Sitagliptin reduces FAP-activity and increases intact FGF21 levels in patients with newly detected glucose abnormalities

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ABSTRACT
Introduction: Fibroblast growth factor 21 (FGF21), a hormone with pleiotropic metabolic effects, is inactivated by fibroblast activation protein (FAP), a member of the dipeptidyl peptidase-IV (DPP-IV) family. We investigate if sitagliptin (DPP-IV inhibitor) inhibits FAP-activity and increases intact FGF21.

Methods: Patients with impaired glucose metabolism were randomized to 100 mg sitagliptin (n = 34) or placebo (n = 37) treatment for 12 weeks. Plasma samples obtained at study entry and at 12-weeks were analysed for FAP-activity, FAP, total FGF21 and intact FGF21.

Results: Sitagliptin significantly inhibited FAP-activity (497 ± 553 vs. 48 ± 712 RFU/min, p < 0.01) and correspondingly increased intact FGF21 levels (253 ± 182 vs 141 ± 80 ng/L, p < 0.01) compared to placebo in plasma. Sitagliptin dose-dependently inhibited the FAP-activity in vitro. Intact FGF21 was higher in patients obtaining a normal glucose tolerance regardless of treatment (p = 0.03).

Conclusion: A sitagliptin-induced increase of intact FGF21 may contribute to an improved metabolic effect in patients with impaired glucose metabolism.

1. Introduction

The role of Fibroblast activation protein (FAP) in the metabolic regulation attract attention, due to its ability to cleave and inactivate fibroblast growth factor 21 (FGF21). FGF21 is a hormone with pleiotropic effects on glucose and fat metabolism and cardio protection (Angelini et al., 2012; Dunshee et al., 2016; Planavila et al., 2013, 2015; Staiger et al., 2017; Yan et al., 2015). Intact FGF21 is required for FGF21 activity, FAP, total FGF21 and intact FGF21.

Serial serum FGF21 levels are found to be elevated in patients with cardiovascular disease (Chow et al., 2013; Lin et al., 2016; Shen et al., 2013), type 2 diabetes (T2D) (Chavez et al., 2009; Keuper et al., 2020) and reported as a marker of disease severity in T2D patients with cardiovascular complications (An et al., 2012; Lin et al., 2014). Additionally, the activity of FAP has been reported to correlate with patient

Abbreviations: acute coronary syndrome, (ACS); acute myocardial infarction, (AMI); dipeptidyl peptidase IV, (DPP-IV); Fibroblast activation protein, (FAP); Fibroblast growth factor 21, (FGF21); impaired glucose tolerance, (IGT); oral glucose tolerance test, (OGTT); Relative fluorescent unit, (RFU); type 2 diabetes, (T2D). The protocol was registered at clinicaltrial.gov (NCT00627744).

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outcome, disease severity and susceptibility to treatment for diseases such as fibrosis and atherosclerosis (De Decker et al., 2019; Lay et al., 2019; Stein et al., 2021). If FAP-activity and total FGF21 increase with disease severity, FGF21 may be upregulated but cleaved and lose its function leading to decreased level of active FGF21. High levels of intact FGF21, either by administration of recombinant hFGF21 or by FAP inhibition, leads to decreased plasma glucose, lower triglyceride levels, less hepatic fat, and increased insulin sensitivity in animal models (Kharitonenkov et al., 2005; Kliwer and Mangelsdorf, 2019). Since FAP belongs to the same enzyme family as DPP-IV, we hypothesize that treatment with a DPP-IV-inhibitor may inhibit the FAP-activity and increase the circulating levels of intact, and therefore bioactive, FGF21. In this study, we investigate our hypothesis in patients with a recent hospitalization for acute coronary syndrome (ACS) and impaired glucose tolerance treated with or without sitagliptin.

