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ORIGINAL ARTICLE

Effects of passage through the digestive tract on incretin secretion: Before and after birth

Seiichi Tomotaki*, Ryosuke Araki, Kouji Motokura, Yutaro Tomobe, Takeru Yamauchi, Shintaro Hanaoka, Hiroko Tomotaki, Kougoro Iwanaga, Fusako Niwa, Junko Takita, Masahiko Kawai

Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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***Correspondence**
Seiichi Tomotaki
Tel.: +81-75-751-3291
Fax: +81-75-751-2361
E-mail address:
stomotaki@kuhp.kyoto-u.ac.jp

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ABSTRACT

Aims/Introduction: It was reported that fetuses secrete endogenous incretin; however, the stimulants of fetal incretin secretion are not fully understood. To investigate the association between the passage of amniotic fluid through the intestinal tract and fetal secretion of incretin, we analyzed umbilical cord incretin levels of infants with duodenum atresia.

Materials and Methods: Infants born from July 2017 to July 2019 (infants with duodenum atresia and normal term or preterm infants) were enrolled. We measured and compared the concentrations of glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP) in the umbilical vein and preprandial blood samples after birth.

Results: A total of 98 infants (47 term, 46 preterm and 5 with duodenum atresia) were included. In patients with duodenum atresia, umbilical vein GLP-1 and GIP levels were the same as those in normal infants. In postnatal samples, there were positive correlations between the amount of enteral feeding and preprandial serum concentrations of GLP-1 (r = 0.47) or GIP (r = 0.49).

Conclusions: Our results show that enteral feeding is important for secretion of GLP-1 and GIP in postnatal infants, whereas the passage of amniotic fluid is not important for fetal secretion of GLP-1 and GIP. The effect of ingested material passing through the digestive tract on incretin secretion might change before and after birth. Other factors might stimulate secretion of GLP-1 and GIP during the fetal period.

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP), known as incretin hormones, are secreted into the bloodstream in response to ingested nutrients, such as glucose or fat[1–8]. Nutrients that pass through the intestinal tract stimulate secretion of incretins[1,5,6]. GLP-1 and GIP stimulate insulin secretion from pancreatic islets through a glucose-dependent process[1,2,9,10].

It was reported that placental transfer of GLP-1 and GIP from mothers to fetuses might be negligible, and fetuses secrete GLP-1 and GIP by themselves[11–13]. However, the stimulants for fetal incretin secretion are not fully understood. Fetuses do not ingest food enterally, but they swallow amniotic fluid. Fetal swallowing of amniotic fluid contributes to intestinal growth and development[14,15]. Studies in fetal sheep and pigs have shown that preventing the fetal swallowing process by esophageal ligation suppressed intestinal growth[16]. Therefore, it is possible that the passage of amniotic fluid through the fetal intestinal tract affects fetal secretion of incretin.

Fetuses swallow amniotic fluid more actively during late gestation than during earlier stages of gestation[17]. Fetuses at 18 weeks of gestation swallow 18–50 mL/kg/day[18], whereas term fetuses swallow 155 mL/kg/day[19]. If the passage of amniotic fluid through the intestinal tract enhances fetal secretion of incretins, the level of incretin secretion should increase with gestational age. However, it was reported that umbilical vein GLP-1 levels in infants during early gestation were higher than those in term infants, and GIP levels in preterm infants were the same as those in term infants[20].

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We hypothesized that the passage of the amniotic fluid through the intestinal tract does not play an important role in fetal secretion of incretin. To investigate this hypothesis, we analyzed umbilical cord incretin levels of infants with duodenum atresia. If incretin levels of infants with duodenum atresia were lower than that of normal infants, the swallowing of amniotic fluid and passage of ingested material through the digestive tract might be necessary for fetal secretion of incretin. Alternatively, the same or higher levels of incretin in infants with duodenum atresia might show that other factors stimulate fetal secretion of GLP-1 and GIP.

METHODS

Study population and design

A single-center prospective study was carried out at Kyoto University Hospital, Kyoto, Japan. Infants born at our hospital from July 2017 to July 2019 were enrolled in the present study (infants with duodenum atresia, and normal term or preterm infants as control). Exclusion criteria were as follows: (i) chromosomal abnormalities (excluding trisomy 21) and other major anomalies (excluding duodenum atresia), such as congenital heart disease (excluding patent ductus arteriosus) or congenital diaphragmatic hernia; (ii) the absence of parental agreement; and (iii) patients whose blood samples could not be obtained. We compared the concentration of hormones in umbilical veins from infants with duodenum atresia with those from normal term or preterm infants. To examine the effects of the passage of nutrients through the intestinal tract on incretin secretion, we also collected blood samples after birth. Clinical information was collected by chart review. Ethical approval was obtained from the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (no. R0095-2). We obtained written informed consent from patients' parents.

