Reduced levels of active GLP-1 in patients with cystic fibrosis with and without diabetes mellitus.

Hillman, Magnus; Eriksson, Leif; Mared, Lena; Helgesson, Karin; Landin-Olsson, Mona

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REDUCED LEVELS OF ACTIVE GLP-1 IN PATIENTS WITH CYSTIC FIBROSIS WITH AND WITHOUT DIABETES MELLITUS

Magnus Hillman, PhD [a,*], Leif Eriksson MD, PhD [a,b], Lena Mared MD [a,b], Karin Helgesson MD [c], and Mona Landin-Olsson MD, PhD [a, c]

[a] Department of Clinical Sciences, Biomedical Center, Lund University, Sweden

[b]Cystic Fibrosis Center, Department of Pulmonary Diseases, Lund University, Skåne University Hospital, Lund, Sweden

[c] Department of Endocrinology, Lund University Hospital, Lund, Sweden

*Corresponding author

Dr. Magnus Hillman
Biomedical Center, B11
Lund University
S214 84 Lund, Sweden

Tfn: +46462220704
Magnus.Hillman@med.lu.se

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Abstract

Glucagon like peptide 1 (GLP-1) is an incretin hormone released as a bioactive peptide from intestinal L-cells in response to eating. It acts on target cells and exerts several functions as stimulating insulin and inhibiting glucagon. It is quickly deactivated by the serine protease dipeptidyl peptidase IV (DPP-IV) as an important regulatory mechanism. GLP-1 analogues are used as antidiabetic drugs in patients with type 2 diabetes.

We served patients with cystic fibrosis (CF, n=29), cystic fibrosis related diabetes (CFRD, n=19) and healthy controls (n=18) a standardized breakfast (23g protein, 25g fat and 76g carbohydrates) after an overnight fasting. Blood samples were collected before meal as well as 15, 30, 45 and 60 min after the meal in tubes prefilled with a DPP-IV inhibitor. The aim of the study was to compare levels of GLP-1 in patients with CF, CFRD and in healthy controls.

We found that active GLP-1 was significantly decreased in patients with CF and CFRD compared to in healthy controls ($p<0.01$). However, levels in patients with CFRD tended to be lower but were not significantly lower than in patients with CF without diabetes ($p=0.06$). Total GLP-1 did not differ between the groups, which points to that the inactive form of GLP-1 is more pronounced in CF patients. The endogenous insulin production (measured by C-peptide) was significantly lower in patients with CFRD as expected. However, levels in non-diabetic CF patients did not differ from the controls.

We suggest that the decreased levels of GLP-1 could affect the progression toward CFRD and that more studies need to be performed in order to evaluate a possible treatment with GLP-1 analogues in CF-patients.

1. Introduction

The frequency of diabetes mellitus as a complication to Cystic fibrosis (CF) is more than 30% in young adult individuals and further increases with age. The prevalence will probably increase since the survival of CF patients is expected to improve(1). Cystic fibrosis related diabetes (CFRD) might be due to the overall pancreatic insufficiency (2) and the ongoing fibrosis in pancreatic tissue that destroys the islets of Langerhan(3). However, the correlation between insulin deficiency and degree of fibrosis has not been found by all (4,5). Insulin resistance induced by chronic pulmonary infections has been proposed as a possible contributor to the development of CFRD (6) although this is also controversial(2). Beta cell specific pancreatic autoantibodies are not more frequent in CFRD compared to in the background population (7) and autoimmunity is not generally considered to be the mechanism as in type 1 diabetes (T1D). However, one study noticed the presence of islet cell antibodies in CF patients who were genetically predisposed with certain HLA-types with increased risk of developing autoimmune diabetes (8).

Beta cell death in CFRD might be due to an increased stress of the endoplasmic reticulum (9) but the process is not as rapid as in T1D. Insulin therapy has been associated with an improvement of lung function and nutritional status in patients with CFRD (10) and even in patients with CF with impaired glucose tolerance (IGT) (11). However, oral hypoglycemic agents, normally effective for the treatment of type 2 diabetes (T2D) have no proven effect in the treatment of CFRD (6) although some data suggest a possible role for sulfonylurea (12).

