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ORIGINAL ARTICLE

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FGF21 and glycemic control in patients with T1D

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Abstract

Purpose Fibroblast growth factor (FGF) 21 is a circulating hormone with an important role in metabolic regulation. FGF21 production in humans responds positively to glucose consumption and we hypothesize that serum FGF21 concentration is associated to glycemic control.

Methods We enrolled 31 patients with type 1 diabetes (T1D) based on their HbA1c (well-regulated (HbA1c <53 mmol/mol), ($n = 18$) or poorly-regulated (HbA1c >69 mmol/mol), ($n = 13$). Twelve patients (39%) were diagnosed with retinopathy. Twenty healthy individuals comparable for age and gender distribution were included as a reference group. Serum FGF21, intact FGF21, fibroblast activation protein (FAP), adiponectin, and C-Reactive Protein (CRP) were measured by immunoassays.

Results No correlation between FGF21 concentration and HbA1c was found. Patients with T1D had lower levels of circulating FGF21 as compared with the reference group, but the difference was nonsignificant ($p = 0.12$). Dividing the patients according to retinopathy, we found that T1D patients with retinopathy had significantly lower FGF21 concentrations (10.0 ng/L) as compared with the healthy reference group (37.1 ng/L), ($p = 0.02$). We found significantly higher levels of the FGF21 cleaving enzyme, FAP, in patients with T1D (97.2 µg/L) as compared with the healthy control group (78.5 µg/L), ($p = 0.006$). Interestingly, serum FAP levels correlated significantly with circulating FGF21 levels in T1D patients, but this correlation was not found in the healthy controls.

Conclusions We found no association between circulating FGF21 levels and HbA1c. T1D patients with retinopathy had significantly lower FGF21 levels as compared with healthy individuals, but it remains unclear if the lower levels of FGF21 are pathogenically related to the development of microvascular complications. Of note, serum FAP levels were significantly higher in all T1D patients as compared with the healthy individuals.

Keywords Fibroblast growth factor 21 · Fibroblast activation protein · Type 1 diabetes mellitus · Glycemic regulation · Microvascular complications · Retinopathy

Introduction

Patients with type 1 diabetes (T1D) suffer from autoimmune beta-cell destruction, and insulin analogs are the first choice for regulation of blood glucose. However, despite insulin treatment, some T1D patients have an insufficient reduction in blood glucose. Fibroblast growth factor 21 (FGF21) is a novel metabolic hormone with glucose- and lipid-lowering effects [1], and FGF21 administration in experimental diabetes-models has shown several beneficial effects [2, 3]. Administration of exogenous FGF21 to rodents lead to improved glycemic control [4]. Treatment with recombinant FGF21 in humans as an alternative or supplement to insulin is thus expected to improve glycemic control, avoid hypoglycemic events, and improve insulin sensitivity. The FGF21 functionality is regulated through proteolytic cleavage of the C-terminal and thus inactivation by the serine dipeptidase fibroblast activating protein (FAP) [5]. Intact FGF21 is required for FGF21 signaling, which occurs through N-terminal binding to FGFR1 and C-terminal binding to the co-receptor β-klotho [6]. So far only a few studies have investigated the FGF21 levels in patients with T1D. In clear contrast to patients with T2D, lower levels of

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FGF21 are reported in patients with T1D when compared with healthy individuals [7, 8]. This is supported by animal models of T1D showing reduced circulating levels of FGF21 [2, 3]. Under normal conditions, FGF21 is produced in the liver, pancreas, and adipose tissue [9]. In vitro studies show that glucose induces FGF21 production in liver cells [10]. In addition, FGF21 secretion has been shown to respond positively to glucose, fructose, and sucrose consumption [11] and 3 days of enriched carbohydrate diet increased plasma FGF21 levels by eightfold [12]. However, the pathophysiological function and regulation of FGF21 is yet not fully understood, and it remains unclear if the state of glycemic regulation is associated with FGF21 levels in T1D. The aim of this study is to assess whether circulating FGF21 levels are associated with glycemic control by measuring FGF21, intact FGF21 and FAP in T1D patients with well- and poorly regulated HbA1c levels.

