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MINI-REVIEW

# Advances in the clinical measurement of glucagon: from diagnosis to therapy

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## Abstract

Glucagon has many functions: it promotes glucose production, fatty acid oxidation, thermogenesis, energy consumption, lipolysis, and myocardial contraction, and suppresses lipogenesis, appetite, and gastrointestinal motility. Which of these functions are physiological and which are pharmacological is not fully understood. Although the Mercodia sandwich ELISA provides significantly higher specificity of glucagon measurement than does conventional competitive RIA, it cannot provide accurate plasma glucagon values in the presence of elevated cross-reacting plasma glicentin. This occurs in patients post-pancreatectomy or bariatric surgery and in around 30% of outpatients suspected for glucose intolerance who have not had surgery. Thus, our newly developed sandwich ELISA with higher specificity and higher sensitivity than the Mercodia sandwich ELISA is needed for accurate measurements of plasma glucagon in diabetic patients. It is expected that the new sandwich ELISA will contribute to personalized medicine for diabetes by its use in clinical tests to accurately diagnose the conditions of diabetic patients in order to design better individual treatment strategies. Meanwhile, clinical trials are being conducted worldwide to apply glucagon/GLP-1 receptor dual agonists and glucagon/GLP-1/GIP receptor triagonists to the treatment of obesity, fatty liver, and diabetes. Most clinical trials have shown that both types of drugs have stronger effects on weight reduction, improving fatty liver, and glucose tolerance than do the single GLP-1 receptor agonists. Glucagon is expected to be used as a new diagnostic marker and in a new therapeutic strategy based on a true understanding of its physiological and pharmacological functions.

**Keywords** Glucagon · Sandwich ELISA · Type 2 diabetes · Glucagon/GLP-1 receptor dual agonist · Glucagon/GLP-1/GIP receptor triagonist

## Introduction

Glucagon is a hormone that was discovered as a blood glucose-elevating substance in 1923, 2 years after the discovery of insulin [1]. For a long time, glucagon has played only a minor role in the field of diabetes, as an antagonistic hormone to insulin. However, the clinical application of antidiabetic drugs such as DPP-4 inhibitors and GLP-1 receptor agonists that suppress glucagon secretion as well as enhance insulin secretion has focused more attention on glucagon in diabetes research [2]. Furthermore, the importance of glucagon in regulating blood glucose levels is further reinforced by the fact that blood glucose levels barely increase even when pancreatic β-cells are diminished by streptozotocin in pancreatic α-cell-deficient mice and glucagon receptor-deficient mice [3, 4]. On the other hand, glucagon research has been delayed compared with insulin research because there has been no accurate assay for plasma glucagon. Accordingly, there is no consensus opinion regarding glucagon abnormalities in the pathophysiology of diabetes, and plasma glucagon levels have rarely been measured in clinical examinations. In this review, we summarize the clinical studies on glucagon that have used the Mercodia sandwich ELISA and then explain the problems with this method. We also introduce our newly developed sandwich ELISA, which was revealed to be more useful than Mercodia sandwich ELISA for the pathophysiological diagnosis of diabetes. Lastly, we refer to the current status of new medicines targeting glucagon. We hope that the new sandwich ELISA will contribute to future personalized medicine for diabetes.

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## Problems in the immunoassays for measuring glucagon in plasma