Glucagon-like protein 1 (GLP-1) is a potent incretin hormone released by intestinal L-cells in the distal ileum in response to food intake (13). It is released as a bioactive peptide $(7\text{-}36)^{amide}$ or (7-37) with a half-life of just a few minutes due to rapid deactivation by dipeptidyl peptidase-IV (DPP-IV), a functional amino peptidase in the CD26 complex (14). DPP-IV targets the N-terminal of peptides where the second amino acid is alanine or proline. The N-terminal dipeptide is cleaved off leaving a peptide without biologic function. Bioactive GLP-1 interacts with the proper receptor on the target

cells and exerts several effects. Among these, inhibition of gastric emptying and gastric acid secretion (15-17) as well as increased glucose-dependent insulin secretion from the beta cells (18,19) are the best described. The glucose lowering effect is further achieved as GLP-1 is also a potent inhibitor of glucagon (19,20). The insulinotropic action of the peptide has made GLP-1 analogues and DPP-IV inhibitors useful as anti-diabetic drugs (21-24).

The aim of this paper was to study levels of active and total GLP-1 in response to a standardized mixed meal in patients with CF and CFRD compared to in healthy controls.

2. Research design and methods

2.1 Subjects and sample collection

Subjects with CF (n=48) and healthy controls (n=18) were recruited by invitation. The Center for Cystic Fibrosis at the Department of Pulmonary Medicine at the Lund University Hospital serves as one of four regional centra where patients with CF are followed. All patients were adults but diagnosed with CF during childhood.

Patients with CFRD (n=19) had a median age of 32 years, range, 23-58 years, were diagnosed by a plasma glucose ≥ 11.0 mmol/L at a 2 h oral 75 g glucose tolerance test (OGTT). A total of 12 men and 7 women were included with CFRD.

Of the remaining 29 patients with CF, 26 had normal OGTT with 2-h plasma glucose levels below 7.8 mmol/L and three responded with impaired glucose tolerance (2-h plasma glucose 7.8-10.9 mmol/L). These three patients were referred to the group of patients without diabetes in the further analyses. The median age of this group was 28 years, range, 20-47 years. A total of 16 men and 13 women were included with CF without diabetes.

Healthy non-diabetic subjects (n=18) served as controls. The median age was 33.5, range 23-60 years. A total of 7 men and 11 women were included as controls.

2.2 Methods

All subjects were fasting overnight and a first blood sample was drawn five minutes before serving breakfast (table 1). Patients with CFRD were instructed to not take insulin during overnight fasting and did not have insulin before all blood samples were collected after the meal. Blood samples were collected 15, 30, 45 and 60 minutes after the meal was finished in EDTA plasma collection tubes (blood volume 7 mL) prepared with 100µl DPP-IV inhibitor (DPP4-010, Linco Research Inc, St. Charles, MO, USA). The fasting sample (0 minutes) was drawn in two EDTA-plasma

tubes with and without the inhibitor. All samples were immediately placed on ice, blood cells were removed by centrifugation (2200 x g) and the plasma was stored in -80°C until analysis.

Diabetes specific autoantibodies were analyzed to define any the autoimmune origin of diabetes in the patients with CF. Glutamic acid decarboxylase 65 (GAD_{65}) is the most prevalent islet specific antigen during autoimmune diabetes in adult subjects and was analyzed by a radioimmuno-precipitating assay. Briefly, ^{35}S-labeled in-vitro translated recombinant antigen was prepared using a plasmid (pGAD65cDNAII) and TNT SP6 Coupled Reticulocyte System Kit (L4600, Promega, USA) according to manufacturer's instructions. Human EDTA-plasma (5µl) without the DPP-IV inhibitor incubated with ^{35}S-labeled GAD_{65} overnight at +4°C. GAD_{65} antibodies in plasma interact with the ^{35}S-labeled antigen and duplicates of 50µl were precipitated on protein A sepharose (50µl) on high affinity protein membrane filter plates (MADVN6550, Molsheim, France) precoated with 1% BSA. Several steps of washing discarded unbound antigen and the ^{35}S activity was measured (Packard Tri-carb 2100 TR, Meriden, CT, USA). The specificity of the assay was 100% and the sensitivity was 70% in the first diabetes antibody standardization program (25). The cut-off limit for positivity was 21.2 WHO units/mL.