Materials and methods

Study design and participants

Patients were included from the outpatient clinic at Aarhus University Hospital. Inclusion criteria were age above 18 years and T1D diagnosed according to WHO criteria. Exclusion criteria were an acute or chronic infection, patients receiving dialysis for kidney failure, pregnancy, breastfeeding, and current or previous cancer disease. Venous blood samples were collected in the morning after an overnight fast (minimum of 12 h) and without diabetes patients receiving their regular morning insulin treatment. Physical examination included blood pressure, body weight measurement, and calculation of BMI. A questionnaire of medical history regarding age at diabetes diagnosis and other medical treatment was obtained. The patients were selected according to their HbA1c measured at the latest visits (every fourth month) at the outpatient clinic and assigned to two groups; "Well regulated" (HbA1c ≤53 mmol/mol) and "Poorly regulated" (HbA1c ≥69 mmol/mol). HbA1c was measured at the sampling day and used for correlation analysis. None of the patients changed group based on the HbA1c at the sampling day. Diagnose of retinopathy was obtained from the medical records. Healthy controls comparable to age and gender distribution of the T1D patients were included through an announcement in the local press.

Laboratory analysis

Serum was collected after centrifugation and stored at −80 °C until analysis. HbA1c, blood glucose, triglycerides, HDL– LDL–, and total cholesterol were analyzed by the routine laboratory at Aarhus University Hospital.

Serum FGF21 was quantified by time-resolved immunofluorometric (TRIFMA) assay as previously described [13]. The limit of detection was 1 ng/L, intra- and interassay coefficient of variation (%CV) were below 6% and 10%, respectively. Intact FGF21 was measured by ELISA (F2131-K01, Eagle Bioscience) according to the instructions provided by the manufacturer. The intra- and interassay %CV were below 6% and 7%, respectively, and the assay sensitivity was 1.7 pg/ml. FAP was quantified by using monoclonal antibodies (R&D System DY3715) modified into an in-house TRIFMA assay. Briefly, wells were coated with 1 µg anti-FAP antibody/ml phosphate buffered saline (PBS) overnight at 4 °C. Residual protein-binding sites in the wells were blocked with 1% Tween20 in PBS for 1 h at room temperature and washed in PBS containing 0.05% Tween 20 (PBS/Tw). Recombinant FAP in the range from 62.5 to 4000 ng/L was used as standard. Serum samples were diluted 100-fold in assaybuffer (PBS, 1% Tween20, 0.5% HSA, 25 µM EDTA, pH 7.4) and incubated overnight at 4 °C. Bound FAP was determined by incubation with 200 ng biotinylated anti-FAP detection antibody/mL assaybuffer at room temperature for 2 h followed by wash and addition of 10 ng Eu^{3+}-labelled streptavidin (Perkin Elmer, Waltham, MA, USA) in 100 µL assaybuffer for 1 h at room temperature. After wash, bound europium was detected by the addition of 200 µL of enhancement solution (Perkin Elmer), followed by 5 min of vigorous shaking and reading the time-resolved fluorescence on a DELFIA fluorometer (Victor^3, Perkin Elmer). The limit of detection was 50 ng/L. The intra- and interassay variations (%CV) were below 7% and 9%, respectively. Serum CRP levels were determined by a high sensitive CRP TRIFMA assay based on commercially available monoclonal antibodies (R&D systems) and calibrated against WHO 85/506 (National Institute for Biological Standards and Control). LOD 50 ng/L, intra- and interassay CVs were <5% and <6%, respectively. Serum Adiponectin (ADPN) levels were determined by a validated in-house TRIFMA based on two monoclonal antibodies and recombinant human adiponectin (R&D Systems, Abingdon, UK). The intra-assay CV averaged <5% and the inter-assay CV < 10% [14].

Statistical analysis

The well- and poorly regulated diabetic groups were analyzed for differences in FGF21, intact FGF21, FAP, and ADPN levels. The data from well- and poorly regulated T1D patients were combined and clinical and laboratory characteristics were compared between the entire diabetic group and controls except from "Duration of diabetes" which is compared between the two diabetic groups (well- and poorly regulated). T1D patients with retinopathy were

identified from the entire diabetic group and compared with healthy controls. Normality was checked by QQ-plots of the residuals. HbA1c, Triglyceride, FGF21, Intact FGF21, FAP, and CRP were non-normally distributed and analyzed by Wilcoxon–Mann–Whitney *U* test and values are given as medians with interquartile ranges. All other continuous variables were normally distributed and analyzed by Student's *t*-test and presented as means ± SD. For non-continuous outcomes (gender and antihypertensive treatment) data were analyzed by Chi-square test. Linear and multiple regression models, to adjust for the confounding effects of BMI, were used to investigate the relationship between FGF21 and various metabolic parameters. Non-normally distributed variables were natural logarithmically transformed before analysis, and the strength of association was analyzed by Pearson correlation or Partial correlation as appropriate. One well-regulated T1D patient had an extremely elevated serum FAP-level of 885.9 µg/L, which was considered an outlier and not included in any statistical analysis containing FAP. *P*-values of <0.05 were considered statistically significant. All statistical analyses and figures were conducted with Stata software version 14.1.