Blood samples

We collected umbilical vein samples from normal term and preterm infants, and infants with duodenum atresia. We obtained umbilical vein samples immediately after delivery of the placenta. We also collected postnatal blood samples preprandially (approximately 3 h after the last feeding) from vein puncture or heel-stick. In term infants, preprandial blood samples were collected 4–6 days after birth, when the daily amount of feeding achieved "full feeding" (defined as >100 mL/kg/day of breast milk or formula orally). In preterm infants, preprandial blood samples were collected at 4–6 and 12–15 days after birth (0–20 mL/kg/day and 20–100 mL/kg/day of breast milk or formula orally, respectively) and after full feeding. In infants with duodenum atresia, preprandial blood samples were collected before the operation (no feeding) and after full feeding (orally). The blood samples were centrifuged for 5 min at 1,000 *g*, and the separated serum samples were stored at –30°C until assays.

Measurements of hormones and blood glucose

We measured serum hormone levels using the bead array system for the Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We measured serum concentrations of three hormones – GIP (total form), GLP-1 (total form) and insulin – according to the manufacturer's instructions. This assay system was a magnetic bead-based multiplex immunoassay that enabled simultaneous assessment of multiple biomarkers with only small amounts of clinical samples. The cross-reactivity of GLP-1 was 0.6% for GIP and 0.1% for insulin. The cross-reactivity of GIP was 0.1% for GLP-1 and 0.0% for insulin. The cross-reactivity of insulin was 0.2% for GLP-1 and 0.3% for GIP. The recovery rates were 94–119%, 87–115% and 85–114% for GLP-1, GIP and insulin, respectively. The intra-assay coefficients of variation were 6%, 3% and 3% for GLP-1, GIP and insulin, respectively. The inter-assay coefficients of variation were 3%, 4% and 5% for GLP-1, GIP and insulin, respectively. This method was carried out in accordance with the Guideline for Incretin Measurement recommended by the Japan Diabetes Society and the Japan Association for Diabetes Education and Care. We measured blood glucose levels by RAPID Point 500 (Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA).

Statistical analysis

Data are expressed as median and interquartile range. We used the χ^2-test to compare the proportions of categorical variables among the groups. The threshold for significance was $P < 0.05$. All statistical analyses were carried out using IBM SPSS software (version 20; IBM Corporation, Armonk, NY, USA).

RESULTS

Patient characteristics

The patient flow diagram is shown in Figure S1. A total of 98 infants (5 with duodenum atresia, 47 term, and 46 preterm) were included in the present study. Characteristics of infants are shown in Table 1. All infants with duodenum atresia had complete atresia (three had complete separation and two had membranous atresia). They underwent duodenum–duodenum anastomosis 2–4 days after birth. One patient had trisomy 21.

Umbilical vein GLP-1 and GIP levels in preterm or term infants

The relationships between umbilical vein incretin levels and gestational age or birth weight are shown in Figure 1a–d. Umbilical vein GLP-1 levels were significantly higher in preterm infants than in term infants ($P < 0.001$; Figure 2a). Umbilical vein GIP levels were similar in preterm and term infants (Figure 2b). There was a negative correlation between gestational age or birthweight and umbilical vein GLP-1 levels ($r = –0.26$, $P = 0.021$, $r = –0.34$, $P = 0.002$, respectively).

Table 1 | Patients' characteristics

	Term infants $n = 47$	Preterm infants $n = 46$	Infants with duodenum atresia $n = 5$
Gestational age (weeks)	38.3 (37.4–39.3)	31.1 (27.6–35.0)	38.0 (37.7–38.0)
Birthweight (g)	2,594 (2,226–2,928)	1,400 (817–2,075)	2,562 (2,452–2,878)

Data are shown as the median (interquartile range).