Glucagon measurement using competitive RIA was established in the 1960s [5, 6]. However, this method is susceptible to problematic cross-reactivity with other, glucagon-related peptides. Proglucagon undergoes processing mainly in the stomach and intestines, and various peptides containing the same amino acid sequence as glucagon are secreted into the circulation (Fig. 1) [7]. Therefore, to avoid cross-reaction with these other peptides, conventional glucagon measurement methods have used an antibody specifically recognizing the C-terminal end of glucagon, as the C-terminal ends of other proglucagon-derived peptides differ from that of glucagon [8]. However, when glucagon is measured by RIA with a glucagon C-terminal antibody on gel-filtration chromatography fractions of plasma, immunoreactive compounds with molecular weights different from that of glucagon are detected in several fractions; this is known as glucagon molecular heterogeneity [9]. To overcome this problem, a sandwich ELISA using both N-terminal and C-terminal glucagon antibodies has been developed. This method theoretically can avoid cross-reactivities with peptides having the same C-terminal ends as glucagon. Indeed, the Mercodia sandwich ELISA (Glucagon ELISA #10-1271-01; Mercodia, Uppsala, Sweden) has been reported to be the most accurate method for measuring plasma glucagon [10, 11]. However, several cross-reactivity tests have revealed that the Mercodia sandwich ELISA still has cross-reactivities with several glucagon-related peptides, constituting at least several percent of the signal [12]. Therefore, we developed a new glucagon assay using liquid chromatography-mass spectrometry (LC–MS/MS) instead of antibodies [13]. Because even glucagon-related peptides that cause cross-reactivity in immunoassays have different molecular weights from glucagon, they are not detected by this method. However, glucagon measurement using LC–MS/MS could not be applied to clinical examinations because it is time consuming and costly. Therefore, we conducted validation tests by comparison with LC–MS/MS and found that the Mercodia sandwich ELISA showed much better correlation with LC–MS/MS than did the conventional competitive RIA, indicating that the Mercodia sandwich ELISA is better than the conventional RIA and thus can be used for clinical examinations [13].

## Evaluation of plasma glucagon levels in type 2 diabetes patients by the Mercodia sandwich ELISA

We evaluated plasma glucagon levels in type 2 diabetic patients, using the Mercodia sandwich ELISA. As shown in Fig. 2, both the glucose tolerance test (left panels) and the meal tolerance test (right panels) were conducted in the same type 2 diabetic patients and the same healthy people. Three significant differences were found in plasma glucagon levels between type 2 diabetic patients and healthy people. (1) Type 2 diabetic patients have higher fasting plasma glucagon levels than do healthy people. (2) Plasma glucagon levels 30 min after glucose loading did not change in type 2 diabetic patients, although they decreased in healthy people. (3) Plasma glucagon levels 30 min after meal loading increased more markedly in type 2 diabetic patients than in healthy people [14]. By contrast, when the same plasma sample were measured by the conventional competitive RIA, these important differences were not observed at all. Furthermore, the difference in the plasma glucagon levels before and 30 min after meal loading (ΔGlucagon 0–0.5) measured by the Mercodia sandwich ELISA was well correlated with the levels of blood glucose and glucose intolerance in type 2 diabetic patients, suggesting that plasma glucagon levels might be associated with pathophysiological conditions in type 2 diabetes [14].