2. Material and methods

2.1. Participants

From the double-blinded randomized clinical trial, the BEta-cell function in Glucose abnormalities and Acute Myocardial Infarction (BEGAMI), post ASC patients in a stable phase 4–23 days (median 6 days) after hospital admission were screened by oral glucose tolerance test (OGTT). The patients were randomly allocated with a block size of four via a computer-generated randomization sequence and a 1:1 ratio to receive either 100 mg sitagliptin (n = 34; JanuviaTM; Merck Sharp & Dohme AB, USA) or placebo (n = 37) once daily for 12 weeks. A detailed description of the study has been presented elsewhere (C. Hage et al., 2013). Based on OGTT results the patients were assigned as impaired glucose tolerant (IGT), type 2 diabetes (T2D) or normal glucose tolerant according to WHO recommendations (Camilla Hage et al., 2013) at study entry and at follow-up. The study was approved by the ethics committee at Karolinska Institutet, Sweden, and performed in accordance with the Declaration of Helsinki. The protocol was registered at clinicaltrials.gov (NCT00627744). Written informed consent was obtained from all subjects before inclusion to the study. Compliance to study drug, estimated by pill counts, was 100% for the sitagliptin group and 99% for the placebo group.

2.2. Biochemical analyses

Fasting blood samples were collected at study entry and after 12 weeks. Samples for each individual were analysed in duplicates and within the same plate. Plasma glucose and insulin, Hba1c levels, cholesterol, HDL, LDL and triglycerides were analysed by routine (C. Hage et al., 2013) and plasma insulin was measured by ELISA (DAKO, Cambridgeshire, UK). Intact GLP-1 was measured by a commercial ELISA kit (ab184857, Abcam) according to the instructions provided by the manufacturer. The intra- and interassay variation (%CV) were below 6 and 10%, respectively.

2.3. Total FGF21 and intact FGF21

Total FGF21 levels were quantified using an in-house time-resolved immunofluorometric (TRIFMA) assay as previously described (Laurentzén et al., 2017). The limit of detection (LOD) was 1 pg/ml and intra- and interassay variations (%CV) were below 6 and 10%, respectively.

The physiological function of FGF21 relies on an active FGF21 protein, and the latter was measured by a commercial sandwich ELISA kit (F2131–K01, Eagle Bioscience) according to the instructions provided by the manufacturer; with this assay, one antibody specifically binds an epitope in the N-terminal part of human FGF21 while the second antibody is specific for the C-terminal human FGF21. The intra- and inter-assay (%CV) were below 6 and 7%, respectively, and the LOD was 1.7 pg/ml.

2.4. FAP and FAP-activity

FAP levels were measured using monoclonal antibodies (R&D System DY3715) modified into an in-house TRIFMA assay as previously described (Arlien-Saborg et al., 2020). The LOD was 50 pg/L and the intra- and interassay variation (%CV) were below 7% and 9%, respectively.

FAP-activity was determined using an in-house modified fluorescence resonance energy transfer (FRET) technique in which fluorescence signal is released when FAP cleaves the FGF21 specific substrate (N-terminal HyLite488–Val–D–Ala–Pro–Ser–Gln–Gly–C-terminal lysine conjugated QXL520, Kaneka Eurogentec S.A., Belgium) between the fluorescence donor HyLite488 and the quencher QXL520 (Bainbridge et al., 2017). Plasma samples were diluted 10-fold in assay buffer (HEPES; pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1 mg/ml BSA) and added in duplicates on a black 96-well plate (Thermo Scientific Nunc, #165305) followed by substrate (1 μM) in each well. The plate was placed on a shaker in 37 °C incubator for 5 min before the first measurement (t0). Fluorescence was measured at 37 °C using PerkinElmer Multimode reader EnVision (EnVision Manager version 1.13.3009 1401) at (t0) and after 1 h (t60). For blank correction relative fluorescence unit (RFU) values obtained at t0 was subtracted from RFU (t60) for each well, this corrected RFU was divided by 60 min for RFU/min. The LOD was 66 RFU/min and the intra- and interassay (%CV) were below 9% and 10%. Human EDTA plasma samples (anonimized samples for assay development with no clinical data available) were used for assay validation and quality controls. Recombinant human FAP, rhFAP, (RnD System) and EDTA plasma dilutions were used for dose-dependency and linearity. Additionally, paired EDTA-plasma and serum samples showed equal FAP-activity allowing for measurements in both serum and plasma, and the assay showed stability up to 10 freeze-thaw cycles.

