

The neuropeptide 26RFa in the human gut and pancreas: potential involvement in glucose homeostasis

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ABSTRACT

Objective: Recent studies performed in mice revealed that the neuropeptide 26RFa regulates glucose homeostasis by acting as an incretin, and by increasing insulin sensitivity. However, in human, an association between 26RFa and the regulation of glucose homeostasis is poorly documented. In this study, we have thus investigated in detail the distribution of 26RFa and its receptor, GPR103, in the gut and the pancreas, and determined the response of this peptidergic system to an oral glucose challenge in obese patients.

Design and Methods: Distribution of 26RFa and GPR103 was examined by immunohistochemistry using gut and pancreas tissue sections. Circulating 26RFa was determined using a specific radioimmunoassay in plasma samples collected during an oral glucose tolerance test.

Results: 26RFa and GPR103 are present all along the gut but are more abundant in the stomach and duodenum. In the stomach, the peptide and its receptor are highly expressed in the gastric glands, whereas in the duodenum, ileum and colon they are present in the enterocytes and the goblet cells. In the pancreatic islets, the 26RFa/GPR103 system is mostly present in the β cells. During an oral glucose tolerance test, plasma 26RFa profile is different between obese patients and healthy volunteers, and we found strong positive correlations between 26RFa blood levels and the BMI, and with various parameters of insulin secretion and insulin resistance.

Conclusion: the present data suggest an involvement of the 26RFa/GPR103 peptidergic system in the control of human glucose homeostasis.

INTRODUCTION

Glucose homeostasis is a crucial parameter to maintain normal body function. Control of blood glucose levels is ensured by a highly sophisticated network of various hormones released by the pancreas, intestine, liver, adipose and muscle tissue, and the brain (1). In particular, accumulating evidence obtained during the last decade support the peripheral role of hypothalamic neuropeptides controlling feeding behaviour such as neuropeptide Y (NPY), orexins, ghrelin, corticotropin-releasing factor (CRF) or apelin, in the regulation of glucose homeostasis (2-6), coining the new concept that hypothalamic neuropeptides may serve as a link between energy and glucose homeostasis, and identifying them therefore as potential therapeutic targets for the treatment of diabetes and obesity (7,8).

26RFa (also referred to as QRFP) is a hypothalamic neuropeptide discovered concurrently by us and others (9-11). Subsequently, 26RFa has been characterized in all the vertebrate phyla including human (12, 13), and identified as the cognate ligand of the human orphan G protein-coupled receptor, GPR103 (10-12, 14). Neuroanatomical observations have revealed that 26RFa- and GPR103-expressing neurons are primarily localized in hypothalamic nuclei involved in the control of feeding behaviour (9, 10, 14-16). Indeed, i.c.v. administration of 26RFa stimulates food intake (9, 14, 17, 18), and the neuropeptide exerts its orexigenic activity by modulating the NPY/POMC system in the arcuate nucleus (Arc) (18). 26RFa also stimulates food intake in birds (19) and fish (20), indicating that 26RFa plays a crucial role in the central regulation of body weight and energy homeostasis in all vertebrates. Consistent with this notion, recent data obtained from 26RFa-deficient mice revealed that the knock-out mice are lean and hypophagic, and that, under a high fat diet, the 26RFa^{-/-} mice show a lower weight gain than the wild-type animals (21). In agreement with this latter observation, a more sustained orexigenic activity of 26RFa has been reported in obese rodents (17, 22), and expression of prepro26RFa mRNA is up-regulated in the hypothalamus of genetically obese

ob/ob and *db/db* mice (14) and in rodents submitted to a high fat diet (17, 22). Altogether, these findings support the notion that 26RFa could play a role in the development and maintenance of the obese status (13).

Recently, an implication of the 26RFa/GPR103 neuropeptidergic system in the control of glucose homeostasis has been reported. It was found that 26RFa and GPR103 are expressed by the pancreatic islets as well as by various insulin-secreting cell lines (23, 24) and that the neuropeptide prevents cell death and apoptosis of β cell lines and isolated pancreatic islets (23). It was also shown that 26RFa is abundantly expressed all along the gut and that i.p. administration of the neuropeptide attenuates glucose-induced hyperglycemia by increasing plasma insulin via a direct insulinotropic effect on the pancreatic β cells, and by increasing insulin sensitivity (24). Finally, it was reported that an oral glucose challenge induces a massive secretion of 26RFa by the gut into the blood, strongly suggesting that this neuropeptide regulates glycemia by acting as an incretin (24).

In human, an association between the 26RFa/GPR103 system and the regulation of glucose homeostasis is far more limited. This observation prompted us to investigate in detail the distribution of 26RFa and its receptor all along the gastrointestinal tract and in the pancreas, and determined the response of this peptidergic system to an oral glucose challenge in obese patients vs healthy volunteers.

MATERIALS AND METHODS

Immunohistochemical procedure

Deparaffinized sections (15- μ m thick) of human pancreas, stomach, duodenum, ileum and colon were used for the immunohistochemical study. Human tissue sections were obtained from our Department of Pathology at the University Hospital of Rouen. All of the tissue procurement protocols were approved by the relevant institutional committees (University of Rouen) and were undertaken under informed consent of each patient and all of the participants. Tissue sections were incubated for 1 h at room temperature with either rabbit polyclonal antibodies against 26RFa (25) diluted 1:400, or GPR103 (#NLS1922; Novus Biologicals, Littleton, CO) diluted 1:100, or mouse monoclonal antibodies against insulin (Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted 1:1000, or goat polyclonal antisomatostatin (Santa Cruz Biotechnology, Dallas, TX) diluted 1:500, or mouse monoclonal antiglucagon (Sigma-Aldrich) diluted 1:400. The sections were incubated with a streptavidin-biotin-peroxydase complex (Dako Corporation, Carpinteria, CA), and the enzymatic activity was revealed with diaminobenzidine. The slices were then counterstained with hematoxylin. Observations were made under a Nikon E 600 light microscope.

For colocalization studies, the following primary antibodies were used: rabbit polyclonal anti-human 26RFa (25) and mouse monoclonal anti-insulin (1:400) (Sigma-Aldrich). Alexa-conjugated antibodies (Invitrogen Life Technologies) including donkey antirabbit (1:100) (DAR-488) and goat antimouse (1:400) (GAM-594) were used as secondary antibodies. Sections were counterstained with 1 μ g/mL 4',6-diamino-2-phenylindole (Sigma-Aldrich) in PBS for 90 seconds. Tissue sections were examined with a Leica SP2 upright confocal laser scanning microscope (DMRAX-UV) equipped with the Acousto-Optico Beam Splitter system (Leica). The specificity of the immunoreactions was controlled by (1) substitution of the primary antibodies with Tris buffer saline (TBS; pH 7.4) and (2) preincubation of the primary

antisera with the corresponding synthetic peptides (10^{-6} M), with the exception of GPR103 whose antigen sequence was unknown. In both cases, no immunoreaction was observed.

