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Ghrelin suppresses insulin secretion in human islets and type 2 diabetes patients have diminished islet ghrelin cell number and lower plasma ghrelin levels

A. Lindqvist[a], L. Shcherbina[a,1], R.B. Prasad[a,1], M.G. Miskelly[a], M. Abels[a], J.A. Martínez-Lopéz[b], R.G. Fred[a], B.J. Nergård[c], J. Hedenbro[a,c], L. Groop[a,d], J. Hjerling-Leffler[b], N. Wierup[a,*]

[a] Lund University Diabetes Centre, Lund University, Malmö, Sweden
[b] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden
[c] Aleris Obesitas, Lund, Sweden
[d] Finnish Institute of Molecular Medicine, Helsinki, Finland

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ABSTRACT

It is not known how ghrelin affects insulin secretion in human islets from patients with type 2 diabetes (T2D) or whether islet ghrelin expression or circulating ghrelin levels are altered in T2D. Here we sought out to identify the effect of ghrelin on insulin secretion in human islets and the impact of T2D on circulating ghrelin levels and on islet ghrelin cells.

The effect of ghrelin on insulin secretion was assessed in human T2D and non-T2D islets. Ghrelin expression was assessed with RNA-sequencing (n = 191) and immunohistochemistry (n = 21). Plasma ghrelin was measured with ELISA in 40 T2D and 40 non-T2D subjects. Ghrelin exerted a glucose-dependent insulin-suppressing effect in islets from both T2D and non-T2D donors. Compared with non-T2D donors, T2D donors had reduced ghrelin mRNA expression and 75% less islet ghrelin cells, and ghrelin mRNA expression correlated negatively with HbA1c. T2D subjects had 25% lower fasting plasma ghrelin levels than matched controls.

Thus, ghrelin has direct insulin-suppressing effects in human islets and T2D patients have lower fasting ghrelin levels, likely as a result of reduced number of islet ghrelin cells. These findings support inhibition of ghrelin signaling as a potential therapeutic avenue for stimulation of insulin secretion in T2D patients.

1. Introduction

Ghrelin is a 28-amino acid peptide originally isolated from rat stomach (Kojima et al., 1999). It is the hormonal product of gastric A-like cells in the rat and P/D1-cells in humans. In rats, the stomach is the major source of circulating ghrelin; 20% of circulating ghrelin remains after fundectomy (Dornonville de la Cour et al., 2001). However, in humans 35–45% of plasma ghrelin remains after total gastrectomy (Ariyasu et al., 2001; Popovic et al., 2005) suggesting that, although the stomach is the major source of circulating ghrelin, other sources such as the pancreas and intestine contribute (Wierup et al., 2007). Several years ago, we identified islet ghrelin cells (epsilon cells) as a fifth islet cell type in human islets (Wierup et al., 2002); mouse islet ghrelin cells were later identified (Heller et al., 2005; Prado et al., 2004; Wierup et al., 2014). In rodents islet ghrelin cells are present primarily during fetal and early postnatal development, whereas in humans islet ghrelin cells remain into adulthood and constitute approximately 1% of all islets cells (Wierup et al., 2014). These species differences complicates extrapolation of rodent data to humans. Although ghrelin clearly plays a role in the regulation of glucose homeostasis (Gray et al., 2019; Dezaki et al., 2008) it is to the best of our knowledge not known whether human islet ghrelin cells are affected in T2D. Little is known about the consequences of long-term hyperglycemia or T2D on circulating ghrelin levels. Low ghrelin levels associate with T2D prevalence (Poykko et al., 2003) and pregnant women with T2D or gestational diabetes have lower ghrelin levels than non-diabetic women (Gomez-Diaz et al., 2016), but the underlying causes are unknown.

