

## RESEARCH

# Fasting decreases plasma FGF21 in obese subjects and the expression of FGF21 receptors in adipose tissue in both lean and obese subjects

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## Abstract

Fibroblast growth factor 21 (FGF21) is a metabolic regulator of energy and lipid metabolism. FGF21 is highly expressed in liver while FGF21 receptors (beta-klotho (KLB) and FGFR1c) are highly expressed in white adipose tissues (WATs). Plasma FGF21 has been shown to be increased after 7–10 days of fasting but oppositely plasma FGF21 is also increased in obesity. The aim of this study was to measure the effect of 60 h of fasting on plasma FGF21 levels in obese and lean subjects and to determine the gene expression of *KLB* and *FGFR1c* in the subcutaneous WAT before, during and after 60 h of fasting. Eight obese (BMI >30 kg/m<sup>2</sup>) and seven lean subjects (BMI <25 kg/m<sup>2</sup>) were fasted for 60 h and blood samples were taken at time 0 and after 12, 36 and 60 h of fasting. A biopsy from the subcutaneous WAT was taken at time 0, 12 and 60 h of fasting. FGF21 was measured in plasma by an ELISA and mRNA expression of *KLB* and *FGFR1c* was measured in WAT by quantitative PCR (qPCR). The fast significantly decreased plasma FGF21 in obese subjects while no change in plasma FGF21 was observed in lean subjects. Interestingly, *KLB* was significantly decreased in WAT in response to fasting in both lean and obese subjects indicating a potential important adaptive regulation of *KLB* in response to fasting.

## Key Words

- ▶ FGF21
- ▶ FGF21 receptors
- ▶ adipose tissue
- ▶ fasting
- ▶ obesity

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## Introduction

Fibroblast growth factor 21 (FGF21) was identified by Nishimura in 2000 (Nishimura *et al.* 2000) and is mainly expressed in liver and pancreas (Fon Tacer *et al.* 2010). FGF21 belongs to the FGF19 subfamily of endocrine FGFs (Fukumoto 2008). FGF21 binds the FGF receptor (FGFR) 1c and 3c but only in the presence of the co-receptor beta-klotho (KLB) (Suzuki *et al.* 2008) and tissue-specific expression of KLB determines the metabolic activity (Ogawa *et al.* 2007, Suzuki *et al.* 2008).

The FGF21 receptors KLB and FGFR1c are highly expressed in the adipose tissue (Fon Tacer *et al.* 2010) and in very specific regions of the CNS (Bookout *et al.* 2013). FGF21 produces strong anti-diabetic, lipid-lowering and weight-reducing effects in pre-clinical models of type 2 diabetes and obesity (Kharitonov *et al.* 2005, 2007, Xu *et al.* 2009, Veniant *et al.* 2012). These findings have led to a significant interest in FGF21 as a therapeutic target for treatment of type 2 diabetes and obesity as reviewed

in (Kharitonov & Adams 2014, Sonoda *et al.* 2017). However, the physiological role of FGF21 is still not fully understood.

In mice, plasma FGF21 has been found to be increased in response to fasting (Inagaki *et al.* 2007) and ketogenic diet (Badman *et al.* 2007) and FGF21 was therefore originally proposed to be the missing link in starvation by inducing lipolysis and ketogenesis (Reitman 2007). Adipocyte-derived free fatty acids (FFA) are strong inducers of hepatic *Fgf21* expression via activation of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) and a feed forward loop was therefore suggested. In contrast, FGF21 has also been suggested to be involved in a negative feedback loop inhibiting lipolysis and in agreement with this hypothesis FGF21-knockout mice have increased lipolytic activity in response to fasting (Hotta *et al.* 2009) and growth hormone-induced lipolysis (Chen *et al.* 2011). Furthermore, in response to injection of FGF21 plasma FFA is acutely decreased in mice (Li *et al.* 2009). Plasma FGF21 is increased while FGF21 receptors in the adipose tissue have been shown to be decreased in obese mice (Fisher *et al.* 2010) in alignment with poor metabolic control and increased flux of FFA in obese mice. Interestingly, FGF21 receptors in adipose tissue have been shown to be decreased in northern elephant seals in response to fasting (Suzuki *et al.* 2015).