Results

Clinical and laboratory characteristics of T1D patients and healthy controls are summarized in Table 1. Patients were comparable with the control group with regard to age and gender but not to BMI. The T1D patients had a significantly higher BMI although the mean BMI of controls and the entire diabetic group are still within "normal weight" (BMI 18.5–24.9). The median diabetes duration was 18 years. Systolic and diastolic blood pressure levels and cholesterol levels were comparable between the groups. Only patients in the diabetic groups were receiving antihypertensive treatment.

Circulating FGF21 levels were lower in patients with T1D (11.7 ng/L [3.6;40.0]) as compared with the healthy controls (37.1 ng/L [8.2;201.7]), although the finding was nonsignificant, $p = 0.12$ (Fig. 1a). Similarly, intact FGF21 was lower in patients with T1D than in healthy controls, but also nonsignificant (Table 1). Serum FAP levels were significantly higher in T1D patients (97.2 µg/L [83.2;112.4]) as compared with healthy controls (78.5 µg/L [73.2;91.0]), $p = 0.006$ (Fig. 1b). T1D patients had higher serum CRP levels (1.85 mg/L [0.54;3.47]) than the controls (0.28 mg/L [0.15;0.80]), ($P = 0.0006$), and further tended to increase when HbA1c ≥69 mmol/mol (3.01 mg/L [1.71;4.34]), ($p = 0.09$). Poor glycemic control, evaluated by HbA1c, did not affect FGF21, intact FGF21, FAP, or ADPN ($p = 0.78$, $p = 0.79$, $p = 0.44$, and $p = 0.83$, respectively), when comparing the well- and poorly regulated diabetic groups. Adjustment for the significant difference in BMI between the patients and the healthy controls did not affect any of the results.

We found no correlation between FGF21 and HbA1c (Table 2), neither in the entire group (T1D patients and the control group) nor in the two groups separately ($r = -0.049$, $p = 0.79$ and $r = 0.404$, $p = 0.08$, respectively). Likewise, no correlation was found between serum FGF21 and intact FGF21, FFA, ADPN, triglycerides, or CRP (Table 2).

We found no overall correlation between FGF21 and FAP (Table 2). But when analyzing the patients with diabetes and the healthy controls separately, a positive correlation between FAP and FGF21 was observed for patients with diabetes ($r = 0.394$, $p = 0.03$, Fig. 2a), but not for the healthy controls (Fig. 2b). No other associations between variables in Table 2 were found when dividing correlation analysis into separate groups of patients with diabetes and controls (data not shown).

Twelve out of thirty-one T1D patients suffered from retinopathy (39%) and had significantly lower FGF21 levels (10.0 ng/L [2.6; 19.4]) than healthy controls (37.1 ng/L [8.2;201.7], ($P = 0.02$) (Fig. 3). Whereas T1D patients without retinopathy had a slightly higher (17.2 ng/L [5.4; 81.6]) but not significantly different level of FGF21 compared with T1D patients with retinopathy ($p = 0.19$). Intact FGF21 in T1D patients with retinopathy was 67.8% (±42.2) of that in healthy controls, although the difference was not significant ($p = 0.77$). The retinopathy group had a longer mean diabetes duration (23.5 years ± 5.5) than diabetic patients without retinopathy (13.9 years ± 9.5), and both T1D patients with and without retinopathy had higher CRP and FAP levels than healthy controls (data not shown). Otherwise, the groups were comparable by BMI, age, gender, systolic- and diastolic blood pressure, cholesterol, HDL, LDL, and triglycerides.

Discussion

In the present study, we analyzed total FGF21, intact FGF21 and FAP in T1D patients grouped according to their HbA1c levels; "Well regulated" (HbA1c ≤53 mmol/mol) and "Poorly regulated" (HbA1c ≥69 mmol/mol). Overall, our study shows, that the patients with T1D had reduced FGF21 levels, less active FGF21, and significant higher FAP concentration in serum as compared with a reference group comparable on age and gender. However, unexpectedly we found no association between the FGF21 concentration and the HbA1c levels.