It is established that ghrelin is a hormone with insulin-suppressing

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GCG, glucagon; GHRL, ghrelin; GHSR, growth hormone secretagogue receptor; INS, insulin; PDX1, pancreatic and duodenal homeobox 1; PPY, pancreatic polypeptide; SST, somatostatin; T2D, type 2 diabetes

* Corresponding author.
E-mail address: Nils.Wierup@med.lu.se (N. Wierup).
[1] Equal contribution.

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properties, both *in vivo* in healthy humans, as well as in isolated rodent islets, single rodent beta cells and beta cell lines (Gray et al., 2019; Dezaki et al., 2008). Blocking ghrelin signaling may be a therapeutic avenue for T2D treatment; an assumption supported by observations that oral administration of an antagonist to the ghrelin receptor, GHSR, improves rodent glucose homeostasis (Esler et al., 2007). Although human islets express GHSR (Segerstolpe et al., 2016) and the mechanistic basis for the insulin-suppressing effect has been thoroughly dissected in rodent models (Yada et al., 2014), it remains to be proven whether ghrelin affects insulin secretion in patients with T2D, as well whether ghrelin has a direct insulin suppressing effect in human islets.

To fill these knowledge gaps, we studied how ghrelin affects insulin secretion in isolated human islets from cadaver donors with or without T2D and how T2D affects circulating ghrelin levels, islet ghrelin cell density and ghrelin mRNA expression in human islets.

2. Materials and methods

2.1. Human specimens

Human islets (www.nordicislets.org) from 191 donors and pancreata from 14 non-T2D and seven T2D donors were used. Donor characteristics have previously been described elsewhere (https://www.biorxiv.org/content/10.1101/435743v2.full). Plasma samples from 40 T2D patients and 40 age- and BMI-matched non-T2D controls were obtained from the PPP Prospective Study (Isomaa et al., 2010). Specimens of gastric corpus mucosa (3 cm distal to the cardia) from T2D patients and non-T2D controls (n = 8 and 9, respectively) were taken during gastric bypass surgery (Nergard et al., 2015). The ethics committees at Uppsala and Lund Universities approved all procedures.

2.2. In vitro islet studies

Islet experiments were carried out in Krebs-Ringer bicarbonate HEPES buffer containing 0.1% fatty acid free BSA (Roche, Basel, Switzerland) with pH 7.4. Islets were pre-incubated in 2.8 mM glucose for 30 min before five islets/well were placed in 96-well plates and incubated for 1h at 37 °C. Human ghrelin (Phoenix Pharmaceuticals, Burlingame, CA) was added in concentrations shown to affect insulin secretion in INS-1 832/13 cells as indicated (Wierup et al., 2004). Experiments were run with eight technical replicates. Information on the donors is provided in Supplementary Table 1.

2.3. Insulin and ghrelin measurement

Insulin and ghrelin secretion was determined by ELISA (Mercodia, Uppsala, Sweden and EMD Millipore, Darmstadt, Germany, respectively).

2.4. RNA-sequencing

Islet RNA from an additional 102 donors was isolated, sequenced and analyzed as previously described for the first subset of 89 donors (Fadista et al., 2014). Data from islets from 191 donors was used for analysis of *GHRL* and *GHSR* gene expression.

2.5. Differential expression analysis

Differential expression between 22 T2D patients (T2D diagnosis and HbA1c ≥ 48 mmol/mol) and 92 non-T2D (HbA1c < 42 mmol/mol) control subjects was assessed using EdgeR; age, sex, BMI, days in culture and purity being covariates. For differential expression between male and female donors, age, BMI, days in culture and purity were covariates.

2.6. Correlation with islet phenotypes

Spearman rank correlation was used for correlating *GHRL* and *GHSR* gene expression with BMI, age and HbA1c in all 191 donors using custom R scripts.