C-peptide was analyzed with an ELISA (10-1136-01, Mercodia, Uppsala, Sweden), with an assay range between 100-4000 pmol/L and a sensitivity of 15 pmol/L, to estimate the insulin response. Total GLP-1 was analyzed with a ^{125}I-radioimmunoassay (GLP1T-36HK, Linco Research, St Charles, Mi, USA) with antibodies targeting the C-terminal. Active GLP-1 was analyzed by fluorescence ELISA (EGLP-35K, Linco Research, St Charles, Mi, USA) with antibodies targeting the N-terminal. All steps were performed according to manufacturer's instructions.

The study was approved by The Regional Ethical Board at Lund University (43/2005).

2.3 Statistical analyses

Kolmogorov-Smirnov test was applied to test for normal distribution. The test is based on the maximum discrepancy between the sample and normal cumulative distribution. Area under the curve (AUC) was compared between the groups for total and active GLP-1 as well as C-peptide. In addition, levels were compared using independent samples T-test or Mann-Whitney U-test where appropriate. All statistical data was analyzed in MedCalc® for Windows version 10.4.0.0. The α-value was set to 5%.

3. Results

Levels of active GLP-1 was significantly lower in patients with CFRD compared to the controls (AUC, p=0.001). Also the patients with CF without diabetes had significantly reduced levels compared to the controls (AUC, p=0.02). The Mann-Whitney U-test at every time point did also indicate a significant difference.

When comparing GLP-1 levels in CF patients with or without the presence of diabetes mellitus the active GLP-1 showed a tendency to be further reduced in CFRD (Fig 1, panel B; table 2), however, this was not statistically significant (p=0.06). Levels of total GLP-1 (including the DPP-IV processed peptide) did not differ at all between patients and controls. Total GLP-1 was also similar in all subjects with CF regardless of diabetic status.

High titers of autoantibodies against glutamic acid decarboxylase 65 (GAD_{65}) was found in a 25 year old woman with CF and with clinical onset of diabetes at the age of 20 yrs. Her fasting C-peptide was within normal range (0.47nM) and she was clinically classified as having CFRD and not T1D. None of the other patients or controls included were considered positive for GAD_{65} or IA-2 autoantibodies.

The C-peptide was significantly reduced in patients with CFRD compared to non-diabetic subjects with CF (AUC, p=0.0001) and controls (AUC, p=0.0001). However, C-peptide did not differ between controls and CF patients without diabetes (AUC, p=0.98) (Fig 3).

4. Discussion

In this study we found that patients with CF had significantly lower levels of active GLP-1 (7-37 and 7-36^{amide}) ($p<0.001$) compared to in controls (Fig 1, panel B). The reduced levels were observed in CF-patients even before the diagnosis of diabetes mellitus even though it was more distinct in patients with CFRD. This pattern was not observed for total GLP-1 (Fig 1, panel A).

A strength of this study is that we have access to a large number of patients with CF, who also are among the oldest CF patients in the world (1). This gives us the exceptional opportunity to study late stage complications as CFRD. We choose to use a standardized breakfast instead of glucose load since we were also interested of the effect mediated by lipids and proteins. A limitation of the study is that the first postprandial sample was collected after 15 minutes. Since the secretion of GLP-1 starts immediately after food intake it would have been interesting to observe the response in the early phase between 1-10 minutes after the meal. Degradation of active GLP-1 is rapid with a half-life of 1-2 minutes and peaks before the first 15 minutes could therefore have been missed.

