. Secondly, to determine whether misclassification of patients with CKD based on the KDIGO guidelines occurs, when one PTH assay is used over the other.

MATERIALS AND METHODS

Samples

A total of 505 residual patient samples were obtained from both in- and outpatients on which a PTH was requested at CMJAH NHLS laboratory between April and September 2022. The following equation was used to derive the sample size: $= \frac{z^2 p(1-p)}{d^2}$ [16]. It utilised a prevalence of 6.4% in South Africa for CKD; where n is the sample size, Z is the statistic corresponding to level of confidence of 95%, P is expected prevalence (6.4 %) and d is precision which reflects the effect

size and was estimated to be 5% [16, 17]. A minimum of 92 samples from CKD patients were required to demonstrate a difference between the two assays.

Samples were collected in BD Vacutainer® EDTA tubes™ (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Samples of sufficient volume and within recommended stability period of 48 hours when stored at 2 - 8 °C were included. The samples were initially analysed using the routine third generation, Roche PTH 1-84 assay and then immediately re-analysed using the newly validated second generation, Roche intact PTH assay. The instrument middleware was set up, to allow analysis of second generation PTH immediately after the requested third generation PTH was analysed to minimise time delays between analyses.

To aid with clinical interpretation of PTH results; data was collected from the NHLS TrakCare™ (InterSystems, Cambridge, MA, USA) laboratory information system (LIS) and included: age, gender, calcium, phosphate and creatinine. The glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) 2009 equation without the race factor [18].

Ethical approval

This study has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificate No. M210475) and performed in line with the Declaration of Helsinki.

Laboratory Analysis

A verification study for second generation PTH assay was performed according to CLSI EP15-A3 guideline [19]. This was done because the second generation PTH assay was not validated on the

Roche Cobas e602 module at CMJAH. Assay performance was within the acceptable limits of the manufacturer's claims with intra-assay and inter-assay variability of 0.9% and 2.2 % respectively.

The PTH assays' analytical performance was compared as per the CLSI EP 09 - A3 guideline [20]. Analyses of samples for PTH were performed using Roche intact PTH (second generation) and PTH 1-84 (third generation) reagents and calibrators on an e602 module of a Cobas 8000© system from Roche Diagnostics (Mannheim, Germany). The analytical characteristics of the PTH assays used are depicted in table 1[21, 22]. Biochemical parameters including creatinine, total calcium and phosphate were measured using standard chemistry methods on the Roche Cobas 8000 analyser 702 module.

Table 1: Analytical Characteristics of Roche PTH Assays used.

	Elecsys Intact PTH (Second generation)	**Elecsys PTH 1-84 (Third generation)**
Methodology	Electrochemiluminescent immunoassay (ECLIA) C terminal epitope: ruthenium labelled monoclonal mouse antibodies reactive with epitopes in the amino acid region 38 - 84. N terminal epitope: biotinylated monoclonal mouse antibodies reactive with epitopes in the amino acid regions 26 - 32 and 37 - 42.	ECLIA Monoclonal mouse antibodies used. A biotinylated monoclonal antibody reacts with the N-terminus of the PTH molecule and a ruthenium labelled monoclonal antibody reacts with the C-terminal fragment PTH. Epitopes not explicitly stated.
Limit of Detection (pmol/L)	0.127	0.583

Measuring Range (pmol/L)	0.127 – 530	0.583 – 244
Calibration Standard	Traceable to a commercial PTH (RIA) assay.	Traceable to the WHO International Standard 95/646.
Reference Interval (pmol/L)	1.6 – 6.9	1.58 – 6.03
Total analytical coefficient of variation (CV) (%)	6.24 %	5.62 %

120

121 **Statistical analysis**

122 The data obtained was captured onto Microsoft Excel® (Microsoft, Seattle, WA, USA) and
123 analysed using MedCalc®, version 200218 (MedCalc Software, Ostend, Belgium). The Shapiro-
124 Wilks test was used to assess for normality. Data were demonstrated to be non-parametric and thus
125 expressed as median values with interquartile range (IQR). Categorical data were expressed as
126 proportion and percentage. The Wilcoxon test was used to compare PTH concentrations between
127 the two assays.

128 Intact PTH was used as the comparative method (X) and PTH 1-84 as the test method (Y). Passing-
129 Bablok regression was used to assess method agreement and the Bland-Altman difference plot was
130 used to determine the magnitude of bias between the two methods. The desirable analytical
131 performance specifications for PTH were derived from the European Federation of Clinical
132 Chemistry and Laboratory Medicine (EFLM) biological variation database [23]. A percentage bias
133 of ±7.1 % was used as the allowable limit of acceptable analytical performance.

134 The McNemar test was used to determine if there were significant differences in the classification
135 of patients and if the clinical interpretation changes when one assay is used over the other. A p-
136 value of <0.05 was considered statistically significant.

CKD patients on dialysis were classified as above, below, or within the PTH target range of 2 – 9 times the upper limit of normal (ULN) as recommended by the KDIGO guidelines, using the two PTH assays. A kappa statistic was used to assess the performance of the classification using both assays with a value of ≥ 0.60 representing acceptable agreement [24].