Oral glucose tolerance test

Eight obese patients ($\text{BMI} > 40 \text{ kg/m}^2$), who performed usual examinations before bariatric surgery, were recruited. Healthy controls (C) underwent standard endocrine tests to exclude any metabolic abnormalities. The nine healthy volunteers had already been recruited for a previous study (26). All of the subjects included in the study gave a written informed consent according to our Ethical Committee instructions, and the study was approved by the Ethical Committee of Haute-Normandie (CCPPRB-HN; 26). The obese patients and the healthy volunteers underwent a 75-g oral glucose tolerance test (OGTT) after a 12-h fasting period. Plasma 26RFa, glucose and insulin concentrations were measured in fasting blood samples obtained at 0, 30, 60, and 120 min after oral glucose loading.

Human plasma assays

Plasma 26RFa levels were measured using a specific radioimmunoassay (RIA) set up in the laboratory that has been previously described in detail (27). For the RIA procedure, each sample was diluted (1:1) in a solution of water/TFA (99.9:0.1; v/v) and pumped at a flow rate of 1.5 ml/min through one Sep-Pak C_{18} cartridge. Bound material was eluted with acetonitrile/water/TFA (50:49.9:0.1; v/v/v) and acetonitrile was evaporated under reduced pressure. Finally, the dried extracts were resuspended in the buffer assay.

Blood glucose levels were measured using a glucose oxidase activity test (LX20 Beckman Coulter, Villepinte, France) and plasma insulin was quantified using an Elecys for automated insulin Assay (COBAS 6000CE, Roche Diagnostics, Meylan, France).

Usual insulin resistance and insulin secretion indices were calculated at fasting levels and during the oral glucose tolerance test:

- Homeostasis Model Assessment of *Insulin* Resistance, HOMA-R index,

$[(\text{fasting glucose (mmol/L)} \times \text{fasting insulin } (\mu\text{U/mL})) / 22.5 \text{ (28)}.$

- Homeostasis Model Assessment of *insulin secretion*, Homa- β : $[20 \times \text{fasting insulin}$

$(\mu\text{U/mL})] / [(\text{fasting glucose (mmol/L)} - 3.5).$

- Index insulinogenic

$(\text{Insulin } 30' - \text{Insulin } 0) / (\text{Glucose } 30' - \text{Glucose } 0)$, units ($\mu\text{U/mL}$) for insulin, mmol/l for glucose (29).

Statistical analysis

Statistical analysis was performed with Statistica (5th version). A Mann Whitney test or ANOVA for repeated measures was used for comparisons between the two groups. A post-hoc comparison using Tukey HSD was applied according to ANOVA results. Statistical significance was considered for an α risk of 5%.

RESULTS

Distribution of 26RFa- and GPR103-like immunoreactivity in the gastrointestinal tract

In the antral part of the stomach, we found that the gastric glands were strongly labeled with the 26RFa antibodies (Fig. 1A, B). At low magnification, it seemed that 26RFa-LI was concentrated in cells of the isthmus and the neck whereas the surface epithelium cells of the gastric pit and the base of the gland were not immunostained (Fig. 1A, B). However, higher magnification revealed that surface epithelium cells contained 26RFa-LI at their basal pole whereas the mucus granules located at the apical pole of the cells were devoid of immunolabeling (Fig. 1C). Transversal section of a gastric gland at the neck level indicated that the 26RFa-positive cells are large and exhibit a round shape identifying them as parietal cells whereas the unlabeled cells are smaller and thus probably correspond to chief cells (Fig. 1D). Preincubation of the 26RFa antibodies with synthetic human 26RFa (10^{-6} M) resulted in a total loss of the immunostaining in the gastric glands (Fig. 1E). Similarly, stomach slices on which the 26RFa antibodies were substituted with Tris buffer were totally devoid of labeling (Fig. 1F).

The localization and distribution of the 26RFa receptor, GPR103, in stomach slices was very similar to that of 26RFa, with the presence of abundant GPR103-like immunoreactivity (LI) in the gastric glands (Fig. 2A). Higher magnification photomicrographs revealed that, in the surface epithelium cells, GPR103-LI was concentrated in the basal pole of the cells whereas the apical pole containing the mucus granules was unstained, as observed for 26RFa (Fig. 2B, C). Similarly, at the neck level of the gland, it appears that the parietal cells (according to their volume and shape) expressed the 26RFa receptor whereas the chief cells did not (Fig. 2D). Longitudinal (Fig. 2E) or transversal sections (Fig. 2F) of gastric glands revealed that substitution of the GPR103 antibodies with Tris buffer results in a total loss of the GPR103 immunolabeling.

In the duodenum, 26RFa-LI was detected in most of the enterocytes and goblet cells of a number of villousities (Fig. 3A). However, we also found that other villousities were devoid of 26RFa immunolabeling (Fig. 3B). At a higher magnification, it clearly appears that, in some villousities, all of the enterocytes expressed 26RFa whereas, in the adjacent villosity, the enterocytes were totally unstained (Fig. 3C). Figure 3D illustrates the strong 26RFa immunolabeling exhibited by some enterocytes and goblet cells of the duodenum.

A low magnification of duodenal villousities clearly shows that the number of enterocytes and goblet cells expressing the 26RFa receptor is more restricted than for 26RFa immunostaining (Fig. 3E). At a higher magnification, it appears that, in some cases, GPR103 immunostaining was concentrated in the basal pole of the enterocytes and goblet cells (Fig. 3F, G) whereas, in other cases, GPR103 labeling was homogeneously distributed in the cytoplasm of the cells (Fig. 3H).

In the ileum, the distribution of 26RFa-LI was very similar to that observed in the duodenum, with an immunolabeling in the enterocytes and goblet cells (Fig. 4A). However, it is to note that the number of 26RFa-labeled cells in the ileum was much lower than in the duodenum (Fig. 4A, B). We also found GPR103 immunostaining in some enterocytes and goblet cells of the ileum, but the number of cells expressing the 26RFa receptor was low (Fig. 4C). However, when the cells were labeled with the GPR103 antibodies, the immunostaining was intense as illustrated by these goblet cells in figure 4D.

In the colon, low magnification of villousities suggested a lack of 26RFa immunostaining (Fig. 4E). However, at a higher magnification, it appears that, in some villousities, enterocytes and goblet cells exhibited a low immunolabeling (Fig. 4E). The 26RFa receptor showed a general distribution, in the colon, closely related to that observed in the ileum with a low number of enterocytes and goblet cells strongly labeled with the GPR103 antibodies (Fig. 4G, H).