2.5. In vitro FAP inhibition by sitagliptin

The bioavailability of sitagliptin has previously been determined to 87%, peaking 4 h after oral administration of a single dose of 100 mg, corresponding to a Cmax of 0.95 μM in plasma (Bergman et al., 2007). Twelve different human plasma samples, serving as internal control for quality assurance and optimization of the FAP-activity assay, were mixed with sitagliptin phosphate (Biotechne, UK) at clinically relevant doses of 0 μM, 1 μM, 5 μM as well as supraphysiological doses of 15 μM and 25 μM sitagliptin. FAP-activity was determined as described above.

2.6. Insulin resistance and glucose tolerance

OGTT and frequently sampled intravenous glucose tolerance test (FSIGT) was performed at study entry and at the 12-week visit, as previously described (C. Hage et al., 2013). The beta cell function was assessed by the insulogenic index (IGI) calculated as Δinsulin0–30/Δglucose0–30 obtained from the OGTT (C. Hage et al., 2013). The Homeostatic Model Assessment (HOMA) was used to estimate insulin resistance HOMA-IR: (fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/L)/22.5). Acute insulin response to glucose (AIRg) was calculated from the FSIGT as the incremental area under the curve from 0 to 10 min and the glucose disappearance constant (Kg) as the slope of the natural logarithm of the difference between the glucose samples at 10- and 20-min Kg = (Δ ln plasma glucose/Δ min) -100.

2.7. FGF21 cleavage site prediction

A cleavage site prediction analysis for FGF21 was performed using the PeptideCutter tool available at expacy.org (Godfrey et al., 2005). This tool searches a protein sequence and predicts potential cleavage sites for a list of proteases and chemicals. The sequence of the peptide in interest,
in this case the 180aa mature circulating FGF21 sequence, was entered into the tool and the potential cleavage sites were identified along with a list of the enzymes or chemicals responsible for the theoretical cleavage.

2.8. Statistical analysis

Normal distributions were evaluated by QQ-plots and histograms. Data with normal distribution are expressed as means ± SD, non-normal distribution as medians [interquartile range], and categorical variables are expressed as numbers and percentages. Paired t-tests were used to evaluate normal distributed baseline and follow-up data, whereas paired samples Wilcoxon tests were used for non-normal distributed data. Unpaired t-tests were used to compare the treatment effects (Δ-values) between the groups, the treatment effects (Δ-values) are reflected by the changes in variables (V) of interest, calculated as delta (Δ) = V(12 weeks) − V(study entry). One patient in the sitagliptin group had total FGF21 levels above 14500 ng/L at both timepoints and were excluded as an outlier (sitagliptin (n = 31) and placebo (n = 35)) and paired intact GLP-1 levels were available in 49 patients (sitagliptin (n = 25) and placebo (n = 24)). Correlations are presented as Pearson R² for parametric variables and Spearman rho (R) for non-parametric variables and p-values. Intact FGF21 delta-values, stratified into groups based on OGTT results at baseline/follow-up (IGT/IGT, IGT/normal, IGT/T2D, T2D/T2D, T2D/IGT and T2D/normal). These groups and the in vitro data were examined using a Dunnett’s test. P < 0.05 was considered statistically significant. All statistics were conducted using RStudio version 1.2.5019.

3. Results

Patient characteristics at study entry and after 12 weeks treatment with sitagliptin or placebo are shown in Table 1. The groups were matched for age and BMI and did not differ in metabolic or hormonal analyses at baseline (Ametz et al., 2015).