In humans, several studies have shown that 3 days of fasting does not alter plasma FGF21 in healthy lean subjects (Dushay *et al.* 2010, Andersen *et al.* 2011), while 7-day fast in patients with rheumatoid arthritis demonstrated a modest 74% increase in FGF21 levels (Galman *et al.* 2008). Moreover, a prolonged fasting of 10 days caused an approximately 3-fold increase in plasma FGF21 in normal to slightly overweight subjects (Galman *et al.* 2008, Fazeli *et al.* 2015). However, the increase in plasma FGF21 observed in response to 10 days of fasting did not increase thermogenic genes in the adipose tissue and a decrease in the metabolic rate and plasma adiponectin were observed (Fazeli *et al.* 2015). Noteworthy, a decrease in KLB in the adipose tissue was observed in response to the prolonged fast (Fazeli *et al.* 2015). Plasma FGF21 is also increased in obese and insulin-resistant subjects (Zhang *et al.* 2008, Chavez *et al.* 2009) and a correlation between FGF21 and the homeostatic model assessment for insulin resistance (HOMA-IR) has been observed in several studies (Zhang *et al.* 2008, Fazeli *et al.* 2015). Therefore, the regulation of plasma FGF21 is rather complex as plasma FGF21 is both increased in response to fasting and in obesity, and it is therefore unknown whether 3 days of fasting change plasma FGF21 in obese subjects. Furthermore, the

potential regulation of FGF21 receptors in the adipose tissue in response to fasting in both lean and obese subjects has not previously been studied.

The aim of this study was to elucidate the effect of 60h of fasting on plasma FGF21 in lean and obese subjects and to elucidate if the fast changes the expression of FGF21 receptors (*BKL* and *FGFR1c*) in subcutaneous white adipose tissue (WAT).

## Materials and methods

The study was part of a previous study (Rasmussen *et al.* 2003). All subjects gave informed consent before participating. The study protocol was reviewed and approved by the Ethics Committee of Copenhagen County and was in compliance with the Declaration of Helsinki.

### Subjects

Eight obese (BMI >30 kg/m<sup>2</sup>) and seven lean subjects (BMI <25 kg/m<sup>2</sup>) male volunteers participated in the study. All subjects were in good health, as assessed by medical history and physical examination and had maintained a stable weight for at least 6 months prior to the study. None of the study participants had diabetes or were taking any medication. The obese subjects tended to have higher fasting insulin levels and had significantly higher systolic blood pressure than the lean subjects (Rasmussen *et al.* 2003).

### Sample collection

The day before the fasting was initiated, a plasma sample was collected 2h after last meal (time 0h). The next day, the prolonged fasting was initiated, and plasma was sampled in the morning after overnight fast (12h), after 36h and after 60h of fasting. Subjects were instructed to avoid all food and drink except water, to avoid excessive exercise and smoking and to come to the laboratory by car or by bus. On each study day, the subjects rested for at least 15 min before samples were obtained. At time 0, 12 and 60h of fasting, a percutaneous abdominal subcutaneous fat sample was obtained under local anesthesia (1% lidocaine without adrenaline) using a liposuction cannula (Tulip 4mm, 30cm; RMI B.V., Breda, The Netherlands). Approx. 150mg of adipose tissue was obtained on each occasion. Samples were frozen in liquid nitrogen within 30s of removal and were stored at  $-80^{\circ}\text{C}$  until assayed.

