JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
ORIGINAL ARTICLE

Phenotyping of type 2 diabetes mellitus at onset on the basis of fasting incretin tone: Results of a two-step cluster analysis

Marco Calogero Amato, Giuseppe Pizzolanti, Vittoria Torregrossa, Felicia Pantò, Carla Giordano*

Biomedical Department of Internal and Specialized Medicine (Di.Bi.M.I.S.), Section of Cardio-Respiratory and Endocrine-Metabolic Diseases, University of Palermo, Palermo, Italy

Keywords
Ghrelin, Glucagon-like peptide-1, Glucose-dependent insulinotropic polypeptide

***Correspondence**
Carla Giordano
Tel.: +39-091-6552109
Fax +39-091-6552123
E-mail address: carla.giordano@unipa.it

J Diabetes Investig 2016; 7: 219–225

doi: 10.1111/jdi.12398

ABSTRACT

Aims/Introduction: According to some authors, in type 2 diabetes there is a reduced postprandial action of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). However, little is known about the role of fasting incretins in glucose homeostasis. Our aim was to evaluate, through a two-step cluster analysis, the possibility of phenotyping patients with type 2 diabetes at onset on the basis of fasting GLP-1, GIP and ghrelin.

Materials and Methods: A total of 96 patients with type 2 diabetes within 6 months of onset (mean age 62.40 ± 6.36 years) were cross-sectionally studied. Clinical, anthropometric and metabolic parameters were evaluated. At fasting the following were carried out: assay of GLP-1, GIP, ghrelin, insulin, C-peptide, glucagon and a panel of adipocytokines (visfatin, resistin, leptin, soluble leptin receptor and adiponectin).

Results: The analysis resulted in two clusters: cluster 1 (63 patients) had significantly lower levels of GLP-1 (4.93 ± 0.98 vs 7.81 ± 1.98 pmol/L; $P < 0.001$), GIP (12.73 ± 9.44 vs 23.88 ± 28.56 pmol/L; $P < 0.001$) and ghrelin (26.54 ± 2.94 vs 39.47 ± 9.84 pmol/L; $P < 0.001$) compared with cluster 2 (33 patients). Between the two clusters, no differences in age, duration of disease, sex, clinical-anthropometric parameters, insulin sensitivity and adipocytokines were highlighted. However, cluster 1 was associated with significantly higher levels of glycated hemoglobin (7.4 ± 0.61 vs 6.68 ± 0.57%, $P = 0.007$), glucagon (232.02 ± 37.27 vs 183.33 ± 97.29 ng/L; $P = 0.001$), fasting glucose (7.85 ± 1.60 vs 6.93 ± 1.01 mmol/L; $P = 0.003$) and significantly lower levels of C-peptide (0.12 ± 0.11 vs 0.20 ± 0.20 nmol/L; $P = 0.017$).

Conclusions: The present study suggests that fasting incretins play an important role in the pathophysiology of type 2 diabetes, which requires to further investigation.

INTRODUCTION

It is known that in normal subjects, oral glucose administration elicits a higher insulin response than intravenous glucose load at identical plasma glucose concentrations; this phenomenon, the incretin effect, is mainly attributed to the postprandial action of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[1–4]. However, in recent years, it has been debated whether post-load GLP-1 and GIP secretion really are lower in patients with type 2 diabetes mellitus compared with matched healthy controls[5,6]. A recent meta-analysis suggested that patients with type 2 diabetes, in general, do not show reduced GLP-1 secretion in response to an oral glucose tolerance test or meal test, and that it is the deterioration of glycemic control that is associated with reduced GLP-1 secretion[7].

Furthermore, in addition to GLP-1 and GIP, another incretin, ghrelin, produced in the gastrointestinal tract, though not involved in the postprandial incretin effect described, plays an important role in glucose homeostasis[8]. The ghrelin effect on plasma insulin and glucose levels in humans, which is produced mainly in the fasting state, is

Received 26 February 2015; revised 15 June 2015; accepted 9 July 2015

still a matter of debate. Some groups have found no changes[9–11], whereas others have found increased fasting blood glucose and decreased plasma insulin after administration of ghrelin[12].