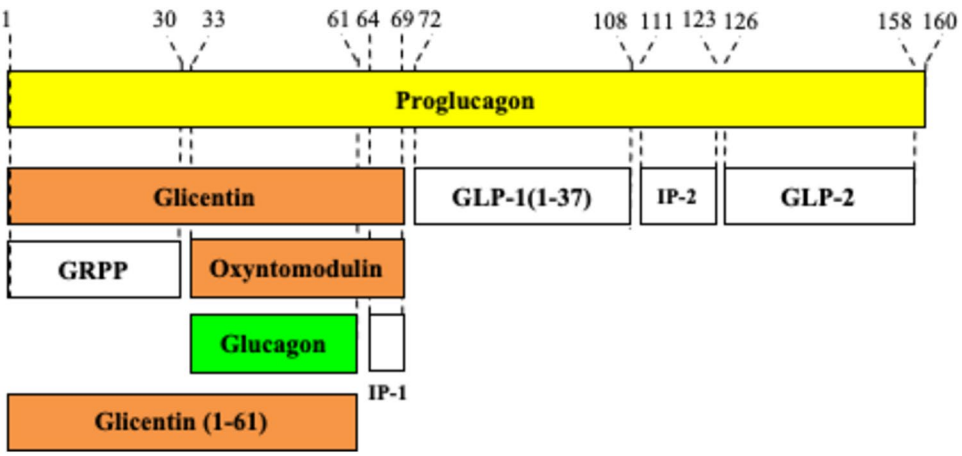



**Fig. 1** Various glucagon-related peptides produced from proglucagon

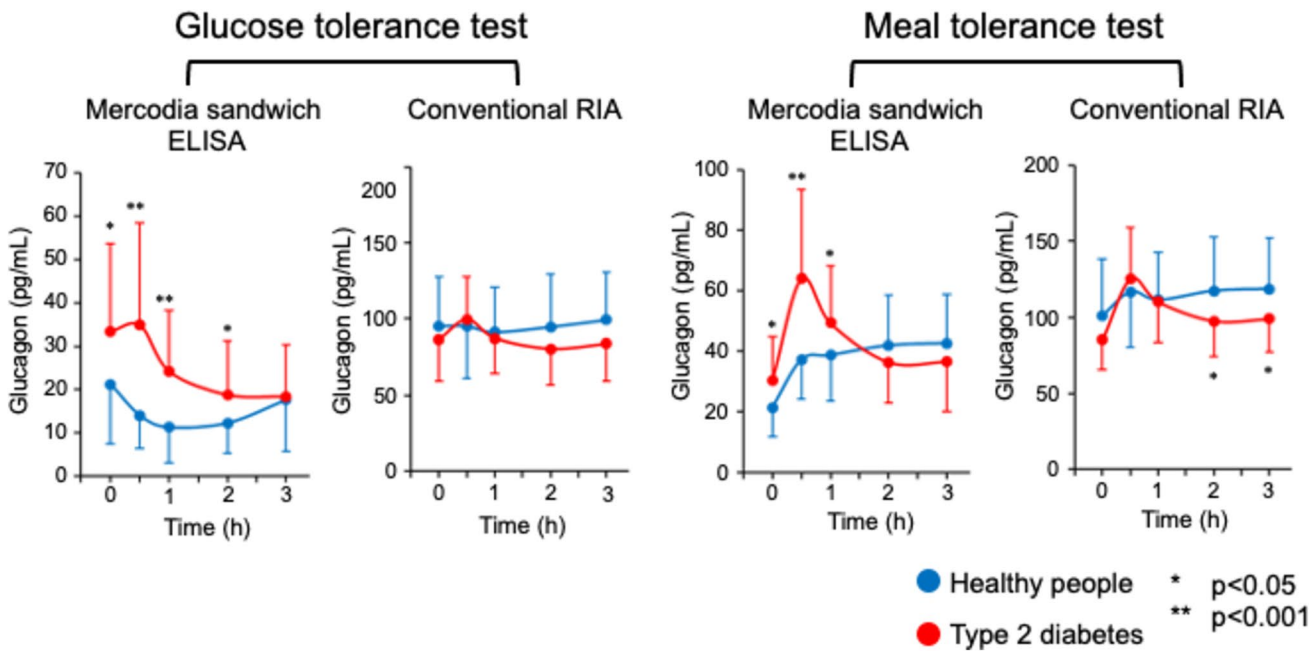



**Fig. 2** Plasma glucagon levels measured by the Mercodia sandwich ELISA (left) or conventional competitive RIA (right) during a glucose tolerance test (left) or meal tolerance test (right) in type 2 diabetic patients (red) and healthy people (blue). Quoted and modified from Ref. [13] with permission from Endocri J

## The Mercodia sandwich ELISA provides falsely high plasma glucagon values in patients post-pancreatectomy and bariatric surgery (laparoscopic sleeve gastrectomy)