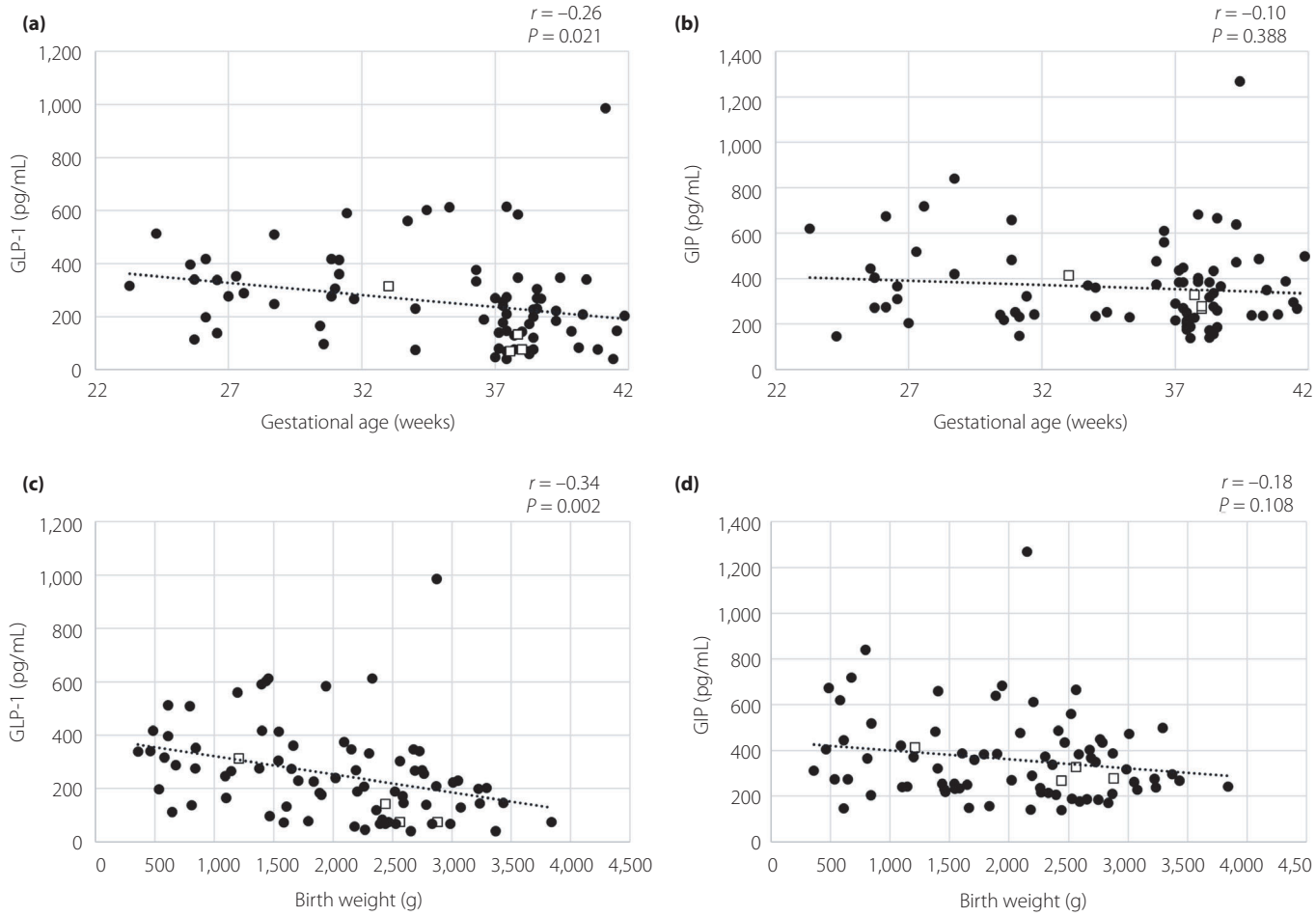

Figure 1 | The relationship between umbilical vein incretin levels and gestational age or birthweight. (a,b) The relationship between umbilical vein incretin levels and gestational age. (c,d) The relationship between umbilical vein incretin levels and birthweight. White squares show umbilical vein glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP) levels in infants with duodenum atresia.

Umbilical vein GLP-1 and GIP in infants with duodenum atresia

Umbilical vein GLP-1 and GIP in infants with duodenum atresia are shown in Figure 1a–d (white squares). Umbilical vein GLP-1 or GIP levels were equivalent in infants with duodenum atresia and normal infants. The median GLP-1 and GIP levels in infants with duodenum atresia were not significantly different from those in normal infants (142.44 vs 228.45 pg/mL, $P = 0.286$; 327.79 vs 314.61 pg/mL, $P = 0.510$; respectively).

Serum GLP-1 and GIP levels after birth

In postnatal samples, serum concentrations of GLP-1 and GIP increased as the amount of enteral feeding increased in both