The pathogenesis of CFRD is unclear. The gene mutations in CF is well described and do not have any known linkage to the any genes associated to T1D as for example certain HLA-haplotypes (26). We have also tested all patients for GAD-antibodies since these are frequently found in patients with T1D. We only found one patient positive for GAD-antibodies, which could be either due to a coexistence of T1D and CF in this specific patient or simply represent the background positivity of GAD-antibodies in the general population (27). The argument against T1D in this patient is that the C-peptide level reflected a good endogenous insulin production. Since no other parameter distinguished the patient from the other CFRD it was decided to not exclude the patient from further analysis. Patients with CF and IGT were also interesting to study and therefore not excluded in this study. These patients were referred to the non-diabetic group and we did not achieve any differences in the results by excluding these subjects.

The low levels of active GLP-1 found in our CF patients could be due to either decreased secretion from L-cells or more rapid degradation by DPP-IV. One interesting observation was that even fasting levels of active GLP-1 were lower in CF patients in agreement with another recent study (28). This study also suggested an impaired secretion of both GLP-1 and GIP in patients with CF after a meal rich in both carbohydrates and lipids. The secretion of GLP-1 was normalized after pancreatic enzyme supplementation and the increase in GLP-1 was parallel with a decrease in gastric emptying in that study. In contrast, we were not able to see effects of pancreatic enzyme supplementation on GLP-1 in our study. Reports on gastric emptying rate in CF is also conflicting and both increased and delayed gastric emptying have been suggested in this group of patients (28-32). Together with gastric dysmobility and malnutrition often observed in these patients it is tempting to speculate on the impact of incretins in this group of patients. Could GLP-1 agonist treatment that is frequently used in T2D be beneficial in CFRD? GLP-1 agonists has the additive effects of delaying gastric emptying, decreased appetite and decreased glucagon secretion. These effects are desirable in T2D but not in patients with CFRD. The positive effect is therefore not transferrable to this patient group. However, the potential with incretin drugs given to CF patients is worth to be further explored.

The decreased levels of active but not total GLP-1 in our CF patients could also indicate a higher rate of degradation, that is an increased DPP-IV activity. The expression of DPP-IV is widely occuring also outside the intestinal tract since it has been demonstrated to be expressed in human lung, liver, spleen, thymus and prostate tissue (33). It has also been suggested to be a costimulator of lymphocyte activation (34). Several hormones, cytokines and chemokines are believed to be processed by this serine peptidase (35-37) which makes the enzyme an important regulator of the immune system. The transcription of DPP-IV is controlled by gamma activated sites (GAS elements) (38), making interferons vital initiators (39). Other proinflammatory cytokines such as tumor necrosis factor (TNFα), IL-2 and IL-12 has been suggested to affect translation and translocation of DPP-IV to the cell surface (40,41). Thus, an increased DPP-IV activity would have several physiological effects beside degradation of glucagon and incretins.

The early onset of chronic inflammation in the CF patients is mainly mediated by neutrophils and even platelets have been suggested (42). Proinflammatory molecules of the innate immune system such as IL-1, IL-6 and IL-8 are elevated in CF, all which could be regulated by serine peptidases. Whether DPP-IV or other related serine peptidases have increased enzymatic activity or not in patients with CF cannot be concluded by this study and needs to be further investigated.

Impaired insulin secretion is common in CF patients with exocrine pancreatic insufficiency (43) but the role of insulin resistance in the development of CFRD is inconsistently reported (44,45). In our study the C-peptide levels were significantly lower in patients with CFRD compared to in healthy controls but also compared to in patients with CF without diabetes. Most of non-diabetic patients with CF have some degree of pancreas insufficiency, however the C-peptide levels did not differ from healthy control subjects (Fig 2, table 3). The insulin peak is normally delayed after meal or glucose load in patients with CF (46) compared to in healthy subjects (47) where the C-peptide peak has been reported to appear within six minutes after stimulation (48). We were not able to observe any early delay in C-peptide peak in our patients since we had the first postprandial sample after 15 minutes.