To date, there is a great deal of literature, although controversial, on the postprandial incretin effect and its role in the pathogenesis of type 2 diabetes; conversely, little is known about the role of fasting incretin tone, and especially about the impact of this on the pathophysiology of type 2 diabetes.

In the present study, through a two-step cluster analysis we evaluated the possibility of phenotyping patients with type 2 diabetes at onset on the basis of fasting serum levels of GLP-1, GIP and ghrelin.

MATERIALS AND METHODS

A total of 96 consecutive Caucasian patients with type 2 diabetes within 6 months of onset (mean age 62.40 ± 6.36 years, range 51–75 years), followed up in our dedicated outpatients clinic at Unit of Endocrinology, Diabetology and Metabolism, University of Palermo, Palermo, Italy (from 1 March 2012 to 31 December 2013), were cross-sectionally studied. Inclusion criteria were type 2 diabetes known for <6 months and in stable treatment for the last 3 months with metformin (1.5–2 g/day). Patients with glycated hemoglobin (HbA1c) ≥8 (64 mmol/mol) were excluded, in order to avoid the effects of severe glucotoxicity on incretins and adipocytokines. The following were excluded: patients with type 1 diabetes and patients with previous treatment with other antidiabetic drugs taken in the past 6 months. To avoid interfering with the Visceral Adiposity Index, patients with morbid obesity, pendulous abdomen, severe hypertriglyceridemia and/or or use of fibrates were excluded[13,14]. Patients with macro- or microvascular complications were excluded (except 8 patients with simple preproliferative retinopathy). Patients were not excluded if they had essential hypertension (62 patients). A complete medical history, a complete physical examination, bodyweight, height, and waist and hip circumference were recorded for the patients included. Waist circumference (WC) was measured midway between the inferior margin of the last rib and the crest of the ilium in a horizontal plane by the measurer sitting by the subject and fitting the tape snugly, but not compressing soft tissues. Hip circumference (HP) was measured around the pelvis at the point of maximal protrusion of the buttocks. Fasting blood samples and a morning spot urine sample were collected for biochemical analyses. All participants were instructed to carry out home glucose monitoring (one full-day glucose profile per week: fasting, 2 h after breakfast, 2 h after lunch and 2 h after dinner).

The study was approved by the institutional review board at the Faculty of Medicine of the University of Palermo. At the time of observation, all patients regularly signed an informed consent for scientific use of their data.

Anthropometric Indices

Body mass index (BMI) was calculated as bodyweight (in kilograms) divided by the square of height (in meters). Waist-to-hip ratio was calculated by dividing the waist circumference by the hip circumference. The Body Adiposity Index (BAI) was calculated using the following formula[15]:

$$\mathrm{BAI} = (((\mathrm{HC})/(\mathrm{height})) \times 1.5) - 1.8$$

Hip circumference (HC) is expressed in centimeters and height in meters.

The Visceral Adiposity Index (VAI) was calculated as described[13,14] using the following sex-specific equation, where triglyceride (TG) levels are expressed in mmol/L and high-density lipoprotein (HDL) cholesterol levels expressed are mmol/L: Females:

$$\mathrm{VAI} = \left(\frac{\mathrm{WC}}{36.58 + (1.89 \times \mathrm{BMI})}\right) \times \left(\frac{\mathrm{TG}}{0.81}\right) \times \left(\frac{1.52}{\mathrm{HDL}}\right)$$

Males:

$$\mathrm{VAI} = \left(\frac{\mathrm{WC}}{39.68 + (1.88 \times \mathrm{BMI})}\right) \times \left(\frac{\mathrm{TG}}{1.03}\right) \times \left(\frac{1.31}{\mathrm{HDL}}\right)$$