RESULTS

Method Comparison

A total of 505 residual patient samples were identified for the study. However, 24 samples were excluded; 17 had results exceeding the measuring range of the Elecsys PTH 1-84 assay and seven had exceeded the stability period. Thus, 481 samples were analysed and utilised for the method comparison study.

The median PTH concentration for third generation was significantly lower at 8.60 pmol/L compared to the second generation median of 9.93 pmol/L, p <0.0001. The regression analysis equation was calculated as $y = 0.713x + 0.887$ (Fig 1). There was good correlation between the two methods with a coefficient correlation (r) = 0.994 and p <0.0001. The intercept was calculated at 0.887 pmol/L (95% confidence interval 0.788 – 1.005) and the slope at 0.713 pmol/L (95% confidence interval 0.703 - 0.723); indicating that there were systematic and proportional differences between the two methods, with increased deviations observed at higher PTH concentrations.

Fig 1: Scatter plot and regression analysis comparing Intact PTH and PTH 1-84 for all samples.

The Bland-Altman analysis showed an average overall difference between the PTH assays of 9.00 pmol/L (18.5%, $p < 0.0001$) among all samples analysed (Fig 2 and 3). The bias in the entire CKD group and predialysis CKD group was 27.2% and 25.3% ($p<0.0001$) respectively (Figures 4 and 5). The dialysis group, however, demonstrated the highest bias of 35.2% ($p<0.0001$) (Figure 6). This is in stark contrast to the difference observed within the non-CKD group with eGFR >60 ml/min/1.73m^2, which was demonstrated to be 5.7% ($p<0.0001$) (Fig 7).

Fig 2: Bland-Altman plot comparing Intact PTH and PTH 1-84 assays for all PTH samples.

Fig 3: The percentage difference plot comparing Intact PTH and PTH 1-84 assays for all PTH samples.

Fig 4: Bland-Altman plot of Intact PTH and PTH 1-84 assays for the entire CKD group.

Fig 5: The percentage difference plot comparing Intact PTH and PTH 1-84 assays in predialysis CKD group.

Fig 6: Bland-Altman plot of Intact PTH and PTH 1-84 assays in the dialysis group.

Fig 7: Bland-Altman plot of Intact PTH and PTH 1-84 assays in the non-CKD group with eGFR >60 ml/min/1.73 m^2

Clinical Interpretation

The clinical performance of the two assays was assessed in the diagnosis of hypoparathyroidism and hyperparathyroidism and in CKD patients, including those on dialysis. A total of 276 patients were used for this purpose as depicted in the flow diagram (Figure 8). The median age was 52 years with majority being female (62%) (Table 2).

Table 2: Demographics and biochemical data for the patient samples used in clinical interpretation.

Parameters	Overall	CKD	Non-CKD
Age (years)	52.50 (49.00 – 54.00)	46.90 (44.61 – 49.19)	55.00 (54.00 – 59.00)
31%)Sex (n)	276	146	130
Female n (%)	170 (62%)	80 (55%)	90 (69%)
Male n (%)	106 (38%)	66 (45%)	40 (31%)
Parathyroid Hormone (PTH)			
Intact PTH	12.33 (10.52 – 16.96)	26.28 (20.84 – 36.28)	6.75 (5.68 – 8.10)
PTH 1-84	10.36 (9.10 – 12.81)	19.95 (15.50 – 27.29)	6.05 (5.00 – 7.04)
Serum Calcium (mmol/L)	2.30 (2.27 – 2.32)	2.26 (2.20 – 2.30)	2.36 (2.31 – 2.41)
Phosphate (mmol/L)	1.26 (1.17 – 1.36)	1.30 (1.19 – 1.42)	1.16 (1.06 – 1.33)
Creatinine (μmol/L)	306.00 (2264.55 – 417.04)	599.00 (428.03 - 697.86)	90.00 (82.08 – 107.93)
eGFR (ml/min/1.73m^2)	16.00 (12.00 – 22.45)	8.00 (6.00 – 10.00)	66.00 (52.23 – 74.92)
CKD Stage 1 (3)		93	
CKD Stage 2 (9)		65.00 (58.41 – 81.03)	
CKD Stage 3a (9)		52 (45.28 – 56.00)	
CKD Stage 3b (9)		36.00 (33.14 – 37.86)	
CKD Stage 4 (18)		19.00 (16.00 – 21.22)	
CKD Stage 5 (70)		5.50 (5.00 – 6.00)	
CKD Stage 5D (28)		5.50 (4.36 – 7.00)	

CKD - chronic kidney disease diagnosed with > 3-month history of eGFR<60 ml/min/1.73 m^2.

Non-CKD - Patients without confirmed history of CKD and with estimated glomerular filtration

rate (eGFR). Values are expressed as median (25th and 75th interquartile) or number of participants (percentage).

Fig 8: Flow diagram of PTH samples used for clinical interpretation.