Our hypothesis was based on human studies that have reported increased FGF21 secretion after glucose, fructose,

Table 1 Clinical and laboratory characteristics of study participants[a]

	Healthy controls, $n = 20$	Well-regulated T1D patients, $n = 18$	Poorly regulated T1D patients, $n = 13$	T1D patients (total), $n = 31$	P- value[b]
Age (years)	36.1 ± 9.9	36.7 ± 12.6	36.1 ± 13.3	36.4 ± 12.7	0.91
Females (%)	45%	44 %	46%	45%	0.99
BMI (kg/m^2)	23.0 ± 2.5	25.2 ± 3.1	24.4 ± 2.6	24.9 ± 2.9	0.02*
Duration of diabetes (years)	–	18.0 ± 10.2	17.0 ± 8.4	17.6 ± 9.4	0.76
HbA1c (mmol/mol)	34 (32;35)	50 (46;54)	73 (66;79)	54 (49;72)	0.000*
Systolic blood pressure (mmHg)	124 ± 14	131 ± 12	128 ± 17	130 ± 14	0.15
Diastolic blood pressure (mmHg)	79 ± 6	80 ± 7	80 ± 8	80 ± 7	0.68
Total cholesterol (mM)	4.7 ± 0.8	4.6 ± 0.7	4.6 ± 0.9	4.6 ± 0.8	0.72
HDL (mM)	1.6 ± 0.4	1.8 ± 0.4	1.6 ± 0.4	1.7 ± 0.4	0.35
LDL (mM)	2.6 ± 0.7	2.4 ± 0.6	2.4 ± 0.9	2.4 ± 0.7	0.34
Triglycerides (mM)	1.0 (0.7;1.2)	0.8 (0.6;0.9)	1.3 (0.8;1.4)	0.8 (0.6;1.4)	0.97
Antihypertensive treatment (%)	0	17	15	16	–
Micro-albuminuria (%)	–	0	8	3	–
Retinopathy (%)					
None	–	78	38	61	–
Simplex	–	22	46	32	–
Proliferative	–	–	15	6	–
ADPN (mg/L)	11.4 ± 5.3	14.0 ± 7.4	13.4 ± 8.5	13.7 ± 7.8	0.24
FGF21 (ng/L)	37.1 (8.2;201.7)	9.6 (2.9;81.6)	13.4 (7.6;25.3)	11.7 (3.6;40.0)	0.12
Intact FGF21 (% of control)	100 ± 92.9	87.0 ± 79.3	66.4 ± 41.2	78.5 ± 66.1	0.85
FAP (µg/L)	78.5 (73.2;91.0)	95.9 (84.4;141.8)	99.7 (81.9;109.9)	97.2 (83.2;112.4)	0.006*
CRP (mg/L)	0.28 (0.15;0.80)	1.07 (0.54;2.85)	3.01 (1.71;4.34)	1.85 (0.54;3.47)	0.0006*
FFA (mmol/l)	0.55 ± 0.26	0.46 ± 0.24	0.39 ± 0.22	0.43 ± 0.23	0.09

BMI body mass index, *HbA1c* hemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ADPN* adiponectin, *FGF21* fibroblast growth factor 21, *FAP* fibroblast activation protein, *CRP* C-reactive protein, *FFA* free fatty acids

[a]Data are means ± SD, median (IQR) or percentage

[b]*P*-value refers to comparison between patients with diabetes and healthy control subjects except from duration of diabetes which refers to comparison between the two diabetic groups Statistically significant results are marked by *

and sucrose consumption or an enriched carbohydrate diet [11, 12] and that glucose-induced FGF21 production in vitro in liver cells [10]. These findings may reflect an acute FGF21 response rather than a sustainable effect on long term from the blood glucose level as assessed by HbA1c.