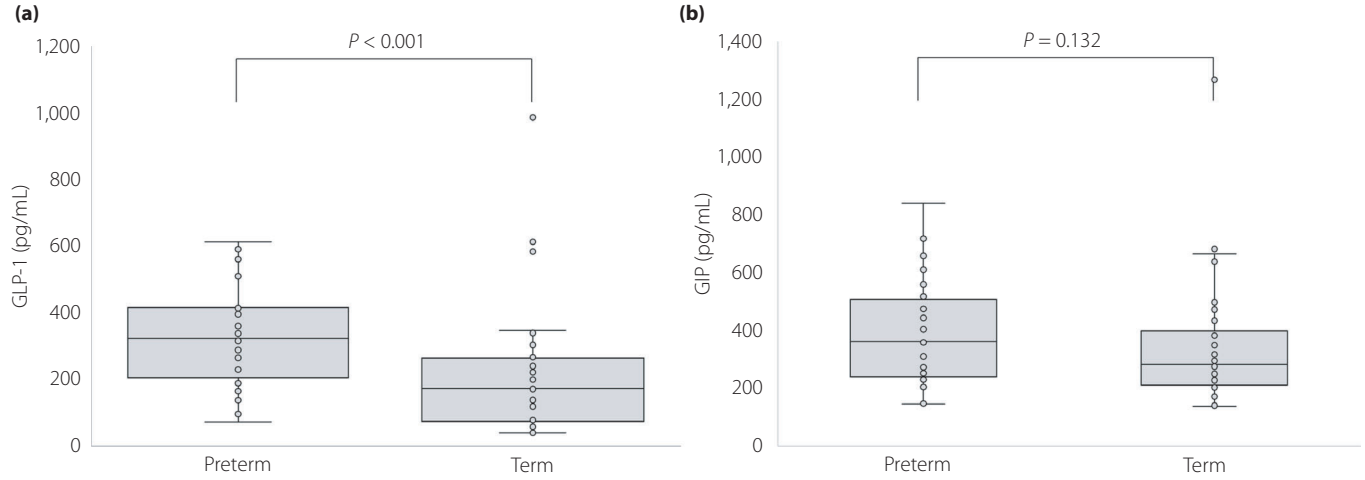

Figure 2 | Comparison of incretin levels between term and preterm infants. (a) Comparison of umbilical vein glucagon-like peptide-1 (GLP-1) levels between preterm and term infants. (b) Comparison of umbilical vein gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP) levels between preterm and term infants.

term and preterm infants (Figure 3a–d). There were positive correlations between the amount of enteral feeding and preprandial serum concentrations of GLP-1 ($r = 0.47$, $P = 0.001$) or GIP ($r = 0.49$, $P < 0.001$) (Figure 3e,f). In infants with duodenum atresia, serum concentrations of GLP-1 and GIP (before starting enteral feeding) were lower than those of umbilical vein samples (Figure 3g,h). After reaching full feeding, infants with duodenum atresia had normal serum concentrations of GLP-1 and GIP.

Associations between umbilical vein GLP-1 or GIP and insulin or blood glucose

The relationships of umbilical vein incretins with insulin or blood glucose are shown in Figure 4a–d. There were no correlations between umbilical vein insulin levels and umbilical vein GLP-1 ($r = 0.12$, $P = 0.303$) and GIP ($r = 0.15$, $P = 0.210$) levels. Also, there were no correlations between umbilical vein glucose levels and umbilical vein GLP-1 ($r = 0.06$, $P = 0.577$) and GIP ($r = 0.17$, $P = 0.139$) levels.

DISCUSSION

This is the first report to investigate the concentration of GLP-1 and GIP in infants with duodenum atresia, and to consider the stimulatory mechanism of fetal GLP-1 and GIP secretion. We showed that umbilical vein GLP-1 and GIP levels in infants with duodenum atresia were the same as those in normal infants.

The present results showed that the passage of something through the fetal digestive tract was not necessary for secretion of GLP-1 and GIP. In the amniotic fluid, the concentrations of stimulants (such as glucose or lipid) for incretin secretion are very low[21–23]. Therefore, it is likely that amniotic fluid does not stimulate the secretion of incretins. Fetuses do not absorb nutrients through the digestive tract; therefore, incretins might not need to be controlled by passage through the digestive tract. The stimulatory factors for the secretion of incretins remain unknown.

In the present study, serum concentrations of GLP-1 and GIP increased after birth, concurrent with the increasing amount of enteral feeding, even in infants with duodenum atresia. Before enteral feeding had started, serum concentrations of GLP-1 and GIP were very low (lower than those in the umbilical vein). These results show that, after birth, the passage of milk through the digestive tract might stimulate secretion of GLP-1 and GIP in infants, like the passage of ingested material in adults. The effect of the passage of material through the digestive tract on incretin secretion might change before and after birth.

We also verified that umbilical vein GLP-1 levels were higher in preterm infants compared with term infants, and umbilical vein GIP levels were equivalent in preterm and term infants. These results are consistent with previous reports[20]. These results indicate that fetuses in early gestation secrete GLP-1 and GIP, and further suggest that the passage of amniotic fluid is not necessary for fetal secretion of GLP-1 and GIP.

To date, there has been no report that investigates the stimulatory factors for fetal incretin secretion. In adults or animals, some factors known to induce incretin secretion (other than ingested material passing through the digestive tract) include acetylcholine, γ-aminobutyric acid, serotonin, progesterone, insulin, interleukin-1β and interleukin-6[24–30]. Some studies showed that a signal from the brain to the gut can modulate GIP release[31–33]. Further studies are required to determine the factors that play a key role in fetal incretin secretion.