Secretory Units of Islets in Transplantation

As a measure of β-cell function, the secretory units of islets in transplantation (SUIT)[16] were calculated from the fasting plasma glucose (mg/dL) and fasting C-peptide (ng/mL) levels using the formula:

$$(1485 \times \text{fasting C-peptide})/(\text{fasting glucose} - 61.8)$$

Assay

The BioPlex Pro Human Diabetes 10-plex assay (BioRad, Milan, Italy) was used to quantitate the following diabetes-related analytes: C-peptide (nmol/L), ghrelin (pg/mL), total GLP-1 (pmol/L), total GIP (pmol/L), leptin (ng/mL), resistin (ng/mL) and visfatin (pg/mL). The BioPlex Pro Human Diabetes Adipsin and Adiponectin duplex assay (BioRad) was used for adipsin (ng/mL) and adiponectin (μg/mL) assays. The intra- and interassay coefficients of variability were 4% and 2% for ghrelin, 3% and 4% for GIP, and 6% and 3% for GLP-1, respectively.

All these assays were run on a BioPlex 200 workstation (BioRad). Human soluble leptin receptor (sOb-R; ng/mL) was assayed using an enzyme-linked immunosorbent assay sandwich enzyme immunoassay (Human leptin receptor ELISA; BioVendor, Heidelberg, Germany). Serum non-esterified fatty acids (mmol/L) were assayed after overnight fasting using an enzymatic colorimetric method (Randox NEFA assay FA115; Randox Laboratories, County Antrim, UK). Blood glucose levels

(mg/dL) were measured using an electrochemical system (Glucocard; Menarini Diagnostics, Florence, Italy). Total cholesterol, HDL cholesterol, triglycerides, and urinary albumin and creatinine were measured in our laboratory using standard assays. HbA1c was determined by high-pressure liquid chromatography with ion-exchange resin (Bio-Rad D-10 HPLC analyzer). Low-density lipoprotein cholesterol levels were calculated with Friedewald's formula. The conversion factors for the International System were the following: glucose (mg/dL vs mmol/L; ×0.0555), insulin (mUI/L vs pmol/L; ×6.945), total cholesterol (mg/dL vs mmol/L; ×0.0259), visfatin (pg/mL vs ng/mL; ×1000), Ghrelin (pg/mL vs pmol/L; ×0.296).

Statistical Analysis

The Statistical Packages for Social Sciences, SPSS version 17 (SPSS, Chicago, IL, USA), was used for data analysis. Baseline characteristics were presented as mean ± standard deviation for continuous variables; rates and proportions were calculated for categorical data. Normality of distribution for quantitative variables was assessed by the Shapiro–Wilk test. Some variables (leptin, leptin/sOb-R ratio, adiponectin and resistin) did not show normal distribution, and the natural logarithmic transformed values of each adipocytokine for statistical analysis were used. Differences between the two groups were detected by the unpaired Student's *t*-test for continuous variables (after testing for equality of variance: Levene test) and by the chi square-test and Fisher's exact test (when appropriate) for categorical variables.

In order to identify the phenotypes of type 2 diabetes based on fasting incretin tone, we carried out a two-step cluster analysis using log-likelihood distance measures. Cluster analysis, also called segmentation analysis, is an explorative analysis that tries to identify structures within the data. More specifically, it tries to identify homogenous groups of cases based on the distribution of some variables (input variables). Cluster analysis is used to identify groups of cases if the grouping is not previously known. In particular, a two-step cluster analysis is more of a tool than a single analysis. It identifies the groupings by running pre-clustering first and then by hierarchical methods. In this respect, it combines the best of both approaches. This technique can detect latent relationships within a complex dataset between patients with multiple distinct characteristics. Cluster analysis was applied using an *a priori* number of fixed clusters (cluster 1 and cluster 2), and the three following continuous variables were included in the analysis (input variables): fasting GLP-1, GIP and ghrelin. The three variables included produced a silhouette coefficient >0.5, indicative of good data partitioning (Figure 1a). In this model, the most important variables in the creation of the two clusters were GLP-1 and GIP (Figure 1a).

For comparison between cluster 1 and cluster 2, the group sizes gave 74.7% power to detect a moderate effect size (Cohen's $d = 0.5$) using unpaired *t*-test, with alpha at 0.05. *Post-hoc* power analysis was carried out using G*POWER Version 3.1.6 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). A *P*-value of <0.05 was considered statistically significant.

