Analytical and clinical comparison between two different analytical methods for the measurement of circulating insulin in patients with metabolic-dysfunction associated steatotic liver disease

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Short title: Insulin measurements and analytical determination

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ABSTRACT

BACKGROUND: Reliable circulating insulin determination in patients with metabolic-dysfunction associated steatotic liver disease (MASLD) is important to assess the entity of insulin resistance, the most important pathogenetic mechanism involved in the onset and progression of the disease. However, one of the most important issues concerning insulin measurement is the lack of standardization across laboratories worldwide. The aim of this study is to compare two different analytical methods for the measurement of circulating insulin in subjects with MASLD and to explore their reliability through the analysis of clinical data.

METHODS: Overall, 160 subjects with MASLD at ultrasound (median age: 51 [95% CI, 49-54]; M/F: 114/46) with available plasma and serum samples were included in the study. Circulating insulin was measured by the fully automated Alinity™ i system (Abbott Laboratories, IL, USA) and by Bio-Plex Luminex™ 200 System (Bio-Rad Laboratories Inc., CA, USA).

RESULTS: Median insulin levels measured by Bio-Plex and Alinity i were not comparable in our study cohort (527 vs. 400 pg/mL, respectively; P<0.001). Passing & Bablok regression analysis showed a correlation coefficient of 0.45 (95% CI: 0.322-0.569), but we observed both systematic and proportional differences between the two methods; furthermore, Bland-Altman plot analysis indicated that the two methods cannot be used interchangeably. Alinity i insulin significantly correlated with BMI, AST, ALT, glucose, triglycerides and HDL-cholesterol levels, while Bio-Plex insulin correlated with AST, ALT and HDL-cholesterol levels. At multivariable regression analysis, both Alinity i and Bio-Plex insulin were significantly associated with liver stiffness (rpartial =0.32, P<0.001 and rpartial=0.17, P=0.032, respectively), but only Alinity i insulin was significantly associated with CAP (rpartial=0.50, P< 0.001).

CONCLUSIONS: Bio-Plex insulin should be used with caution and only for research purposes, while Alinity system is confirmed as the most reliable platform in the clinical setting. However, it is important to continue through the process of insulin harmonization to ensure reliable, comparable and reproducible results.

Key words: Alinity i; Bio-Plex; insulin; MASLD; steatosis

Introduction

Insulin is an important hormone released from pancreatic beta cells after a meal that regulates glucose metabolism. Insulin is initially synthesized as proinsulin and subsequently cleaved to insulin and C-peptide. After secretion, a large amount of insulin is cleared by the liver and its half-life in the blood ranges from 4 to 6 minutes [1]. Insulin reduces circulating glucose levels maintaining its homeostasis in various conditions. High levels of insulin are associated with type 2 diabetes mellitus (T2DM), a condition characterized by a status of chronic hyperglycemia affecting about 12% of the general population [2-3]. Along with T2DM, metabolic-dysfunction associated steatotic liver disease (MASLD) is a clinical condition characterized by both glucose and lipid derangement. In this context, insulin resistance (IR) is considered one of the most important determinants in the onset of hepatic steatosis and its progression to metabolic-dysfunction associated steatohepatitis (MASH) and hepatic fibrosis [4]. The homeostasis model assessment (HOMA)-IR index is a surrogate index widely used in the clinical setting to estimate the entity of IR. Specifically, the HOMA-IR is calculated by the product of the fasting insulin and fasting glucose divided by a constant [5]. MASLD subjects with a HOMA-IR value higher or equal to 2.7 are characterized by a more severe liver damage in terms of liver fibrosis [6-7]. Beyond the HOMA-IR index, the oral glucose insulin sensitivity (OGIS) index is another score that has been proposed as a promising tool for patients' risk stratification in subjects with MASLD [8]. Overall, the reliability of these scores depends on the accuracy of both insulin and glucose measurements.