3.1. FAP-activity is reduced in patients treated with sitagliptin

The total levels of FAP were similar at study entry (placebo 67 ± 18 μg/L vs. sitagliptin 73 ± 29 μg/L, p = 0.35) and at the follow-up visit (placebo 84 ± 19 μg/L vs. sitagliptin 85 ± 33 μg/L, p = 0.8). Thus, total FAP levels increased equally in both groups (Δ placebo 15 ± 13 μg/L vs Δ sitagliptin 12 ± 18 μg/L, p = 0.33), (Fig. 1A). In contrast, the FAP-activity increased significantly in the placebo group compared to the sitagliptin group (Δ placebo 497 ± 553 RFU/min vs. Δ sitagliptin 48 ± 712 RFU/min, p = 0.0049 (Fig. 1B). This difference persisted after adjustment for total FAP concentration (Δ placebo 18 ± 4 RFU/min/μg/L vs. Δ sitagliptin 16 ± 7 RFU/min/μg/L, p = 0.014), (Fig. 1C), despite the significantly higher FAP-activity at study entry in the sitagliptin group compared to the placebo group (951 ± 511 RFU/min vs. 1213 ± 541 RFU/min, p = 0.04). Even after adjusting the FAP-activity for the total FAP concentration, the FAP-activity/FAP concentration was still significantly higher in the sitagliptin group compared to the placebo group at study entry (14 ± 7 RFU/min/μg/L vs. 18 ± 8 RFU/min/μg/L, p = 0.05) (Table 1).

3.2. Intact FGF21 is increased in patients treated with sitagliptin

The total FGF21 levels, reflecting both intact and cleaved FGF21, were similar at study entry (placebo 208 ± 490 ng/L vs sitagliptin 365 ± 992, p = 0.4) and after 12 weeks treatment (placebo 191 ± 321 ng/L vs. sitagliptin 414 ± 1141 ng/L, p = 0.28), leading to no treatment difference (Δplacebo – 17 ± 185 ng/L vs Δsitagliptin 49 ± 169 ng/L, p = 0.1) (Fig. 1D). Intact FGF21, were similar in both groups at study entry (placebo 147 ± 95 ng/L vs. sitagliptin 141 ± 80 ng/L, p = 0.8) but in contrast to the total FGF21, intact FGF21 increased significantly in the sitagliptin group compared to the placebo group (placebo 159 ± 80 ng/L vs sitagliptin 253 ± 182 ng/L, p = 0.008) reflecting a significant increased treatment effect of sitagliptin (Δplacebo 12 ± 79 ng/L vs Δsitagliptin 113 ± 160 ng/L, p = 0.002 (Fig. 1E).

Table 1. Patient characteristics at study entry and after 12 weeks treatment with sitagliptin or placebo. Paired t-tests with mean [SD], Wilcoxon signed rank with median [Q1; Q3] tests between study entry and 12-week follow-up visit for selected variables. Acute insulin response to glucose (AIRg), area under the curve (AUC), Fibroblast activation protein (FAP), Fibroblast growth factor 21 (FGF21), fasting plasma insulin (FPI), fasting plasma glucose (FGP), Glucagon Like Peptide 1 (GLP-1), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), Insulinogenic Index (IGI), impaired glucose tolerance (IGT), Type 2 diabetes (T2D), *P < 0.05.

