Original Article

European Biological Variation Study (EuBIVAS): within- and between-subject biological variation estimates for serum biointact parathyroid hormone based on weekly samplings from 91 healthy participants

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Background: The European Biological Variation Study (EuBIVAS) was created by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group on Biological Variation to establish high-quality biological variation (BV) estimates for clinically important measurands. In this study, the aim was to deliver reliable BV estimates for the biointact parathyroid hormone (PTH 1-84).

Methods: Serum samples were obtained from a population of 91 healthy individuals (38 men, 43 pre-menopausal women, and 10 post-menopausal women; 21–69 years) from 5 European countries, with all samples stored at −80 ℃ prior to analysis. PTH 1-84 analysis was performed at the San Raffaele Hospital (Milan, Italy) on the Roche Cobas e801. All samples from each individual were analysed in duplicate within a single run. CV-ANOVA was applied, after analysis of variance homogeneity and outliers, to obtain BV estimates for PTH 1-84 with 95% CIs.

Results: The within-subject BV [CV_I (95% CI)] estimates were significantly different between men and women [13.0% (12.1–14.2%) and 15.2% (14.3–16.3%), respectively], while the between-subject estimates [CV_G (95% CI)] were similar (men: 26.8% (21.4–35.1%), pre-menopausal women: 27.8% (22.7–36.1%)], allowing for delivery of updated analytical performance specifications and reference change values.

Conclusions: Updated BV estimates for serum PTH 1-84 based on the large-scale EuBIVAS may be beneficial for the diagnosis and management of parathyroid glands and bone turnover pathologies.

Keywords: Biological variation (BV); parathyroid hormone (PTH); PTH 1-84; reference change values

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Introduction

Parathyroid hormone (PTH) is the secreted product of the parathyroid glands chief cells, and its production and secretion is predominantly regulated by the extracellular calcium concentration. In fact, extracellular calcium binds the Calcium Sensing Receptor (CaSR), situated at the level of parathyroid cell membrane, which activates an intracellular signalling that results in the inhibition of PTH secretion. Also, the active 1,25-dihydroxyvitamin D is able to inhibit PTH production. Once secreted, PTH plays an essential role in regulating extracellular calcium and phosphate homeostasis (1). Reduced extracellular calcium concentration induces PTH production which in turn directly enhances calcium and inhibits phosphate reabsorption by the kidneys. In the kidney, PTH also stimulates renal 1-α hydroxylase, thus inducing the conversion of the inactive 25-hydroxyvitamin D to the active 1,25-dihydroxyvitamin D, which then promotes calcium absorption at the intestinal level. At the same time, PTH acts on bone remodelling: persistent increased PTH levels, caused by reduced extracellular calcium concentration, induce bone resorption resulting in calcium and phosphate release from bone. PTH exerts also an anabolic activity on bone, in fact, it is known that an intermittent PTH administration may also stimulate bone formation in osteoporosis patients (1). In clinical practice, PTH in blood is an essential and routinely used biomarker for the assessment of primary/secondary hyperparathyroidism, hypoparathyroidism, calcium-phosphate metabolism disorders and, as recommended by the Kidney Disease Improving Global Outcomes (KDIGO) guidelines (2), for monitoring patients with chronic kidney disease (CKD) and the associated bone mineral pathologies (MBD) (1,3,4). In the bloodstream, PTH circulates in different molecular forms, as: (I) PTH 1-84, also described as biointact PTH, which is a peptide composed by 84 amino acids and the most bioactive form of the hormone; and as (II) numerous truncated forms, such as PTH 7-84 and other smaller fragments. The available assays for PTH measurement recognise the truncated forms to different extent, thus leading to significant between-method differences in PTH evaluation. The second-generation assays, defined as intact PTH assays, recognise both PTH 1-84 and truncated fragments, especially PTH 7-84, whereas third-generation assays, defined as biointact PTH assays, specifically detect only PTH 1-84 (3). The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Committee for Bone Metabolism (C-BM) is presently working on the standardization of PTH measurement in order to obtain reliable decision limits (3,4). The availability of reliable biological variation (BV) data for PTH is essential for defining analytical performance specifications (APS), which are utilized to assess the suitability and the quality of analytical methods (5,6). Additionally, BV data are used to establish reference change value (RCV), which may be applied to assess the significance of change when performing serial measurements in a subject, as a tool for patient monitoring (7). The within-subject (CV_I) and between-subject (CV_G) BV estimates with the associated APS for the most routinely used analytes have been available in a historical online 2014 BV database (https://www.westgard.com/biodatabase1.htm) (8,9). Now, this database has been superseded by the newly published European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Biological Variation Database, available at https://biologicalvariation.eu/ (10). As of March 2020, 165 analytes have been published, with the review being finalized or under way (10). In this database, studies are appraised by the Biological Variation Data Critical Appraisal Checklist (BIVAC), which is based on 14 Quality Items (QIs), each of which is associated with a score A, B, C or D (11). For PTH, ten studies have been identified by systematic literature review (12-21), all of which have received a BIVAC grade C. Five of these fulfil the inclusion criteria for meta-analysis (healthy adults, sampling from biweekly to monthly and > two samples included per participant, second or third-generation assay) from which relevant data sets have been derived and used to deliver the global CV_I and CV_G estimates presented in the EFLM BV Database (13-16,21).