Concerning insulin determination, immunoassay-based methods are the most commonly used analytical methods in the laboratories due to their high sensitivity and relatively low costs. However, one of the most important issues related to the insulin measurement is the lack of standardization across laboratories worldwide [9]. In addition, no specific guidelines are available to standardize the different methods used despite many efforts have been made in this regard [9-11]. The aim of this study is to compare two different methods (one used for research purposes and the other used in the routine clinical setting) for the measurement of insulin in plasma and serum

samples of subjects with MASLD and to explore their reliability through the analysis of clinical data.

Materials and Methods

Patients

One hundred and sixty subjects with MASLD were consecutively enrolled from February 2022 to February 2024 at the Division of Gastroenterology and Hepatology, Città della Salute e della Scienza of Torino. Hepatic steatosis was diagnosed by ultrasound and subsequently quantified by Fibroscan™ (controlled attenuation parameter, CAP). Clinical and biochemical characteristics were collected at the time of enrollment. T2DM was diagnosed in the presence of fasting glucose levels higher than 126 mg/dL and/or HbA1c > 6.5% confirmed by repeated testing or random plasma glucose higher than 200 mg/dL. Plasma and serum samples were collected at the time of enrollment in 2 mL polypropylene tubes and stored at -80 °C until analytical determination. Hemolyzed blood samples were excluded from the analysis. The study protocol was conducted according to the Helsinki Declaration and all subjects signed a written informed consent for the participation in the study (Protocol number: 0117567, 09/11/2021).

Fasting insulin measurements

Serum fasting insulin was measured by the fully automated Alinity™ i system (Abbott Laboratories, IL, USA) and by Bio-Plex Luminex™ 200 System (Bio-Rad Laboratories Inc., CA, USA). Both instruments required a low amount of sample for insulin determination (ranging from 150 μL to 20 μL for the Alinity system and Bio-Plex, respectively). Specifically, the automated Alinity™ i system method is based on chemiluminescent detection technology with an assay design "free from biotin" interference. This peculiarity allows providing greater confidence in results. Conversely, Bio-Plex is based on the quantitative suspension immunofluorescence. The automated Alinity™ i system is located in the Clinical Biochemistry Laboratory of the Città della Salute e della Scienza University Hospital and is used for routine practice. The Bio-Plex instrument is located in the Hepatology and Gastroenterology Laboratory of the Department of Medical Sciences (University of Turin) and is used for research purposes. The details of the procedures are depicted in Figure 1.

Statistical analysis

Continuous variables were reported as mean ± standard deviation (SD) or median (interquartile range) as appropriate. To assess the analytical performance and concordance of the analytical

methods, we performed the nonparametric Passing & Bablok regression analysis. The Bland-Altman plot was used to assess the agreement between the two methods. The strength of agreement between the two techniques was assessed by the concordance correlation coefficient (ρc); ρc is characterized by the measurement of both precision (ρ) and accuracy (Cb) according to the following formula: ρc = ρ x Cb. To compare paired and unpaired variables we used the Wilcoxon or the Mann-Whitney test, respectively. The Kruskal-Wallis Test was used to compare more than two different groups. Correlation analysis were performed using the Spearman correlation analysis (*rs*). Multivariable linear regression analysis were performed to assess the association between circulating insulin and hepatic fibrosis and steatosis by Fibroscan™. The statistical analysis were performed with MedCalc® Software version 18.9.1 (MedCalc Software bvba, Ostend, Belgium).

Results

One hundred and sixty MASLD patients (male, 114 [71.2%]; mean age, 50 ± 12 years) with available plasma samples and complete clinical and biochemical data were included in the study. Clinical and biochemical characteristics of the study cohort are reported in Table 1. Most of the subjects were obese (61.2%), 27 subjects (17%) had T2DM and 55 subjects (34.6%) had arterial hypertension. The median values of serum insulin were 400 pg/mL (range 355-440 pg/mL) and 527 pg/mL (range 444-640 pg/mL), assessed by Alinity™ and Bio-Plex Luminex™, respectively.