### Measurement of plasma FGF21

FGF21 plasma levels were measured using a commercial FGF21-specific human ELISA kit (BioVendor, Prague, Czech Republic). Plasma samples were diluted 1:1 and analyzed according to manufacturer's instructions. The sensitivity was 7.0 pg/mL and both the intra-assay and interassay variability was 4.0%. For unknown reasons, one obese subject had no measurable FGF21 in any of the plasma samples and was therefore excluded from all data sets.

### Quantitative real-time PCR

Total RNA was extracted from the adipose tissue using TRIzol (Invitrogen) and RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized using iScript reverse transcription kit (BioRad). Quantitative real-time PCR was performed on an ABI 7900 Sequence Detection System (Applied Biosystems) using a LNA probe-based system from Roche. Primers were designed using Primer3 software (Table 1). All samples were run in triplicates and expression was calculated using the  $\Delta\Delta C_T$  method. Samples were normalized to the mean expression of *GAPDH*, *18S* and *Cyclophilin B (CYPB)* expression.

### Statistical analysis

Statistical analyses of plasma FGF21 as well as *BKL* and *FGFR1c* mRNA expression were performed using SAS/STAT 14.3. Data were analyzed by a repeated-measures analysis with fixed effects of time, BMI status (obese, lean) and interaction between the two factors. An unstructured  $4 \times 4$  covariance matrix was modeled. For each BMI status, the difference between time 60 and baseline (time 0) was estimated, and the difference in change from baseline was compared between BMI status. For additional information, the difference between time 36 and baseline and between time 12 and baseline were also estimated and compared between BMI statuses. Tissue samples were missing from 1 to 2 subjects in each group (varies between subjects and time points), which was taken into account using SAS/STAT 14.3. FGF21 and FFA plasma correlations were analyzed by Pearson correlation test using GraphPad Prism version 7 (GraphPad Software). Data analysis of plasma glucose, C-peptide, triglycerides (TGs) and FFA was performed with repeated-measures ANOVA with Dunnett's *post hoc* test using GraphPad Prism, version 7. Significance was compared with the corresponding group

and time point 0. Data are presented as means  $\pm$  s.e.m., lean  $n=6-7$ , obese  $n=6-7$ , and the results were considered statistically significant for \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$ .

### Results

The changes in plasma glucose, C-peptide, TG and FFA in response to the 60h of fasting in lean and obese subjects can be seen in Table 2. The prolonged fasting caused a gradual decrease in blood glucose in both lean and obese subjects (Rasmussen *et al.* 2003). Plasma FFA and beta-hydroxybutyrate were increased in both lean and obese subjects; however, a greater increase was observed in the lean subjects (Rasmussen *et al.* 2003).

#### Fasting decreases plasma FGF21 in obese subjects

Plasma FGF21 was increased albeit not significantly ( $P=0.07$ ) in obese subjects compared to lean subjects at time point 0 (Fig. 1A). In response to fasting, no significant changes in plasma FGF21 was observed in lean subjects (Fig. 1B) while plasma FGF21 gradually declined in obese subjects  $296 \pm 29$  pg/mL (0h),  $230 \pm 43$  pg/mL (12h),  $154 \pm 21$  pg/mL (36h) and  $128 \pm 18$  pg/mL (60h) (Fig. 1C). The decline in plasma FGF21 was significantly decreased after 36 and 60h of fasting. At 60h of fasting, plasma FGF21 tended to be higher in lean subjects ( $205 \pm 45$  pg/mL) compared to obese subject ( $128 \pm 18$  pg/mL), but this was not statistically significant. Also, a trend toward an increase in plasma FGF21 was observed in the lean subjects from 36h ( $145 \pm 34$  pg/mL) to 60h ( $205 \pm 45$  pg/mL) of fasting. This was however driven by one subject in the lean group where FGF21 increased from 86 pg/mL at  $t=0$ h to 383 pg/mL after 60h of fasting as seen in Fig. 1D. Individual graphs of plasma FGF21 during fasting in obese subjects are shown in Fig. 1E.