To further facilitate the delivery of updated and reliable BV estimates for all clinically important measurands, the

ELFM WG-BV, in agreement to the requirements of the checklist published by Bartlett *et al.* (22), designed the European Biological Variation Study (EuBIVAS). EuBIVAS is a large-scale, fully BIVAC compliant BV study (all 14 quality items were scored as A), including 91 participants from 5 different European countries, and from which a number of studies have been already published (11,23-29). The aim of the present paper is to provide EuBIVAS-based BV data and the associated APS and RCV for serum PTH in its biointact form (PTH 1-84).

Methods

The EuBIVAS project

Detailed information about involved laboratories, exclusion/inclusion criteria for subjects' enrolment, and protocols for sample collection, handling, and storage, has been previously published in detail (23). Briefly, 91 healthy subjects (38 men, 43 pre-menopausal women aged <50, and 10 post-menopausal women aged >50; overall age range, 21–69 years) were enrolled by 6 different laboratories in 5 European countries (Italy, Spain, Norway, the Netherlands, and Turkey). At the first visit, participants were asked to fill an enrolment questionnaire on lifestyle habits, medical, and family history, in order to be able to confirm their state of well-being. Subjects who fulfilled the inclusion criteria underwent phlebotomy for ten consecutive weeks (April–June 2015). Seventy-seven participants completed all 10 collections, 10 completed 9, 2 completed 8, and 2 completed 7. Serum was obtained from fasting blood collected in serum tubes with clot activator, silicone coated, plastic, 10 mL (16×100 mm^2) [Becton Dickinson, USA, code 367820] kept for 30 min up to 2 h at room temperature and then centrifuged for 10 min at 3,000 *g*. Samples were aliquoted and stored at –80 ℃, before being shipped frozen to San Raffaele Hospital, the coordinating centre.

The study was approved by the Institutional Ethical Review board of San Raffaele Hospital (Milan, Italy) (protocol number: WG-BV project #001, 50/INT 2014) in agreement with the World Medical Association Declaration of Helsinki (as revised in 2013) and by the Ethical board/regional Ethics Committee for each involved centre (protocol number: WG-BV project #001, PI-1993. April 2015 for Spain; WG-BV project #001, 2014-26 for The Netherlands; WG-BV project #001, 3452/AO/15 for PD Italy; 2015-3/17 for Turkey; 2014/1988 for Norway). Informed consent was taken from all the patients.

Analytical method

Quantitative determination of PTH 1-84 was performed in December 2016 at the San Raffaele Hospital (Milan, Italy) on the Roche Cobas e801, using the PTH 1-84 ELECSYS E2G 100 reagent (code 07027745190) and the PTH 1-84 CS ELECSYS calibrator (code 5608554190).

The PTH 1-84 immunoassay is a third-generation test aimed at quantifying the biointact PTH form (PTH 1-84); in fact, the antibodies cross reactions with the 1-34 and 7-84 PTH fragments are ≤0.1%, thus highly specific for the intact bioactive hormone (3). According to the manufacturer, the reference interval of the assay is 14.9–56.9 ng/L (2.5° and 97.5° percentile) with 31.3 ng/L as the mean value. Samples from each subject were analysed in duplicate within a single run. PreciControl Varia ELECSYS 1 and 2 (code 5618860190) were used as internal controls of quality for PTH 1-84 and were evaluated in duplicate for each run.

Data analysis

The CV-ANOVA, an ANOVA method based on the CV-transformation of data (30), was used to analyze PTH 1-84 data and to produce estimates of analytical variation (CV_A) and CV_I. Assessment for outliers between replicates (for CV_A) and for variance homogeneity (for CV_I) was performed by Bartlett's test (31) and by the Cochran test (32), respectively. The steady state of the participants was evaluated using the linear regression of the 180 values mean for each blood drawing 1, 2, ... 10 (pooled mean group sample concentrations) *vs.* the blood drawing number [1–10]. The CV_G estimates were obtained using ANOVA on the natural log-transformed data after outliers between subjects were identified by the Dixon q-test (33) and the verification of the normality assumption by the Shapiro-Wilk test (34).

The BV components were estimated for the overall group as well as separately for women and men; with women being also divided in two groups: women <50 (only pre-menopausal women) and women >50 (post-menopausal women). The 95% CI for BV estimates (35) and mean concentrations were calculated. If the 95% CI between the PTH 1-84 mean values, CV_G, and CV_I estimates of men and women or female subgroups did not overlap, they were

Table 1 Numbers of excluded results of biointact parathyroid hormone (PTH 1-84) and the reasons for exclusion

	Number of results excluded				Numbers of results used to estimate CV_I		Number of outliers (%)
	Homogeneity (Bartlett and Cochran's tests)			Reed and Dixon			
	Replicate (analytical homogeneity)	Samples (within homogeneity)	Subjects (within homogeneity)	Subjects (between)	Results	Subjects	
All data	6	26	0	0	1,721	91	3.3
Men	0	12	0	0	716	38	3.2
Women	6	20	0	0	993	53	4.4
Women <50 years	0	19	0	0	802	43	4.5
Women >50 years	6	1	0	0	191	10	4.0

CV_I, within-subject biological variation.