Correlation between assays

Bio-Plex Luminex™ insulin values were significantly higher compared to Alinity™ insulin values ($p<0.001$). For the comparison between Alinity™ and Bio-Plex Luminex™ analytical methods, we performed the Passing & Bablok regression analysis. The correlation coefficient was 0.45 (95% CI: 0.322 - 0.569). The scatter diagram with the regression line is reported in Figure 2A. We found both systematic (intercept A: 190.657 [95% CI: 148 - 230]) and proportional differences (intercept B: 0.463 [95% CI: 0.374 - 0.546]) between the two methods; furthermore, we observed random differences (residual standard deviation: 196 [-384 - 384]). Bland-Altman plot confirmed that the differences between the two methods did not fell within ± 1.96 SD of the mean, indicating that they may not be used interchangeably (Figure 2B). Concordance analysis showed a ρ 0.398 (95% CI: 0.656 - 0.809), with ρ = 0.524 and Cb = 0.759.

Correlations between insulin measurements and clinical and biochemical parameters

Overall, both Alinity™ and Bio-Plex Luminex™ insulin levels did not discriminate MASLD patients with T2DM from those without (Figure 3A-B). Alinity™, but non Bio-Plex Luminex™

insulin levels, were significantly higher in obese patients compared to lean/overweight subjects (444 pg/mL vs. 351 pg/mL, P = 0.007) and in patients with arterial hypertension compared to those without (360 pg/mL vs. 476 pg/mL, P = 0.004), Figure 3C-F.

Alinity™ insulin significantly correlated with glucose levels (rs = 0.34, P < 0.001), BMI (rs = 0.29, P < 0.001), triglycerides levels (rs = 0.24, P = 0.002), AST levels (rs = 0.34, P = 0.002), ALT levels (rs = 0.38, P < 0.001) and HDL-cholesterol levels (rs = -0.25, P = 0.001). On the contrary, Bio-Plex Luminex™ insulin showed no correlation with fasting glucose, BMI and triglycerides levels while significantly correlated with AST, ALT and HDL-cholesterol levels (rs = 0.27, P < 0.001; rs = 0.30, P < 0.001; rs = -0.19, P = 0.018; respectively), according with Alinity™ Table 2.

Association between insulin measurements and histological features of MASLD

To assess the clinical utility of insulin levels measured by both Alinity™ and Bio-Plex Luminex™, we explored their correlation with the amount of liver steatosis and with the severity of hepatic fibrosis by Fibroscan™ (CAP and liver stiffness). Alinity™ insulin showed a significant correlation with both liver stiffness and CAP, while Bio-Plex Luminex™ insulin showed a borderline correlation with CAP, Figure 4. At multivariable regression analysis adjusted for age, sex, BMI and T2DM, both Alinity™ and Bio-Plex Luminex™ insulin was significantly associated with liver stiffness ($r_{partial}$ = 0.32, P < 0.001 and $r_{partial}$ = 0.17, P = 0.032, respectively) while only Alinity™ insulin was significantly associated with CAP ($r_{partial}$ = 0.50, P < 0.001), Table 3.

Discussion

In this study, we compared the analytical and clinical performance of two different analytical methods for the measurement of insulin in plasma and serum samples of patients with MASLD. The two methods are used in different contexts: the use of Alinity platform is intended for the routine clinical biochemistry, while Luminex system is used for research purposes. From our analysis, we observed that i) the two methods are not comparable and that ii) chemiluminescence by Alinity i is the most accurate and reliable analytical method for the measurement of circulating insulin. Alinity i system by Abbott is a platform designed to simplify diagnostics and to ensure the most reliable results through assays design free from biotin interference. Recently the Food and Drug Administration (FDA) released a Safety Communication warning the public that “biotin supplementation may interfere with laboratory tests” [12]. In the study by Katzman et al., the authors administered a survey questionnaires to 4000 outpatients reporting a high biotin concentration in about 7% of them (>10 ng/mL or >40.9 nmol/L) suggesting both laboratorians and clinicians to develop effective strategies to mitigate the negative impact of biotin in clinical

laboratory determinations. [13]. In fact, laboratory tests that use streptavidin–biotin binding mechanisms may be affected by high circulating biotin concentrations, leading to positive and negative interference in biotinylated competitive and noncompetitive (sandwich) immunoassays, respectively [14,15]. For this reason, harmonization of laboratory tests is a very important endpoint allowing the comparison of results obtained in different laboratories independent of when the analysis are performed.

