

# Pseudo-hyperglucagonemia was observed in pancreatctomized patients when measured by glucagon sandwich enzyme-linked immunosorbent assay

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## Keywords

Glicentin, Glucagon, Pancreatectomy

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## ABSTRACT

Glucagon is detected in plasma even after total pancreatectomy, and it is debated whether this glucagon is derived from the gastrointestinal tract. Here, we applied sandwich enzyme-linked immunosorbent assay (ELISA) and liquid chromatography–high-resolution mass spectrometry to measure plasma glucagon levels in one patient after partial pancreatectomy (one-seventh of the pancreas remaining) and three patients after total pancreatectomy. Sandwich ELISA detected higher glucagon levels in pancreatectomy patients than in healthy individuals. In contrast, liquid chromatography–high-resolution mass spectrometry showed that plasma glucagon levels in pancreatectomy patients were below the lower limit of quantification. Plasma glucagon measured by sandwich ELISA showed a striking correlation with plasma glicentin, suggesting cross-reaction with this gastrointestinal glucagon-related peptide. These results indicated that pancreatectomized patients falsely showed pseudo-hyperglucagonemia when measured by glucagon sandwich ELISA.

## INTRODUCTION

Glucagon is a 29-amino-acid peptide that is associated with diabetes pathophysiology<sup>1</sup>. Although it is predominantly produced by and secreted from pancreatic  $\alpha$ -cells, glucagon is also produced in the gastrointestinal tract<sup>2</sup>. Plasma glucagon has been detected in patients even after total pancreatectomy<sup>3</sup>, and reports of post-pancreatectomy changes in plasma glucagon levels are controversial. Bajorunas *et al.* reported that total pancreatectomy greatly reduced plasma glucagon levels, as measured by radioimmunoassay with gel chromatography<sup>4</sup>. However, conventional radioimmunoassay lacked sufficient sensitivity and specificity for measuring glucagon in plasma<sup>5</sup>, and thus more accurate sandwich enzyme-linked immunosorbent assay (ELISA) kits were developed<sup>6</sup>. Using sandwich ELISA,

Lund *et al.*<sup>7</sup> showed much higher plasma glucagon levels after glucose loading in total pancreatectomy patients compared with healthy individuals. More recently, we have developed a new glucagon measurement system using liquid chromatography–high-resolution mass spectrometry (LC-HRMS)<sup>8</sup>, which enables more accurate quantification of glucagon values in plasma by excluding the cross-reactivity with other molecules that might affect immunoassay results, including sandwich ELISA measurements. Here, we report the evaluation of plasma glucagon levels using LC-HRMS, revealing hypoglucagonemia in four pancreatectomized patients, with values that clearly differed from the measurements obtained using sandwich ELISA.

## CASE REPORT

We measured plasma glucagon levels in four pancreatectomized patients who gave their consent. Table S1 and Data S1 provide information regarding the patients and study methods. This

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study was carried out under approval by the ethical review committees of Gunma University (#2017-170), University of Tokyo Hospital (#11857e) and Kurume University School of Medicine (#20010).

Patient 1 was partially pancreatectomized, with one-seventh of the pancreas remaining, and was subjected to glucose and meal tolerance tests. All plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification, except for at 30 min after meal loading. In contrast, sandwich ELISA results showed that plasma glucagon levels dramatically increased after both glucose and meal loading (Table 1). Notably, the peak plasma glucagon level after glucose loading in patient 1 was approximately 15-fold higher than in healthy individuals (Figure S1). The results showed similar dramatic increases in plasma glicentin and glucagon-like peptide-1 levels in patient 1 (Table 1; Figure S1). Plasma gastric inhibitory polypeptide level also increased after glucose loading in patient 1, but this increase was also observed in the healthy individuals, suggesting that the gastric inhibitory polypeptide response after glucose loading was not affected by pancreatectomy (Figure S1).

Patients 2–4 had undergone total pancreatectomy, and their plasma glucagon levels were measured before and after

breakfast. As observed in patient 1, plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification, whereas the levels measured by sandwich ELISA were comparable to those in healthy individuals (Table 2).

Even though the plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification value in all four pancreatectomized patients, the sandwich ELISA results showed incorrectly high glucagon values in these patients. One possible reason for the incorrect measurement was that sandwich ELISA might have been affected by cross-reaction with glucagon-related peptides, including glicentin<sup>9</sup>. We found no significant correlation between the plasma concentrations of glucagon and glicentin measured by sandwich ELISA in healthy individuals; however, we detected a highly positive correlation between these two hormones in pancreatectomized patients (Figure S2). This suggested that pancreatectomy increased glicentin secretion, whereas markedly reduced glucagon secretion, which might have been reflected as a good correlation between glicentin and glucagon measured by sandwich ELISA. Another possible explanation was that the sandwich ELISA system per se is generally affected by pancreatectomy. However, plasma gastric inhibitory polypeptide levels measured by sandwich ELISA and LC-triple quadrupole mass spectrometry showed a

**Table 1** | Blood glucose and various plasma hormone levels during glucose and meal tolerance tests in patient 1 (partially pancreatectomized)