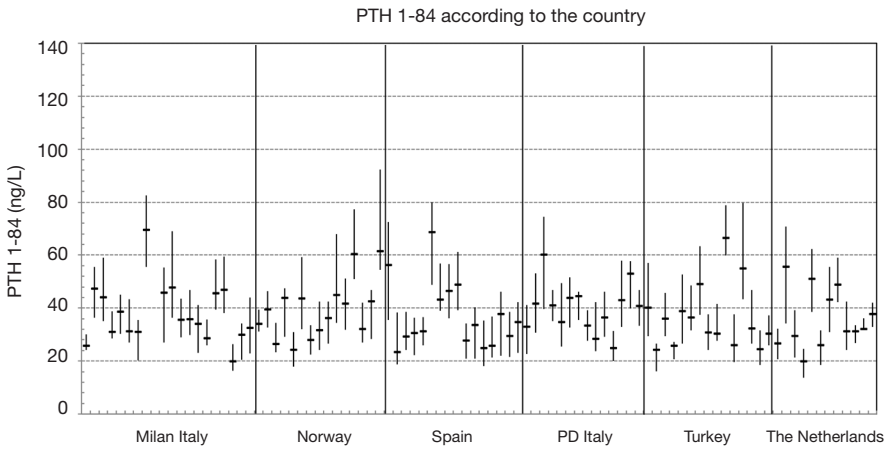

Figure 1 Median values (horizontal bars) and range (minimum-maximum) of biointact parathyroid hormone (PTH 1-84) for each participant after exclusion of outliers, ordered by country.

considered significantly different. In addition, correlations between mean concentrations and age or body mass index (BMI) were evaluated for women <50 and men subgroups.

The APS for the analytical imprecision (CV_{APS}) and analytical bias (B_{APS}), and the RCV were calculated as described in (27,36).

Data were analyzed using Excel 2016, XLSTAT (Statistical software for Excel), and IBM SPSS Statistic (version 20).

Results

In the subgroups of men and pre-menopausal women, the median age was 35 years and 34 years and median BMI 24.4 and 21.3 kg/m^2, respectively (see *Table S1* for further details). The 91 participants reported the following level of physical exercise: 18% did <3 h/week of physical activity while 36% did >3 h/week. The alcohol intake was moderate and drug consumption was limited. Three of the 91 subjects were heavy smokers (10–20 cigarettes/day), while 17 were moderate smokers (<10 cigarettes/day) (*Table S2*). To fulfil criteria for variance homogeneity, 3.3% of results were excluded, but no outliers were identified by the Dixon test (*Table 1*). Based on the Shapiro-Wilk test, PTH 1-84 data for the whole population as well as for men and women were normally distributed only if ln-transformed.

Table 2 Within-subject (CV_I) and between-subject (CV_G) biological variation (BV) estimates for biointact parathyroid hormone (PTH 1-84) with 95% confidence intervals (CIs); analytical performance specification (APS) for imprecision (CV_{APS}) and bias (B_{APS}), and reference change values (RCV) for PTH 1-84 based on the BV estimates[1]

	Number of individuals	Total number of results	Mean number of samples/ individuals	Mean number of replicates/ samples	Mean value, ng/L (95% CI)	CV_A % (95% CI)[2]	CV_I % (95% CI)	CV_G % (95% CI)	CV_{APS} %[3]	B_{APS} %[4]	RCV %[5] decrease; increase
All subjects	91	1,721	9.51	1.98	37.9 (37.2–38.6)	3.3 (3.1–3.4)	14.7 (14.0–15.5)		6.5	7.5	–26.7; 36.5
Men	38	716	9.45	1.99	39.3 (38.3–40.3)		13.0 (12.1–14.2)	26.8 (21.4–35.1)			
Women	53	993	9.43	1.97	36.7 (35.8–37.6)		15.2 (14.3–16.3)				
<50 years	43	802	9.33	2.00	35.5 (34.6–36.4)		15.5 (14.4–16.7)	27.8 (22.7–36.1)			
>50 years	10	191	9.90	1.87	41.7 (39.4–43.9)		14.2 (12.3–16.6)	30.8 (21.3–62.3)			

[1], Results were assessed for men, women, women <50, and women >50. Results in bold were used to estimate analytical performance specification (APS) and reference change value (RCV) for the whole population; [2], Analytical variation (CV_A) estimates were based on CV-ANOVA of duplicate analysis of all study samples; [3], APS for imprecision, $CV_{APS} = ½\ CV_I$; [4], APS for bias, $B_{APS} = 0.25(CV_I^2 + CV_G^2)^{0.5}$; [5], RCV were calculated as described in the text delivering asymmetric values for rise and fall at the probability level of 95% for significant unidirectional change, applying CV_A estimates based on duplicate measurement of all study samples.