Distribution of 26RFa- and GPR103-like immunoreactivity in the pancreatic islets

Treatment of human pancreas sections with the 26RFa antibodies revealed that the neuropeptide was present in the endocrine islets (Fig. 5A). In contrast, the exocrine tissue was virtually devoid of 26RFa-LI (Fig. 5A). A higher magnification showed that almost all the cells of the islet were strongly labeled with the 26RFa antibodies (Fig. 5B). Similarly, we found an intense GPR103-immunostaining in the human pancreatic islets (Fig. 5C). However, a high magnification of the islets revealed that some islet cells were strongly labeled with the 26RFa receptor antibodies whereas others were devoid of immunostaining (Fig. 5D). Figures 5E and F revealed that preincubation of the 26RFa antibodies with synthetic human 26RFa (10^{-6} M) resulted in a total loss of the immunostaining in the pancreatic islets. Similarly, pancreas slices on which the GPR103 antibodies were substituted with Tris buffer were totally devoid of labeling in the islets (Fig. 5G, H).

In addition, treatment of consecutive sections with the 26RFa antibodies and insulin antibodies revealed that the histological distribution of 26RFa-LI was similar to that of insulin (Fig. 6A, B). Supporting this observation, a double immunolabeling experiment with 26RFa and insulin antibodies showed a colocalization of the neuropeptide and the hormone in most of the pancreatic islet cells (Fig. 6C). However, it is to note that some cells were only labeled with the 26RFa antibodies whereas others only exhibited insulin-LI (Fig. 6C). Consecutive pancreas sections were also treated with the 26RFa antibodies and glucagon antibodies, and suggest that in some cases glucagon cells express the neuropeptide (Fig. 6D, E). Similarly, treatment of consecutive sections with the 26RFa antibodies and somatostatin antibodies revealed that some somatostatin-producing cells expressed 26RFa (Fig. 6F, G).

Concurrently, treatment of consecutive sections with the GPR103 antibodies and insulin antibodies showed that the histological distribution of GPR103-LI was closely related to that

of insulin (Fig. 7A, B). Consecutive pancreas sections were also treated with either the GPR103 antibodies or glucagon antibodies and the observations revealed that some glucagon-positive cells also expressed the 26RFa receptor (Fig. 7C, D). Similarly, treatment of consecutive sections with the GPR103 antibodies and somatostatin antibodies suggested that a few somatostatin-producing cells expressed the 26RFa receptor (Fig. 7E, F).

Oral glucose tolerance test in human

An oral glucose tolerance test was performed on 8 obese patients and 9 healthy volunteers. Age was not significantly different between the two groups (43 ± 3.7 for obese patients vs 35.3 ± 3.7 years for the healthy volunteers). By contrast and as expected, BMI and insulin fasting levels were significantly higher in the obese patients as compared to controls (BMI: 46.4 ± 1.9 vs 21.1 ± 0.7 kg/m², $p < 0.0001$; insulin: 115.8 ± 22.1 vs 40.7 ± 7 pmol/L, $p = 0.0012$).

During the oral glucose tolerance test, blood glucose and insulin levels rose to values significantly higher in obese patients in comparison to the healthy volunteer group (Fig. 8A, B). In controls, plasma 26RFa concentrations were stable during the first 90 min of the test and increased significantly at 120 min ($p < 0.05$; Fig. 8C). By contrast, there was not significant alteration of the plasma 26RFa concentrations all along the test in obese patients (Fig. 8C). In addition, it is to note that, during the test, 26RFa blood levels were significantly higher in the obese patients ($p < 0.05$; Fig. 8C), as assessed by the AUC analysis (Fig. 8D).

Correlation analysis revealed that AUC of plasma 26RFA concentrations were positively correlated with BMI (Fig. 9A), fasting insulinemia (Fig. 9B) and with a marker of insulin resistance represented by the HOMA R index (Fig. 9C). Moreover, markers of insulin

secretion such as HOMA B and insulinogenic index were also strongly correlated with the 26RFa AUC (Fig. 9D, E).

DISCUSSION

The neuropeptide 26RFa and its receptor GPR103 have been discovered simultaneously by us, and two pharmaceutical groups in 2003 (9-11). Animal studies accumulated during the last decade have revealed that, centrally, this neuropeptidergic system plays a key role in the control of feeding behavior (12), and that, at the periphery, it is involved in the regulation of glucose homeostasis (13). However, our knowledge on the 26RFa/GPR103 system in human is far more limited. In the present study, we took advantage of a collaboration with different Departments of the University Hospital to obtain human tissue and plasma samples to investigate the 26RFa/GPR103 system in human with regard to its implication in the peripheral regulation of glucose homeostasis.

The availability of various human tissue sections gave us the opportunity to decipher the distribution and localization of 26RFa and GPR103 in the gut and the pancreas. Our observations indicate that 26RFa is present all along the gastro-intestinal tract, from the stomach to the colon. However, the neuropeptide is much more abundant in the stomach and duodenum than in the ileum and the colon confirming preliminary data obtained previously by our team (24). The high expression of 26RFa in the stomach is very interesting. Indeed, 26RFa strongly stimulates food intake when injected centrally by modulating the NPY/POMC system of the Arc (18, 30). The stomach is well known to produce another important orexigenic peptide that is ghrelin (31, 32). Ghrelin is secreted by the endocrine cells of the stomach in the general circulation during the preprandrial period to initiate food intake by targeting the NPY neurons of the Arc that express the ghrelin receptor, GHSR (33). The NPY neurons have been previously shown to express GPR103, the 26RFa receptor, and to mediate the orexigenic activity of 26RFa (18). Our team has shown that, in human, high levels of 26RFa are detected in the blood (24, present study), and that the neuropeptide is able to cross the brain-blood-barrier in rats (unpublished data). Together, these observations suggest that

26RFa, in as much as ghrelin, may be released by the stomach into the blood and cross the brain-blood-barrier to target the NPY neurons of the Arc to exert its orexigenic activity. In addition, it also raises the exciting hypothesis that the stomach is a major source of orexigenic peptides that trigger the initiation of food intake. However, we observed that 26RFa is present in abundance in the parietal cells whereas ghrelin is specifically produced by the endocrine cells, suggesting that the two orexigenic peptides are produced by distinct cells of the stomach gastric glands.

The present study also reveals that 26RFa is present in a number of enterocytes and goblet cells in the human duodenum. In mice, we have previously shown that 26RFa is highly expressed by the enterocytes of the duodenum that release the peptide into the blood after an oral glucose load, leading to the incretin effect of 26RFa (24). We show here that, in healthy volunteers, plasma 26RFa levels are increased 90 min after an oral glucose load, suggesting that, in human as observed in mice, duodenal 26RFa may be secreted by the gut after a glucose ingestion.