The low level of FGF21 in T1D found in this study is in concordance with the few other human T1D studies [7, 8]. Xiao et al. reported that FGF21 levels increased progressively from T1D, latent autoimmune diabetes in adults to T2D [7]. Mouse models for T1D show a similar reduction in FGF21 levels, as mice with STZ-induced diabetes have low FGF21 plasma levels and reduced fgf21 expression in the liver, as compared with WT mice [2]. In addition, the STZ mice had an impaired ability to upregulate FGF21 levels during fasting. Under normal conditions, FGF21 is primarily produced by the liver but is also expressed by the pancreas and the adipose tissue [8]. FGF21 in the pancreas has been reported to act as a compensatory defensive response in order to protect the beta cells [15] and acinar cell *Fgf21 gene* expression has been reported to rise compensatory to induction of pancreatitis in mice [16]. Akita mice, who have a spontaneous mutation in the insulin gene that leads to loss of function, FGF21 levels were lower as compared with WT mice [17]. But, in LIRKO mice, with a selective inactivation of the insulin receptor in the liver, circulating FGF21 levels were similar to the levels in WT mice, indicating the hepatic regulation of FGF21 in mice is not controlled by insulin [18]. Contrary, Samms et al. reported, that insulin rather than glucose increases human FGF21 levels [19]. The blood samples in our study were collected after an overnight fast and before the T1D patients took their regular insulin in the morning. Thus, the lack of insulin may contribute to the very low levels of FGF21 measured in our T1D patients. Also, it cannot be excluded, that the effects of insulin on FGF21 production might differ depending on the site of administration, as exogenous insulin administration in T1D might result in different FGF21 responses than the same amount of insulin released directly from the pancreas in healthy individuals. We found a significant correlation between FAP concentration and total FGF21 in T1D patients, but not in the healthy reference group. Overall, this may indicate an abnormal production or function of FGF21 related to T1D.