RESULTS

The 96 patients studied were 51 women and 45 men; the clinical characteristics, and the incretin and adipocytokine patterns of the patients are summarized in Table 1.

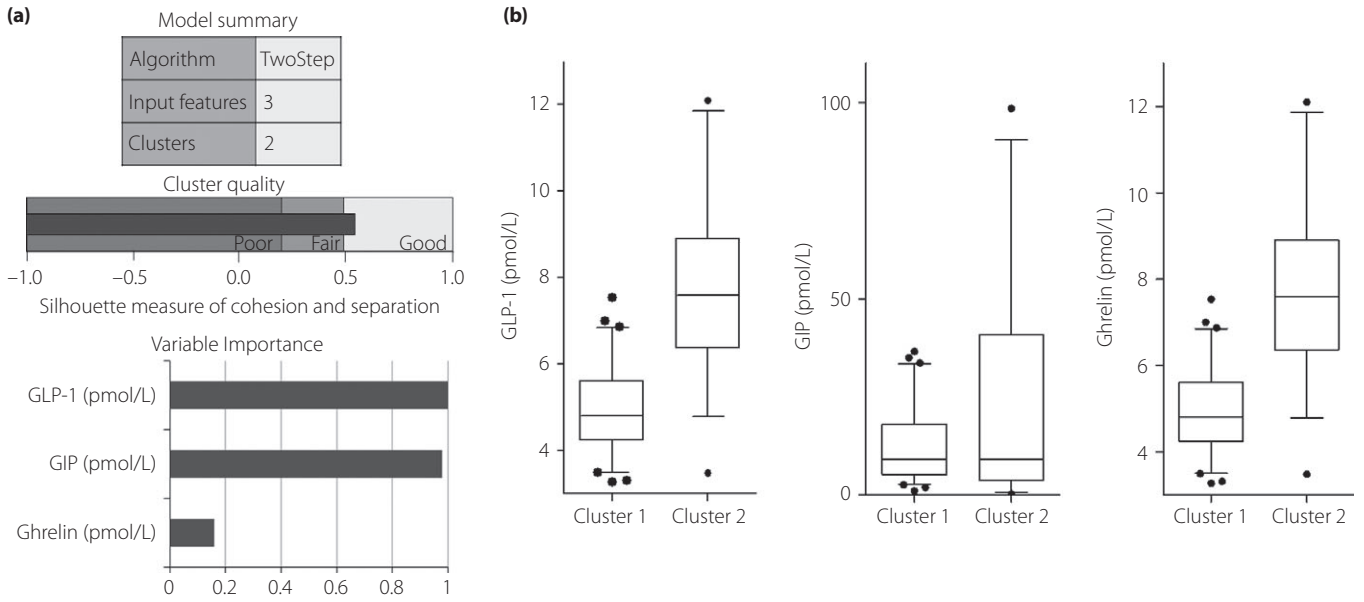

Figure 1 | (a) Model summary of the two-step cluster analysis with a graph of the quality of clusters and importance of the three input variables. (b) Differences in serum levels of glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and ghrelin between cluster 1 and cluster 2; the ends of the whiskers represent the 5th and 95th percentiles.

The two clusters derived from the cluster analysis showed the following fasting incretin patterns: compared with cluster 2 (33 patients), cluster 1 (63 patients) showed significantly lower levels of GLP-1 (4.93 ± 0.98 vs 7.81 ± 1.98 pmol/L; $P < 0.001$), GIP (12.73 ± 9.44 vs 23.88 ± 28.56 pmol/L; $P < 0.001$) and ghrelin (26.54 ± 2.94 vs 39.47 ± 9.84 pmol/L; $P < 0.001$; Figure 1b).

Regarding the clinical and anamnestic characteristics of the patients, we did not find any significant differences between the two clusters, except for a greater prevalence of patients practicing physical activity in cluster 2 (27.3 vs 11.1%; $P = 0.044$; Table 2). Similarly, no significant differences in anthropometric parameters were found (Table 2).