Our histological observations also reveal a high expression of the 26RFa receptor all along the gastro-intestinal tract, notably in the stomach and the duodenum. In fact, in the gut, GPR103 exhibits a cell localization and distribution very similar to that of its ligand, suggesting that 26RFa may act in the gastro-intestinal tract *via* an autocrine mechanism. The presence of GPR103 in the gastric glands and the enterocytes and goblet cells suggests an involvement of the 26RFa/GPR103 system in the secreting activity of the gut although to our knowledge such an activity of this peptidergic system has never been reported. However, it is to note that the role of various peptides/hormones of the gut such as cholecystokinin, secretin, GIP, VIP, in the secretion of gastrin, pepsin, HCl is clearly established and widely documented (34), and that some of them such as cholecystokinin, GIP or secretin are also involved in the control of feeding behavior and glucose homeostasis. Collectively, these

observations support the idea that 26RFa is a gut hormone able to modulate gut functions and regulate energy and glucose metabolism.

In the pancreas, we found that 26RFa and its receptor are strictly localized in the pancreatic islets. In addition, double labeling experiments reveal that the 26RFa/GPR103 system is mainly expressed by the β cells that produce and secrete insulin, suggesting a role of this peptidergic system in the production and secretion of insulin. Consistent with this hypothesis, previous studies performed in mice have shown that 26RFa can directly stimulate insulin secretion by the β cells by activating GPR103, which is expressed by the same cells (24). It is thus probable that, in human, 26RFa displays the same insulin-secreting activity than that observed in mice. In addition, the presence of 26RFa, together with GPR103, in the β cells suggests that the peptide may regulate insulin secretion via an autocrine mechanism. Finally, 26RFa has also been shown to prevent cell death and apoptosis induced by serum starvation, cytokine synergism and glucolipototoxicity in a model of isolated human pancreatic islets (23). This latter observation indicates that, in addition to its insulinotropic action, the 26RFa/GPR103 system is able to promote survival of β cells. The present double labeling experiments also reveal that both 26RFa and GPR103 are expressed in non insulin-producing cells in the pancreatic islets that may correspond to glucagon- and somatostatin-producing cells, suggesting a role of the peptidergic system in the metabolism and/or secreting activity of the α and δ cells. A single study, using perfused rat pancreas, report that infusion of 26RFa has no effect on glucagon output (35). Therefore, the role of the 26RFa/GPR103 system in the glucagon and somatostatin cells deserves further investigation.

In addition to the above data that precisely determined the location of the system 26RFa/GPR103 in the gastro-intestinal tract, we thought it was important to examine the role of this peptidergic system in the regulation of glucose homeostasis. In fact, previous experiments performed in mice by our team have led to the conclusion that 26RFa regulates

glucose homeostasis by acting as an incretin, and that the global anti-hyperglycemic effect of the peptide was due to an increase of insulin secretion associated with an enhanced insulin sensitivity (24). In the present work, we have evaluated plasma 26RFa concentrations in healthy volunteers and in obese patients during an oral glucose tolerance test. We found that 26RFa blood levels are significantly higher in the obese patients as compared to the healthy volunteers, and that the 26RFa secretion kinetics are different in the two groups during the glucose load. This result is in agreement and complete previous data reporting a significant increase of fasting plasma 26RFa concentrations in obese subjects and obese diabetic patients (24), raising the hypothesis that this up-regulation of the 26RFa system may play a role in the development and maintenance of the obese status. We may also speculate that the rise of plasma 26RFa observed in the obese patients is due to a loss of effect of 26RFa on the regulation of glucose homeostasis. Indeed, we have recently found that, in high fat-induced obese mice, the antihyperglycemic effect exerted by 26RFa is totally vanished, due to a total loss of effect of the peptide on insulin secretion and a strongly diminished effect on insulin sensitivity (36). In addition, we observed that this loss of effect of 26RFa in the regulation of glycemia is associated with a decreased expression of GPR103 in insulin target organs and pancreatic islets. Consequently, we hypothesize that the increase of 26RFa production could reflect the implementation of a compensatory mechanism to counterbalance the low expression levels of GPR103 induced by the high fat diet. Further analysis of GPR103 expression in human adipose tissue and muscle obtained from both healthy controls and obese patients is needed to confirm this hypothesis.

In addition, we found strong positive correlations between 26RFa blood levels and the BMI, and with various parameters of insulin secretion and insulin resistance. These findings are in agreement with previous data obtained from a large cohort of subjects including healthy controls, obese, diabetic and obese diabetic patients, revealing that fasting 26RFa blood

concentrations were correlated with BMI and fasting insulinemia (24). The strong correlation between the 26RFA OGGT AUC and fasting insulinemia or HOMAR index suggests that the 26RFa peptidergic system may be implicated in the occurrence of the decreased insulin sensitivity and increased insulin resistance observed in a number obese patients. Studies are planned in a near future to determine the role of the 26RFa/GPR103 on the insulin signaling pathway.

In conclusion, the present histological and physiological data obtained in human reveal that the 26RFa/GPR103 peptidergic system is abundantly expressed in the gut and the pancreas and suggest an involvement of this peptidergic system in the control of human glucose homeostasis.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR CONTRIBUTIONS

G.P. and N.C. contributed to the study design and interpretation, and wrote the manuscript. M.A.L.S., M.M. and M.P. contributed to the immunohistochemical experiments. S.C. and H. B. performed the 26RFa radioimmunoassays. A.B. and E.N. realized the insulin assays. J. L. produced 26RFa. F.G. provided the human tissue sections. V.B., H.L. and Y. A. revised and approved the final version of the manuscript. N.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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LEGENDS TO FIGURES

Figure 1. Distribution of 26RFa-like immunoreactivity in the human stomach. **(A, B)** Low magnification photomicrographs showing a dense immunolabeling in the gastric glands. **(C, D)** Higher magnification photomicrographs reveal that, in the surface epithelium cells, the immunostaining is restricted to the basal pole of the cells (C). In the neck part of the gastric glands, 26RFa-positive cells are large and exhibit a round shape identifying them therefore as parietal cells (D). **(E, F)** Control sections showing that preincubation of the 26RFa antibodies with synthetic human 26RFa (10^{-6} M) (E) or substitution of the primary antibodies with Tris buffer saline (F) results in a total loss of the immunostaining. Scale bars: (A, B), 200 μ m; (E, F), 50 μ m; (D), 25 μ m; (C), 10 μ m.

Figure 2. Distribution of GPR103-like immunoreactivity in the human stomach. **(A)** Low magnification photomicrograph showing a dense immunolabeling in the gastric glands. **(B-D)** Higher magnification photomicrographs reveal that, in the surface epithelium cells, the immunostaining for the 26RFa receptor is restricted to the basal pole of the cells (B, C). In the neck part of the gastric glands, GPR103-positive cells are large and exhibit a round shape identifying them therefore as parietal cells (D). **(E, F)** Control sections showing that substitution of the GPR103 primary antibodies with Tris buffer saline results in a total loss of the immunostaining. Scale bars: (A), 200 μ m; (E, F), 50 μ m; (B, D), 25 μ m; (C), 10 μ m.