No significant trend of the mean PTH 1-84 values during the 10 weeks of collection was identified. Mean PTH 1-84 concentrations among the 5 countries were similar (*Figure 1*). The PTH 1-84 mean value of the women <50 years subgroup significantly differed from both men and women >50 years (*Table 2*, *Figure 2*). Considering separately the mean concentrations of men and women <50 years, data analysis did not reveal a significant correlation with age, however, PTH 1-84 values of men were positively correlated with the BMI (*Figure 3*). CV_G estimates were calculated for the three subgroups; men, women above and below 50 years (*Table 2*). Pre- and post-menopausal women had similar CV_I estimates (*Table 2*), and thus a common CV_I estimate was calculated for all women at 15.2%, (95% CI: 14.3–16.3%), which was significantly higher than the CV_I estimate of 13.0% (95% CI: 12.1–14.2%) derived in men. As women <50 years and men had significantly different mean PTH 1-84 concentrations, the lowest CV_G estimate was applied in calculation of APS (*Table 2*). In the case of RCV calculation (*Table 2*), the men's CV_I estimate was applied because it was the lowest. Estimates from the women >50 years subgroup were not applied due to the exiguous number of subjects in this group. The EuBIVAS BV estimates for PTH 1-84 were compared to previously published estimates from studies appraised and included in the EFLM BV database (*Table 3*) (10). In addition, the potential impact of the EuBIVAS CV_I estimate in patient monitoring was evaluated creating a probability plot of percentage unidirectional change that showed the percentage increase between two consecutive PTH 1-84 results necessary, at any given probability, to evidence a significant difference (*Figure 4*).

Discussion

PTH is a key biomarker for diagnosing of parathyroid glands' pathologies, calcium-phosphate metabolism disorders and for monitoring chronic kidney disease mineral and bone disorder (CKD-MBD). In this paper, the use of the most suitable and updated methodology for BV estimation of PTH, in its biointact form (PTH 1-84), allowed us to obtain robust and reliable BV data with implications for the calculation of the APS for bias and imprecision, and RCV. The differences in PTH 1-84 mean values between the women <50 years and both men and women >50 years (*Table 2*, *Figure 2*) underline that PTH 1-84 concentrations are affected by sex and pre-menopausal status, as previously described (37,38). In addition, the positive correlation between PTH 1-84 and BMI in men (see *Figure 3*) is a feature already described in literature (39). In addition, our data indicate that there is a

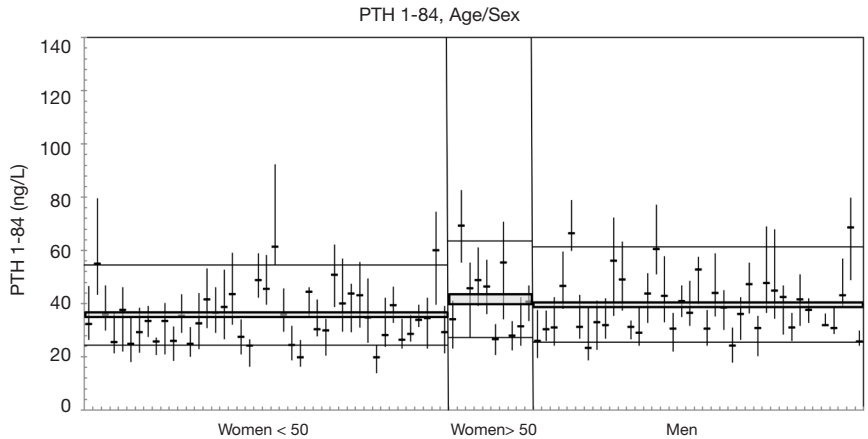

Figure 2 Median values (horizontal bars) and range (minimum-maximum) of biointact parathyroid hormone (PTH 1-84) for each participant after exclusion of outliers, ordered by sex and age. Continuous lines indicate the 95% CI of the mean, the 5th and the 95th percentiles for women and men.

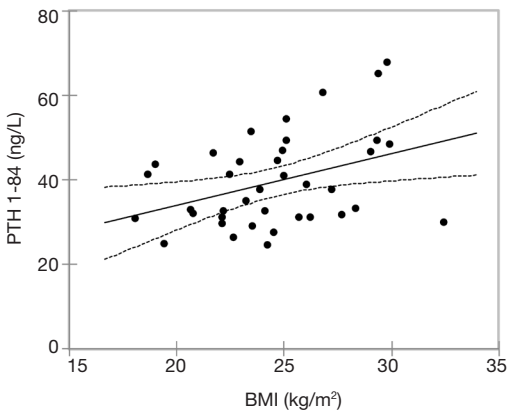

Figure 3 Effect of body max index (BMI) on biointact parathyroid hormone (PTH 1-84) concentration in men. Linear regression equation: PTH 1-84 (ng/L) = 1.2 (0.2–2.2) × BMI + 9.2 (−15.5–33.9).

weak correlation between the individual CV_I estimates of total calcium and PTH 1-84, which is strengthened when including only subjects with PTH 1-84 mean values higher than the median of the whole population (see *Figure S1*). CV_I estimates were calculated also for subpopulations of smokers *vs.* non-smokers, and for subjects who had an alcohol intake of 1–2 U/day *vs.* those who had less alcohol intake; no significant differences in the CV_I values were found (data not shown). In the EFLM BV database 10 studies delivering BV estimates for PTH in healthy (12-16,19-21) or diseased populations (15,17,18,20) are included and meta-analysis-derived BV estimate based on BIVAC compliant studies fulfilling the set criteria are published. Considering the studies performed in healthy populations: 2 declared to assess the biointact PTH (PTH 1-84) BV using third-generation assays (15,19); 4 the intact PTH BV using second-generation assays (12,15,19,21), whereas 4 did not specify the assay used for PTH evaluation and thus it is not clear whether the obtained BV data refer to intact or biointact PTH (13,14,16,20).