Cluster 1 showed a worse glyco-metabolic profile compared with cluster 2: higher levels of HbA1c (7.41 ± 0.61 vs 6.68 ± 0.57%; $P = 0.007$) and fasting plasma glucose (7.85 ± 1.60 vs 6.93 ± 1.01 mmol/L; $P = 0.003$) were found; no difference was found between the two clusters in regard to postprandial glucose levels. Regarding pancreatic function, cluster 1 showed significantly higher levels of fasting flucagon (232.02 ± 37.27 vs 183.33 ± 97.29 ng/L; $P = 0.001$), lower

Table 1 | Clinical-anthropometric characteristics, incretin and adipocytokine patterns in 96 patients with type 2 diabetes

Clinical-anthropometric characteristics	*n* (%)
Women	51 (53.1)
Men	45 (46.9)
	Mean ± SD
Age (years)	62.40 ± 6.36
Duration of disease (months)	4.41 ± 1.66
BMI (kg/m^2)	29.29 ± 4.65
Waist circumference (cm)	102.08 ± 11.31
Hip circumference (cm)	107.23 ± 10.28
Waist-to-hip ratio	0.95 ± 0.08
Body Adiposity Index	33.94 ± 6.47
Visceral Adiposity Index	2.28 ± 1.33
HbA1c	
%	6.92 ± 0.62
mmol/mol	52 ± 6.80
HOMA-IR	3.08 ± 3.22
Fasting insulin (pmol/L)	64.32 ± 67.26
Fasting C-peptide (nmol/L)	0.15 ± 0.15
Fasting glucagon (ng/L)	215.28 ± 68.08
Urinary albumin-to-creatinine ratio (mg/g)	16.69 ± 38.10
Fasting lipids	
Total cholesterol (mmol/L)	4.58 ± 0.85
HDL cholesterol (mmol/L)	1.25 ± 0.32
LDL cholesterol (mmol/L)	2.76 ± 0.84
Triglycerides (mmol/L)	1.41 ± 0.56
NEFA (mmol/L)	0.46 ± 0.27
Fasting incretins	
GLP-1 (pmol/L)	5.92 ± 1.96
GIP (pmol/L)	16.56 ± 19.01
Ghrelin (pmol/L)	30.99 ± 8.74
Fasting adipocytokines	
Leptin (ng/dL)	8.83 ± 10.45
sOb-R (ng/mL)	21.70 ± 6.56
Leptin/sOb-R ratio	0.55 ± 0.88
Adiponectin (μg/mL)	11.75 ± 12.94
Visfatin (ng/mL)	0.62 ± 0.52
Resistin (ng/mL)	4.01 ± 4.68

BMI, body mass index; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model addessment of insulin resistance; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; SD, standard deviation; sOB-R, soluble leptin receptor.

Table 2 | Differences in clinical and anthropometric characteristics between cluster 1 and cluster 2

	Cluster 1 *n* (%) *n* = 63	Cluster 2 *n* (%) *n* = 33	*P*
Clinical characteristics			
Sex			
Male	30 (47.6)	15 (45.5)	0.840
Female	33 (52.4)	18 (54.5)	
Family history of diabetes	51 (81)	24 (72.7)	0.437
Dietary compliance	43 (68.3)	21 (63.6)	0.819
Physical activity	7 (11.1)	9 (27.3)	0.044
Smoking			
Current	10 (15.9)	4 (12.1)	0.845
Former	13 (20.6)	8 (24.2)	
Metabolic syndrome*	46 (73)	22 (66.7)	0.516
High blood pressure	42 (66.7)	20 (60.6)	0.555
High triglycerides	20 (31.7)	6 (18.2)	0.155
Low HDL cholesterol	40 (63.5)	21 (63.6)	0.989
Increased WC	41 (65.1)	19 (57.6)	0.471
	Mean ± SD	Mean ± SD	
Age (years)	61.94 ± 6.14	63.27 ± 6.78	0.348
Duration of disease (months)	4.51 ± 1.66	4.21 ± 1.67	0.413
Anthropometric parameters			
BMI (kg/m^2)	29.56 ± 4.49	28.79 ± 4.98	0.461
WC (cm)	103.63 ± 10.62	99.12 ± 12.13	0.076
Hip circumference (cm)	107.60 ± 9.92	106.54 ± 11.05	0.647
Waist-to-hip ratio	0.96 ± 0.09	0.93 ± 0.08	0.067
Body Adiposity Index	33.90 ± 6.20	34.02 ± 7.04	0.931
Visceral Adiposity Index	2.36 ± 1.43	2.12 ± 1.12	0.364

Student's *t*-test after testing for equality of variance (Levene test).
*According to Adult Treatment Panel (ATP) III criteria. BMI, body mass index; HDL, high-density lipoprotein; SD, standard deviation; WC, waist circumference.