Figure 3. Distribution of 26RFa- and GPR103-like immunoreactivity in the human duodenum. **(A-D)** Photomicrographs showing that some villusities are abundantly labeled with the 26RFa antibodies (A, C), whereas others are totally unstained (B, C). High magnification indicates that the enterocytes as well as the goblet cells display intense 26RFa-like immunoreactivity (D). **(E-H)** Low magnification photomicrographs showing that, in the duodenal villusities, the immunolabeling for the 26RFa receptor is less important than for

26RFa (E, F). Higher magnification photomicrographs reveal that, in some cases, GPR103-like immunoreactivity is concentrated in the basal pole of the enterocytes and the goblet cells (G), whereas, in other cases, the labeling is homogeneously distributed throughout the cytoplasm of the cells (H). Scale bars: (A, B, E), 200 μm ; (F), 50 μm ; (C, G), 25 μm ; (D, H), 10 μm .

Figure 4. Distribution of 26RFa- and GPR103-like immunoreactivity in the human ileum and colon. **(A, B)** In the ileum, 26RFa-immunolabeling is present in scattered enterocytes and goblet cells of the villus (A). A higher magnification photomicrograph shows that a number of villi are, indeed, devoid of immunostaining (B). **(C, D)** Similarly, GPR103-immunolabeling is present in a few enterocytes and goblet cells of the ileum (C), but when the cells are labeled, the immunostaining is intense (D). **(E, F)** In the colon, most of the villi are devoid of 26RFa-immunolabeling (E). However, in some cases, weakly immunostained enterocytes can be found (F). **(G, H)** Distribution and localization of the 26RFa receptor in the colon is very similar to that observed in the ileum with an intense immunostaining in the enterocytes and goblet cells of some villi. Scale bars: (A, C, E, G), 200 μm ; (B), 50 μm ; (F, H), 25 μm ; (D), 10 μm .

Figure 5. Distribution of 26RFa-like immunoreactivity in the human pancreas. **(A, B)** Low magnification photomicrograph showing that the 26RFa labeling is present in the endocrine islets, whereas the exocrine tissue is virtually devoid of 26RFa-like immunoreactivity (A). A higher magnification reveals that almost all the cells of the islet are strongly labeled with the 26RFa antibodies (B). **(C, D)** Similarly, the pancreatic islets are strongly labeled with the GPR103 antibodies (C). However, a higher magnification of the islets reveals that some islet cells are strongly labeled (arrows) whereas others are devoid of immunostaining (arrowheads) (D). **(E, F)** Photomicrographs showing that preincubation of the 26RFa antibodies with synthetic human 26RFa (10^{-6} M) results in a total loss of the immunostaining in the pancreatic

islets (F). **(G, H)** Similarly, substitution of the GPR103 antibodies with Tris buffer results in a total loss of the immunostaining in the pancreatic islets (H). Scale bars: (E-H), 200 μm ; (A, C), 50 μm ; (B, D), 10 μm .

Figure 6. Co-distribution of 26RFa-like immunoreactivity (LI) with insulin, glucagon and somatostatin in the human pancreas. **(A, B)** Consecutive sections treated with the 26RFa antibodies or the insulin antibodies showing that the histological distribution of 26RFa-LI (A) is similar to that of insulin (B). **(C)** Double immunolabeling experiment with the 26RFa and the insulin antibodies shows a colocalization of the neuropeptide and the hormone in most of the pancreatic islet cells (small arrowhead in the merge photomicrograph for instance). However, some cells are only labeled with the 26RFa antibodies (arrows), whereas others only exhibit insulin-like immunoreactivity (large arrowhead). **(D, E)** Consecutive sections treated with the 26RFa antibodies and glucagon antibodies suggesting that in some cases glucagon-positive cells (arrow) (E) display 26RFa-like immunoreactivity (arrow) (D). **(F, G)** Consecutive sections treated with the 26RFa antibodies and somatostatin antibodies suggesting that in some cases somatostatin-positive cells (arrow) (G) display 26RFa-like immunoreactivity (arrow) (F). Scale bars: (A, B, D-G), 25 μm ; (C), 50 μm .

Figure 7. Co-distribution of GPR103-like immunoreactivity (LI) with insulin, glucagon and somatostatin in the human pancreas. **(A, B)** Consecutive sections treated with the GPR103 antibodies and insulin antibodies reveal that the histological distribution of GPR103-LI (A) is closely related to that of insulin (B). **(C, D)** Consecutive sections treated with the GPR103 antibodies and glucagon antibodies suggesting that, in some cases, glucagon-positive cells (arrow) (D) display GPR103-like immunoreactivity (arrow) (C). **(E, F)** Consecutive sections treated with the GPR103 antibodies and somatostatin antibodies suggesting that, in some cases, somatostatin-positive cells (arrow) (F) display 26RFa-like immunoreactivity (arrow) (E). Scale bars: 25 μm .

Figure 8. Plasma profiles of glucose, insulin and 26RFa during an oral glucose tolerance test in healthy volunteers and obese patients. **(A, B)** As expected, in obese patients (n=8), blood glucose (A) and insulin (B) levels rose to values significantly higher to those measured in healthy volunteers (n=9). **(C, D)** Plasma 26RFa concentrations in the healthy volunteers are stable during the first 90 min of the glucose tolerance test and significantly increase at 120 min (C). By contrast, there is no significant alteration of the plasma 26RFa concentrations all along the test in the obese patients (C). However, it is to note that, during the test, 26RFa blood levels are significantly higher in the obese patients as assessed by the AUC analysis (D). *, $p < 0.05$; ** $p < 0.01$ obese patients vs healthy volunteers. #, $p < 0.05$ T120 vs T90 in healthy volunteers.

Figure 9. Correlation analysis of the AUC of plasma 26RFA concentrations during the oral glucose tolerance test with various glycemic and metabolic parameters. Plasma 26RFA AUC is positively correlated with BMI (A), fasting insulinemia (B) and with the marker of insulin resistance, the HOMA R index (C). Moreover, markers of insulin secretion such as HOMA B and insulinogenic index are also strongly correlated with the 26RFa AUC (D, E).