There has been a long debate as to whether plasma glucagon can no longer be detected if the pancreas is removed. In the past, it has been reported that glucagon could not be detected by RIA in gel-filtration chromatography fractions of plasma from pancreatectomized patients [15]. However, recently it was reported that plasma glucagon levels could be measured in patients with pancreatectomy when assayed by sandwich ELISA. Moreover, they were elevated after glucose loading more drastically than in healthy people [16]. Therefore, in order to put an end to this controversy, we measured plasma glucagon levels after glucose loading in the patients with pancreatectomy, using LC–MS/MS and the Mercodia sandwich ELISA. As shown in the top panels in Fig. 3, while plasma glucagon levels in patients with pancreatectomy were below the limit of quantification by LC–MS/MS, they could apparently be measured by the Mercodia sandwich ELISA and were dramatically elevated after glucose loading [17]. Intriguingly, plasma glicentin also increased after pancreatectomy, and the pattern of plasma glicentin increase after glucose loading was similar to the pattern of plasma glucagon measured by the Mercodia sandwich ELISA. As mentioned earlier, cross-reactivity of several percent with glicentin has been observed in the Mercodia sandwich ELISA, and considering that the plasma concentration of glicentin was approximately 20 times higher than the plasma glucagon concentration, the measurement values using the Mercodia sandwich ELISA are likely false values due to cross-reaction with glicentin [17]. Although not elucidated as glicentin, previous report also suggested the increase in immunoreactive glucagon (probably glucagon-related peptides including glicentin) in the plasma of pancreatectomized patients [18]. Additionally, a similar phenomenon was observed in the patients who underwent bariatric surgery, laparoscopic sleeve gastrectomy (LSG), and Roux-en-Y gastric bypass [19, 20]. As shown in the lower panels in Fig. 3, both before and after LSG, glucagon levels measured by LC–MS/MS decreased after glucose loading, whereas glucagon levels measured by the Mercodia sandwich ELISA paradoxically increased after surgery. Because the plasma glicentin levels were also markedly increased after surgery, the glucagon values measured by the Mercodia sandwich ELISA are likely false, due to cross-reaction with glicentin [19]. Therefore, although the Mercodia sandwich ELISA is more accurate than the conventional competitive RIA, it gives incorrect values after resection of the pancreas or gastrointestinal tract. Thus, it was considered necessary to develop a more specific measurement method that reduces cross-reactivity with glucagon-related peptides such as glicentin.