Table 3 | Differences in glycometabolic profiles and adipocytokine pattern between cluster 1 and cluster 2

	Cluster 1 $n = 63$ Mean ± SD	Cluster 2 $n = 33$ Mean ± SD	*P*
Glycometabolic profile			
HbA1c (%)	7.41 ± 0.61	6.68 ± 0.57	0.007
HbA1c (mmol/mol)	57 ± 6.7	49 ± 6.2	0.007
HOMA-IR	2.94 ± 3.08	3.35 ± 3.50	0.566
Fasting insulin (pmol/L)	59.07 ± 60.32	74.34 ± 78.88	0.293
Fasting glucagon (ng/L)	232.02 ± 37.27	183.33 ± 97.29	0.001
Fasting C-peptide (nmol/L)	0.12 ± 0.11	0.20 ± 0.20	0.017
Fasting glucose (mmol/L)	7.85 ± 1.60	6.93 ± 1.01	0.003
SUIT	7.66 ± 6.35	16.00 ± 16.78	0.001
2 h after breakfast glucose (mmol/L)	6.96 ± 1.69	6.47 ± 0.93	0.124
2 h after lunch glucose (mmol/L)	7.74 ± 1.88	7.23 ± 1.11	0.133
2 h after dinner glucose (mmol/L)	7.98 ± 2.06	7.33 ± 1.48	0.082
Urinary albumin-to-creatinine ratio (mg/g)	19.18 ± 45.61	11.93 ± 15.62	0.257
Fasting lipids			
Total cholesterol (mmol/L)	4.55 ± 0.86	4.65 ± 0.84	0.603
HDL cholesterol (mmol/L)	1.24 ± 0.30	1.26 ± 0.36	0.753
LDL cholesterol (mmol/L)	2.71 ± 0.84	2.85 ± 0.83	0.445
Triglycerides (mmol/L)	1.45 ± 0.58	1.33 ± 0.50	0.324
NEFA (mmol/L)	0.50 ± 0.29	0.39 ± 0.21	0.051
Fasting adipocytokine profile			
Leptin (ng/dL)*	9.77 ± 9.61	7.03 ± 11.84	0.257
sOb-R (ng/mL)*	22.20 ± 6.71	20.76 ± 6.28	0.077
Leptin/sOb-R ratio*	0.56 ± 0.69	0.54 ± 1.17	0.928
Adiponectin (μg/mL)*	10.46 ± 11.14	14.22 ± 15.70	0.953
Visfatin (ng/mL)	0.60 ± 0.51	0.64 ± 0.55	0.788
Resistin (ng/mL)*	4.28 ± 5.05	3.49 ± 3.90	0.089

Student's *t*-test after testing for equality of variance (Levene test). *The natural logarithmic transformed (Ln) values were used for the Student's *t*-test. BMI, body mass index; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model addessment of insulin resistance; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; SD, standard deviation; sOB-R, soluble leptin receptor; SUIT, secretory units of islets in transplantation.

levels of fasting C-peptide (0.12 ± 0.11 vs 0.20 ± 0.20 nmol/L; $P = 0.017$) and secretory units of islets in transplantation (7.66 ± 6.35 vs 16.00 ± 16.78; $P = 0.001$; Table 3).

The lipid pattern appeared comparable between the two clusters. No difference was found between the two clusters in regard to adipocytokine profile (Table 3).

DISCUSSION

In the present study we showed that it is possible to phenotype patients with type 2 diabetes at onset based on the values of fasting GLP-1, GIP and ghrelin; patients with reduced fasting incretin tone are characterized by worse HbA1c and fasting glucose, probably secondary to reduced fasting β-cell activity associated with increased α-cell activity.