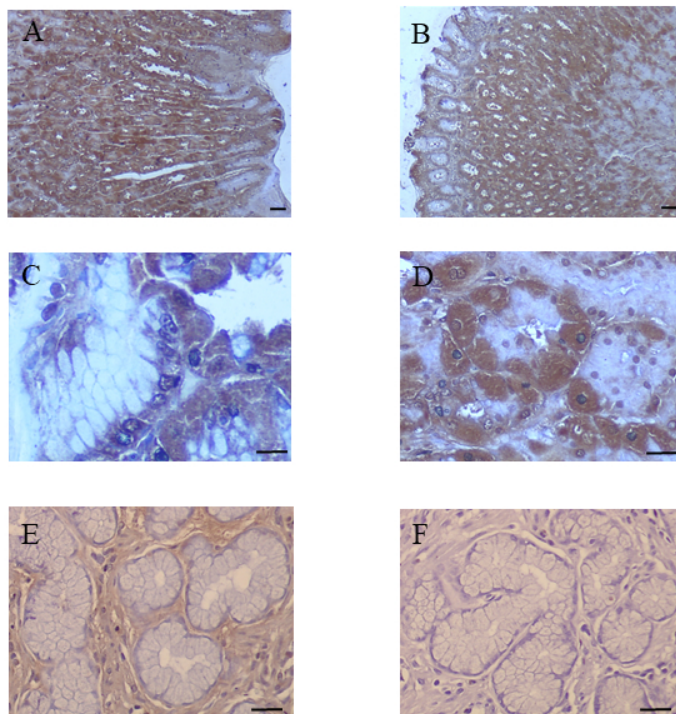


Figure 1

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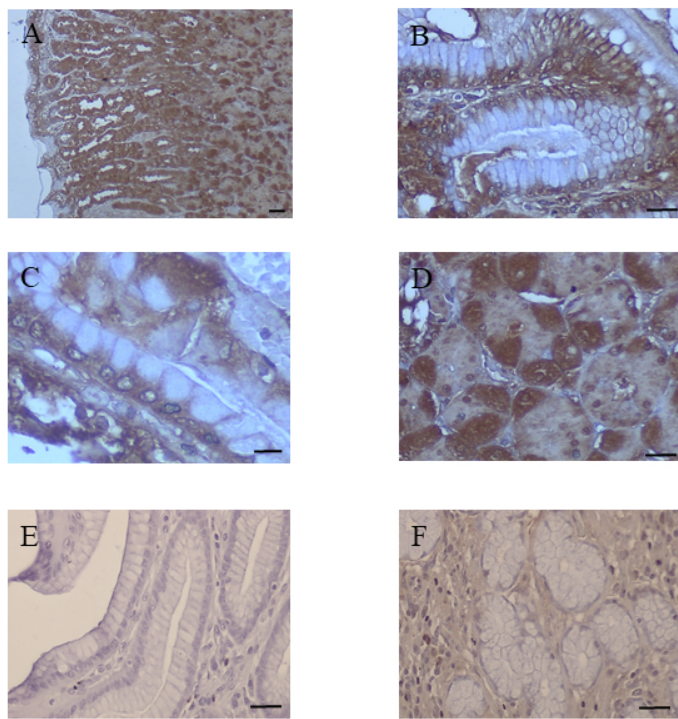


Figure 2

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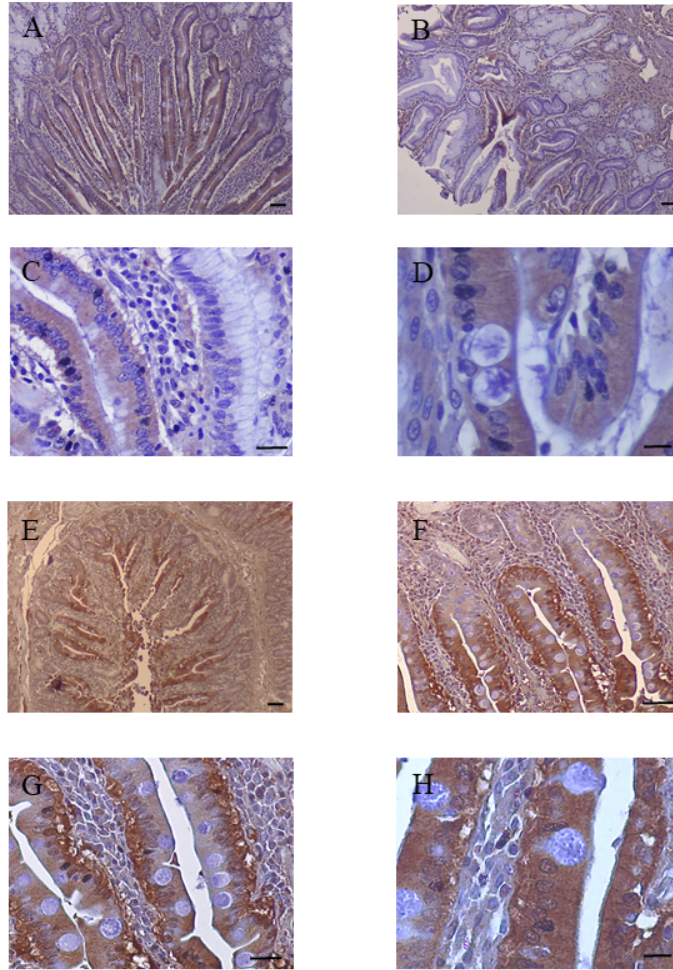


Figure 3

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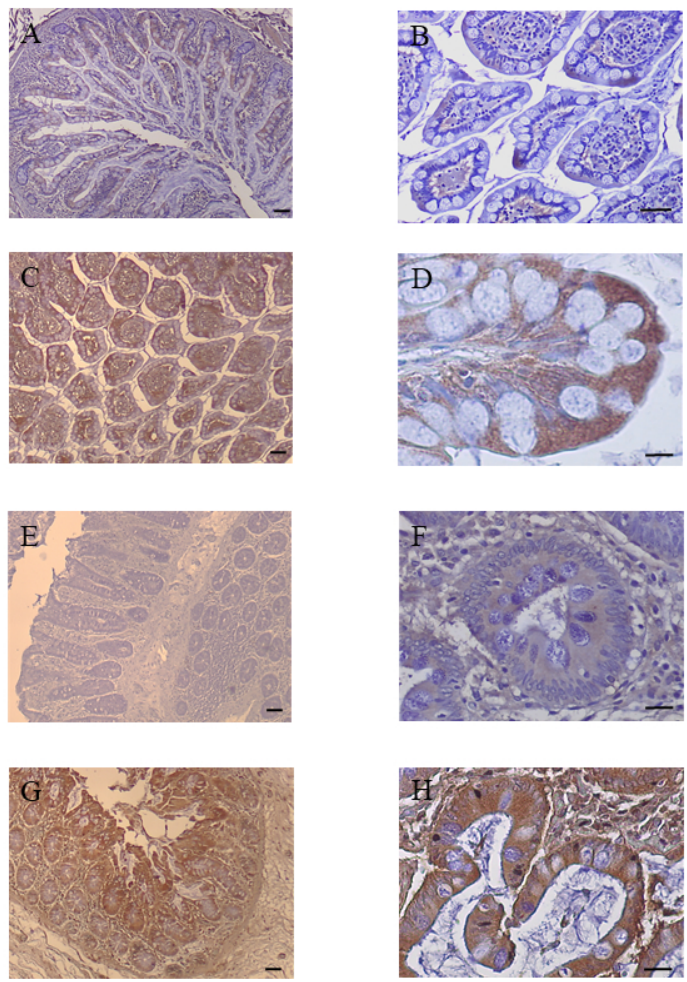


