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Running title: c-peptide assays comparison

ABSTRACT

BACKGROUND: Connecting peptide (C-peptide) is the cleavage product of proinsulin and is released in blood in equimolar amounts to insulin. Compared to the latter, C-peptide has a longer plasma half-life and is less affected by haemolysis, therefore could be a useful marker of insulin production. We aimed to compare the analytical performance of two different chemiluminescent enzyme immunoassays (CLEIA) for the measurement of C-peptide in serum.

METHODS: Overall, 106 subjects (median age: 51 [20 – 75]; M/F: 72/34) with available serum samples were included in the study; 14 (13.2%) had a diagnosis of impaired fasting glucose (IFG) and 28 (26.4%) of type 2 diabetes mellitus (T2DM). C-peptide was measured in serum by Elecsys® C-Peptide (Roche, Mannheim, Germany) and by Lumipulse® C-Peptide (Fujirebio, Tokyo, Japan) CLEIAs.

RESULTS: Median C-peptide levels measured by Elecsys® and Lumipulse® were comparable in our study cohort (2.6 [0.3 – 13.3] ng/mL vs. 2.54 (0.01 – 10.50) ng/mL, respectively; $p = 0.665$). No random differences were observed between the two methods; the analytical agreement between both was satisfactory. C-peptide serum values were strongly correlated to insulin concentration ($r_s = 0.626$, $p < 0.001$, for Elecsys®; $r_s = 0.719$, $p < 0.001$, for Lumipulse®) and increased in a stepwise manner from patients with normal glucose tolerance to those with IFG and T2DM ($p < 0.001$). Both CLEIAs showed an area under the curve > 0.7 for the discrimination between patients with and without overt T2DM.

CONCLUSIONS: The Elecsys® and Lumipulse® C-Peptide CLEIAs showed an adequate analytical agreement. The measurement of serum C-peptide may represent a valid surrogate of pancreatic β-cell function with a potential useful application in the clinical setting.

Key words: c-peptide – CLEIA – impaired fasting glucose – type 2 diabetes mellitus

Introduction

The connecting peptide (C-peptide) is the cleavage product of proinsulin, the precursor of insulin molecule. Both C-peptide and insulin are stored in the secretory granules of pancreatic β-cells and are released into the bloodstream in equimolar amounts.[1] Following secretion, the majority of insulin is cleared by the liver, whereas C-peptide has negligible hepatic clearance.[1] As a consequence, C-peptide levels in the circulation are significantly increased compared to insulin levels showing constant blood values [1, 2] and reflecting the real amount of insulin secretion. While the physiological role of insulin is well known,[3] the functions of C-peptide need to be thoroughly investigated.

The advantage of measuring C-peptide instead of insulin, as a marker of β-cells secretion, is due to several factors: first of all, C-peptide has a longer plasma half-life *in vivo* compared to insulin (20 – 30 minutes *vs.* 3 – 5 minutes, respectively); further, it is less affected by haemolysis.[4] Despite the fact that several studies already showed the potential value of C-peptide as a biomarker of β-cells function, current national and international guidelines do not advocate its use in the clinical setting.[5]

Notwithstanding this, the assessment of insulin secretion by C-peptide in clinical practice, can be useful to discriminate between type 1 and type 2 diabetes mellitus (T2DM),[6, 7] to diagnose insulinoma[8] or to understand the causes of hypoglycaemia. Increasing evidence suggests that C-peptide may also be useful to assess glycaemic control,[9] the response to hypoglycaemic agents,[10-12] and the risk to develop diabetes complications.[13-15] Furthermore, the American Diabetes Association (ADA) suggests the use of C-peptide to monitor the residual insulin secretion rate during clinical trials investigating new strategy to preserve β-cells function.[16, 17]

Despite the use of C-peptide in the clinical setting is becoming increasingly important, its accuracy as well as its reproducibility between different laboratories has not thoroughly evaluated yet. The aim of this work is to compare the analytical and clinical performance of two different chemiluminescent enzyme immunoassays (CLEIA) for the measurement of C-peptide in serum.

Materials and Methods

Patients

A total of 175 outpatients from the Division of Gastroenterology, Città della Scalute e della Scienza di Torino were consecutively recruited in our retrospective study between January 2011 to December 2016. Demographic, clinical and biochemical data were collected anonymously in an electronic medical record. For the purpose of this study patients were classified according to diabetic status. Patients with fasting glucose between 100 and 125 mg/dL and/or elevated haemoglobin A1c (HbA1c) level between 5.7% and 6.4% were classified as pre-diabetic (impaired fasting glucose [IFG]). A diagnosis of T2DM was established according to the following criteria: fasting plasma glucose >126 mg/dL and/or HbA1c >6.5% confirmed by repeated testing or random plasma glucose >200 mg/dL in symptomatic patients.[18, 19]

Serum samples were collected in polypropylene 2 ml tubes labelled with the study participant identification code and stored at -80°C until analysis. Haemolysed blood samples, which can produce unreliable laboratory results, were excluded. The study protocol was compliant to the Declaration of Helsinki and was approved by the Institutional Ethics Committee. All subjects signed a written informed consent prior to recruitment.