Postprandial responses of regulatory enteral peptides have been studied before but the results have been sometimes conflicting (46,47,49,50). Reduced levels of gastric inhibitory peptide (GIP) after stimulation with milk or standard test meal have been reported (46,49) but no change was observed after stimulation with glucose only (47). Conflicting results of postprandial responses may be due to the content of the meal. This could therefore explain why we found decreased levels of active GLP-1 in patients with CF when stimulating with a standard test meal (carbohydrates, proteins and lipids) while Lanng and collegues found active GLP-1 to be unaffected when stimulating with oral glucose (47).

The low level of active GLP-1 in response to meal that was demonstrated in all patients with CF, regardless of the presence of diabetes, could on the other hand indicate an early disturbance of food-induced stimulation of GLP-1 in these patients. The disappearance of the peak of active GLP-1

precedes the decrease in insulin secretion, since low levels of active GLP-1 were seen in all patients with CF but with simultaneous low levels of C-peptide only in these with manifest diabetes. Insulin secretion must therefore be maintained by other stimuli than GLP-1 as for example by small imperceptible elevations in glucose concentration before diabetes is diagnosed. The failure of GLP-1 peaking could also explain previous findings of a delayed insulin peak after enteral stimulation in patients with CF (46).

In conclusion, bioactive GLP-1 in patients with CFRD and CF was found to be reduced compared to in healthy controls after meal stimulation. Low levels of active GLP-1 preceded the decrease in endogenous insulin secretion in CF patients, since low GLP-1 levels and normal C-peptides were observed in CF patients not yet diagnosed with diabetes. These findings supported the hypothesis that the disturbance of active GLP-1 could be closely connected to the primary cause of CFRD. With regard to new therapeutic agents in the treatment of diabetes in form of GLP-1 agonists and DPP-IV inhibitors these results are important. The possibility to treat CFRD with these new drugs is worth to investigate and calls for new clinical trials.

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Table 1. Contents of the meal.

Breakfast (Energy 617 kcal, protein 23g, fat 25g, carbohydrates 76g)	
50g	White wheat bread
50g	Dark whole meal bread
10g	Butter (80% fat)
30g	Cheese (28% fat)
40g	Orange marmalade
200g	Milk (3% fat)

Table 2. Active GLP-1 concentrations (pmol/L) in controls, patients with cystic fibrosis without diabetes (CF), and patients with cystic fibrosis related diabetes (CFRD). Data are presented as median levels followed by interquartile range (IQR).

	O min	15 min	30 min	45 min	60 min
Controls (n=18)	5.2 (3.1-8.4)	7.0 (4.1-9.5)	6.2 (4.9-8.7)	5.5 (4.6-8.8)	5.7 (4.6-8.8)
CF (n=29)	1.9 (1.0-3.9)	4.2 (3.5-5.8)	4.0 (3.4-6.6)	4.0 (2.3-5.3)	3.7 (2.3-4.6)
CFRD (n=19)	1.7 (1.1-2.9)	3.7 (2.7-5.3)	3.1 (1.3-5.6)	2.5 (1.7-4.5)	2.6 (1.3-4.9)

Table 3. Total GLP-1 concentrations (pmol/L) in levels in controls, patients with cystic fibrosis without diabetes (CF), and patients with cystic fibrosis related diabetes (CFRD). Data are presented as median levels followed by interquartile range (IQR).

	O min	15 min	30 min	45 min	60 min
Controls (n=18)	12.7 (8.0-17.1)	16.6 (9.4-21.9)	17.9 (11.1-23.4)	15.6 (8.0-24.0)	17.4 (13.0-24.1)
CF (n=29)	11.4 (5.6-19.6)	16.7 (10.2-28.4)	15.7 (11.2-27.3)	14.6 (9.1-20.3)	11.8 (8.6-22.8)
CFRD (n=19)	9.4 (7.6-22.2)	16.4 (10.4-34.4)	12.9 (8.7-31.5)	15.7 (8.7-31.5)	16.1 (7.1-34.7)

Table 4. C-peptide concentrations (nmol/L) in controls, patients with cystic fibrosis without diabetes (CF), and patients with cystic fibrosis related diabetes (CFRD). Data are presented as median levels followed by interquartile range (IQR).