#### Fasting decreases the expression of *KLB* and *FGFR1c* in subcutaneous WAT in lean and obese subjects

The mRNA expression of *KLB* and *FGFR1c* were similar between lean and obese subjects in the basal state (0h). In both lean and obese subjects *KLB* mRNA expression significantly decreased with fasting (Fig. 2A and B). A five-fold decrease was observed after 60h of fasting in obese subjects, while a six-fold decrease was observed in lean subjects. Fasting also caused a small decrease in *FGFR1c* expression in both lean and obese subjects (Fig. 2C and

**Table 1** Primer sequences used for quantitative real-time PCR.

Gene	5'-3' sequences <sup>a</sup>
<i>KLB</i>	F: ACGGCCATCTACATGATGAA R: CGTATTCATCTAACCTTATTGCTTG
<i>FGFR1c</i>	F: ACAACCTGCCTTATGTCCAGA R: TCCATCTCTTTGTCGGTGGT
<i>FGF21</i>	F: ATGGGGCCCTGTATGGAT R: AAACATTGTATCCGTCTCAAGA
<i>GAPDH</i>	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC
<i>18S</i>	F: GCAATTATCCCCATGAACG R: GGGACTTAATCAACGCAAGC
<i>CYPB</i>	F: CCCAGTTCTTCATCAGACA R: GTCTTGGTGCTCTCCACCTT

<sup>a</sup>F, forward primer; R, reverse primer.

D), but the changes were only significant after 60h of fasting. Almost none of the samples had any expression of FGF21 before, during or after the 60-h fast (data not shown).

### Plasma FGF21 correlations

As FGF21 has been proposed to be a negative regulator of FFA (Chen *et al.* 2011, Park *et al.* 2016), plasma FGF21 levels were correlated to plasma FFA at time 0, 12, 36 and 60h of fasting. However, as seen in Fig. 3 no significant correlations between plasma FGF21 and FFA was found at any given time points in any of the groups. However, in general at  $t=0$ h higher FFA trended to correlate with higher FGF21, but this trend was lost during fasting and at 60h of fasting an inverse correlation between FGF21 and FFA was seen in obese subjects (Fig. 3D), this did however not reach significance ( $P=0.0788$ ).

Furthermore, no significant correlations were observed between plasma FGF21 and glucose, plasma FGF21 and C-peptide or plasma FGF21 and TG at any time points in lean or obese subjects (data not shown).