| Variables                  | Placebo | Sitagliptin | Δ values (12 weeks follow-up -Study entry) |
|----------------------------|---------|-------------|------------------------------------------|
| Age (years)                | 66.5 [9.0] | 67.7 [8.7]  | -                                        |
| Males/Females (%)          | 29/8 (78/22) | 29/5 (85/15) | -                                        |
| T2D/IGT/normal (%)         | 38/62/0 | 30/70/0     | -                                        |
| Weight (kg)                | 83 ± 13 | 84 ± 11     | 0.01*                                    |
| BMI (kg/m²)                | 26.8 ± 3 | 27.4 ± 3     | <0.01*                                   |
| HBAlc (IFCC mmol/mol)      | 40 [37; 43] | 40 [37; 42] | 0.2                                      |
| FPG (mmol/L)               | 6.2 ± 0.7 | 6.1 ± 0.7    | 0.04*                                    |
| FPI (mmol/L)               | 10 ± 5 | 11 ± 7      | 0.9                                      |
| OGTT glucose AUC (mmol/ L min) | 20 ± 2.9 | 20 ± 2.9 | 0.8                                      |
| OGTT insulin AUC (mmol/L min) | 651 [450; 911] | 673 [605; 1227] | 0.03*                                    |
| IGI (mmol/mmol)            | 66 [37; 89] | 70 [50; 115] | 0.01*                                    |
| HOMA-IR (μL/mmol-L)        | 2.3 [1.7; 3.4] | 2.4 [1.8; 3.2] | 0.4                                      |
| AIRg (mmol/L/min)          | 1106 [630; 2131] | 1392 [925; 1877] | 0.6                                      |
| Intact FGF21 (ng/L)        | 147 ± 95 | 141 ± 80     | <0.01*                                   |
| Total FGF21 (ng/L)         | 208 ± 490 | 235 ± 182    | <0.01*                                   |
| Total FAP (μg/L)           | 67 ± 18 | 73 ± 29      | <0.01*                                   |
| FAP-activity (RFU/min)     | 951 ± 511 | 1231 ± 541 | <0.01*                                   |
| Intact GLP-1 (pg/mL)       | 769 ± 606 | 671 ± 307  | -0.04*                                   |
|                          | 642 ± 352 | 859 ± 432  | 0.1                                      |

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3.3. Intact FGF21 levels are higher in patients with improved glucose tolerance

After 12 weeks, 59% of the patients (26 patients from the sitagliptin group and 15 from the placebo group), who were at study entry stratified by OGTT as either IGT or T2D, had obtained normal glucose tolerance and these patients presented with increased levels of intact FGF21 (Fig. 2). A significant increase in intact FGF21 was observed in the patients who improved their glucose tolerance from T2D to normal (T2D/normal) at 12 weeks as compared with those who showed no improvement (T2D/T2D). For post-hoc testing, the groups were stratified according to the OGTT assigned group at study entry; IGT or T2D, allowing a true reference group (the group shifting to normal). No significant differences vs. the reference group were observed in the group assigned IGT at study entry. No change in either total FGF21 nor FAP-activity were found when stratifying by group shift (data not shown).

3.4. Levels of intact FGF21 correlated with FAP-activity but not with metabolic parameters

The FAP-activity was negatively correlated with intact FGF21 after stratification according to the OGTT assigned group at study entry; IGT or T2D, allowing a true reference group (the group shifting to normal). No significant differences vs. the reference group were observed in the group assigned IGT at study entry. No change in either total FGF21 nor FAP-activity were found when stratifying by group shift (data not shown).

Fig. 2. Treatment effect on intact FGF21 (\(\Delta = V(12 \text{ weeks}) - V(\text{study entry})\)) stratified by group shift. The group shift was based on changes in OGTT result from study entry (IGT or T2D) to 12-week follow-up (normal, IGT or T2D). A significant increase in intact FGF21 was found for the group with the largest OGTT improvement (“T2D to normal”) compared to the group that did not improve in OGTT (“T2D to T2D”) (Dunnett’s test \(p = 0.03\)).
12 weeks in the placebo group \( (R^2 = -0.48, p = 0.0025) \) but not in the sitagliptin treated group \( (\text{Fig. 3a}) \). We found no correlation between treatment effects of intact FGF21 \( (\Delta \text{-values}) \) with any of the metabolic improvements found after sitagliptin treatment \( (\text{Table 2}) \). The change in adjusted FAP-activity/FAP level \( (\Delta \text{-values}) \) correlated positively with the difference in HbA1c \( (R = 0.39, p = 0.03) \) in the placebo group. No other correlations between treatment effects and FAP-activity/FAP level were found \( (\text{Table 2}) \). Intact GLP-1 correlated positively with intact FGF21 after treatment with sitagliptin \( (\text{Fig. 3b}) \), however not significantly, most likely due to the reduced sample size, as we had insufficient amount of sample-volume for the GLP-1 analysis \( (\text{placebo group } n = 24 \text{ and sitagliptin group } n = 25) \). No correlations were found with the OGTT results or HOMA-IR.