The two studies performed in healthy subjects using a third-generation assay for biointact PTH (PTH 1-84) evaluation (see *Table 3*), reported the following CV_I: 23.8% (95% CI: 21.2–27.1%) (15) and 24.0% (95% CI: 22.0–26.4%) (19), both significantly higher than the EuBIVAS CV_I estimates. These two papers have received a BIVAC grade C, typically for the QI related to statistical handling (variance homogeneity and outlier evaluation). This difference in statistical approach may potentially explain the higher CV_I results found in these studies compared to the EuBIVAS. Standard statistical approaches used for estimating BV, such as the CV-ANOVA applied in our study, are sensitive to "noisy data" and assume homogeneity of the within-participant CV. Thus, the EuBIVAS data set has been trimmed to achieve this for PTH 1-84, requiring 3–4% of data to be excluded. A recently published Bayesian model for estimating BV is robust to "noisy data" and is an approach for delivering BV estimates without the need for data trimming (40).

The CV_I estimates from the 7 studies on healthy subjects that had applied second-generation assays (for intact

Table 3 Overview of within-subject (CV_I) and between-subject (CV_G) biological variation (BV) estimates and analytical variation (CV_A) published for parathyroid hormone (PTH)

Study	Gender	State of well being	Number of subjects	Matrix	Sampling	Assay	CV_A % (95% CI)	CV_I % (95% CI)	CV_G % (95% CI)	SCORE
Takahashi *et al.*, 2002 (12)	Females	Healthy	10	Serum	1 sample/day for 5 days	Second-generation PTH assay	–	16.4	–	$C_{5,7,8,10,11,12,13}$
Viljoen *et al.*, 2008 (13)	Mixed	Healthy	20	Plasma	1 sample/ week for 5 weeks	Immunoassay	3.3 (2.9–3.8)[§]	25.3 (21.9–30.0)[§]	43.4 (32.4–63.8)[§]	$C_{5,7,8,10,12,13}$
Ankrah-Tetteh *et al.*, 2008 (14)	Mixed	Healthy	10	Serum	1 sample/ week for 6 weeks	Immunoassay	5.0 (3.2–11.0)[§]	25.9 (21.3–32.4)[§]	23.8 (14.5–44.5)[§]	$C_{7,8,10,13}$
Gardham *et al.*, 2010 (15)	Mixed	Healthy	12	Plasma	2 samples/ week for 6 weeks	Second-generation PTH assay	3.5 (3.2–4.0)[§]	19.2 (17.1–21.9)[§]	–	C_{10}
						Third-generation PTH assay	4.2 (3.8–4.8)[§]	23.8 (21.2–27.1)[§]	–	
		All hemodialysis patients	22 (19 for BV estimation)			Second-generation PTH assay	3.6 (3.3–4.0)[§]	25.6 (23.3–28.3)[§]	–	
						Third-generation PTH assay	6.3 (5.8–7.0)[§]	30.2 (27.5–33.5)[§]	–	
		Hemodialysis patients with mean PTH concentration in the tertile 1	7			Second-generation PTH assay	3.7 (3.2–4.4)[§]	31.7 (27.4–37.7)[§]	–	
		Hemodialysis patients with mean PTH concentration in the tertile 2	8				3.4 (2.9–3.9)[§]	27.2 (23.7–31.9)[§]	–	
		Hemodialysis patients with mean PTH concentration in the tertile 3	7				3.9 (3.4–4.6)[§]	4.5 (3.6–5.6)[§]	–	
		Hemodialysis patients with mean PTH concentration <300 ng/L	13				3.5 (3.1–4.0)[§]	31.2 (28.2–37.3)[§]	–	
		Hemodialysis patients with mean PTH concentration ≥300 ng/L	9				3.9 (3.4–4.5)[§]	13.9 (12.1–16.2)[§]	–	
		Hemodialysis patients who dialyzed in the morning	9				3.5 (3.1–4.0)[§]	32.1 (3.2–37.3)[§]	–	
		Hemodialysis patients who dialyzed in the afternoon	13				3.7 (3.4–4.2)[§]	19.8 (17.7–22.4)[§]	–	
Smith *et al.*, 2012 (16)	Mixed	Healthy	12	Plasma	1 sample/week for 6 weeks	Immunoassay	2.5 (2.2–3.0)[§]	20.2 (17.1–24.6)[§]	21.8 (14.4–37.6)[§]	$C_{10,12}$
Cavalier *et al.*, 2013 (17)	Mixed	Hemodialysis	17	Serum	2 samples/ week for 6 weeks	Second-generation PTH assay	2.3 (2.1–2.6)[§]	13.8 (12.5–15.4)[§]	–	$C_{5,7,8,10,12,13}$
						Third-generation PTH assay	4.5 (4.1–5.0)[§]	14.9 (13.5–16.7)[§]	–	

Table 3 (*continued*)

Table 3 (*continued*)