Time (min)		0	30	60	120
<i>Oral glucose tolerance test</i>					
Glucagon (pmol/L)	LC-HRMS	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	Sandwich ELISA	5.6	65.0	56.1	28.0
Glucose (mmol/L)		4.89	12.5	16.4	11.1
Insulin (pmol/L)		6.09	54.8	97.4	36.5
C-peptide (pmol/L)		0.1	0.3	0.6	0.7
Glicentin (pmol/L)		29.4	441	513	251
Total GLP-1 (pmol/L)		2.93	62.4	39.8	22.5
Total GIP (pmol/L)	LC-TQMS	25.1	288	196	85.4
	Sandwich ELISA	24.8	238	221	61.0
Active GIP (pmol/L)	LC-TQMS	6.09	57.2	36.5	7.85
	Sandwich ELISA	4.56	67.4	48.8	4.41
<i>Meal tolerance test</i>					
Glucagon (pmol/L)	LC-HRMS	<LLOQ	0.7	<LLOQ	<LLOQ
	Sandwich ELISA	4.6	15.7	30.7	26.4
Glucose (mmol/L)		4.94	10.4	12.9	8.72
Insulin (pmol/L)		6.09	79.2	146	85.3
C-peptide (pmol/L)		0.1	0.4	1.0	1.2
Glicentin (pmol/L)		44.5	237	276	257
Total GLP-1 (pmol/L)		4.25	21.7	25.5	16.4
Total GIP (pmol/L)	LC-TQMS	17.7	265	313	219
	Sandwich ELISA	32.5	357	390	214
Active GIP (pmol/L)	LC-TQMS	5.61	80.2	61.7	24.6
	Sandwich ELISA	4.53	110	88.2	30.4

ELISA, enzyme-linked immunosorbent assay; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; LC-TQMS, liquid chromatography–triple quadrupole mass spectrometry; LLOQ, lower limit of quantification in glucagon measurement (0.5 pmol/L with liquid chromatography–high-resolution mass spectrometry).

**Table 2** | Blood glucose and various plasma hormone levels before and after breakfast in patients 2–4 (totally pancreatectomized)

Time (min)		0	30	60	90	120
<i>Patient 2</i>						
Glucagon (pmol/L)	LC-HRMS	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	Sandwich ELISA	<LLOQ	1.60	1.67	4.01	4.60
Glicentin (pmol/L)		32.5	105	119	177	199
Total GLP-1 (pmol/L)		3.61	14.7	6.08	25.1	16.8
Total GIP (pmol/L)	LC-TQMS	45.0	86.2	110	73.5	56.5
	Sandwich ELISA	32.6	86.8	88.1	77.8	68.3
Active GIP (pmol/L)	LC-TQMS	7.36	30.2	14.3	12.4	8.82
	Sandwich ELISA	3.70	24.9	16.3	16.1	12.1
<i>Patient 3</i>						
Glucagon (pmol/L)	LC-HRMS	<LLOQ	<LLOQ	–	–	–
	Sandwich ELISA	3.16	4.06	–	–	–
Glicentin (pmol/L)		158	174	–	–	–
Total GLP-1 (pmol/L)		10.1	11.3	–	–	–
Total GIP (pmol/L)	LC-TQMS	146	141	–	–	–
	Sandwich ELISA	191	133	–	–	–
Active GIP (pmol/L)	LC-TQMS	50.5	23.8	–	–	–
	Sandwich ELISA	58.4	27.7	–	–	–
<i>Patient 4</i>						
Glucagon (pmol/L)	LC-HRMS	–	<LLOQ	–	–	–
	Sandwich ELISA	–	1.94	–	–	–
Glicentin (pmol/L)		–	129	–	–	–
Total GLP-1 (pmol/L)		–	8.76	–	–	–
Total GIP (pmol/L)	LC-TQMS	–	77.1	–	–	–
	Sandwich ELISA	–	99.2	–	–	–
Active GIP (pmol/L)	LC-TQMS	–	26.6	–	–	–
	Sandwich ELISA	–	33.7	–	–	–

Horizontal bar indicates no sample collected. ELISA, enzyme-linked immunosorbent assay; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; LC-TQMS, liquid chromatography–triple quadrupole mass spectrometry; LLOQ, lower limit of quantification in glucagon measurement (0.5 pmol/L with liquid chromatography–high-resolution mass spectrometry; 1.6 pmol/L with sandwich enzyme-linked immunosorbent assay).

high correlation in the pancreatectomized patients (Figure S3), which probably excluded the latter possibility.

## DISCUSSION

In the present report, LC-HRMS showed that plasma glucagon levels were much lower in pancreatectomized patients compared with those in healthy individuals. In contrast, sandwich ELISA results showed incorrectly high plasma glucagon levels in pancreatectomized patients, most likely due to cross-reaction with glicentin.