From a clinical point of view, both Alinity™ and Bio-Plex Luminex™ insulin levels did not discriminate MASLD patients with T2DM from those without; this is because diabetic patients were treated with glucose lowering drugs and antidiabetic treatments normalize hyperglycemia affecting the results. Conversely, only Alinity™ insulin but not Bio-Plex Luminex™ insulin, was able to discriminate obese from normal weight/overweight subjects and MASLD patient with arterial hypertension from those without. When we analyzed the correlation between insulin levels and hepatic fibrosis and steatosis by Fibroscan™ we showed that Alinity™ insulin significantly correlated with both liver stiffness and CAP while insulin by Bio-Plex Luminex™ significantly (but weakly) correlated only with liver stiffness. The correlation between Bio-Plex Luminex™ insulin and CAP did not reach statistical significance casting doubts on the reliability of the method. In fact, the correlation between insulin levels and hepatic steatosis is common in patients with MASLD considering that high insulin levels and insulin resistance are two hallmarks of these patients. Multivariable linear regression analysis confirmed that the most reliable association between insulin levels and both liver stiffness and CAP by Fibroscan™ derived by the use of Alinity™ analytical system.

The most important limitation of the study is the use of thawed samples for the measurement of Bio-Plex Luminex™ insulin. Despite this, samples were thawed only once and stored at -80°C until use. Moreover, these samples are part of the same blood drawn used for insulin measurement with Alinity™. Another important difference between the two measurements is that insulin by Bio-Plex Luminex™ was not measured as single analyte, but using a panel including additional seven analytes (interleukin [IL]-1b, IL-6, IL-8, leptin, monocyte chemoattractant protein-1, nerve growth factor and tumor necrosis factor-alpha). Although the analytical kit is optimized for avoiding cross-reactions among different analytes, we cannot completely exclude that insulin was affected by other variables. Notwithstanding this, both low and high internal controls were in the correct range after each analytical session. Finally, Alinity™ insulin was measured on serum samples while Bio-Plex Luminex™ insulin was measured on plasma samples. We were aware of the potential confounder due to the differences between biological matrices but both methods allow the measurement in serum and plasma.

Our results suggest that Bio-Plex insulin data should be used with caution and only for research purposes, while the automated and validated system Alinity™ allows obtaining reliable results that can be used in the clinical setting. However, it is important to continue through the process of insulin harmonization to ensure reliable, comparable and reproducible results.

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Notes

Conflicts of interest: The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript

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Tables.

Table 1. Clinical and biochemical characteristics of the study cohort (N = 160).

Variables	**N = 160**
Age, y	51 (49-54)
Sex M/F, n (%)	114/46 (71%/29%)
BMI, kg/m^2	30.8 (30.1-32.3)
Obesity, n (%)	98 (61.2%)
T2DM, n (%)	28 (17.5%)
Arterial Hypertension, n (%)	55 (34.4%)
AST, iU/L	28 (26-30)
ALT, iU/L	36 (31-41)
Albumin, g/L	45 /45-46)
Fasting glucose, mg/dL	85 (83-87)
Fasting insulin, pg/mL (Alinity™)	400 (355-440)
Fasting insulin, pg/mL (Bio-Plex Luminex™)	527 (444-640)
Total cholesterol, mg/dL	202 (195-209)
LDL-cholesterol, mg/dL	135 (128-139)
HDL-cholesterol, mg/dL	52 (49-55)
Triglycerides, mg/dL	108 (99-117)

Data are reported as median and 95% confidence interval (CI) for continuous variables and as number and percentage (%) for dicotomous variables. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; HDL, high density lipoproteins; LDL, low density lipoproteins; M, male; T2DM, type 2 diabetes mellitus.