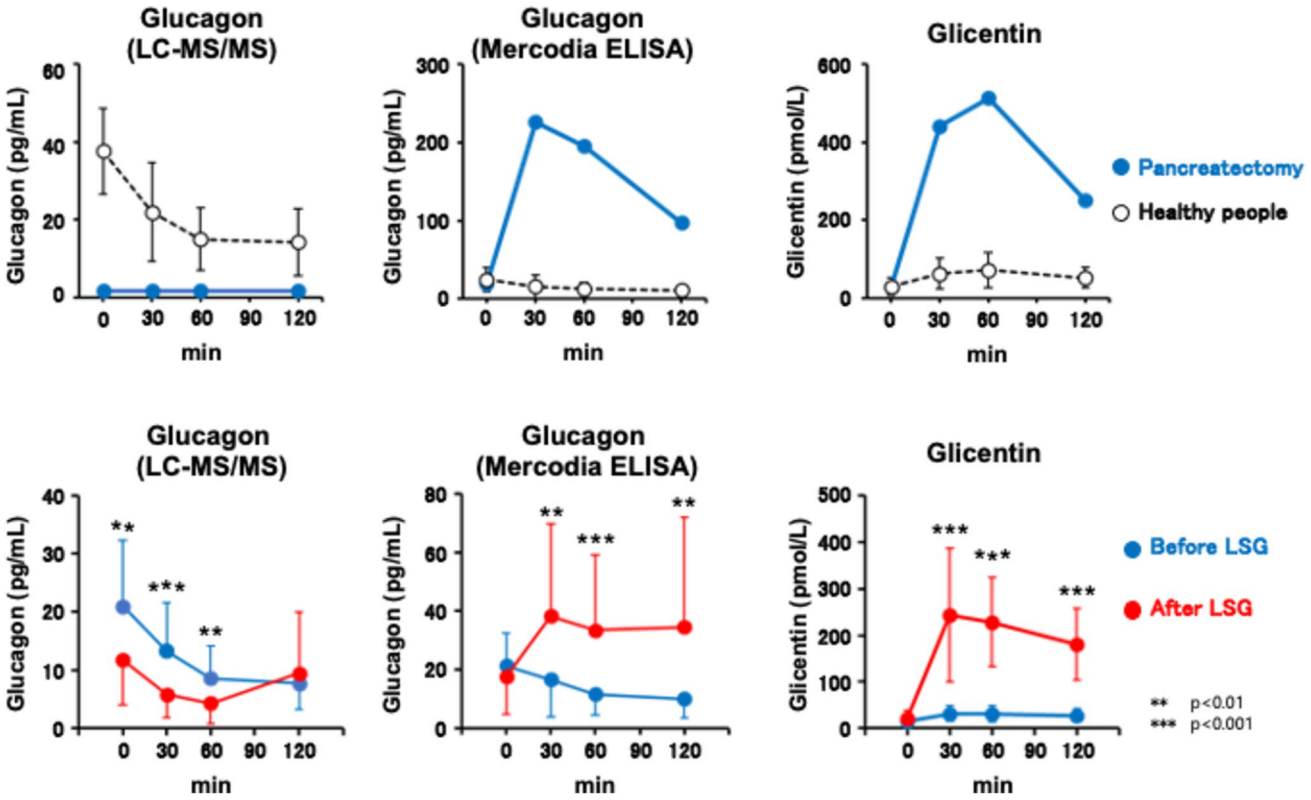


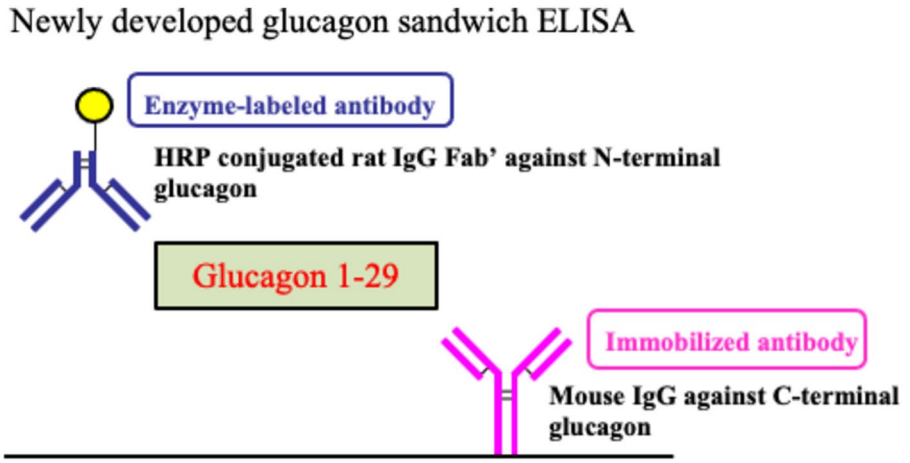

**Fig. 3** Top: Plasma glucagon levels measured by LC–MS/MS (left) or the Mercodia sandwich ELISA (middle) and plasma glicentin levels (right) during glucose tolerance tests in patients with pancreatectomy (blue lines). The dashed white lines indicate the pattern in healthy people for comparison. Bottom: Plasma glucagon levels measured by LC–MS/MS (left) or the Mercodia sandwich ELISA (middle) and plasma glicentin levels (right) during glucose tolerance tests in patients before (blue) and after (red) laparoscopic sleeve gastrectomy (LSG). Quoted and modified from Refs. [16, 17] with permission from J Diabetes Investig

## Development of a new glucagon sandwich ELISA having higher specificity and higher sensitivity than the Mercodia sandwich ELISA

As mentioned before, the Mercodia sandwich ELISA cross-reacts with various glucagon-related peptides by several percent [12]. Some may think that several percent cross-reactivity is within an acceptable error range, but because the cross-reacting peptides exist at high concentrations in plasma after pancreatectomy or bariatric surgery, the issue cannot be ignored. Therefore, to develop a more specific glucagon sandwich ELISA in which cross-reactivities with the other glucagon-related peptides would be reduced drastically, we generated two monoclonal antibodies having high specificity and high sensitivity to the N-terminal and C-terminal end of glucagon, respectively, and constructed a sandwich ELISA system (the design scheme is shown in the top panel of Fig. 4). As shown in the bottom panel in Fig. 4, cross-reactivity tests revealed that there was almost no cross-reactivity with glucagon-related peptides including glicentin in the new sandwich ELISA, in striking contrast to the Mercodia sandwich ELISA where several percent cross-reactivity with the other peptides remained [19]. In fact, as shown in the right-hand panels in Fig. 4, the new sandwich ELISA showed much better correlation with LC–MS/MS than did the Mercodia sandwich ELISA, even in the samples after LSG [19].