3.5. FAP-activity assay

At study entry total FAP levels and the FAP-activity correlate positively \( (R^2 = 0.41, p < 0.001) \) \( (\text{Fig. 4a}) \). FAP-activity increased dose-dependently with addition of rhFAP or with higher plasma concentration \( (\text{Fig. 4b}) \). However, the rhFAP was less enzymatically active than the endogenous plasma FAP, and supraphysiological levels of rhFAP, 10-fold higher, were needed to reveal this effect \( (\text{Fig. 4b}) \). Prediction of theoretical cleavage sites for the mature human FGF21 \( (180 \text{ aa}) \) was performed using the database expasy.org \( (\text{https://web.expasy.org/peptide_cutter/}) \). The intracellular oligopeptidase prolyl endopeptidase PREP, the DPPIV enzyme most similar to FAP, was the only other enzyme on the list that could in theory cleave the FGF21 sequence. The database also generated a list of enzymes not able to cleave human FGF21, including caspases 1–10, Factor Xa, Granzyme B (GRAB), proline endo peptidase (PPCE), Thrombin and Trypsin.

3.6. Sitagliptin inhibition of FAP-activity in vitro

Adding increasing concentration of sitagliptin to plasma samples from healthy individuals showed a dose-dependent inhibition of the FAP-activity \( (\text{Fig. 5}) \). A clinically relevant dose of 1 μM sitagliptin did not significantly decrease the FAP-activity \( (1433 \pm 366 \text{ RFU/min}) \) in plasma compared to the control with no sitagliptin \( (1509 \pm 366 \text{ RFU/min}) \), whereas higher concentrations of sitagliptin showed a dose-dependent inhibition of the FAP-activity in plasma \( (5 \mu \text{M sitagliptin}) \); FAP-activity \( = 1263 \pm 306 \text{ RFU/min}, p = 0.15 \); 15 μM sitagliptin; FAP-activity \( = 1133 \pm 208 \text{ RFU/min}, p = 0.01 \) and 25 μM sitagliptin; FAP-activity \( = 785 \pm 240 \text{ RFU/min}, p < 0.00001 \).

A similar effect was found with a fixed amount of sitagliptin in different plasma dilutions, or with addition of rhFAP, however the latter was only found in high concentrations \( (\text{data not shown}) \).

4. Discussion

12 weeks of sitagliptin treatment inhibits plasma FAP-activity in patients with a recent ACS and impaired glucose regulation. Correspondingly, increased level of circulating intact, and thus bioactive, FGF21 was available as compared with patients treated with placebo. The level of circulating FAP protein was not affected by sitagliptin treatment compared to placebo, revealing that only the FAP-activity was altered by sitagliptin treatment. The fact that an increased FAP-activity was present in the sitagliptin group at study entry may even underestimate the reported inhibitory effect of FAP by sitagliptin. Additionally, total FGF21 levels were similar between the groups, highlighting the necessity of including measurements of intact FGF21.