Measurement of c-peptide by Cobas e 801 analyser

Elecsys® C-Peptide immunoassay (Roche, Mannheim, Germany) is a quantitative CLEIA for the determination of C-peptide in serum or plasma on the fully automated Cobas® e 801 immunoanalyser (Roche, Mannheim, Germany). The Elecsys® C-Peptide requires 12 µL of sample for each determination. According to manufacturer, the reported median concentration of C-peptide in apparently healthy controls is 1.96 (5° – 95° percentile: 1.1 – 4.4) ng/mL. The precision of the assay was ≤3.6%. The limit of blank was 0.01 ng/mL, the limit of sensitivity 0.02 ng/mL and the limit of quantitation 0.15 ng/mL. The range of measurement corresponded to 0.02 – 40 ng/mL.

Measurement of c-peptide by Lumipulse G600II system

Lumipulse® C-Peptide immunoassay (Fujirebio, Tokyo, Japan) for the quantitative determination of C-peptide in serum or plasma specimens, is based on CLEIA technology by a two-step sandwich immunoassay method on the fully automated Lumipulse® *G* System (Fujirebio, Tokyo, Japan). The Lumipulse® C-Peptide uses 30 µL of specimen for each assay. As reported by the manufacturer, the observed range of C-peptide values in apparently healthy subjects was 0.71 – 2.58 ng/mL. The precision of Lumipulse® C-Peptide resulted ≤5.1%; Lower Limit of Detection (LLoD) and Quantitation (LLoQ) corresponded to 0.0109 ng/mL. Dilution testing reported that linearity was found in a range of 0.57 – 30.00 ng/mL.

Statistical analysis

Data normality was assessed by D'Agostino-Pearson test. According to data distribution, quantitative variables were reported as mean ± standard deviation (SD), median (range) or median and 95% confidence interval (CI) where appropriate. To assess the analytical performance and concordance of the two CLEIAs for the measurement of c-peptide in serum, the following analyses were performed. Correlation between the different assays was calculated by nonparametric Passing Bablok regression analysis. A Bland-Altman plot was constructed to analyse the agreement between the quantitative results obtained with the two CLEIA methods. The concordance correlation coefficient (ρ_c) was calculated to evaluate the strength of agreement between the two quantitative determinations; ρ_c contains the measurement of precision (ρ) and accuracy (C_b) according to the following formula: $\rho_c = \rho \times C_b$.

To compare paired and unpaired variables we used Wilcoxon or Mann-Whitney test, respectively. To compare more than two groups of variables, Kruskal-Wallis test was used. Correlation between biochemical parameters was investigated by Spearman correlation analysis (r_s). Receiver Operator Characteristic (ROC) analysis was performed to evaluate the clinical

performance of the two assays for the measurement of C-peptide in patients with or without T2DM. The optimal cut-off that maximizes sensitivity (Se) and specificity (Sp) was selected by means of Youden's J statistic.

For all the analyses was considered significant a p value <0.05. All statistical analyses were performed by MedCalc® Software version 18.9.1 (MedCalc Software bvba, Ostend, Belgium).

Results

Overall, 106 patients with available serum samples and complete of demographic, biochemical and clinical data were included in the final analysis. The main characteristics of the patients are reported in Table I. The majority of patients were males (n = 72, 67.9%), with a median age of 51 years; more than one third of the study cohort had a diagnosis of IFG or T2DM (n = 42; 39.6%). The median values of serum C-peptide were 2.6 (0.3 – 13.3) ng/mL and 2.54 (0.01 – 10.50) ng/mL, assessed by Elecsys® and Lumipulse®, respectively (p = 0.665).

Correlation between assays

Passing Bablok regression was run to compare Elecsys® and Lumipulse® C-peptide assays. The corresponding scatter diagram and regression line is reported in Figure 1. The analysis revealed that there were both systematic (intercept A: -0.839 [95% CI -1.462 – -0.393]) and proportional differences (intercept B: 1.403 [95% CI 1.227 – 1.605]) between the two methods; however, no random differences were observed (residual standard deviation: 0.866 [-1.698 – 1.698]). Bland-Altman plot (Figure 2) showed that the differences between the two methods fell within ± 1.96 SD of the mean, indicating that the two methods may be used interchangeably. Concordance analysis showed a $\rho_c = 0.742$ (95% CI 0.656 – 0.809), with $\rho = 0.783$ and $C_b = 0.948$.