The McNemar test did not demonstrate any statistically significant differences in clinical interpretation as shown by p values above 0.05 in the detection of low, normal and high PTH using both assays. In both the CKD and dialysis group, no significant differences in interpretation were found using McNemar as shown in table 3.

Table 3: Differences in clinical interpretation using manufacturer reference range.

	Category	Intact PTH (n, %)	PTH 1-84 (n, %)	p value
Overall	PHPT	15 (5.4%)	16 (5.8%)	1.0000
	SHPT	185 (66.5%)	184 (66.2%)	1.0000
	Normal	57 (20.5%)	58 (20.9%)	1.0000
	Hypoparathyroidism	20 (7.2%)	21 (7.6%)	1.0000
CKD	SHPT	133 (91.1%)	135 (92.5%)	0.500
	Normal	11 (7.5%)	9 (6.2%)	0.500
	Hypoparathyroidism	2 (1.4%)	2 (1.4%)	UTC
Dialysis	SHPT	28 (100%)	28(100%)	UTC

PHPT – primary hyperparathyroidism; SHPT – secondary hyperparathyroidism; UTC – unable to calculate and perform test.

The clinical impact of using either second or third generation in the classification of CKD patients on dialysis, in terms of the KDIGO guidelines was assessed. Patients were classified as below,

above or within the recommended PTH target range of 2 to 9 times the upper limit of normal. For the kappa analysis, 29 confirmed patients on dialysis were included. Classification according to KDIGO revealed minimal discordance between the two generations, with a κ coefficient of 0.79 (Table 4).

Table 4: Classification According to KDIGO guidelines for Intact PTH and PTH 1-84 Assays

Manufacturer ULN (pmol/L)	< 2 x ULN	2 – 9 x ULN	>9 x ULN
Intact PTH (1.6 – 6.9)	0 (0.0%)	15 (53.6%)	13 (46.4%)
PTH 1-84 (1.6 – 6.0)	1 (3.6%)	16 (57.1)	11 (39.3%)

ULN – upper limit of normal.; Weighted Kappa = 0.79710 (0.594 – 1.000)

DISCUSSION

Currently two different generations of PTH assays exist in the market, with significant inter-assay variability [3]. The lack of standardisation between these assays has created some confusion for clinicians especially in the management of CKD patients.

We found that there was a significant inter-assay bias between the second and third generation PTH assays. However, there was no significant change in the clinical classification of hypoparathyroidism, PHPT, and secondary hyperparathyroidism in predialysis and dialysis CKD patients when one assay was used over the other.

With regards to analytical performance, the methods were strongly correlated. However, as the percentage mean bias surpassed the EFLM biological variation goal, this implies that these assays are not interchangeable nor comparable. Our study showed that for the entire patient group, the third generation PTH assay produced results that were on average approximately 20% lower than the second generation assay, and even lower at 27% and 35% in CKD and dialysis patients, respectively. This is in keeping with other reported research [3, 14, 15, 25-27]. Studies by Hashim et al and O'Flaherty et al, both using Roche PTH assays found that third generation assays were lower by 30% and 40% compared to second generation assays [28, 29]. In contrast, in the non-CKD group with eGFR above 60 ml/min/1.73 m^2, the observed bias was 5.7%. This indicates that in patients with normal GFR, there are minimal differences between second and third generation assays, which is keeping with other reported studies [26, 28].

Although the observed differences between the two PTH assays were significantly higher in both the predialysis and dialysis CKD groups, there was no change in interpretation of secondary hyperparathyroidism with either assay. The KDIGO guidelines do not state the target PTH range in predialysis CKD patients [15]. Furthermore, we did not find studies that assessed the impact on clinical interpretation in this group.

There is a paucity of studies that have compared second and third generation PTH assays in dialysis patients using the KDIGO classification of 2 – 9 fold of the ULN [26, 30]. In the dialysis group, using the manufacturer's ULN, classification using KDIGO did not demonstrate statistically significant discordance between the two assays. This study was limited by a small sample size of confirmed dialysis patients which reflects the limited dialysis resources in our country. However, our finding was in contrast to a few published studies; including that by Dupuy et al, which demonstrated a high percentage of discordant results, with a κ coefficient of <0.20 [26]. In addition,

a study by Beko et al, found that using the manufacturer's ULN, the clinical classification was altered in up to 23% of dialysis patients because of the switch from second to third generation PTH assay [30]. Presence of discordance is important as PTH concentrations are used in the management of CKD-MBD. Hence, the use of second over third generation PTH assays may result in misclassification leading to inadequate management [18].

There have been various attempts to reduce the discrepancies between the second and third generation PTH assays in dialysis patients; by applying a correction factor or deriving an equation to allow interconversion between the two assays [31]. Some authors have suggested the use of 1–84/7–84 PTH ratio to report PTH results to minimise potential misdiagnosis of CKD-BMD in CKD patients [31]. However, further validation studies are needed, and these efforts are not currently adopted by clinical guidelines.