Study	Gender	State of well being	Number of subjects	Matrix	Sampling	Assay	CV_A % (95% CI)	CV_I % (95% CI)	CV_G % (95% CI)	SCORE
Lutsey *et al.*, 2016 (18)	Mixed	Atherosclerosis	160	Serum	2 samples/ week for 6 weeks	Immunoassay	5.1 (4.1–6.8)[§]	16.7 (14.8–18.9)[§]	37.9 (33.7–43.0)[§]	$C_{5,7,8,10,11,13}$
Schleck *et al.*, 2017 (19)	Mixed	Healthy	22	Plasma	2 samples on the Mondays, Wednesdays, and Fridays of 2 consecutive weeks	Second-generation PTH assay	1.5[†] (1.4–1.6)[§]	19.0 (17.4–20.9)[§]	–	$C_{7,8,10,12,13}$
						Third-generation PTH assay	2.5[†] (2.3–2.7)[§]	24.0 (22.0–26.4)[§]	–	
				Serum		Second-generation PTH assay	1.6[†] (1.5–1.8)[§]	19.0 (17.4–20.9)[§]	–	
						Third-generation PTH assay	2.0 (1.8–2.2)[§]	24.0 (22.0–26.4)[§]	–	
Meijers *et al.*, 2017 (20)	Mixed	Healthy	28	Not specified	1 sample every 4 weeks for 20 weeks	Not specified	1.1 (0.8–1.7)[§]	16.7 (14.8–19.2)[§]	39.8 (31.3–54.3)[§]	$C_{3,4,8,10}$
		Chronic heart failure	83	Not specified	1 sample every 3 weeks for 9 weeks	Not specified	1.1 (0.9–1.5)[§]	22.5 (20.3–25.2)[§]	49.2 (42.2–58.6)[§]	
Ercan *et al.*, 2019 (21)	Mixed	Healthy	20	Serum	1 sample/ week for 10 weeks	Second-generation PTH assay	3.8 (3.3–4.4)	21.1 (19.4–23.2)	24.9 (18.4–37.0)	$C_{7,8,10}$
	Men		10					18.5 (15.8–21.5)	26.2 (17.5–48.7)	
	Women		10					24.0 (20.9–28.2)[§]	18.6 (11.7–34.4)[§]	
The EuBIVAS study	Mixed	Healthy	91	Serum	1 sample/ week for 10 weeks	Third-generation PTH assay	3.3 (3.1–3.4)	14.7 (14.0–15.5)		-
	Men		38					13.0 (12.1–14.2)	26.8 (21.4–35.1)	
	Women		53					15.2 (14.3–16.3)		
	Women <50 years		43					15.5 (14.4–16.7)	27.8 (22.7–36.1)	
	Women >50 years		10					14.2 (12.3–16.6)	30.8 (21.3–62.3)	

[†], analytical variation (CV_A) with 95% CI calculated by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation database (10); [§], 95% CI calculated by the EFLM biological variation database (10).

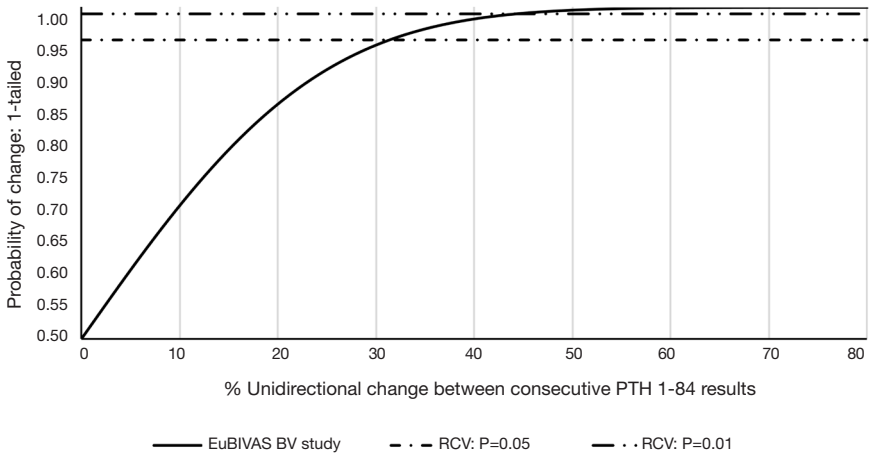

Figure 4 Probability plots of unidirectional change between two consecutive biointact parathyroid hormone (PTH 1-84) results obtained from the European biological variation study (EuBIVAS). The plot represents a simulation obtained using the formula $Z = \text{change}/[2^{1/2} (CV_A^2 + CV_I^2)^{1/2}]$ (CV_A: analytical variability, CV_I: within-subject biological variation). The obtained Z values are converted to a 1-tailed probability and then plotted against the percentage of change used for generating Z values. The curve crosses the probability lines set at 0.95 and 0.99 indicating a 1-tailed RCV (reference change value) calculation of 31.2% (P=0.05) and 44.2% (P=0.01), respectively.

PTH) or that had not specified the assay employed for PTH evaluation ranged from 16.4% (12) to 25.9% (95% CI: 21.3–32.4%) (14). The very recently published study performed by Ercan and colleagues (21) reported intact PTH BV data for a population of 20 healthy subjects and also BV estimates in subgroups of men and women. The CV_I estimates for the entire population and for the female subgroup were significantly higher than the EuBIVAS ones, while the male CV_I estimate was slightly higher (*Table 3*). In this case, the higher CV_I estimates reported by the Ercan study could be related to the inclusion of different data as variance homogeneity and outlier evaluation were not performed in the Ercan study. Furthermore, this study applied a second-generation assay for intact PTH analysis. Two studies have analysed their samples in parallel with both second-generation and third-generation assays, where one does not demonstrate any difference in BV estimates delivered by the two methods (15), while the other reports generation-dependent differences in PTH estimates (19).