2.7. Immunohistochemistry

Immunohistochemistry was performed as previously described (Wierup et al., 2002). Primary antibodies used were: anti-goat ghrelin (code sc10368, dilution 1:1000, Santa Cruz Biotechnology, Houston, TX) (Wierup et al., 2007) and anti-guinea pig insulin (code M9003, dilution 1:5000, EuroDiagnostika, Malmö, Sweden). Secondary antibodies used were donkey anti-guinea pig Texas Red and donkey anti-goat Cy2. Both secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were diluted 1:400 in PBS (pH 7.2 with 0.25% BSA and 0.25% Triton X-100). All ghrelin cells were counted in all islets in three sections of frozen isolated islet preparations.

3. Results

3.1. Ghrelin suppresses insulin secretion in human islets

To study the impact of ghrelin (1–100 nM) on insulin secretion in isolated human islets we performed static incubations. Non-T2D islets responded to increased glucose (16.7 vs 2.8 mM) with 4-fold increased insulin secretion, compared to 3-fold in T2D donors. Ghrelin had no effect at 2.8 mM glucose, but at 16.7 mM glucose 100 nM ghrelin reduced insulin secretion by 32% in non-T2D (Fig. 1A). In T2D donors ghrelin dose dependently decreased (30–50% reduction) insulin secretion (Fig. 1B).

3.2. Reduced ghrelin cell density and fasting circulating levels in T2D patients

Next, we investigated ghrelin cell density in pancreas sections immunostained for ghrelin from T2D- and non-T2D donors. Notably, ghrelin cell density was reduced by 75% in T2D donors ($p < 0.05$; Fig. 1C and D). In line with the observed reduction of ghrelin cells in T2D donors, T2D patients from a different cohort had 25% lower fasting ghrelin levels ($p < 0.05$; Fig. 1E). To assess whether this reduction could be explained by reduced gastric ghrelin expression (the main source of circulating ghrelin(9)) we quantified ghrelin cell density in corpus mucosa specimens from obese subjects with T2D as well matched non-T2D controls. This revealed that gastric ghrelin cell density was unaffected by glycemic status (Fig. 1F).

3.3. RNA-sequencing of human islets

We assessed islet *GHRL* expression in RNA-sequencing data from 191 donors (technical replication in microarray data from 89 islet donors) (Taneera et al., 2012). *GHRL* mRNA was robustly expressed in human islets and did not correlate with BMI (Fig. 2A), but correlated negatively with age (rho = −0.26, p = 0.0002; Fig. 2B) and HbA1c (Fig. 2C; rho = −0.15, p = 0.044). *GHRL* expression was lower in T2D donors compared with non-T2D donors (Fig. 2D; p = 0.036). Finally, *GHRL* expression was higher in female than in male donors (Fig. 2E; $p = 1.27 \times 10^{-7}$).

GHSR mRNA (encoding the ghrelin receptor) was also assessed in the same data set. *GHSR* expression did not correlate with BMI (Fig. 3A), but correlated positively with age (Fig. 3B; rho = 0.16, p = 0.025). *GHSR* expression was similar in T2D and non-T2D donors (Fig. 3D), and not affected by sex (Fig. 3E). Finally, *GHSR* expression correlated positively with expression of *INS* (Fig. 3F; rho = 0.58, $p = 5.45 \times 10^{-19}$) and *SST* (Fig. 3H; rho = 0.68, $p = 1.28 \times 10^{-27}$).