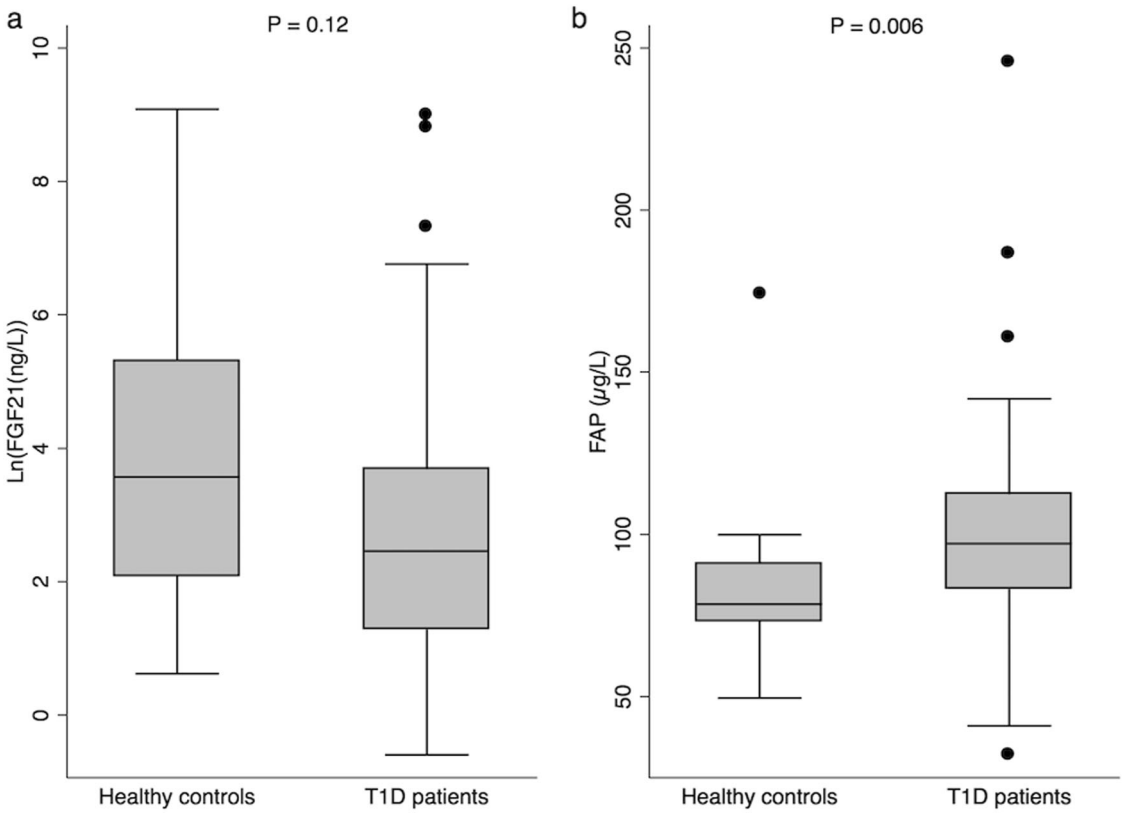

Fig. 1 Serum fibroblast growth factor 21 (FGF21) and fibroblast activation protein (FAP) levels in type 1 diabetes (T1D) patients and healthy control subjects. Horizontal bars represent medians, columns indicate IQR, and vertical bars indicate the 10th and 90th percentiles. Statistical analysis is by Wilcoxon–Mann–Whitney *U* test for differences between groups. **a** Comparison of serum FGF21 levels between T1D patients and healthy controls, $p = 0.12$. *Y*-axis is on a natural logarithmic (Ln) scale for visual reasons. **b** Comparison of serum FAP levels between T1D patients and healthy controls, $p = 0.006$

Table 2 Correlations of serum FGF21[b] levels and various metabolic parameters[a]

Variables	*r*	*p*
HbA1c[b] (mmol/mol)	−0.128	0.37
Intact FGF21[b] (ng/L)	0.193	0.19
FAP[b] (µg/L)	0.150	0.30
FFA (mmol/l)	0.061	0.67
ADPN (mg/L)	−0.069	0.63
Triglycerides[b] (mM)	−0.108	0.45
CRP[b] (mg/L)	−0.017	0.91

*FGF*21 fibroblast growth factor 21, *HbA1c* hemoglobin A1c, *FAP* fibroblast activation protein, *FFA* free fatty acids, *ADPN* adiponectin, *CRP* C-reactive protein

[a]Correlations were analyzed with both T1D and healthy controls together

[b]Natural logarithmically transformed before analysis

Our patients had significantly increased CRP levels as compared with the healthy reference group. Hoffman et al. showed that increased glucose variability, accessed by a continuous glucose monitor was associated with elevated CRP levels in T1D patients [20]. Furthermore, a positive correlation between CRP and FGF21 levels has been reported in a group of newly diagnosed patients with T2D [21]. However, this is not supported by our findings in patients with T1D.

FAP levels have been reported to be increased in patients with T2D [19], but to our knowledge, this is the first report that shows elevated FAP levels in patients with T1D. FGF21 is a substrate of FAP [22] and through deletion of the FGF21 C-terminal, FAP attenuates the binding of FGF21 to the co-receptor β-klotho and thus diminishes the action of FGF21. Interestingly, FAP is homolog to the serine protease dipeptidyl peptidase-4 (DDP-4), which regulates the bioavailability of GIP and GLP-1. In concordance with our FAP results, increased DPP-4 activity was reported in patients with T1D compared with healthy individuals and DPP-4 activity was associated with albuminuria [23].

Circulating FGF21 has been associated with both micro- and macrovascular complications. A u-shaped relationship between FGF21 and micro- and macrovascular complications has been reported in patients with T2D [24, 25], and FGF21 is suggested to have potential as a cardiovascular prognostic biomarker in T2D [26]. This suggests that