Regarding the importance of the three variables in the process of clusterization, the results of the cluster analysis show that we should give priority to GLP-1 and GIP. In this connection, fasting ghrelin, although it proved to be significantly lower in the group with reduced fasting incretin tone, was not decisive in the formation of the two clusters. At present, the effects of fasting plasma ghrelin on fasting plasma insulin and glucose levels in humans are still a matter of debate. Some authors have found no changes[9–11], whereas others have found that acyl ghrelin increased fasting blood glucose and decreased plasma insulin after its administration[12,17,18]. Unfortunately, the present study is not able to contribute to the clarification of these aspects, both because of the slight weight of ghrelin in the formation of the clusters, and because of the limit of having assayed total ghrelin and not acyl ghrelin.

Instead, a more important role in glucose homeostasis in patients with type 2 diabetes seems to be played by fasting levels of GLP-1 and GIP. It is known that the secretion of GLP-1 (mainly synthesized by L-cells in the duodenum, and small and large intestine) in the gastrointestinal tract is influenced by glucose and fatty acids after food ingestion or as a result of vagus nerve stimulation[19]. The main GLP-1 action mechanisms involve stimulating insulin secretion by β-cells in the islets of Langerhans and inhibiting glucagon secretion by α-cells; increased insulin secretion is the result of its increased synthesis[20–22]. The present study has shown that the basal secretion of GLP-1 also plays an important role in glucose homeostasis, although with our data we were not able to show whether this is the result of secretion by L-cells or of degradation by dipeptidyl peptidase-4 (DPP-4)[23,24]. Future studies evaluating the concentration or enzyme activity of DPP-4 in patients with type 2 diabetes might be clarifiers. Analogous considerations must be made for GIP (a peptide secreted by K-cells in the mucosa of the duodenum, jejunum and the proximal portion of the ileum) about its incretin activity stimulating food intake-mediated insulin secretion by β-cells in the islets of Langerhans[24,25]. In this case too, although the postprandial levels of GIP depend on the basic nutrient content of a meal (higher values are observed after the ingestion of carbohydrates, compared with proteins)[26], regarding the fasting serum levels we do not know if they are the result of secretion by K-cells or of degradation by DPP-4[23].

In the present study, what we believe to be of particular interest is the similarity between the two groups in regard to the clinical-anthropometric characteristics and the insulin-sensitivity.

Even adipose tissue function was similar, as shown by the lack of significant differences between the two clusters in relation to the VAI values, leptin, sOb-R, leptin/sOb-R ratio, adiponectin, visfatin and resistin. Although today it is known that adipocytokines form an important part of an 'adipoinsular

axis,' dysregulation of which could contribute to β-cell failure and hence to type 2 diabetes[27], the present data confirm that the fasting level of incretin hormones do not influence this axis; we still cannot exclude a possible role in postprandial phases. Indeed, it is known that postprandial GLP-1 amplifies insulin-mediated glucose uptake in adipocytes[27], and this certainly contributes to normal adipocyte function. The same goes for GIP, which appears to provide a benefit by not only stimulating postprandial intestinal glucose transport and releasing insulin to facilitate nutrient storage, but also through its insulin-mimetic properties, including enhanced uptake of glucose by adipocytes[28].

In conclusion, although the physiological importance of the decreased fasting level of incretins in patients with type 2 diabetes requires further clarification, the present study suggests that a simple phenotyping of patients at onset is possible based on these parameters. In the future, similar studies of larger size could also suggest the appropriate cut-off of GLP-1 and GIP pointing to reduced incretin tone, in order to better select patients who require treatment with GLP-1 analogs or DPP4-inhibitors.

ACKNOWLEDGMENTS

In memory of our great teacher Professor Aldo Galluzzo. We thank Dr Gabriella Misiano and Dr Salvatore Milano for past collaboration. This study was partially supported by the University of Palermo 'Fondo Ricerca di Ateneo 2012' (ID: 2012-ATE-0302).

DISCLOSURE

The authors declare no conflict of interest.

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