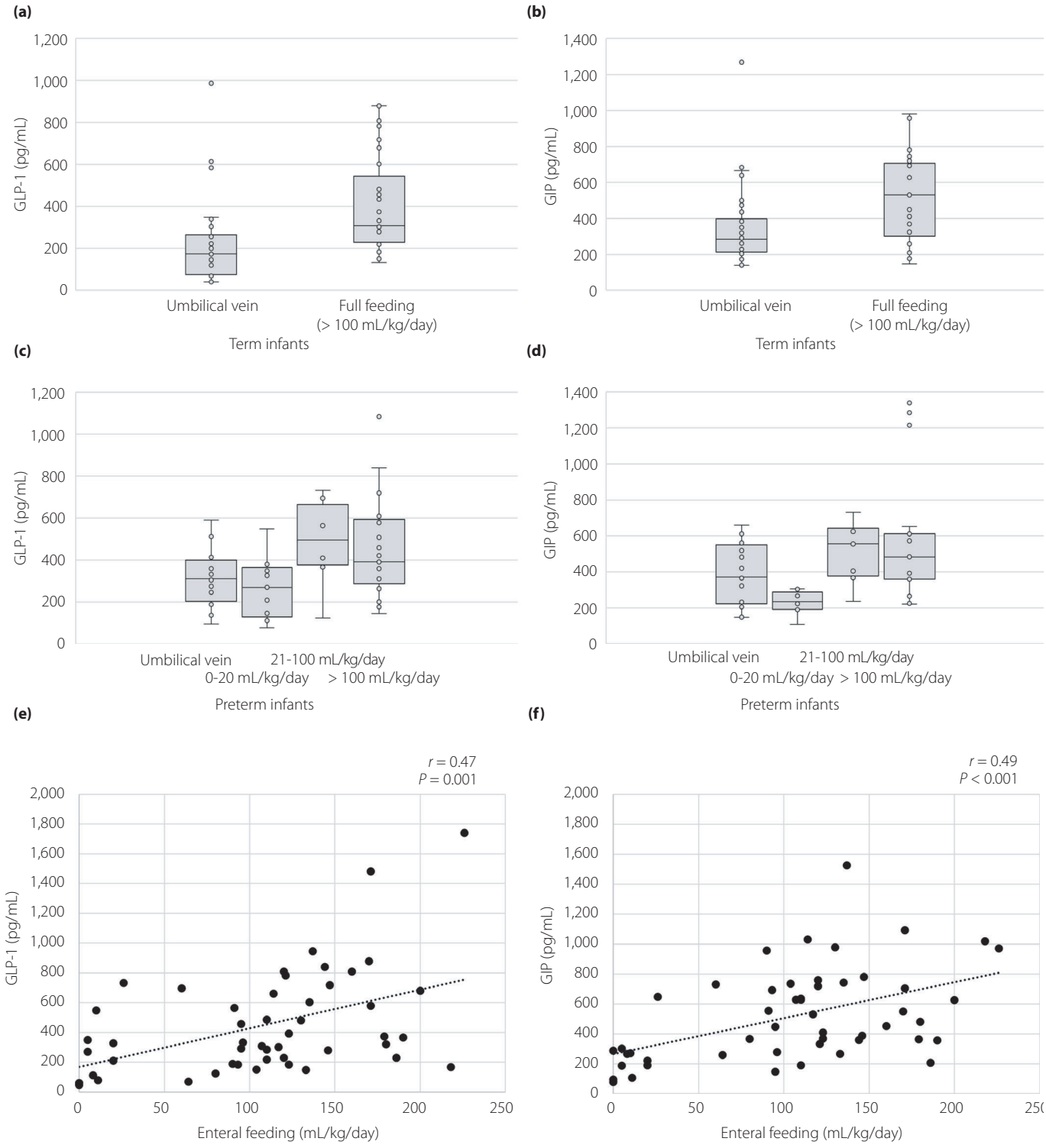

Figure 3 | Serum concentrations of incretins after birth. (a–d) Change in incretin levels with the amount of enteral feeding in preterm or term infants. (e,f) Relationship between incretin levels and the amount of enteral feeding. (g,h) Change in incretin levels with the state of enteral feeding in infants with duodenum atresia. GIP, gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1.

The present study showed that fetuses secrete GLP-1 and GIP, like newborn infants, showing that GLP-1 and GIP might provide some biological functions. In our study, umbilical vein levels of GLP-1 and GIP did not correlate with umbilical vein levels of insulin or blood glucose. These results show that fetal GLP-1 and GIP might not play a role in the regulation of