Overestimation of PTH by second generation assays because of PTH fragment cross-reactivity is of negligible consequence in patients with primary hyperparathyroidism (PHPT). Nonetheless, the question whether third generation PTH assays offer superior diagnostic efficacy remains. In our study, there were no differences in interpretation between the two assays with regards to diagnosis of PHPT. This is consistent with other published studies [32, 33]. Taking into consideration published evidence, the recent international guideline on evaluation and management of PHPT, recommend the use of either second or third generation PTH assays [34].

The IFCC Committee for Bone Metabolism has established a working group for the purpose of creating PTH standardisation to make the results comparable among different assays [35]. In the absence of a reference measurement method, the IFCC has recommended the use of the WHO standard, but this has not been completely implemented due to lack of commutability [9, 10]. Even though second generation PTH assays are the most utilised assays, third generation PTH assays

offer the best hope for harmonisation of PTH because majority are traceable to the WHO standard [35].

To the best of our knowledge, this is the first study in Africa to evaluate the differences in analytical performance and impact on clinical interpretation of second and third generation PTH assays. However, our study had a few limitations. Firstly, we had a small sample size of patients on dialysis. Secondly, the study was based on information available on LIS with limited clinical information.

CONCLUSION

The second and third generation PTH assays correlate very well, however there was significant bias observed between the two methods which increases with rising PTH concentrations that prevail in CKD patients. However, using the manufacturer's assay specific reference intervals and KDIGO guideline on PTH classification, there were no major changes in clinical interpretation when either assay was used. Thus, the decision to use third over second generation PTH should take all this into account. We recommend that assays not be used interchangeably. Therefore, as stated by the manufacturer and in accordance with the KDIGO recommendations, clinical laboratories should state the generation of PTH assay employed and inform clinicians of any change in clinical methods to facilitate appropriate interpretation of PTH results.

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ORIGINALITY REPORT

8%	3%	9%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Anne Marie Dupuy, Anne Sophie Bargnoux, Marion Morena, Emilie Lauret et al. "Moving from the second to the third generation Roche PTH assays: what are the consequences for clinical practice?", Clinical Chemistry and Laboratory Medicine (CCLM), 2018 Publication	2%
2	Flaherty, D.O', A. Sankaralingam, P. Scully, P. Manghat, D. Goldsmith, and G. Hampson. "The relationship between intact PTH and biointact PTH (1-84) with bone and mineral metabolism in pre-dialysis chronic kidney disease (CKD)", Clinical Biochemistry, 2013. Publication	1%
3	Kittrawee Kritmetapak, Chatlert Pongchaiyakul. "Parathyroid Hormone Measurement in Chronic Kidney Disease: From Basics to Clinical Implications", International Journal of Nephrology, 2019 Publication	1%

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UNIVERSITY OF THE WITWATERSRAND

FACULTY OF HEALTH SCIENCES

SCHOOL OF PATHOLOGY

Comparison of 2nd and 3rd generation Parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic Hospital – Are they fit for purpose?

Research Proposal

In partial fulfilment of the requirements for the degree Master of Medicine:

Chemical Pathology

Submitted by

Dr Nokuthula Nhlapo

Student number: 305014

Supervisor:

Dr M Maphayi

Co - supervisor:

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Comparison of 2nd and 3rd generation Parathyroid hormone (PTH) assays performed at Charlotte Maxeke Johannesburg Academic hospital – Are they fit for purpose?

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BACKGROUND

Chronic kidney disease (CKD) is a global health problem with a prevalence of 5 - 10% (1). In Africa, CKD poses a major public health concern particularly against a background of limited resources (2). The rapid epidemiological transition and increasing incidence of hypertension, HIV, and diabetes, which are independently associated with CKD, are the driving force fuelling this disorder (2, 3). Patients with this disease have an increased risk of CKD-mineral and bone disorder (CKD-MBD) which is associated with high morbidity and mortality (1). CKD-MBD is defined as a systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following: (i) abnormalities of calcium, phosphorus, parathyroid hormone (PTH), or vitamin D metabolism; (ii) abnormalities in bone turnover, mineralization, volume, linear growth, or strength; or (iii) vascular or other soft tissue calcification (1). Various clinical guidelines have been proposed in an effort to reduce the adverse clinical outcomes associated with CKD-MBD. These guidelines make use of serum biomarkers including parathyroid hormone to assess, monitor and manage this disorder appropriately (1).

PTH is a key regulator of calcium and phosphate haemostasis and bone remodelling (4). PTH determination is crucial in the diagnostic workup of hypocalcaemia and hypercalcaemia; assessment of secondary hyperparathyroidism in CKD patients who are at risk of developing CKD-MBD; and in the perioperative assessment in thyroid or parathyroid surgery (1, 4). Therefore, accurate and reliable PTH assays are needed.