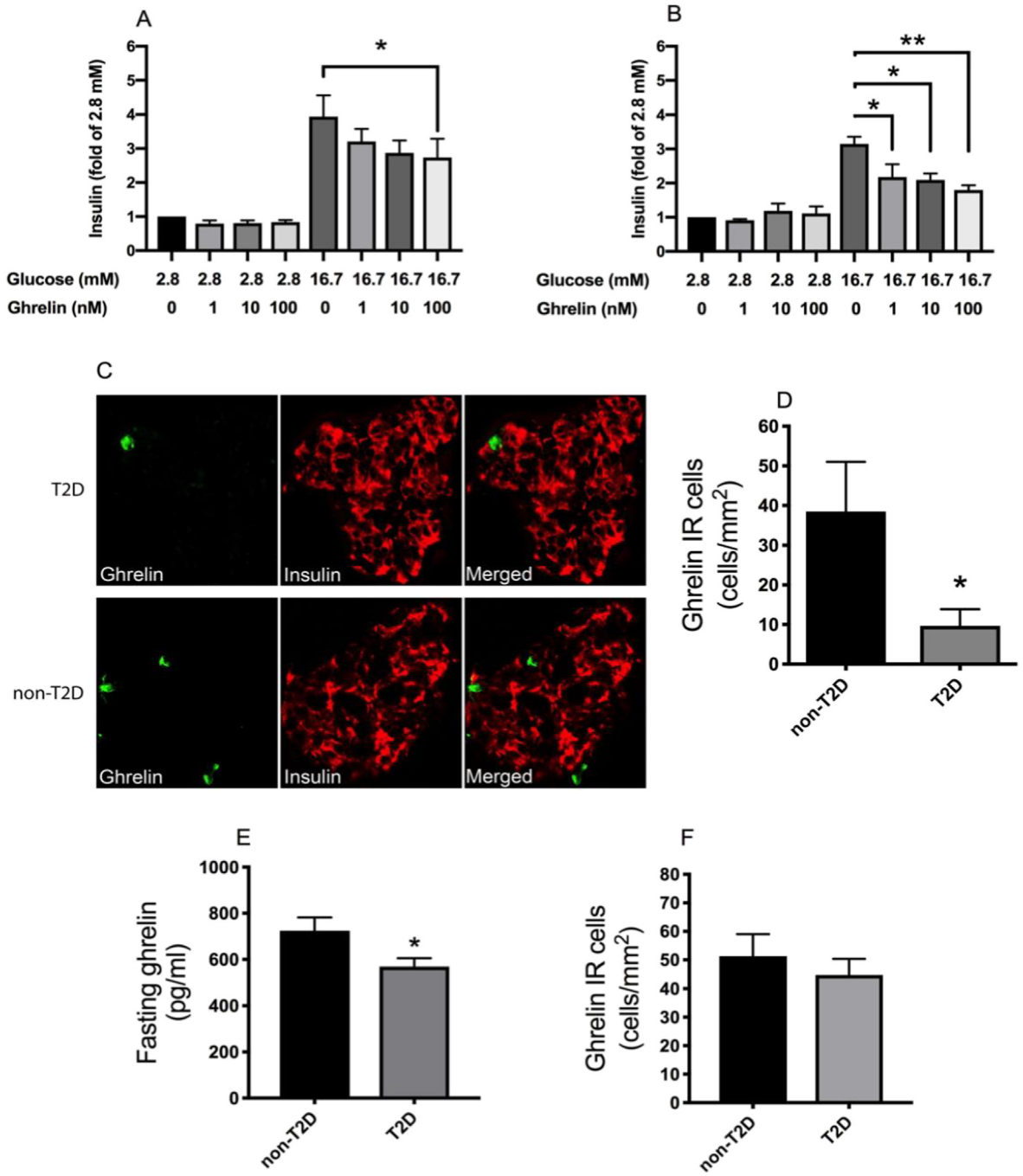

Fig. 1. Ghrelin inhibits glucose-stimulated insulin secretion in islets from non-T2D donors (A) and donors with T2D (B). Ghrelin has no effect on insulin secretion at 2.8 mM glucose. All experiments were performed in 8 replicates with n = 6 for (A) and n = 5 for (B). Double-immunostaining for ghrelin (green) and insulin (red) in T2D (top) and non-T2D (bottom) donors (C). Donors with T2D (n = 7) have 75% lower pancreatic density of ghrelin immunoreactive (IR) cells compared with non-T2D donors (n = 14) (D). Circulating ghrelin levels are lower in subjects with T2D compared with that of matched non-T2D controls (n = 40 for both groups) (E). Density of ghrelin IR cells in the gastric mucosa is unaffected by T2D (n = 8 for T2D and n = 9 for non-T2D) (F). * denotes $p < 0.05$ using one-way ANOVA with Tukey's test for multiple comparisons, or unpaired Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Here we show that ghrelin directly suppresses insulin secretion in human islets and that T2D patients have reduced number of islet ghrelin cells, islet ghrelin mRNA expression, and circulating fasting ghrelin levels.