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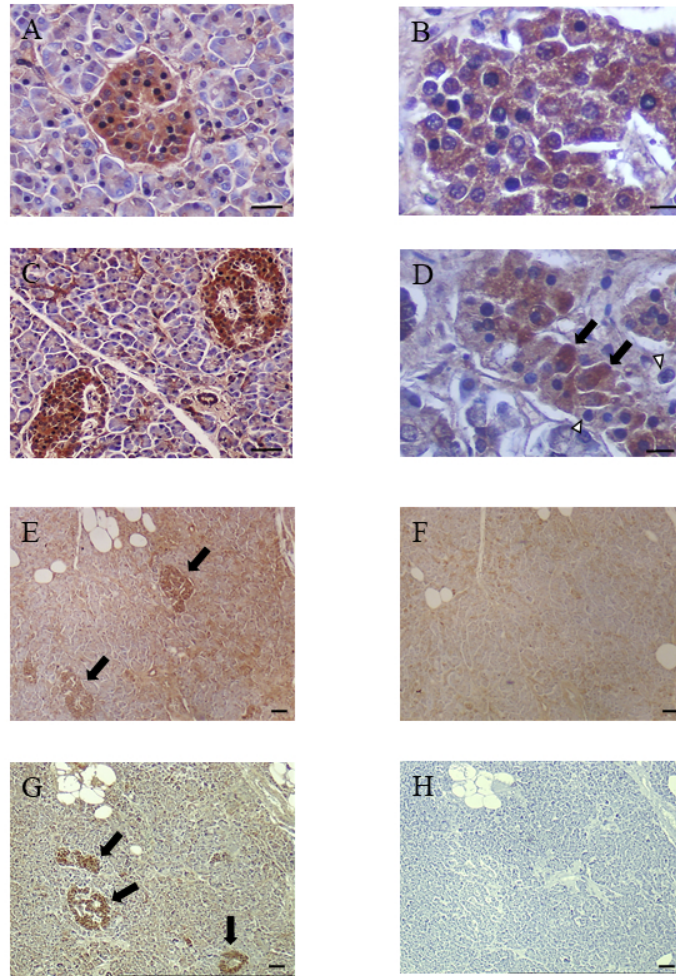


Figure 5

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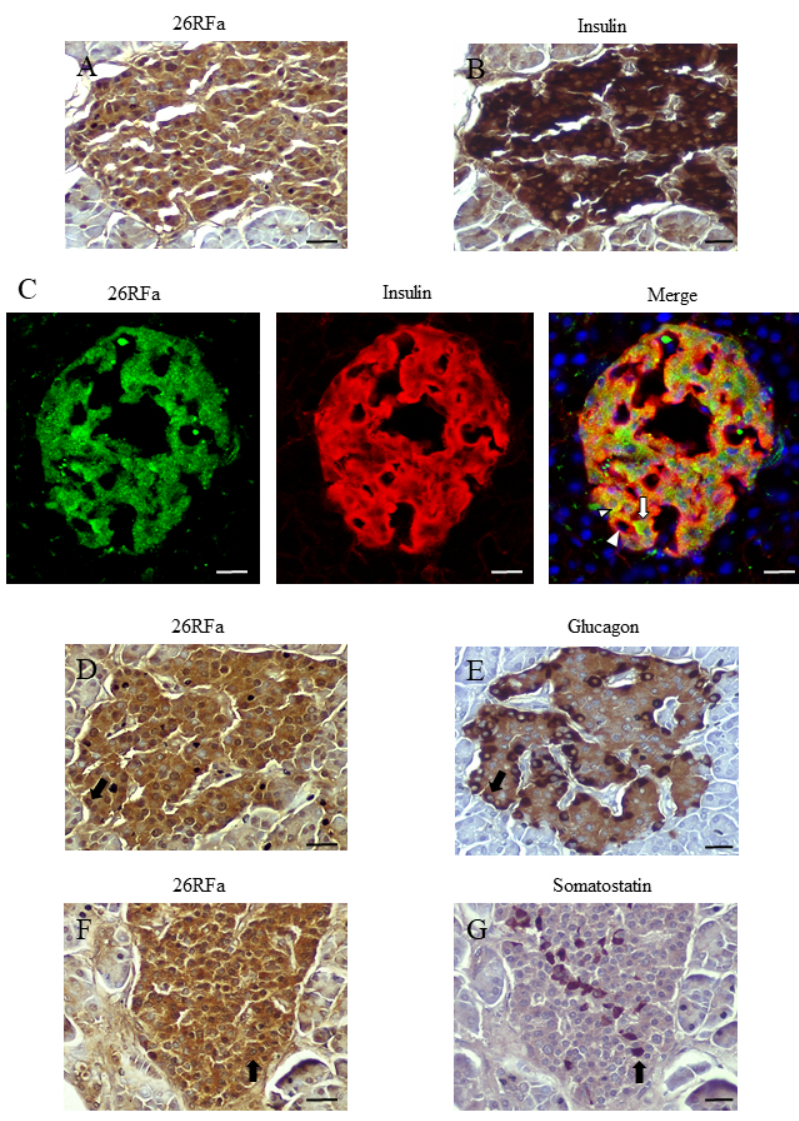


Figure 6

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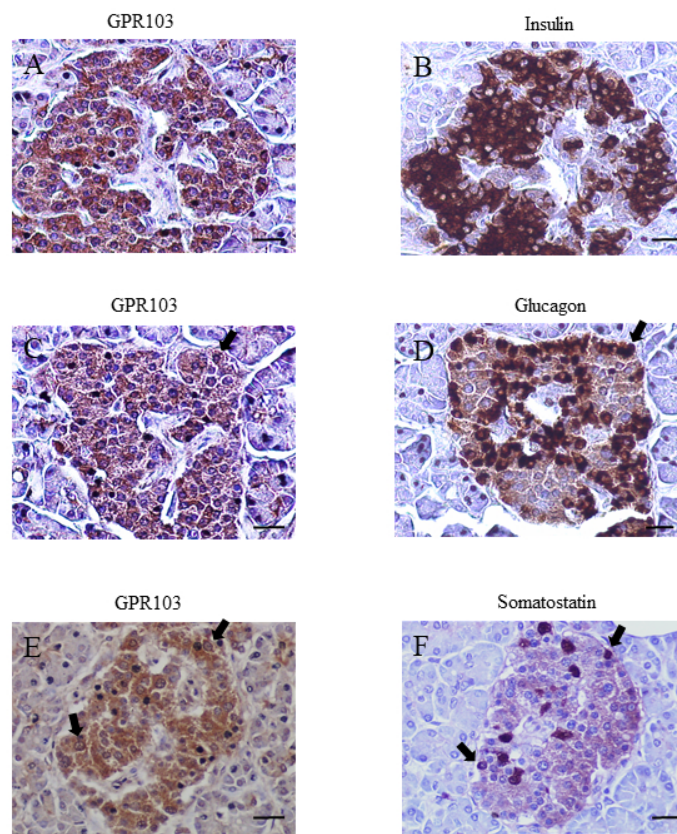