## The new sandwich ELISA is expected to contribute to the diagnosis of pathophysiological conditions of individual diabetic patients

As we have demonstrated, the newly developed sandwich ELISA is indispensable for accurate measurements of plasma glucagon in patients who have undergone pancreatectomy or bariatric surgery. However, most diabetic patients as well as healthy people have not undergone such surgeries, so there seemed to be no problem with using the Mercodia sandwich ELISA for the measurement in these individuals. In fact, we and others have also reported that plasma glucagon values measured by the Mercodia




Cross-reactivity tests

| Peptides | Millipore RIA | Mercodia ELISA | New ELISA |
|---|---|---|---|
| Glucagon (1-29) | 100 | 100 | 100 |
| Glucagon (19-29) | >100 | <0.01 | ND |
| Glucagon (3-29) | 34.7 | 4.6 | ND |
| Glicentin (1-61) | >100 | 5.6 | 0.05 |
| Oxyntomodulin | <1 | 6.5 | 0.06 |
| Glicentin | <1 | 5.3 | ND |

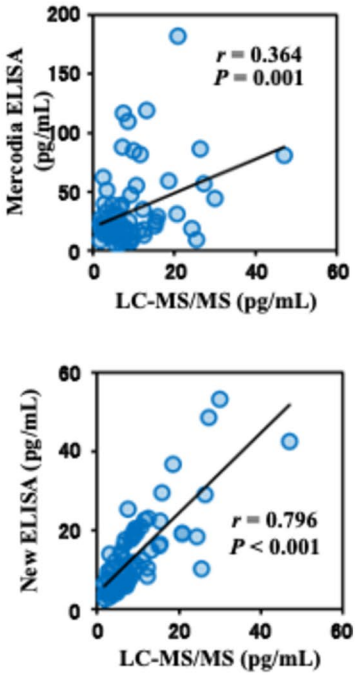



**Fig. 4** Left: Schematic diagram of the newly developed glucagon sandwich ELISA (top) and the results of cross-reactivity tests in the Mercodia sandwich ELISA and new sandwich ELISA (bottom). Right: Correlations between plasma glucagon values derived by LC–MS/MS and the Mercodia sandwich ELISA (top) or the new sandwich ELISA (bottom) in plasma samples after bariatric surgery. Quoted and modified from Ref. [17] with permission from J Diabetes Investig

sandwich ELISA show good correlation with those measured by LC–MS/MS in healthy people and mild type 2 diabetic patients [13, 21]. However, we recently obtained the following surprising results. In a joint clinical study with the diabetes specialist clinic, we measured plasma glucagon levels in 104 outpatients suspected for glucose intolerance by using the new sandwich ELISA and the Mercodia sandwich ELISA and analyzed the correlations in the plasma glucagon values between these two methods. Although good correlations were found between these two ELISAs in many patients, in some patients apparently poor correlations were observed (Fig. 5, left top panel). We then classified these patients according to their correlation coefficients and found that 74 out of 104 patients had high correlation coefficients (above 0.8), but the remaining 30 patients had very low and variable correlation coefficients (Fig. 5, middle top panel and right-hand panels). Therefore, we divided these patients into two groups having either high (> 0.8) or low (< 0.8) correlation coefficients. Although we found that plasma glucagon levels measured using the new sandwich ELISA decreased after glucose loading in both groups (Fig. 5, left bottom panel), plasma glucagon levels paradoxically increased after glucose loading in the latter group when measured by the Mercodia sandwich ELISA (Fig. 5, middle bottom panel). Importantly, plasma glicentin levels also increased drastically after glucose loading in the latter group, suggesting that the glucagon values measured by the Mercodia sandwich ELISA in the latter group were false values due to cross-reaction with glicentin [19]. At present, it is unclear why plasma glicentin levels were so high in these 30 patients; however, approximately 30% of type 2 diabetic patients may have high plasma glicentin levels that could lead to falsely high plasma glucagon levels when measured by the Mercodia sandwich ELISA. Thus, we concluded that the new sandwich ELISA is indispensable for accurate assessments of plasma glucagon levels in type 2 diabetic patients [19]. We lastly inform that the new sandwich ELISA has been already commercially available from Immuno-Biological Laboratories Co. Ltd as a research reagent. Also, this kit is planning to be adopted as a clinical examination in Japan.