PTH is composed of a single polypeptide produced exclusively by the chief cells of parathyroid glands, with a molecular weight of ~9.5 kDa (5). PTH is synthesised as a pre-prohormone consisting of 115 amino acids that is sequentially cleaved at the amino terminal to yield the 90 amino acid prohormone and the mature biologically active 84 amino acid molecule (5). The latter form, (1-84) PTH, consists of two main parts; the amino

(N) terminal made up of the first 34 amino acids and the carboxyl (C) terminal that contains the remaining 50 amino acids. PTH exerts its classical biological effects on kidney and bone by binding of the first 34 amino acids to the type 1 PTH receptor (PTHR1) in target tissues (6).

Post synthesis, PTH is secreted predominantly as intact (1-84) PTH, which is rapidly degraded to produce multiple fragments. This is a result of intraglandular secretion by the parathyroid gland in a calcium dependent manner and peripheral metabolism in the liver Kuppfer cells and kidneys (6). In circulation, PTH exists as intact PTH and fragments i.e., N-terminal, C-terminal and mid region PTH. The plasma half-life of intact PTH is between 2 and 4 minutes (6). In comparison, C-terminal PTH (C-PTH) fragments have a longer half-life of several hours as they undergo renal clearance. They may account for more than 20% of circulating PTH in normal individuals and up to 45% in patients with renal failure due to the decline in glomerular filtration (6). Thus, laboratory measurement of PTH has been made difficult by the heterogeneity of PTH fragments.

Measurement of PTH

Pre-analytical Considerations

Optimisation of the pre-analytical phase is crucial for correct PTH determination and accurate interpretation of results as PTH is relatively labile. Pre-analytical factors that can influence PTH analysis include: specimen type, storage, stability, sampling site and time (7). PTH has been measured in whole blood, serum or Ethylenediaminetetracetic acid (EDTA) plasma (7). The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group on PTH, strongly recommends the use of EDTA plasma due to the stability of PTH when compared to serum (7, 8). PTH is more stable in plasma than in serum as the presence of EDTA chelates divalent ions required for adequate functioning of metallo-proteases that digest the PTH peptide. Additionally, the clotting

process that occurs in serum, results in the release of thrombin that can cleave PTH, interfering with antibody binding in immunoassays (9). The major disadvantage of plasma is that it cannot be used for simultaneous PTH and calcium determination, which is possible with serum (8, 9).

PTH Standardisation

Currently, there is no reference method for PTH measurement. Liquid chromatography coupled with mass spectrometric (LC-MS) or tandem MS (LC-MS/MS) detection methods for PTH quantitation have been developed (8, 9). Thus, MS based techniques may be developed as candidate reference measurement procedure but more studies are still required in this regard (10). The IFCC working group on PTH standardisation has proposed the use of a single recognised international standard e.g. WHO PTH 95/646 (9). However, it is yet to be implemented, as commutability has not been formally established. All 3 generations of PTH assays developed have been calibrated using different standards. This has contributed to a lack of comparability between results obtained using different PTH assays (8).

PTH Assays

In 1963, Berson et al, described the radioimmunoassay (RIA) as the first generation assay for PTH analysis (11). It was a competitive assay that utilised a single polyclonal antibody generated against epitopes in the mid or C-terminal end of the PTH peptide (7, 11). The RIAs had several limitations, including cross reactivity with the biologically inactive C-fragments, which led to poor sensitivity and specificity (10, 11). This was more evident in patients with CKD, where decreased renal clearance of the C-fragments leads to their plasma accumulation and thus overestimation of PTH levels (11). To overcome this issue, the immunometric assays were developed.

Second generation assays were introduced in 1987 (7, 11). They are non-competitive, sandwich immunoassays that make use of two antibodies. The first is a solid phase capture antibody generated against epitopes located within the C-terminal end of PTH (amino acid position 39 – 84) and a second detection antibody directed against either the proximal (amino acid position 12 – 24) or distal (amino acid position 26 – 32) portion of the N-terminal region (7). It was assumed that this assay detected the bioactive (1-84) PTH and avoided cross reactivity with C-fragments thus it was referred to as the "intact" PTH (I-PTH) assay (11, 12).

Brossaud et al showed that I-PTH assay overestimated values in patients with renal failure compared to PTH values in normal subjects due to the accumulation of a non (1-84) PTH molecular form (11). This consisted of large C-terminal PTH fragments with a partially preserved N-terminus, and were therefore termed N-truncated PTH fragments. The most abundant is (7-84) PTH, so called as it co-eluted with synthetic (7-84) PTH during high pressure liquid chromatography (HPLC) (4). These fragments were found to accumulate in renal failure where they represent up to 45% of circulating I-PTH but only 20% in those with normal renal function (6). Further studies, revealed that (7-84) PTH, unlike most C-fragments, has biological activity (5). It was shown that this form antagonises the biological action of (1-84) PTH in bone and kidney (5, 9). This would partially explain the PTH resistance that is seen in CKD (9).

The 3rd generation PTH assays were introduced in the late 1990s. They are sandwich immunoassays that use a similar capture antibody as 2nd generation assays. However, the detection antibody is directed against epitopes in the first 4 amino acids of the N-terminus, which avoids cross reactivity with (7-84) PTH fragments (7, 11). The 3rd generation assays were termed bio-intact (bio-PTH) or whole PTH assays, as they were more specific for (1-84) PTH. However, they also detect post-translationally modified PTH forms including amino-PTH; Amour et al described it as having a phosphorylated N-

terminus serine residue (7, 11). It is overproduced in severe hyperparathyroidism and parathyroid carcinomas and has been found to cross-react with 3rd generation but not 2nd generation assays (11).