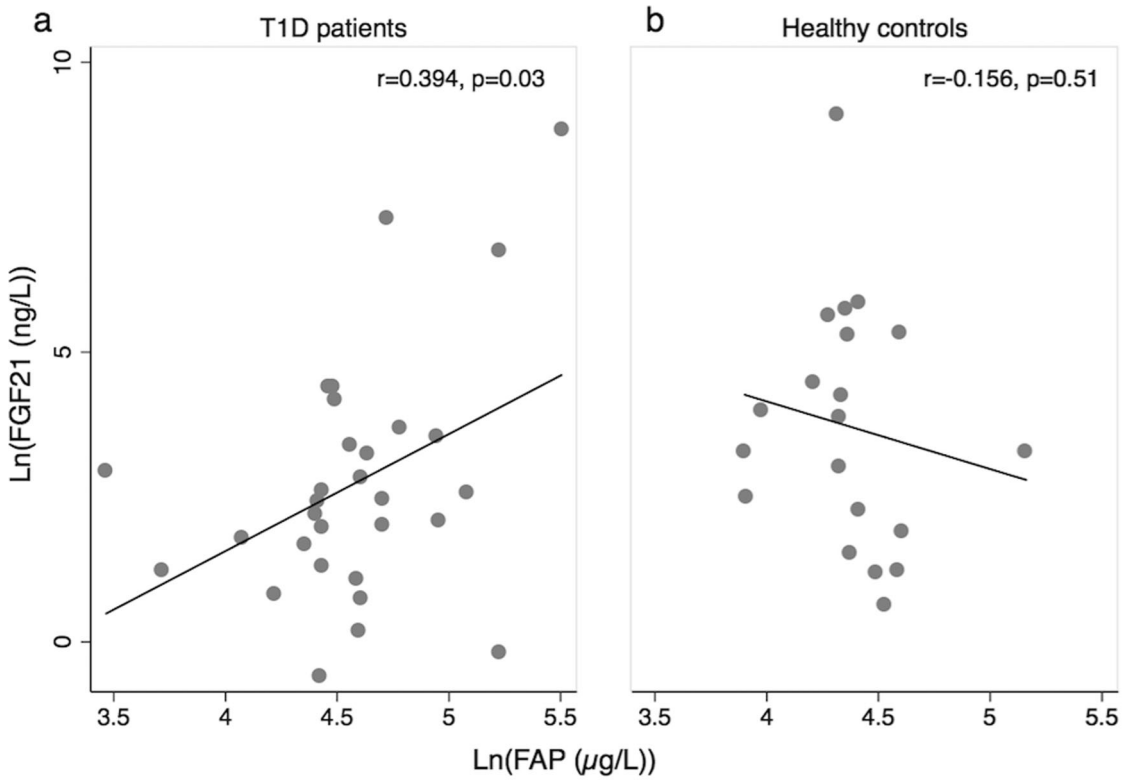

Fig. 2 Correlations between fibroblast growth factor 21 (FGF21) and serum fibroblast activation protein (FAP) levels in **a** type 1 diabetes (T1D) patients ($n = 30$) and **b** healthy controls ($n = 20$). Ln, Natural logarithm

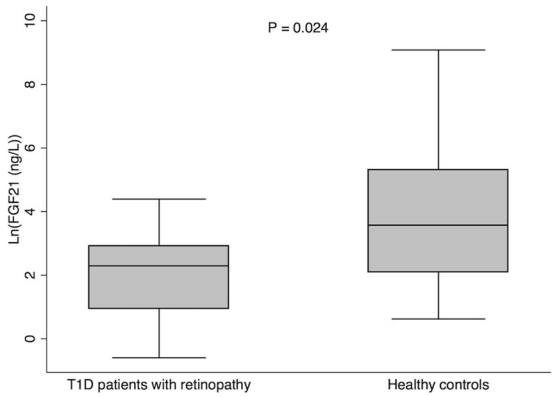

Fig. 3 Distribution of serum fibroblast growth factor 21 (FGF21) concentrations in healthy control subjects ($n = 20$) and type 1 diabetes (T1D) patients with retinopathy ($n = 12$). *Y*-axis is on a natural logarithmic (Ln) scale for visual reasons, but the analysis is nonparametric by Wilcoxon–Mann–Whitney *U* tests for differences between groups, $p = 0.024$. Horizontal bars represent medians, columns indicate IQR, and vertical bars indicate the 10th and 90th percentiles

neither very low nor very high levels of FGF21 seem to be beneficial, when it comes to diabetic complications in T2D, and our data support a relation between low FGF21 levels and retinopathy in patients with T1D. Administration of recombinant FGF21 in akita mice reversed diabetes-induced changes in the photoreceptors [17], which are early retinal signs of diabetic retinopathy, and thus supports our data.

Our study has some limitations that must be taken into accounts when interpreting the results, in particular, the relatively low number of patients. Despite the large inter-individual variation in FGF21 levels, we find significant lower FGF21 levels in T1D patients presenting with retinopathy. Even though the retinopathy group ended up being comparable to the healthy controls, the study was not designed to detect differences as to diabetic complications such as retinopathy. In addition, the patients with retinopathy had increased duration of diabetes, and it might be this causing the lower levels of FGF21 and not the retinopathy itself.

In conclusion, our study found no association between FGF21 levels and HbA1c. Serum FAP levels were significantly higher in T1D patients as compared with healthy controls, whereas serum FGF21 tended to be lower in T1D patients. FAP levels positively correlated with FGF21 levels in patients with T1D, but not in healthy controls. We suggest that the physiological pattern of FGF21 regulation is impaired in T1D possible due to increased FAP action and abnormal capacity for FGF21 production. Additional studies are required to explore the regulation and

pathophysiology of FGF21 in relation to T1D and microvascular complications more clearly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of The Central Denmark Region Committees on Health Research Ethics (1-10-72-255-15) and of the Danish Data Protection Agency (1-16-02-83-16) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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