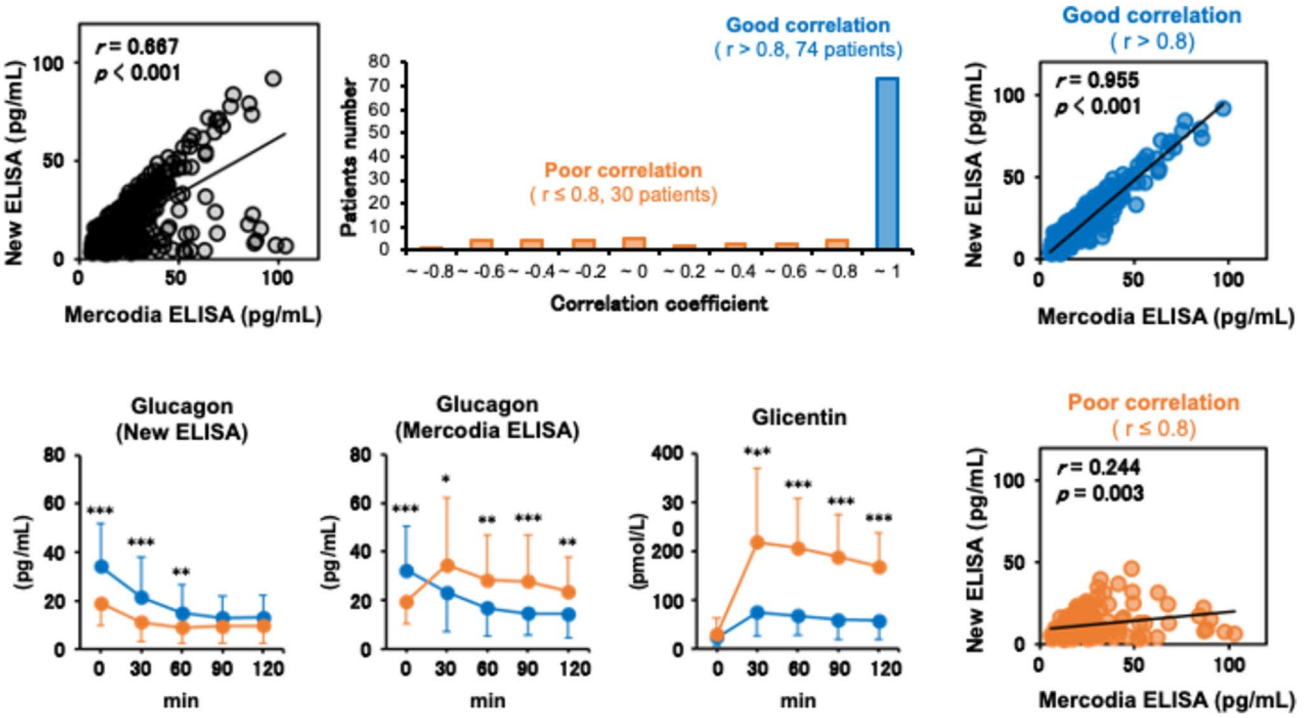



**Fig. 5** Top left: Correlations between plasma glucagon levels measured by the new sandwich ELISA versus the Mercodia sandwich ELISA in 104 patients suspected for impaired glucose tolerance. Top middle: distribution diagram for each correlation coefficient in 104 patients. Bottom: Plasma glucagon levels measured by LC–MS/MS (left) or the Mercodia sandwich ELISA (middle) and plasma glicentin levels (right) during glucose tolerance tests in the patients having high (> 0.8) correlation coefficients (blue) or low (< 0.8) correlation coefficients (orange). Right: Correlations between plasma glucagon levels measured by the new sandwich ELISA versus the Mercodia sandwich ELISA in the patients with high correlation coefficients (blue) or low correlation coefficients (orange). Quoted and modified from Ref. [17] with permission from J Diabetes Investig

## Current status of clinical applications of glucagon as a therapeutic target

### Current glucagon formulations

Injectable glucagon (intramuscular or intravenous) has been used as an emergency treatment for severe hypoglycemia and as a preparation for gastric endoscopy. These are clinical applications that utilize glucagon's functions of promoting glucose production and suppressing gastrointestinal motility, respectively. A glucagon nasal spray, which is easier to use, has recently also been used clinically as an emergency treatment for hypoglycemia [22].