Another post-translational modification that has been demonstrated is oxidation, which occurs on methionine at positions 8 and 18 (4). Oxidised PTH is not biologically active and has been found to accumulate in renal failure, due to the elevated levels of oxidative stress present in this condition. A recent study has shown that measuring non-oxidised PTH correlated better with clinical outcomes, including mortality than measuring both oxidised and non-oxidised forms (4). Since both 2nd and 3rd generation assays cannot differentiate between oxidised and non-oxidised PTH there may be a need for a 'fourth' generation PTH assay (5).

Rationale for the Study

The 2nd generation assays have provided the basis for the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines (12). Studies have shown that there is significant inter-method variability between 2nd generation assays; thus, they are incomparable among one another (13). Souberbielle et al tested 15 different commercial PTH assays, of which 2 were 3rd generation assays. The Allegro intact PTH assay was used as a reference method in the study. Their results showed that the median bias between the Allegro and the other assays was between -44.9 - 123.9% and that when the serum PTH concentration was 150 or 300 ng/L, as measured by the reference assay, it ranged from 83 - 323 ng/L and 160 - 638 ng/L when compared to the other assays (13). Taking this into consideration, the recent KDIGO guidelines proposed utilisation of multiples of 2-9 fold of the upper limit of normal for a given PTH assay instead of absolute values (12). The application of the KDIGO guidelines using the manufacturers' published reference ranges has improved the inconsistencies in classification of dialysis patients.

However, Cavalier et al, found 36% of patients were still misclassified when 10 different PTH assays were used (12, 14).

Research has shown 3rd generation assays produce results that are about 50 - 60% lower in CKD patients and 15% lower in non CKD subjects when compared to 2nd generation assays (10). Dupuy et al, compared 2nd and 3rd generation assays by Roche in healthy, pre-dialysed and haemodialysed patients. The results revealed that in healthy subjects the PTH concentration did not differ among the two assays and that at concentration > 200 pg/ml there was an overestimation of PTH with 2nd generation assays (15).

In a study by Hecking et al, involving haemodialysis patients, I-PTH was compared to two bio-intact assays from Diasorin and Roche. PTH results from bio-intact assays were a third lower with increased deviations at higher concentrations (16). Bonansea et al investigated the usefulness of 3rd generation assays in diagnosis of primary hyperparathyroidism (PHPT) (17). Bio-PTH was compared to I-PTH utilising Roche assays. In PHPT, median PTH values from 3rd generation assays were lower than those from 2nd generation. Although there was good correlation ($r = 0.952$, $p < 0.0001$), Bland-Altman analysis revealed poor agreement between the assays with observed differences increasing with higher concentrations. They concluded that 3rd generation assays are not superior to the 2nd generation assays in the diagnosis of PHPT; however, the sample size was small for this conclusion.

Third generation assays are more specific than 2nd generation PTH assays and should theoretically be more accurate in the evaluation of PTH status. Yet, there is still debate as to which assay is preferable and whether the newer assays offer improved clinical value (4). Bio-intact assays are seemingly ideal as they exclusively measure full length PTH (1–84). However, there is insufficient research, when compared to that of I-PTH assays, for the 3rd generation to be universally adopted in clinical practice (17). Clinical studies have

not demonstrated the superiority of 3rd generation assays over 2nd generation assays in the assessment of bone turnover in CKD (4). I-PTH assays are readily available, cost efficient, extensively researched and part of current clinical guidelines. In contrast, bio-PTH are not well researched and commonly used (12). A table summarising 2nd and 3rd generation assays has been provided (see Annexure).

In the Johannesburg area, two academic hospitals currently use either the 2nd or 3rd generation assays. At Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), the National Health Laboratory Services (NHLS) uses the 3rd generation assay whereas Chris Hani Baragwanath Academic Hospital (CHBAH) utilises the 2nd generation assay. Interestingly, only 4.7% of laboratories surveyed as part of the CMJAH laboratory's proficiency scheme use the 3rd generation assays. There is considerable movement of patients between the two hospitals, which may affect PTH interpretation, and clinical decision making depending on the assay used. The reference ranges between the 2nd and 3rd generation PTH assays offered at CHBAH and CMJAH are not that dissimilar; at 1.6 – 6.9 pmol/L and 1.6 – 6.0 pmol/L respectively (18,19).

To our knowledge, there are no studies comparing the performance of 2nd and 3rd generation PTH assays in the South African population, which has an increasing incidence of CKD

AIM OF STUDY

The aim is to compare the 2nd generation and 3rd generation PTH assays to ascertain whether they are fit for purpose in terms of analytical and clinical performance.