Figure 7

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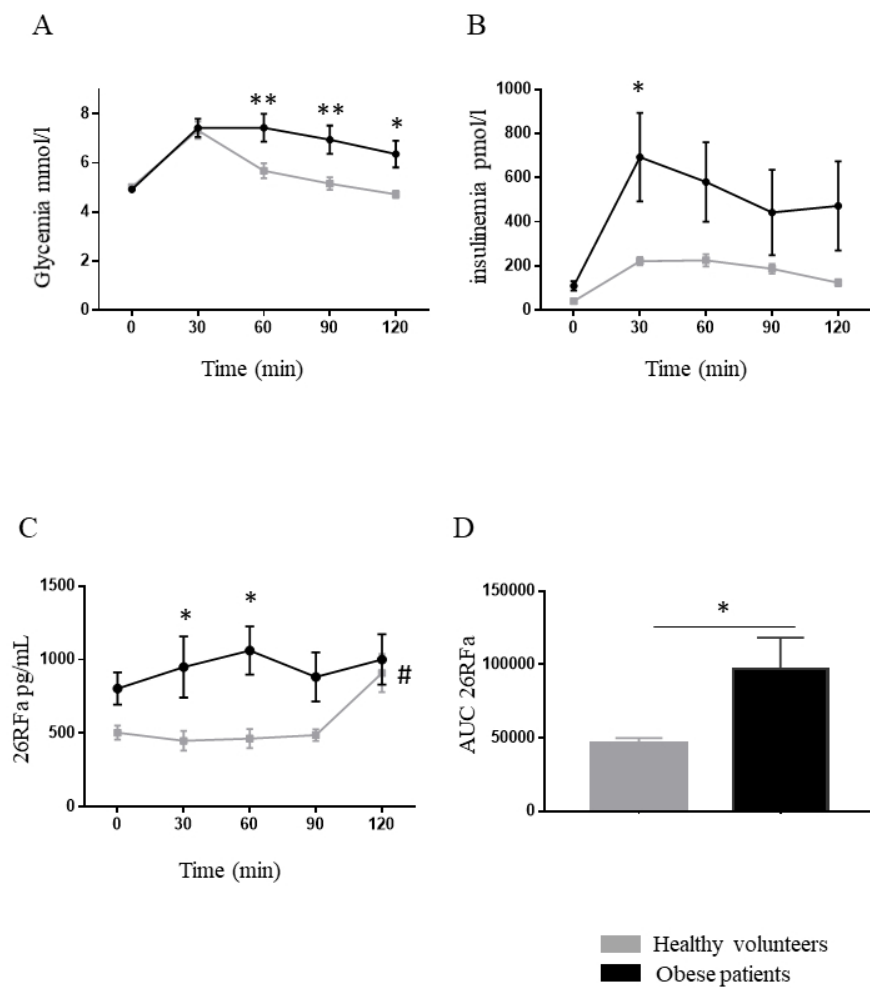


Figure 8

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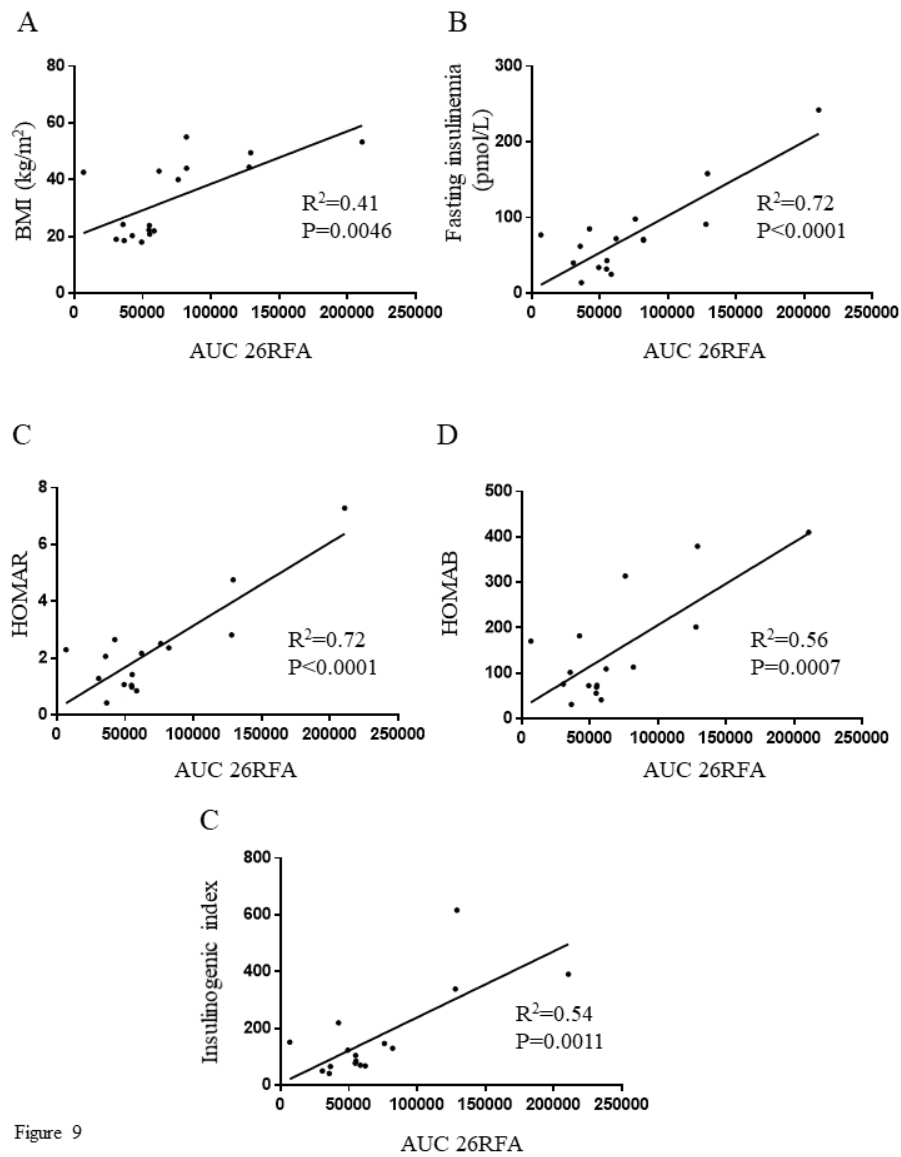


Figure 9

Figure 9

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