The two studies performed by Cavalier *et al.* (17) and Gardham *et al.* (15) also evaluated the BV components for PTH in haemodialysis patients (*Table 3*). In these cases, the CV_I estimates for the entire diseased population were derived using both the second- and the third-generation assays.

The EuBIVAS CV_A estimate, as well as CV_A estimates reported in the other two biointact PTH (PTH 1-84) BV studies, are lower than the EuBIVAS APS for imprecision (*Tables 2,3*). This indicates that, even if the EuBIVAS CV_A estimate is based on duplicate analysis of study samples, PTH 1-84 analysis satisfy the analytical quality requirements. The APS for PTH 1-84 obtained from this EuBIVAS study may provide important information about the desirable performance thus being able to select a suitable method for PTH estimations in different laboratories.

It is interesting to evaluate how the EuBIVAS CV_I estimates can impact the RCV calculation and the potential consequences when monitoring patients. In fact, RCV may be a helpful tool for the interpretation of the PTH 1-84 concentration changes observed between two consecutive measurements from a subject. An important clinical question (e.g., in CKD-MBD subjects) is when an increase in two consecutive PTH 1-84 evaluations is significant. In this case, the RCV formula should be adapted for obtaining a Z value and thus for the 1-tailed change value calculation, which can be considered as the critical increase between two consecutive results of an analyte, using the following formula: $Z = \text{change}/[2^{1/2} (CV_A^2 + CV_I^2)^{1/2}]$ (41). The obtained Z value can be converted in a 1-tailed probability allowing the construction of the percentage increase plot between consecutive results against the calculated 1-tailed probability. This type of plot can be useful for evaluating

the potential impact of EuBIVAS BV estimates when monitoring patients and thus to understand if a change between two consecutive results can be explained by biological and analytical variations (*Figure 4*). In this case, using the analytical imprecision calculated in this study, the obtained 1-tailed value for PTH 1-84 percentage increase, set at 95%, was 31.2% (30.4–32.0%). In clinical practice, considering a subject with a PTH 1-84 of 60.7 ng/L, a rise to 83.3 ng/L could be explained by biological and analytical variations (1-sided z-score, α=0.025). It is important to take into account that the EuBIVAS RCV for PTH 1-84 were obtained using the specific EuBIVAS CV_A based on analysis of duplicate samples, and, for this reason, cannot be considered as universal values thus underlining that each laboratory has to calculate its own PTH 1-84 RCV using relevant CV_A estimates.

In conclusion, this EuBIVAS results for PTH 1-84, provided using the most suitable and updated methodology for BV estimation, led to the availability of reliable and robust CV_I estimates, APS for analytical imprecision, and RCV. The EuBIVAS CV_I estimates were lower than those delivered by previously published papers on biointact PTH, possibly related to different statistical approaches and to the strict control of the fasting status, thus minimizing possible effects of the ingestion of calcium-containing nutrients (42). These EuBIVAS BV estimates, together with a suitable interpretation of the PTH 1-84 concentration changes, represent a key tool in medical practice for a correct diagnosis and monitoring of bone turnover and parathyroid glands pathologies, for patient management, for creating standardized protocols for the pre-analytical, analytical, and post-analytical stages of PTH evaluation, and for giving information about the analytical quality of the method used for PTH 1-84 evaluation.

Study limitations

Long samples storage before analysis, but the samples were always continuously stored at –80 ℃ and thawed only once prior to analysis. The PTH 1-84 analysis was performed using only one manufacturer's reagents, but it is unlikely that the BV estimates were affected by this for the same measurand. In addition, the analyses have been performed on serum kept at room temperature for a maximum of two hours before centrifugation, this procedure may have introduced some small degradation of PTH 1-84 that reasonably can be considered acceptable [as reported by Hanon *et al.* (43) and Dupuy *et al.* (44)]. In any case, considering that the possible PTH 1-84 degradation would be proportional in each sample, it is improbable that it may have increased the within-subject BV.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-19-4498). The authors have no conflicts of interest to declare.

Ethical Statement: the authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Ethical Review board of San Raffaele Hospital (Milan, Italy) (protocol number: WG-BV project #001, 50/INT 2014) in agreement with the World Medical Association Declaration of Helsinki (as revised in 2013) and by the Ethical board/regional Ethics Committee for each involved centre (protocol number: WG-BV project #001, PI-1993. April 2015 for Spain; WG-BV project #001, 2014-26 for The Netherlands; WG-BV project #001, 3452/AO/15 for PD Italy; 2015-3/17 for Turkey; 2014/1988 for

Norway). Informed consent was taken from all the patients.

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Supplementary

Table S1 Gender, number, age, and body max index (BMI) of men, women <50, and women >50 years enrolled by each center