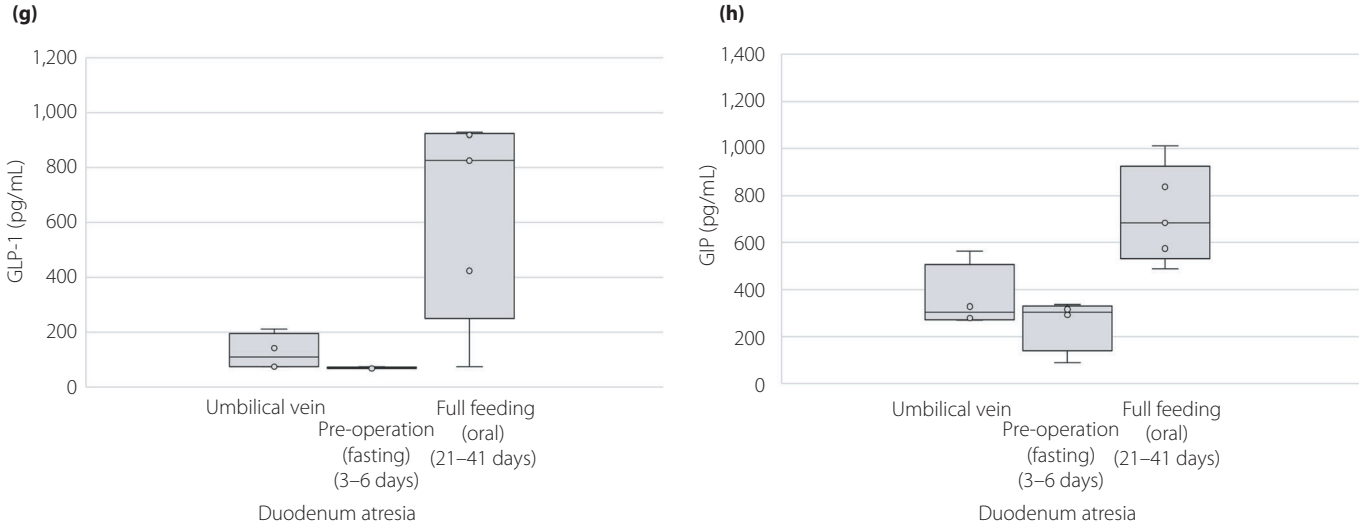

Figure 3 | (Continued)

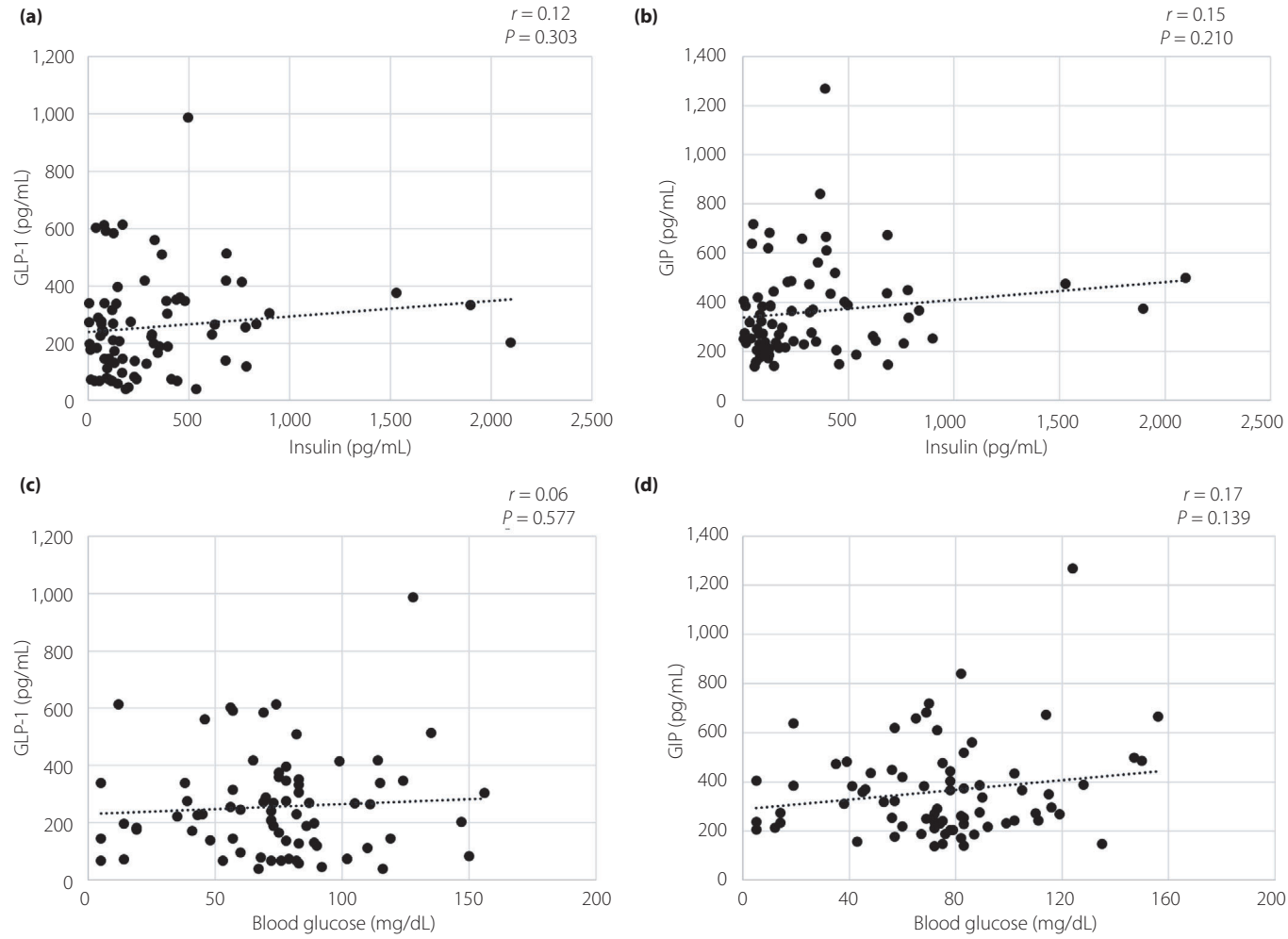

Figure 4 | Relationship between umbilical vein incretin levels and insulin or blood glucose. A, B; Relationship between umbilical vein incretin levels and insulin. C, D; Relationship between umbilical vein incretin levels and blood glucose. GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide

insulin and blood glucose secretion. Fetal blood glucose is passively regulated by maternal blood glucose, and fetal endogenous insulin has roles in anabolism and growth, but not in the regulation of blood glucose[34]. Considering that fetuses do not ingest food enterally, GLP-1 and GIP might not be required for the regulation of fetal insulin and blood glucose. Recently, many extrapancreatic actions of GLP-1 and GIP have been reported[35–37], such as fat and bone metabolism, and the prevention of cardiovascular diseases related to diabetes. Extrapancreatic functions that prevent macrovascular and microvascular diseases related to diabetes are receiving considerable research attention. For fetal growth, GLP-1 and GIP might have important roles in bone metabolism. GLP-1 and GIP levels in cord blood were found to be negatively correlated with 25-hydroxyvitamin D levels in both maternal and cord blood[38]. Further studies are required to elucidate the role of fetal incretin.

The main limitation of the present study was the small number of participants (especially infants with duodenum atresia). The lack of a statistically significant difference in the incretin levels between infants with and without duodenum atresia might reflect the small sample size. However, our analysis of this extreme condition (duodenum atresia) has provided valuable information. Regardless of sample size, the present results show that fetuses secrete incretins without the passage of amniotic fluid. Another limitation was that the study used infants with duodenum atresia. In infants with duodenum atresia, swallowed amniotic fluid can reach the stomach. Incretin secretion might be indirectly stimulated by amniotic fluid reaching the stomach and then actions of other stomach-derived factors (such as hormones or neurotransmitters). We did not investigate other factors from the digestive tract. Esophageal atresia might be considered a better candidate for models lacking the passage of amniotic fluid. However, >90% of patients with esophageal atresia show a connection between the lower esophagus and trachea, so amniotic fluid may pass through the digestive tract. Therefore, esophageal atresia was not a suitable model for the present study; so we selected duodenum atresia.

In conclusion, the present results show that enteral feeding is important for the secretion of GLP-1 and GIP in postnatal infants, but the passage of amniotic fluid is not important for fetal GLP-1 and GIP secretion. The effect of the passage of ingested material through the digestive tract on incretin secretion might change before and after birth, and other factors might stimulate secretion of GLP-1 and GIP during the fetal period. Further studies are required to elucidate the mechanism of secretion, and the role of fetal GLP-1 and GIP.

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DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Schmidt WE, Siegel EG, Creutzfeldt W. Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. *Diabetologia* 1985; 28: 704–707.
2. Kreymann B, Williams G, Ghatei MA, *et al*. Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* 1987; 2: 1300–1304.
3. Brown JC, Pederson RA, Jorpes E, *et al*. Preparation of highly active enterogastrone. *Can J Physiol Pharmacol* 1969; 47: 113–114.
4. Brown JC, Mutt V, Pederson RA. Further purification of a polypeptide demonstrating enterogastrone activity. *J Physiol* 1970; 209: 57–64.
5. Creutzfeldt W, Ebert R. New developments in the incretin concept. *Diabetologia* 1985; 28: 565–573.
6. Creutzfeldt W. The incretin concept today. *Diabetologia* 1979; 16: 75–85.
7. Meier JJ, Nauck MA. Glucagon-like peptide 1 (GLP-1) in biology and pathology. *Diabetes Metab Res Rev* 2005; 21: 91–117.
8. Drucker DJ. Glucagon-like peptides. *Diabetes* 1998; 47: 159–169.
9. Dupre J, Ross SA, Watson D, *et al*. Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab* 1973; 37: 826–828.
10. Takemura J, Seino Y, Yamamura T, *et al*. The role of endogenous gastric inhibitory polypeptide in the enteroinsular axis. *J Clin Endocrinol Metab* 1982; 54: 909–913.
11. Adrian TE, Soltesz G, MacKenzie IZ, *et al*. Gastrointestinal and pancreatic hormones in the human fetus and mother at 18–21 weeks of gestation. *Biol Neonate* 1995; 67: 47–53.
12. Otonkoski T, Hayek A. Constitution of a biphasic insulin response to glucose in human fetal pancreatic beta-cells with glucagon-like peptide 1. *J Clin Endocrinol Metab* 1995; 80: 3779–3783.
13. Jackson Huang TH, Brubaker PL. Synthesis and secretion of glucagon-like peptide-1 by fetal rat intestinal cells in culture. *Endocrine* 1995; 3: 499–503.
14. Burrin DG, *et al*. Trophic factors and regulation of gastrointestinal tract and liver development. In: Polin RA, Abman SH, Rowitch DH), (eds). Fetal and neonatal physiology, 5th edn. Elsevier, Philadelphia, 2017; 855–860.
15. Ross MG, Nijland MJM. Development of ingestive behavior. *Am J Physiol* 1998; 274: R879–R893.
16. Sangild PT. Gut responses to enteral nutrition in preterm infants and animals. *Exp Biol Med* 2006; 231: 1695–1711.
17. Mann SE, Nijland MJM, Ross MG. Mathematical modeling of human amniotic fluid dynamics. *Am J Obstet Gynecol* 1996; 175: 937–944.