A direct effect of ghrelin on insulin secretion from human islets has not previously been shown, but agrees with a body of evidence showing insulin-suppressing action of ghrelin in several experimental models, and *in vivo* in humans. Furthermore, the effect of ghrelin on insulin secretion has so far not been assessed in patients with T2D (Yada et al., 2014). Notably, ghrelin dose dependently reduced insulin secretion in islet of T2D donors. This proof-of-concept finding suggests that blockade of GHSR could be used to release the "ghrelin brake" on insulin secretion also in T2D patients. The potential for antagonizing GHSR as insulin stimulatory treatment of T2D subjects with impaired insulin secretion should be evaluated. Our trancriptomic analyses showed that *GHSR* expression was not correlated with HbA1c and

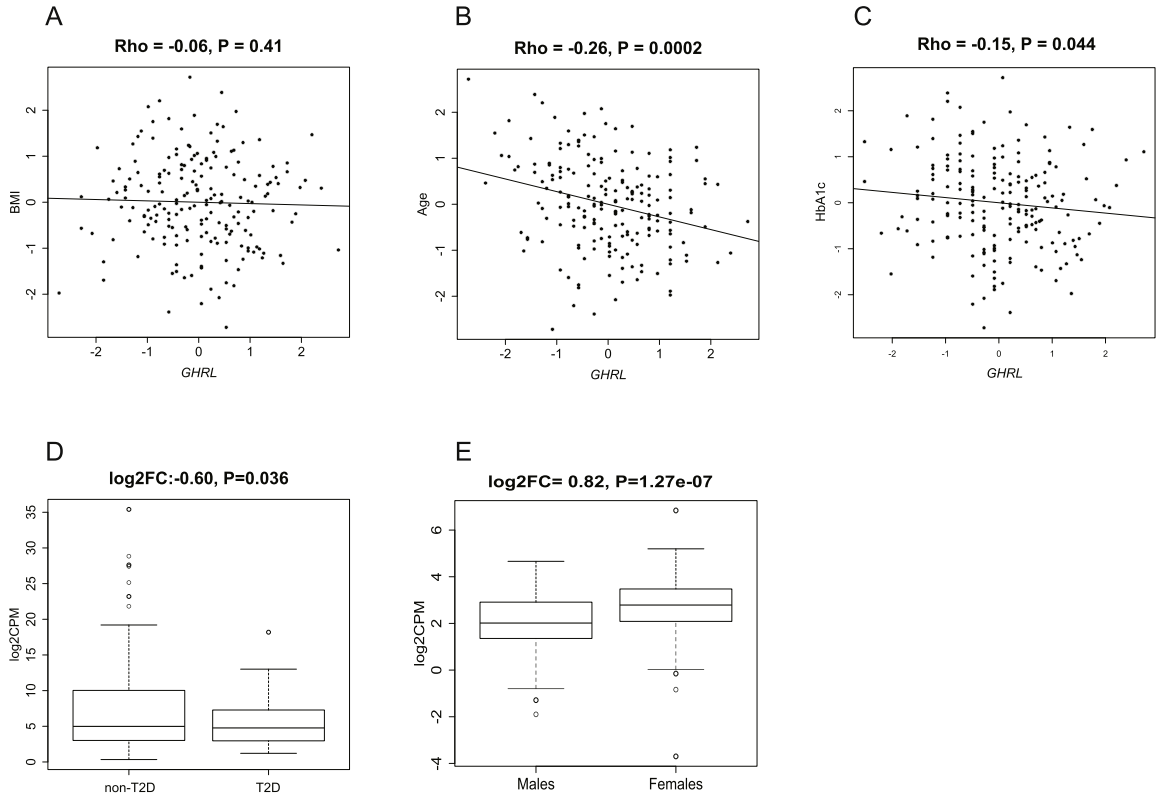