	Men (age range, 20–60 years)	Men median age, Years [age range]	Men median BMI, kg/m^2 (BMI range)	Women <50 (age range, 20–50 years)	Women <50 median age, years (age range)	Women <50 median BMI, kg/m^2 (BMI range)	Women >50 (age range, 50–70 years)	Women >50 median age, years (age range)	Women >50 median BMI, kg/m^2 (BMI range)
Italy-Milan (19 persons)	9	38 [24–59]	25.2 (20.8–30.0)	7	34 (24–48)	22.7 (17.6–23.9)	3	58 (55–59)	22.8 (19.4–27.5)
Norway (15 persons)	7	37 [28–42]	24.3 (18.1–26.3)	6	39 (29–49)	21.7 (18.7–24.4)	2	63	24.6 (23.7–25.5)
Spain (16 persons)	7	34 [26–54]	25.1 (19.5–32.5)	7	26 (24–48)	21.7 (17.9–23.1)	2	60	21.3 (21.2–21.4)
Italy-Padua (14 persons)	5	32 [27–35]	22.5 (19.0–23.5)	8	33 (27–49)	19.8 (18.7–23.2)	1	69	18.6
Turkey (15 persons)	6	27 [22–35]	27.5 (22.2–29.9)	9	33 (21–38)	21.1 (18.3–27.3)	–	–	–
The Netherlands (12 persons)	4	36 [23–45]	24.0 (18.1–26.3)	6	39 (29–49)	21.7 (20.9–24.2)	2	60 (59–60)	23.0 (20.7–25.3)
Total (91 persons)	38	35 [22–59]	24.4 (18.1–32.5)	43	34 (21–49)	21.3 (17.6–27.3)	10	60 (55–69)	22.1 (18.6–27.5)

Table S2 Smoking habits, alcohol intake, drug consumption, and physical activity done by men and women enrolled by each center

		Physical activity			Smoking habits			Drug consumption		Alcohol intake[†]		
		No physical activity	<3 h/week	>3 h/week	0 cigarettes/day	<10 cigarettes/day	10–20 cigarettes/day	No drug	Type of drug	0 U/day	< 1 U/day	1–2 U/day
Italy – Milan (19 persons)	Men (n: 9)	5	1	3	6	1	2	8	1 antihistamine (as needed)	1	2	6
	Women (n: 10)	3	1	6	9	1	0	8	1 antihistamine; 1 antibiotic (for 1 week)	1	9	0
Norway (15 persons)	Men (n: 7)	1	0	6	7	0	0	6	1 (not specified)	1	3	3
	Women (n: 8)	2	2	4	6	2	0	8	–	0	5	3
Spain (16 persons)	Men (n: 7)	3	1	3	6	1	0	6	1 omeprazole	2	4	1
	Women (n: 9)	1	5	3	6	2	1	6	2 Oral contraceptives; 1 Antihistaminic and diuretics	0	9	0
Italy – Padua (14 persons)	Men (n: 5)	1	1	3	3	2	0	4	1 antifungal	1	3	1
	Women (n: 9)	3	2	4	9	0	0	7	1 Ketoprofene; 1 antihistamine (as needed)	5	4	0
Turkey (15 persons)	Men (n: 6)	5	1	0	4	2	0	6	–	5	1	0
	Women (n: 9)	7	1	1	4	5	0	9	–	3	6	0
The Netherlands (12 persons)	Men (n: 4)	1	1	2	4	0	0	4	–	1	3	0
	Women (n: 8)	0	0	8	7	1	0	6	2 (not specified)	0	0	8
Total (91 persons)	Men (n: 38)	16	5	17	30	6	2	34	4	11	16	11
	Women (n: 53)	16	11	16	41	11	1	44	9	9	33	11

[†], one alcohol unit (U) correspond to 10 mL, equivalent to 8 grams, of pure alcohol (https://www.drinkaware.co.uk/alcohol-facts/alcoholic-drinks-units/what-is-an-alcohol-unit/).

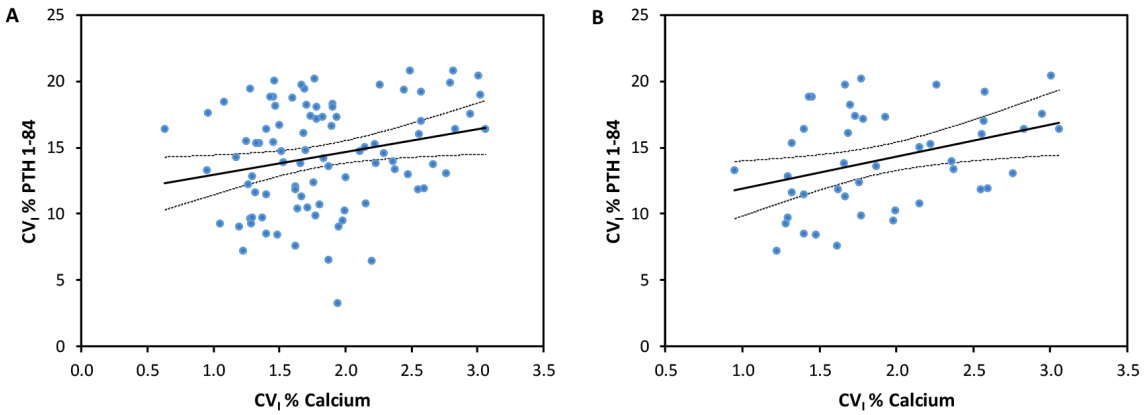

Figure S1 Relation between total calcium and biointact parathyroid hormone (PTH 1-84) within-subject biological variation (CV_I) estimates in individual study subjects for the entire European biological variation study (EuBIVAS) population (A) and for the subjects with PTH 1-84 mean values higher than the median (35.16 ng/L) (B). Linear regression equation: (A) CV_I % PTH 1-84=1.7(0.2–3.2)*CV_I % Calcium+11.2(8.3–14.1); (B) CV_I % PTH 1-84=2.4(0.5–4.4)*CV_I % Calcium+9.5(5.6–13.4).