### Glucagon receptor antagonists as antidiabetic agents

Progress has been made in the development of glucagon receptor antagonists that suppress glucose production in the liver and decrease blood glucose levels. However, because these drugs also inhibit the other beneficial effects of glucagon and have side effects of weight gain and accelerating fatty liver, their development has been discontinued [23]. In the future, it will be necessary to develop fine-tuning drugs that can reduce the excessive glucagon actions to the appropriate levels in diabetic patients.

### Glucagon/GLP-1 receptor dual agonists

Recent new findings demonstrate that the promotion of lipolysis in hepatocytes by glucagon is essential for β-oxidation and gluconeogenesis in mitochondria, and the mechanism of glucagon's beneficial action on fatty liver has been clarified [24]. Utilizing these beneficial effects of glucagon, the development of glucagon receptor agonists for fatty liver and obesity is progressing. However, because glucose production in the liver increases and the blood glucose level rises, these are not single agonists but dual agonists with the GLP-1 receptor. They are expected to have countervailing effects through GLP-1 on blood glucose levels, and additive effects through GLP-1 on appetite suppression, weight loss, and improvement of fatty liver. It was first reported in 2009 that the glucagon/GLP-1 dual agonist reduced body weight and improved glucose intolerance in an obese mouse model by suppressing food intake and increasing energy expenditure [25]. Furthermore, in a clinical study, administration

of the glucagon/GLP-1 dual agonist to obese type 2 diabetic patients resulted in decreasing blood glucose levels and body weight, as well as improvements in lipid markers. In this study, although gastrointestinal symptoms such as nausea and vomiting were reported as side effects, they were mild [26]. Since then, several clinical trials have been conducted, including one on Asian subjects, and all have shown positive results on body weight. However, there are reports showing that the dual agonist had no beneficial effect on blood glucose levels or HbA1c [27]. Thus, depending on the affinity ratio between GLP-1 receptors and glucagon receptors, glucagon's effect of promoting glucose production may predominate, and therefore, those dual agonists could be unsuitable as diabetic drugs. On the other hand, because glucagon receptors are most abundantly expressed in hepatocytes, and because glucagon promotes lipolysis while it suppresses lipogenesis in hepatocytes, strong improvement of fatty liver can be expected. In fact, it was reported that the beneficial effect of the dual agonist on fatty liver was nearly twice that of the single GLP-1 receptor agonist, with the comparable weight reduction effects [28]. Currently, the development and clinical trials of glucagon/GLP-1 dual agonists are underway around the world, and clinical applications are expected within the next few years.

### Glucagon/GLP-1/GIP receptor triagonists

The results of the first glucagon/GLP-1/GIP triagonist trial were announced in 2015. The triagonist reduced body weight more strongly in an obese mouse model than did the GLP-1/GIP dual agonist [29]. Furthermore, the results of the clinical trials of triagonist have recently been published one after another, showing significant weight reduction and improved glucose intolerance in obese type 2 diabetic patients [30]. However, it remains unclear whether the triagonists are significantly more effective than the GLP-1/GIP dual agonists and glucagon/GLP-1 dual agonists, and whether their side effects such as gastrointestinal symptoms are comparable to those of the dual agonists. The results of the comparative analysis of these clinical trials are awaited.

## Conclusions

The new sandwich ELISA is expected to be more useful than the Mercodia sandwich ELISA for glucagon assessment in type 2 diabetic patients. In the future it will be important to clarify what background characteristics and pathological factors in diabetic patients are associated with abnormal glucagon secretion. Once the drugs that mainly target glucagon are applied clinically, there will be more opportunities to evaluate plasma glucagon levels, and therefore, the importance of accurate glucagon measurements using the new sandwich ELISA should increase. In the future, we hope that accurate glucagon measurement will lead to a correct understanding of the pathophysiological conditions of diabetic patients and thus contribute to personalized medicine for diabetes.

**Data availability** The data that support the findings of this study are available from the corresponding author T.K. upon reasonable request.

## Declarations

**Conflict of interest** The authors have no conflict of interest to disclose.

**Research involving human and animals participants** Name of institutional or national ethical committee on human experimentation: The Ethical Review Committees of Gunma University. Date of approval: not applicable. Approval number: not applicable.

**Informed consent** Informed consent was obtained from all patients for being included in this study.

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