Fig. 2. *GHRL* expression modulation and correlation with donor phenotypes. RNA-sequencing analyses of islet expression data from 191 cadaver donors show that ghrelin expression does not correlate with BMI (A), but correlates negatively with age (B) and HbA1c (C). *GHRL* expression is lower in T2D donors (n = 22) than in non-T2D donors (n = 92) (D). *GHRL* expression is higher in female donors (E).

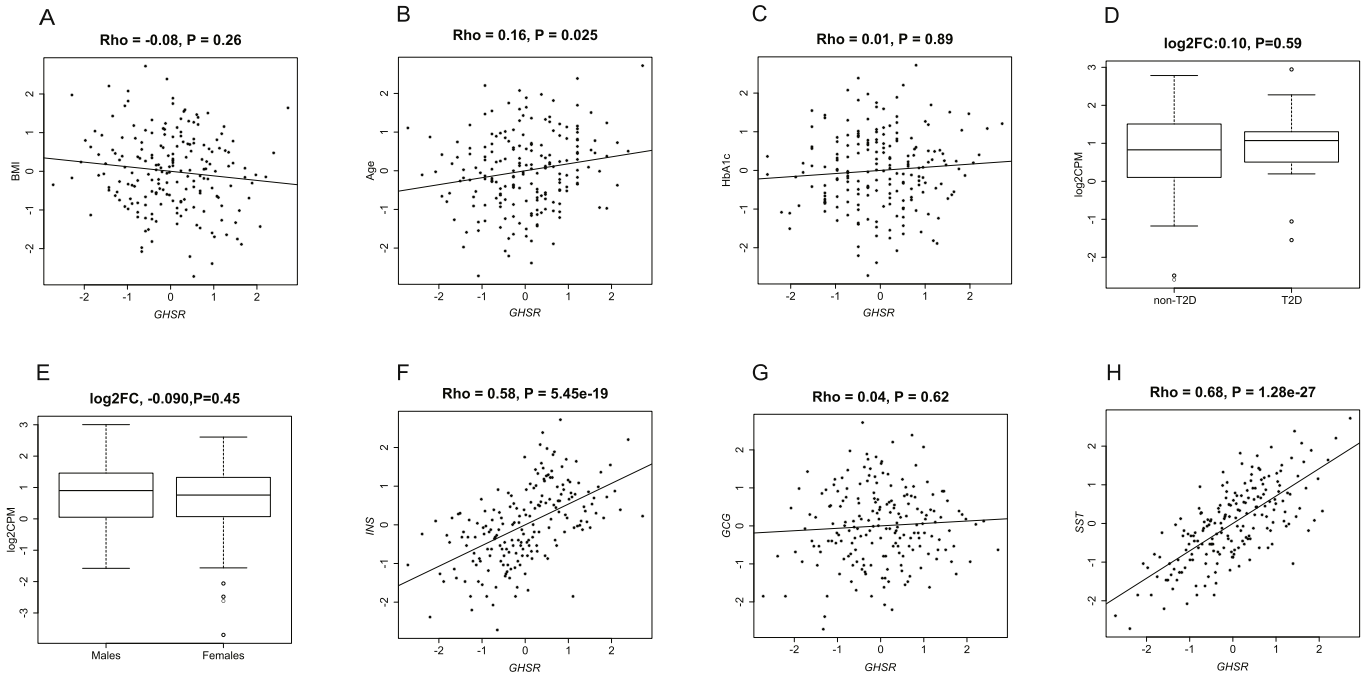

Fig. 3. *GHSR* expression modulation and correlation with donor phenotypes. RNA-sequencing analyses of expression data from 191 islet donors show that *GHSR* expression does not correlate with BMI (A) or HbA1c (C), but correlates positively with age (B). *GHSR* expression is similar in T2D and non-T2D donors (D), and not affected by sex (E). *GHSR* expression correlates positively with expression of *INS* (F) and *SST* (H), but not with *GCG* (G).