18. Abramovich DR. Fetal factors influencing the volume and composition of liquor amnii. *J Obstet Gynaecol Br Commonw* 1970; 77: 865–877.
19. Pritchard JA. Deglutition of normal and anencephalic fetuses. *Obstet Gynecol* 1965; 25: 289–297.
20. Kawamata R, Suzuki Y, Yada Y, *et al*. Gut hormone profiles in preterm and term infants during the first 2 months of life. *J Pediatr Endocrinol Metab* 2014; 27: 717–723.
21. Kirshon B, Rosenfeld B, Mari G, *et al*. Amniotic fluid glucose and intraamniotic infection. *Am J Obstet Gynecol* 1991; 164: 818–820.
22. Velika B, Birkova A, Dudic R, *et al*. Selected physicochemical properties of amniotic fluid according to week of pregnancy. *Bratisl Lek Listy* 2018; 119: 175–179.
23. Das SK, Foster HW, Adhikary PK, *et al*. Gestational variation of fatty acid composition of human amniotic fluid lipids. *Obstet Gynecol* 1975; 45: 425–432.
24. Anini Y, Hansotia T, Brubaker PL. Muscarinic receptors control postprandial release of glucagon-like peptide-1: in vivo and in vitro studies in rats. *Endocrinology* 2002; 143: 2420–2426.
25. Gameiro A, Reimann F, Habib AM, *et al*. The neurotransmitters glycine and GABA stimulate glucagon-like peptide-1 release from the GLUTag cell line. *J Physiol* 2005; 569: 761–772.
26. Ripken D, van der Wielen N, Wortelboer HM, *et al*. Nutrient-induced glucagon like peptide-1 release is modulated by serotonin. *J Nutr Biochem* 2016; 32: 142–150.
27. Flock GB, Cao X, Maziarz M, *et al*. Activation of enteroendocrine membrane progesterone receptors promotes incretin secretion and improves glucose tolerance in mice. *Diabetes* 2013; 62: 283–290.
28. Lim GE, Huang GJ, Flora N, *et al*. Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell. *Endocrinology* 2009; 150: 580–591.
29. Kahles F, Meyer C, Diebold S, *et al*. Glucose-dependent insulinotropic peptide secretion is induced by inflammatory stimuli in an interleukin-1-dependent manner in mice. *Diabetes Obes Metab* 2016; 18: 1147–1151.
30. Ellingsgaard H, Hauselmann I, Schuler B, *et al*. Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. *Nat Med* 2011; 17: 1481–1489.
31. Yavropoulou MP, Kotsa K, Kesisoglou I, *et al*. Intracerebroventricular infusion of neuropeptide Y increases glucose dependent-insulinotropic peptide secretion in the fasting conscious dog. *Peptides* 2008; 29: 2281–2285.
32. Yavropoulou MP, Kotsa K, Kesisoglou I, *et al*. Effect of intracerebroventricular infusion of neurotensin on glucose-dependent insulinotropic peptide secretion in dogs. *Peptides* 2010; 31: 150–154.
33. Yavropoulou MP, Kotsa K, Anastasiou OE, *et al*. Intracerebroventricular infusion of bombesin modulates GIP secretion in conscious dogs. *Neuropharmacology* 2010; 58: 226–232.
34. Kalhan SC, *et al*. Metabolism of glucose and methods of investigation in the fetus and newborn. In: Polin RA, Abman SH, Rowitch DH), (eds). Fetal and neonatal physiology, 5th edn. Elsevier, Philadelphia, 2017; 390–403.
35. Yabe D, Seino Y. Incretin actions beyond the pancreas: lessons from knockout mice. *Curr Opin Pharmacol* 2013; 13: 946–953.
36. Seino Y, Yabe D. Glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1: incretin actions beyond the pancreas. *J Diabetes Investig* 2013; 4: 108–130.
37. Campbell JE, Drucker DJ. Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metab* 2013; 17: 819–837.
38. Niwa S, Mezawa H, Kobayashi N, *et al*. Inverse association between maternal 25OHD level and cord GLP-1/GIP concentrations. *Pediatr Res* 2016; 79: 536–542.

SUPPORTING INFORMATION

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

Figure S1 | Patient flow diagram.