	O min	15 min	30 min	45 min	60 min
Controls (n=18)	0.45 (0.32-0.63)	1.03 (0.71-1.47)	1.09 (0.97-1.75)	1.20 (0.86-1.26)	1.07 (0.78-1.25)
CF (n=29)	0.40 (0.30-0.58)	0.93 (0.58-1.20)	1.33 (1.05-1.61)	1.40 (1.10-1.60)	1.32 (1.13-1.83)
CFRD (n=19)	0.25 (0.19-0.47)	0.35 (0.24-0.47)	0.45 (0.25-0.81)	0.56 (0.30-0.77)	0.56 (0.36-1.00)

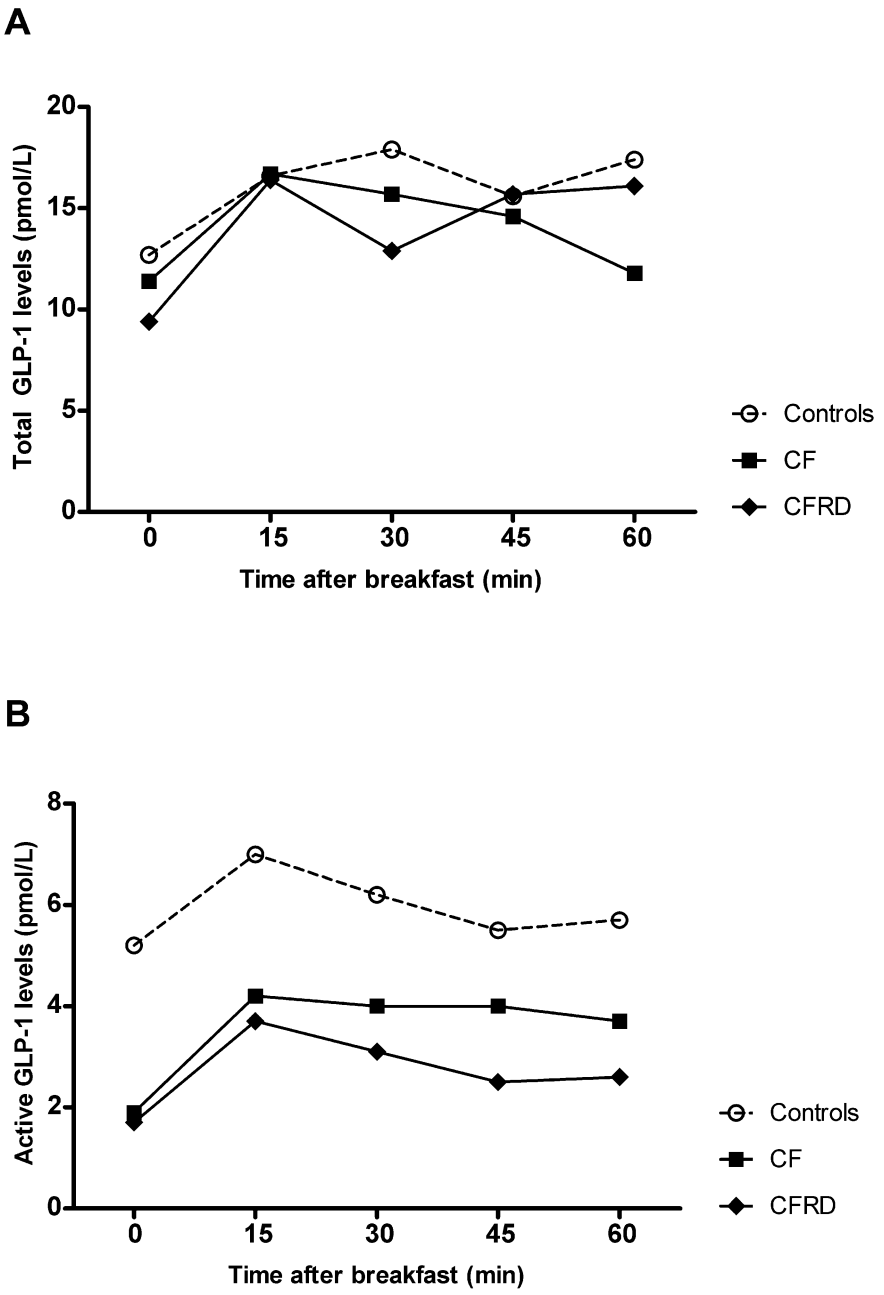

Figure 1. The lines show the median GLP-1 levels at different points of time after ingestion of the meal. Panel A shows the level of total GLP-1 and panel B the level of active GLP-1 in healthy control subjects (O, n=18), in patients with cystic fibrosis without the presence of diabetes (■, n=29) and in patients with