FAP-activity is reported to correlate with the concentration of the FAP protein \( (\text{Zhen et al., 2016b}) \), which our data supports. DPP-IV and FAP exhibit overlapping dipeptidyl peptidase activity and cleave a subset of similar substrates, e.g. neuropeptide Y, substance P and brain natriuretic peptide \( (\text{Keane et al., 2011}) \). The endopeptidase activity of FAP results in cleavage of denatured collagen type I and III, \( \beta \)-klotho \( \alpha \)-anti-plasmin and FGF21 \( (\text{Lee et al., 2006; Sánchez-Garrido et al., 2016; Zhen et al., 2016a}) \). Despite their similarities, FAP does not inactivate GLP-1 and DPP-IV does not cleave and inactivate FGF21, supporting that FAP is the only enzyme in the circulation that cleaves FGF21 and that FGF21 is not a substrate for DPP-IV \( (\text{Dunseh et al., 2016}) \). In addition, we performed a cleavage site prediction analysis using expasy.org, which revealed a theoretical cleavage site for the intracellular oligopeptidase prolyl endopeptidase PREP, the DPP-IV enzyme most similar to FAP, but no other enzymes found in blood circulation \( (\text{included in the database}) \) were capable of cleaving the 180 aa human FGF21 sequence. However \( (\text{Coppel et al., 2016}) \), showed that human FGF21 is only digested by FAP but not PREP. We use a highly FAP specific substrate, which is not cleaved by PREP and thus our results support that FAP is responsible for the cleavage of FGF21 as shown in \( \text{Fig. 3a and b} \). Like GLP-1, FGF21 is highly susceptible to cleavage and inactivation. Intact FGF21 is required for simultaneous binding to FGFR1 and \( \beta \)-klotho \( (\text{Miconovic et al., 2009}) \) to obtain FGF21 cell-signalling \( (\text{Sánchez-Garrido et al., 2016}) \), thus elevation of intact FGF21 is an attractive new treatment strategy.

Assessment of specific FAP-activity is challenging as many of the activity-based probes lack selectivity with respect to FAP related peptidases, especially the endopeptidase prolyl oligopeptidase (PREP) \( (\text{De Fig. 3. A) Correlation between FAP-activity and intact FGF21 after 12 weeks in the placebo group (Pearson } R = -0.48, p < 0.0025), \) but not in the sitagliptin treated group \( (R = 0.15, p = 0.42) \). B) Correlation between intact GLP-1 and intact FGF21 after 12 weeks in the placebo group \( (R = 0.063, p = 0.75) \) or in the sitagliptin treated group \( (R = 0.31, p = 0.1) \).
In order to determine the FGF21 specific FAP-activity we have taken advantage of the FRET technology using an FGF21 peptide including a slightly modified FAP cleaving site as a substrate (Bainbridge et al., 2017). Bainbrigde et al. elegantly showed, that substitution of the P2 Gly with D-Ala in the FGF21 peptide, revealed a FAP-specific substrate, not cleaved by any of the other DPP-family substrate (Bainbridge et al., 2017).
Despite the reported beneficial effect of FGF21 on the glucose and lipid metabolism (Zhang et al., 2008), T2D (Chavez et al., 2009; Keuper et al., 2020), which may also explain the lower FAP-activity we found in subjects with BMI was reported (Zhen et al., 2016a), which our data support. The short half-life of the exogenous compound with high FAP inhibitory effect and blood glucose reduction in mice (Jung et al., 2021), and BR103354 with increased intact FGF21 level in obese mice and in cynomolgus monkey (Cho et al., 2020), supports this strategy.

Importantly, we showed that patients who improved their glucose tolerance had higher levels of intact FGF21. This finding is supported by in vitro studies in adipocytes and liver cells, which reported that FGF21 signalling stimulate glucose uptake through upregulation of GLUT-1 (Ge et al., 2011; Liu et al., 2018). We did not find any association between intact FGF21 and the rate of disappearance for glucose, insulinogenic index or beta cell function, although these metabolic parameters were significantly improved in the BEGAMI patients after treatment with sitagliptin (C. Hage et al., 2013). However, we found a negative correlation between delta HbA1c and the adjusted FAP-activity/FAP level in the placebo group. This indicates that FAP-activity is associated with HbA1c, supporting the hypothesis that FAP-activity and reduced active FGF21 is involved in the pathogenesis of impaired glucose tolerance.