STUDY OBJECTIVES

Primary objectives:

- To evaluate the analytical performance of the 2nd generation assay using quality control materials according to CLSI EP 15 protocol.
- To determine the analytical performance and agreement between the two PTH assays using patient samples according to CLSI 09 protocol.

The secondary objectives

- To determine the clinical interpretation of PTH results when 2nd and 3rd generation assays are used.
- To determine misclassification of patients with CKD based on the KDIGO guidelines, with both assays.

MATERIALS AND METHODS

STUDY DESIGN

Experimental method comparison study

STUDY POPULATION

The study will be conducted on remnant patient EDTA plasma samples with a PTH requested at CMJAH.

Inclusion criteria

Patient samples from CMJAH with enough volume. Collected in a suitable tube and within the stability period i.e. 24 hours or less at 2 - 8°C. Concomitant serum calcium, phosphate, creatinine, estimated glomerular filtration rate (eGFR) or 25 hydroxyvitamin D results to aid in the interpretation of PTH results. These results will be obtained from TrakCare via the Academic Affairs, Research, and Quality Assurance (AARQA) approval.

Exclusion criteria

Samples that have exceeded the stability period or have insufficient volume. Samples from other referral sites.

SAMPLE SIZE

According to CLSI EP-09, in a method comparison study, a minimum of 40 specimens spanning the measuring range is adequate. A minimum of 147 samples from CKD patients will be required to demonstrate a difference between the two assays, using a prevalence of 10.7% in Sub-Saharan Africa (3). This value was derived using the following equation

$n = \frac{Z^2 P(1-P)}{d^2}$; where n is the sample size, Z is the statistic corresponding to level of confidence of 95%, P is expected prevalence (10.7%) and d is precision which reflects the effect size and was estimated to be 5% (20).

MEASUREMENT OF PTH

PTH measurements will be performed on a Roche Cobas 8000 analyser using EDTA plasma samples that have been stored at 2 - 8 ˚C for no longer 48 hours. Quality control will be done prior to analysis of specimens and the instrument will be calibrated according to the manufacturer's instructions. Samples will initially be run using the 3rd generation assay and then re-analysed using a validated 2nd generation assay. This will be set-up to run automatically, on the instrument middleware, to prevent delays.

The Roche Elecsys intact PTH assay is a 2nd generation electrochemiluminescence immunoassay (ECLIA). It utilises a sandwich principle in which a biotinylated monoclonal antibody reacts with the N-terminal and a monoclonal antibody labelled with a ruthenium complex reacts with the C-terminal (18). The antibodies used are reactive with epitopes in the amino acid regions 26-32 and 37-42 respectively. It has been standardised against a commercial RIA.

The Roche Elecsys PTH (1-84) assay is a 3rd generation sandwich ECLIA. It uses a biotinylated monoclonal antibody against the N-terminal amino acids and a ruthenium complex-labelled monoclonal antibody that reacts with the C-terminal amino acid residues (19). The precise location of the N-terminal epitope is not stated by the manufacturer. It is standardised against the WHO international standard 95/646.

DATA ANALYSIS

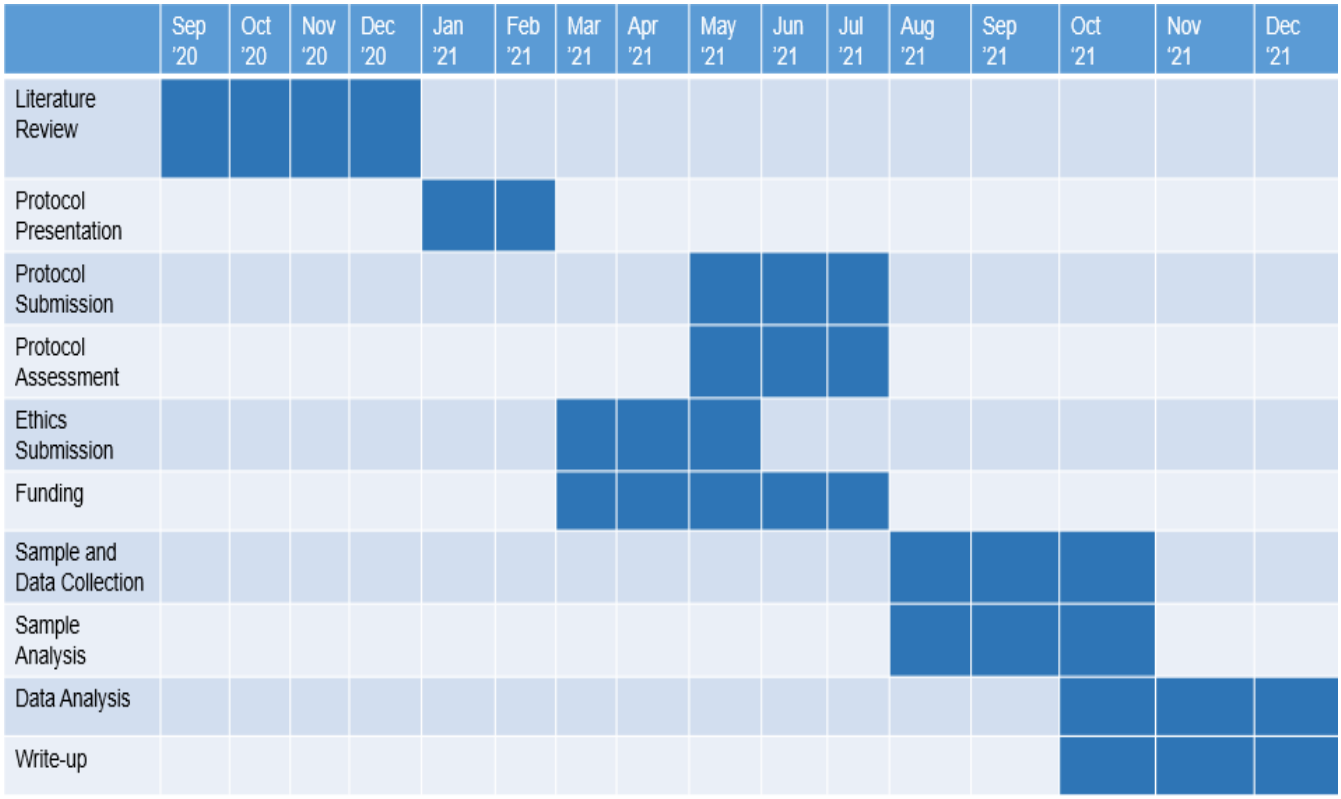
The data obtained will be captured onto Microsoft Excel®. For statistical analysis, MedCalc software version 19.4.1 (MedCalc Software Ltd, Ostend, Belgium) will be used. Normally distributed data will be presented as mean and standard deviation (SD) and non-parametric data as median and interquartile range.