similar expression of *GHSR* expression was seen in T2D and non-T2D donors. These observations, speak in favor of similar ghrelin responsiveness in both groups. Recent data obtained using single-cell RNA-sequencing show that *GHSR* is predominantly expressed in human delta cells (Segerstolpe et al., 2016), suggesting that the effect of ghrelin on insulin secretion is mediated via release of somatostatin. High expression of *GHSR* in delta cells, gain support from our observation of a strong correlation between expression of *GHSR* and *SST* in human islets. Previous rodent studies (Wierup et al., 2004) suggest *GHSR* expression, albeit at lower levels also in beta cells. Thus, the available data suggests that the insulin-suppressing effect of ghrelin could be mediated via release of somatostatin, or via a direct action on the beta cell. The latter is supported by elegant studies in single rat (Dezaki et al., 2004, 2007) and mouse (Kurashina et al., 2015) beta cells, in which ghrelin inhibits glucose-induced Ca^{2+} signaling in a GHSR dependent manner.

Notably, ghrelin mRNA expression was lower in T2D donor islets and ghrelin expression correlated negatively with HbA1c. In agreement, ghrelin cell density was markedly lower in T2D donors. This is a novel finding, not previously reported in humans or in experimental models; the latter likely due to lack of experimental models with islet ghrelin cells during adulthood (Gray et al., 2019; Wierup et al., 2004).

Also, fasting circulating ghrelin levels were lower in T2D patients. This agrees with observations of lower circulating ghrelin levels in pregnant women with T2D or gestational diabetes (Gomez-Diaz et al., 2016). Supporting reduced islet ghrelin expression as an explanation for the reduced plasma ghrelin levels, we found that the density of gastric ghrelin cells, the main source of circulating ghrelin, was unaffected by T2D. Based on the insulin-suppressing actions of ghrelin, the observed reduction in islet ghrelin expression in T2D is likely an adaptation to the increased insulin demand in these subjects.

A few limitations of the study should be recognized. Firstly, the relatively low number of donors used for insulin secretion experiments may be a limiting factor. Secondly, we do not, for ethical reasons, have full access to the medical history of the donors. Thirdly, the doses of ghrelin used were higher than circulating levels of ghrelin. This notwithstanding, it is not inconceivable that such ghrelin levels can be reached locally within the islets. The doses of ghrelin used here were chosen based on having insulin-suppressing effects in INS-1 832/13 cells (Wierup et al., 2004).

5. Conclusions

We provide evidence for direct, glucose- and dose-dependent insulin-suppressing actions of ghrelin in human T2D islets, and that T2D patients have reduced pancreatic ghrelin expression, and lower circulating ghrelin levels. Our findings point at antagonizing islet ghrelin action as a potential strategy for new T2D treatments.

CRediT authorship contribution statement

A. Lindqvist: Methodology, Writing - original draft. **L. Shcherbina:** Methodology. **R.B. Prasad:** Software, Formal analysis. **M.G. Miskelly:** Methodology. **M. Abels:** Methodology. **J.A. Martínez-Lopéz:** Writing - original draft. **R.G. Fred:** Methodology. **B.J. Nergård:** Writing - original draft. **J. Hedenbro:** Writing - original draft. **L. Groop:** Writing - original draft. **J. Hjerling-Leffler:** Writing - original draft. **N. Wierup:** Conceptualization, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110835.

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