Clinical performance

Overall, Elecsys® C-peptide showed a poor correlation with fasting glucose (r_s = 0.169 [95% CI -0.023 – 0.348], p = 0.084) and a good correlation with fasting insulin (r_s = 0.626 [95% CI 0.493 – 0.731], p < 0.001) (Figure 3A and 3B). Lumipulse® C-peptide showed a moderate correlation with fasting glucose (r_s = 0.368 [95% CI 0.190 – 0.522], p < 0.001) and a good correlation with fasting insulin (r_s = 0.719 [95% CI 0.611 – 0.801], p < 0.001) (Figure 3C and 3D). Median Elecsys® C-peptide and Lumipulse® C-peptide were significant different among patients with normal glucose tolerance (NGT), IFG and T2DM (p < 0.001 for both assays); direct comparison between the different groups of patients is reported in Figure 4.

For the discrimination between patients with or without T2DM, ROC curve analysis showed an area under the curve (AUC) value of 0.719 (95% CI 0.523 – 0.802) for Elecsys® C-peptide and an AUC value of 0.778 (0.687 – 0.853) for Lumipulse® C-peptide (Figure 5). For Elecsys® C-peptide, at a cut-off of 3.4 ng/mL corresponded Se = 57.1%, Sp = 85.9%, positive likelihood ratio (+LR) = 4.05 and -LR = 0.50; for Lumipulse® C-peptide, at a cut-off of 3.32 ng/mL corresponded Se = 60.7%, Sp = 87.2%, +LR = 4.74 and -LR = 0.45. The flowchart reporting the number of patients correctly classified according to the cut-off values of C-peptide is depicted in Figure 6.

Discussion

In the present study, we compared the analytical and clinical performance of two different CLEIAs for the measurement of C-peptide in serum. Overall, the two methods showed a good reproducibility and moderate diagnostic accuracy for the identification of patients with T2DM; furthermore, the strong correlation observed between C-peptide and blood insulin levels may point up new potential applications for the measurement of C-peptide in the setting of clinical and laboratory medicine.

One of the advantages of measuring C-peptide as a marker of β-cells function, referred to its longer plasma half-life *in vivo* compared to insulin.[4] Despite current national and international guidelines do not advocate the use of C-peptide in the clinical setting,[5] recent insights revealed its

potential use in different algorithms aiming to evaluate the degree of insulin resistance. For example, the use of C-peptide-based indices seem to have a higher performance in identifying subjects more prone to develop T2DM compared to insulin-based indices.[20-21] Moreover, C-peptide levels were recently associated with both hepatic inflammation and fibrosis in T2DM patients with non-alcoholic fatty liver disease, that with a current trend towards the most relevant increase in hepatology, suggesting a potential role in the onset and progression of liver damage in chronic liver diseases.[22-26]

Based on the data of the two CLEIAs reported by their manufacturers, both assays are characterized by high precision (equal or below a 5% coefficient of variation), a wide dynamic range of measurement and a similar limit of sensitivity. In addition, both assays require a low amount of specimen for each testing. From a direct comparison, in spite of conspicuous systematic and proportional differences, no random differences were observed between the two methods. Furthermore, Bland-Altman analysis suggested an interchangeability between Elecsys® and Lumipulse® C-peptide assays; this feature is essential to ensure reproducibility of the measurement both for care and research purposes.

From a clinical point of view, C-peptide serum values were strongly correlated to insulin concentration; in particular, Lumipulse® assay showed a correlation coefficient above 0.7. As a matter of fact, the measurement of fasting insulin may be clinically relevant not only in the setting of diabetes, but also in other pathological conditions in which metabolic syndrome represents the common risk determinant.[27-29] The availability of a surrogate of pancreatic β-cell function that could overcome the limitations of fasting insulin measurement may be of great clinical value. Furthermore, in our series, the serum levels of C-peptide showed a stepwise increase from NGT to IFG patients, and further rose in patients with overt T2DM. Both CLEIAs proved a moderate diagnostic accuracy for the identification of T2DM patients; of note, Lumipulse® C-peptide showed a diagnostic accuracy of 0.778 with a percentage of patients correctly classified of 79%.