Consistent with the present results, Lund *et al.*<sup>7</sup> used sandwich ELISA, and reported that oxyntomodulin, glucagon-like peptide-1 and glucagon levels after glucose loading were higher in total pancreatectomy patients compared with those in healthy individuals. They concluded that this hyperglucagonemia could result from extrapancreatic glucagon secretion<sup>7</sup>. They also carried out more precise analysis using gel chromatography and mass spectrometry, which showed glucagon presence in the plasma even after total pancreatectomy, but they did not compare these measurements between pancreatectomized

patients and healthy individuals. Although glucagon sandwich ELISA shows higher specificity than conventional glucagon radioimmunoassays, there can still be cross-reactivity with glucagon-related peptides, such as glicentin and oxyntomodulin<sup>9</sup>. Therefore, as the plasma glicentin level is extremely elevated after pancreatectomy, sandwich ELISA might show incorrect glucagon values as a result of cross-reaction with glicentin. Similar observations have been reported in the patients after bariatric surgery<sup>10</sup>. It will be of importance to elucidate how glicentin secretion is enhanced by pancreatectomy or bariatric surgery.

Among the four pancreatectomized patients, only patient 1 had a partial pancreatectomy and therefore pancreatic exocrine function, as well as endogenous insulin secretion, remained, whereas patients 2–4 had total pancreatectomy and thereafter received daily insulin injections (Table S1), which might have affected the incretin secretion and accounted for the different correlation coefficient of glucagon and glicentin between patient 1 and patients 2–4. Furthermore, because patient 1 had a nephrectomy, and patient 2 had a resection of the small

intestine, impaired renal function and intestinal function might have affected the stability and secretion of incretin in these patients (Table S1). These are the limitations of this clinical report.

In conclusion, LC-HRMS measurement showed that glucagon secreted from the gastrointestinal tract was very scarce in the pancreatectomized patients; nevertheless, sandwich ELISA falsely showed pseudo-hyperglucagonemia as a result of cross-reaction with gastrointestinal glucagon-related peptides.

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## DISCLOSURE

The authors declare no conflict of interest.

## REFERENCES

1. Unger RH, Orci L. The essential role of glucagon in the pathogenesis of diabetes mellitus. *Lancet* 1975; 1: 14–16.
2. Sasaki H, Rubalcava B, Baetens D, *et al.* Identification of glucagon in the gastrointestinal tract. *J Clin Invest* 1975; 56: 135–145.
3. Holst JJ, Pedersen JH, Baldissera F, *et al.* Circulating glucagon after total pancreatectomy in man. *Diabetologia* 1983; 25: 396–399.
4. Bajorunas DR, Fortner JG, Jaspan JB. Glucagon Immunoreactivity and Chromatographic Profiles in Pancreatectomized Humans: Paradoxical Response to Oral Glucose. *Diabetes* 1986; 35: 886–893.
5. Holst JJ, Christensen M, Lund A, *et al.* Regulation of glucagon secretion by incretins. *Diabetes Obes Metab* 2011; 13(Suppl 1): 89–94.
6. Wewer Albrechtsen NJ, Hartmann B, Veedfald S, *et al.* Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? *Diabetologia* 2014; 57: 1919–1926.
7. Lund A, Bagger JI, Wewer Albrechtsen NJ, *et al.* Evidence of Extraprostatic Glucagon Secretion in Man. *Diabetes* 2016; 65: 585–597.
8. Miyachi A, Kobayashi M, Mieno E, *et al.* Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays. *Anal Bioanal Chem* 2017; 409: 5911–5918.
9. Matsuo T, Miyagawa J, Kusunoki Y, *et al.* Postabsorptive hyperglucagonemia in patients with type 2 diabetes mellitus analyzed with a novel enzyme-linked immunosorbent assay. *J Diabetes Investig* 2016; 7: 324–331.
10. Roberts GP, Kay RG, Howard J, *et al.* Gastrectomy with Roux-en-Y reconstruction as a lean model of bariatric surgery. *Surg Obes Relat Dis* 2018; 14: 562–568.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** | Comparisons of blood glucose and various plasma hormone levels during glucose tolerance testing between patient 1 and healthy subjects. For comparison, data from healthy subjects are presented, some of which were published in Reference 8 and others were unpublished. We used the criteria for healthy as those who are not taking regular medication and whose fasting blood glucose <6.1 mmol/L and 2 h blood glucose <7.8 mmol/L during OGTT according to the WHO guideline. Data from healthy subjects are shown as mean  $\pm$  SD. The number of healthy subjects is indicated on each panel. Red horizontal line indicates the lower limit of quantitation (0.5 pM).

**Figure S2** | Correlations between the plasma glicentin levels and glucagon levels measured by sandwich ELISA in the pancreatectomized patients. For comparison, we present data from healthy subjects—some published in Reference 8 and others unpublished (n = 85).

**Figure S3** | Correlations between liquid chromatography–triple quadrupole mass spectrometry and sandwich enzyme-linked immunosorbent assay results in terms of measured plasma levels of total gastric inhibitory polypeptide (left) and active gastric inhibitory polypeptide (right) in pancreatectomized patients.

**Table S1** | Clinical characteristics of the pancreatectomized patients.

**Data S1** | Supplementary methods.