Interplay of FGF21 and GLP-1 in hepatic glucose metabolism has been reported (Liu et al., 2019). The GLP-1 analogue, Liraglutide, were found to stimulate FGF21 mRNA expression in both liver and adipose tissue, as well as increased circulating FGF21 plasma levels, in adiponecin deficient/ApoE knockout mice on a high-fat-diet (Yang et al., 2012) whereas the GLP-1R antagonist, Exenatide, reduced the total FGF21 levels, which correlated negatively with fasting insulin (Hu et al., 2016). Interestingly, the two large randomized clinical trials; LEADER (Liraglutide) and EXSCEL (exenatide) showed different effects of GLP-1 receptor agonist treatment on cardiovascular protection. The LEADER study showed that Liraglutide reduced the risk of cardiovascular events compared to placebo, whereas the EXSCEL study showed no difference in cardiovascular outcomes between exenatide and placebo (Holman et al., 2017; Marso et al., 2016). One could speculate if this was associated with the ability of liraglutide to increase FGF21 and exenatide to reduce the levels of FGF21. Our data support, that in addition to inhibition of DPP-IV and thus improving endogenous GLP-1 function, treatment with sitagliptin also increase the levels of intact FGF21 (Table 1), through inhibition of FAP-activity, which may in combination lead to improved OGTT. In mice, the increased levels of intact GLP-1 and FGF21 in combination were reported to improve insulin sensitivity and thereby improve oral glucose tolerance (Yang et al., 2012). Recently (Liu et al., 2019), reported that GLP-1 stimulate hepatic FGF21 production in vivo in db/db mice and in vitro in human HepG2 cells and showed that FGF21 and GLP-1 participate in hepatic glucose metabolism, thus suggesting a new glucose-lowering mechanism of GLP-1. The complementary functions of GLP-1 and FGF21 are highly beneficial for T2D patients and dual-treatment have recently been suggested (Gilroy et al., 2020; Pan et al., 2021) with beneficial effect in mice and a treatment effect superior to GLP-1 or FGF21 alone. Accumulating knowledge suggest that FGF21 and GLP-1 may cross-talk and provide synergism, at least in rodents and in vitro. We do see a trend supporting a positive correlation between intact GLP-1 and intact FGF21 after treatment with sitagliptin, but we do not find a combined effect on glucose tolerance or HOMA-IR. Thus, additional studies are needed in patients with diabetes to determine an additive effect of increasing both intact GLP-1 and intact FGF21.

The BEGAMI study was not designed to investigate FAP-activity nor changes in FGF21 levels and taking the well-known inter-individual difference in human FGF21 levels into account (Gálman et al., 2008), a clinical translation of our findings may have benefitted from a larger sample size. A longer treatment period with sitagliptin may have reduced the FAP-activity even further and stimulated an improvement in the metabolic endpoints. The patients in this study were recovering from proteolytic degradation mediated by FAP.
ACS and we do not know how this affects FAP-activity and FGF21. An improvement in glucose tolerance (evaluated by OGTT) was observed in both groups, possibly affected by similar lifestyle counselling. However, the improvement was considerably higher in the sitagliptin group as compared to the placebo group (Table 1).

5. Conclusion
Sitagliptin treatment prevented FAP-activity increase in response to ACS and increased levels of intact FGF21 in a placebo-controlled design. Higher levels of intact FGF21 were found in patients with improved glucose tolerance. Inhibition of FAP may be an alternative approach to increase endogenous intact FGF21 levels to obtain better metabolic regulation. Metabolic improvements achieved by DPP-IV inhibitor, e.g., sitagliptin treatment, may arise from a dual inhibition of DPP-IV and FAP, resulting in increased levels of both intact GLP-1 and intact FGF21 in circulation.

Declaration of interest
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AKNP, NJ and MB have no conflict of interest.

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CRediT authorship contribution statement
Anne K.N. Pedersen: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Camilla Hage: Investigation, Writing – review & editing. Niels Jessen: Supervision, Resources, Writing – review & editing. Linda Mellbin: Investigation, Writing – review & editing. Mette Bjerring: Funding acquisition, Supervision, Resources, Writing – original draft, Writing – review & editing.

Data availability
The data that has been used is confidential.

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