In conclusion, our results on the analytical and clinical performance of the Elecsys® and Lumipulse® C-Peptide CLEIAs showed an adequate analytical agreement and a moderate clinical accuracy. The measurement of serum C-peptide may represent a valid surrogate of pancreatic β-cell function with a potential useful application in the clinical setting.

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Table I. *Characteristics of the patients included in the study.*

Characteristics	
Number of patients	106
Age, years (median and range)	51 (20 – 75)
Gender, M/F	72/34
BMI, kg/m^2 (mean ± SD)	28.1 ± 4.1
IFG (n, %)	14 (13.2%)
T2DM (n, %)	28 (26.4%)
Hb, g/dL (median and range)	14.9 (8.5 – 17.3)
Platelets, 10^9 x L (median and range)	216 (111 – 664)
ALT, U/L (median and range)	53 (13 – 323)
Total bilirubin, mg/dL (median and range)	0.8 (0.2 – 2.5)
Albumin, g/dL (median and range)	4.4 (3.3 – 5.3)
Fasting glucose, mg/dL (median and range)	95 (72 – 204)
HbA1c, % (median and range)	5.8 (3.6 – 8.9)
Fasting insulin, MU/L (median and range)	13.6 (2.0 – 76.5)
Total cholesterol, mg/dL (median and range)	184 (85 – 300)
Cholesterol HDL, mg/dL (median and range)	45 (24 – 83)
Triglycerides, mg/dL (median and range)	118 (28 – 701)

Abbreviations: ALT: alanine aminotransferase; BMI: body mass index; F: female; Hb: haemoglobin; HDL: high-density lipoprotein; IFG: impaired fasting glucose; M: male; SD: standard deviation; T2DM: type 2 diabetes mellitus.

Figure 1. Scatter diagram with regression line of Passing Bablok analysis.

Intercept A: -0.839 (95% CI -1.462 – -0.393); intercept B: 1.403 (95% CI 1.227 – 1.605); residual standard deviation: 0.866 (-1.698 – 1.698). The intercept A is a measure of the systematic differences between the two methods. The 95% confidence interval for the intercept A can be used to test the hypothesis that A = 0. This hypothesis is accepted if the confidence interval for A contains the value 0. If the hypothesis is rejected, then it is concluded that A is significantly different from 0 and both methods differ at least by a constant amount. The slope B is a measure of the proportional differences between the two methods. The 95% confidence interval for the slope B can be used to test the hypothesis that B = 1. This hypothesis is accepted if the confidence interval for B contains the value 1. If the hypothesis is rejected, then it is concluded that B is significantly different from 1 and there is at least a proportional difference between the two methods. The residual standard deviation (RSD) is a measure of the random differences between the two methods; 95% of random differences are expected to lie in the interval -1.96 RSD to +1.96 RSD. If this interval is large, the two methods may not be in agreement.

Abbreviations: C-peptide: connecting peptide.

Figure 2. Bland-Altman plot.

The differences between the two techniques are plotted against the averages of the two techniques.

Abbreviations: C-peptide: connecting peptide.

Figure 3. Correlation between Elecsys® C-peptide and fasting glucose (A) and fasting insulin (B), and between Lumipulse® C-peptide and fasting glucose (C) and fasting insulin (D).

Abbreviations: C-peptide: connecting peptide; CI: confidence interval; r_s: Spearman correlation coefficient.

Figure 4. Elecsys® (A) and Lumipulse® C-peptide (B) among patients with NGT, IFG and T2DM.

Median Elecsys® C-peptide values were 2.0 (0.3 – 13.3) ng/mL in NGT patients, 3.4 (0.5 – 6.5) ng/mL in IGT patients and 3.6 (0.6 – 10.1) ng/mL in patients with T2DM (A). Median Lumipulse® C-peptide values were 2.08 (0.01 – 10.5) ng/mL in NGT patients, 2.95 (0.01 – 4.96) ng/mL in IGT patients and 3.60 (1.08 – 7.35) ng/mL in patients with T2DM (B).

Abbreviations: C-peptide: connecting peptide; IFG: impaired fasting glucose; NGT: normal glucose tolerance; T2DM: type 2 diabetes mellitus.

Figure 5. ROC curves of Elecsys® and Lumipulse® c-peptide for the discrimination between patients with and without T2DM.

Abbreviations: AUC: area under the curve.

Figure 6. Flowchart of the correctly classified cases according to C-peptide cut-off by Elecsys® and Lumipulse®.

The rate of true positive, true negative, false positive and false negative cases was calculated according to the ability of C-peptide cut-off values (by Elecsys® and Lumipulse®) to correctly classified the diabetes status.

Abbreviations: FN: false negative; FP: false positive; N: number of cases; TN: true negative; TP: true positive.