cystic fibrosis related diabetes, CFRD (◆, n=19). Panel B, Area under the curve (AUC) analysis showed significantly higher levels of active GLP-1 in healthy subjects compared to in CFRD (p=0.001) as well as in CF patients without diabetes (p=0.02).

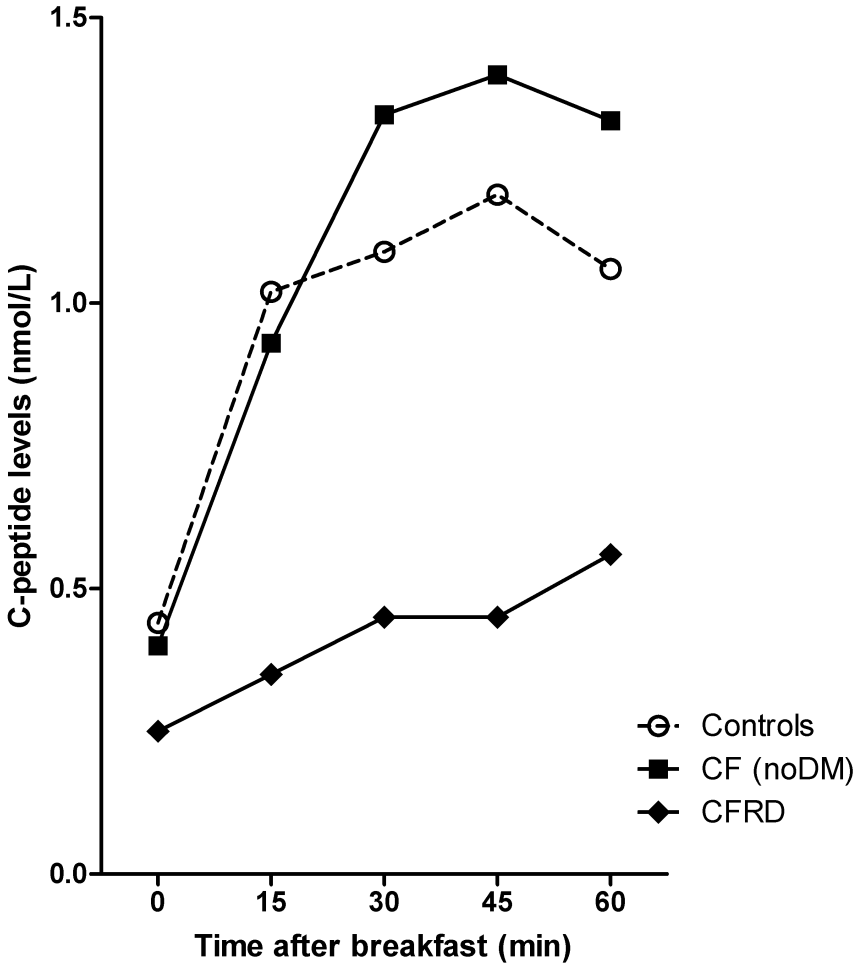

Figure 2. The lines show the median C-peptide levels at different points of time after ingestion of the meal. Patients with Cystic fibrosis related diabetes, CFRD (◆, n=19) had significantly lower levels of C-peptide compared to non diabetic subjects with CF (■, n=29) and healthy controls (O, n=18). No significant difference was observed between healthy controls and CF patients without diabetes.