Table 2. Correlations between Alinity™ and Bio-Plex Luminex™ insulin levels with biochemical parameters.

		Age	BMI	AST	ALT	Albumin	Glucose	Total chol	HDL-chol	LDL-chol	Triglyc
Alinity™ insulin	r_S	0.027	0.293	0.341	0.377	-0.069	0.34	-0.071	-0.251	-0.096	0.238
	P	0.736	<0.001	<0.001	<0.001	0.389	<0.001	0.371	0.001	0.227	0.002
Bio-Plex Luminex™ insulin	r_S	-0.103	0.135	0.273	0.297	-0.059	0.018	-0.045	-0.187	0.026	0.11
	P	0.194	0.088	<0.001	<0.001	0.459	0.822	0.569	0.018	0.747	0.165

Correlations are described by the Spearman r coefficient (r_S) for non-parametric continuous variables. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; chol, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; triglyc, triglycerides.

Table 3. Multiple regression analysis.

	Liver stiffness			CAP		
Variables	**Coefficient**	**P value**	**r_p**	**Coefficient**	**P value**	**r_p**
Age, y	0.02105	0.012	0.203	0.3114	0.231	0.097
Sex M/F, n (%)	0.4404	0.053	0.157	1.0195	0.885	0.012
BMI, kg/m^2	0.04208	0.181	0.109	3.4815	<0.001	0.278
T2DM, n (%)	-0.1057	0.679	-0.034	-8,1249	0.310	-0.083
Fasting insulin, pg/mL (Alinity™)	0.001719	<0.001	0.315	0.09446	<0.001	0.504
Age, y	0.01922	0.026	0.179	0.1968	0.507	0.054
Sex M/F, n (%)	0.3005	0.203	0.104	-5.0194	0.537	-0.050
BMI, kg/m^2	0.06669	0.037	0.169	4.9549	<0.001	0.345
T2DM, n (%)	-0.1007	0.705	-0.031	-9.0712	0.324	-0.080
Fasting insulin, pg/mL (Bio-Plex Luminex™)	0.0005111	0.032	0.174	0.01459	0.075	0.144

BMI, body mass index; CAP, controlled attenuation parameter; F, female; M, male; T2DM, type 2 diabetes mellitus; y, years.

Figures

Figure 1. Analytical method comparison between Bio-Plex 200 system (A) and Alinity i platform (B).

Footnote. Bio-Plex 200 system and Alinity i platform are characterize by two different analytical methods for the measurement of circulating insulin. Specifically, the automated Alinity™ i system method is based on chemiluminescent detection technology with an assay design "free from biotin" interference, while Bio-Plex method is based on the quantitative suspension immunofluorescence. This figure is created with BioRender.

Figure 2. Scatter diagram with regression line by Passing & Bablok analysis (A) and Bland-Altman plot (B).

Footnote. Insulin measurements are reported in pg/mL.

Figure 3. Alinity™ and Bio-Plex Luminex™ insulin levels according to metabolic comorbidities.

Footnote. Alinity™ and Bio-Plex Luminex™ insulin levels according to type 2 diabetes (A-B), obesity (C-D) and arterial hypertension (E-F). AH, arterial hypertension; NW, normal weight; OB, obese; OW, overweight; T2DM, type 2 diabetes mellitus.

Figure 4. Correlations between Alinity™ and Bio-Plex Luminex™ insulin levels with hepatic fibrosis (A-B) and steatosis (C-D) by Fibroscan™.

Footnote. The severity of liver fibrosis and steatosis were assessed by Fibroscan™ (Echosense). CAP, controlled attenuation parameter; LS, liver stiffness.