Passing-Bablok regression analysis will be performed to evaluate the relationship between the two assays. Bland-Altman difference plot will be used to determine the magnitude of bias between the two methods. A paired t-test will be used to compare the difference between the means. Should data not be normally distributed, a non-parametric equivalent test (Wilcoxon signed rank test) will be used. The McNemar test will be used to determine if there is a change in clinical interpretation when one assay is used over the other. A p-value of <0.05 will be considered statistically significant.

ETHICAL CONSIDERATION

An application for ethics clearance from the University of the Witwatersrand Human Research Ethics Committee (Medical) will be submitted. No patient consent is required as remnant samples will be used.

TIMELINE



BUDGET AND FUNDING

Item	Cost	Quantity	Total Cost
Reagent Kit (100 tests/kit)	R2824	3	R8472
Calibrator	R1042.73	3	R3128.19
Controls	R1498.44	3	R4495.32
			R16095.51

An application for funding will be submitted to WITS Postgraduate Funding and the NHLS K-funding.

POTENTIAL LIMITATIONS OF THE STUDY

- Patient's clinical data as obtained from patient files will not be utilised.
- Vitamin D levels may not be available for all patient samples.
- Not getting sufficient samples to cover the measuring range.

- Due to temporary closure of CMJAH, sample collection may be delayed until the hospital is operational.

OUTCOMES OF THE STUDY

- Submission as part of requirements for Master of medicine (MMed) degree.
- Publication in a peer reviewed accredited journal.
- Possible future laboratory recommendations for the use of 2nd or 3rd generation assays by NHLS based on the study findings.
- Potential guidance for clinical utility.

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Annexure1: Table summarising studies on 2nd and 3rd generation PTH assays

First Author	Study population	PTH assay used: 2nd (intact) or 3rd (bio-intact) generation	Main Findings
Souberbielle J-C (13)	47 serum pools from dialysis patients.	Comparison of 15 commercial PTH assays of which; 13 - 2nd generation 2 - 3rd generation	Wide inter –method variability within the 2nd generation assays.
Cavalier E (14)	149 haemodialysed patients 240 healthy subjects	Compared PTH results from 10 PTH assays: 8 were 2nd generation 2 were 3rd generation.	36% of patients were still misclassified when 10 different PTH assays were used.
Dupuy AM (15)	46 healthy subjects 103 pre-dialysis 73 haemodialysed patients	Comparison of 2nd and 3rd generation Roche PTH assays	In healthy subjects, the PTH concentration did not vary. In CKD there were observed differences between the generations with increasing PTH levels and decreasing GFR.

Hecking M (16)	121 haemodialysed patients	Two 3rd generation PTH assays from different manufactures (Roche and DiaSorin) and Roche 2nd generation assay.	Roche and DiaSorin bio-intact PTH assays were well correlated, but showed increased deviations at higher concentrations. Bio-intact PTH assays were roughly two thirds of the intact PTH.
Bonansea TC (17)	41 primary hyperparathyroidism (PHPT) patients 543 Healthy control subjects	2nd and 3rd generation Roche assays.	In PHPT, median PTH values from 3rd generation assays were lower than those from 2nd generation. Although there was good correlation ($r = 0.952$, $p < 0.0001$), Bland-Altman analysis revealed poor agreement between the assays with observed differences increasing with higher concentrations