### Discussion

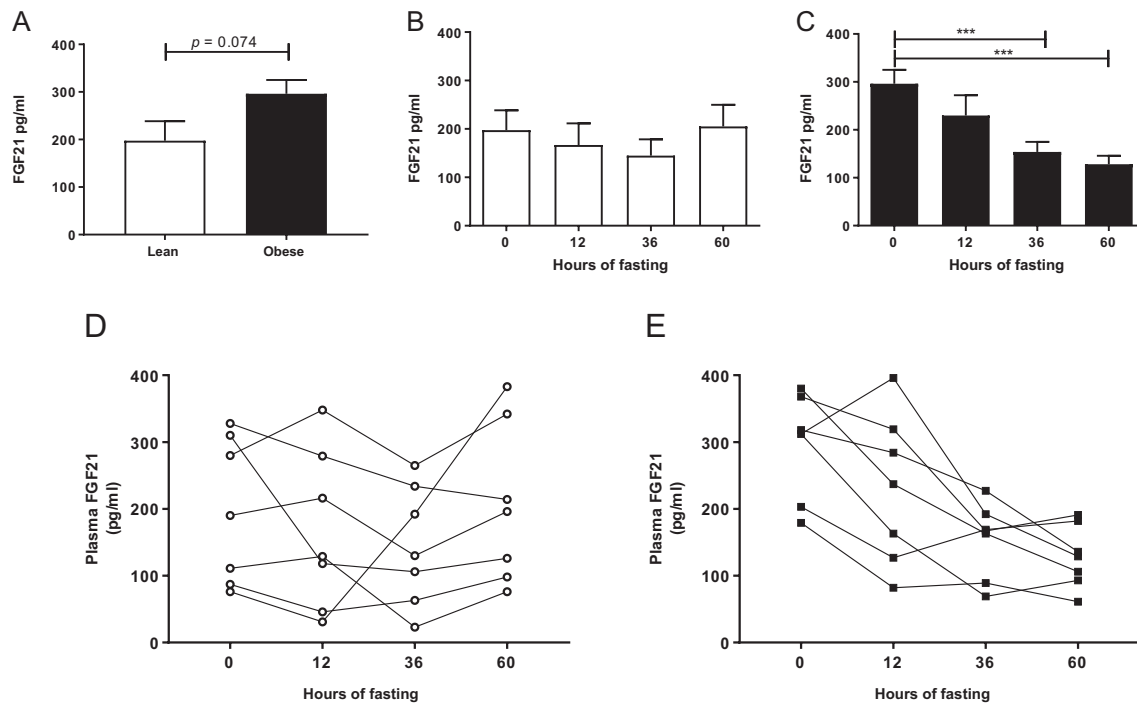
This study shows that plasma FGF21 is differently regulated in lean and obese subjects upon short-term fasting. In agreement with previous studies, 3 days of fasting in humans did not change plasma FGF21 in lean subjects. However, in obese subjects, fasting decreased plasma FGF21 to the levels observed in lean subjects and at 60h the plasma FGF21 levels were even slightly lower in the obese subjects compared to lean subjects. This seems however to be due to a slight increase in plasma FGF21 from time 36 to 60h in the lean subjects but the increase was driven by one subject in the lean group.

The decrease in plasma FGF21 in obese subjects may reflect an improvement in the metabolic status as observed during short-term fasting (Patterson & Sears 2017) or after bariatric surgery (Runkel & Brydniek 2016). In response to bariatric surgery, a decrease in fasting plasma FGF21 has also been described (Fjeldborg *et al.* 2017). The decrease in plasma FGF21 in our study occurs despite an approximately three-fold increase in FFA (Rasmussen *et al.* 2003) in obese subjects during the 60h of fasting and indicate that other factors such as hepatic steatosis may regulate plasma FGF21 (Li *et al.* 2010, Yan *et al.* 2011). In agreement with this plasma TG, which must arise from the liver in the fasted state, are decreased approximately two-fold in the obese subjects while plasma triglycerides

**Table 2** Metabolic changes in plasma in response to 60-h fasting in lean and obese subjects.

	0h	12h	36h	60h
Lean subjects				
Age	29.3±3.6 years			
BMI	22.8±0.6 kg/m <sup>2</sup>			
Glucose (mM)	4.6±0.3	4.4±0.2	3.9±0.1	3.2±0.2***
C-peptide (pM)	953±169	374±47*	207±64***	223±46***
NEFA (mM)	0.20±0.04	0.34±0.03*	0.97±0.16**	1.31±0.15***
Triglycerides (mM)	1.3±0.2	1.1±0.2	0.8±0.1*	0.9±0.1
Obese subjects				
Age	29.8±2.9 years			
BMI	33.4±1.0 kg/m <sup>2</sup>			
Glucose (mM)	5.2±0.4	4.6±0.3*	4.5±0.3*	3.6±0.2***
C-peptide (pM)	1333±216	674±77*	499±71**	390±57**
NEFA (mM)	0.38±0.08	0.55±0.08	0.87±0.03***	1.15±0.07***
Triglycerides (mM)	1.9±0.2	1.60±0.3	1.4±0.2	1.1±0.2*

Results are mean±s.e.m.,  $n=7$ . Significance is compared with the corresponding group and time 0h, \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$  (repeated-measures ANOVA with Dunnett's *post hoc* test).

**Figure 1**

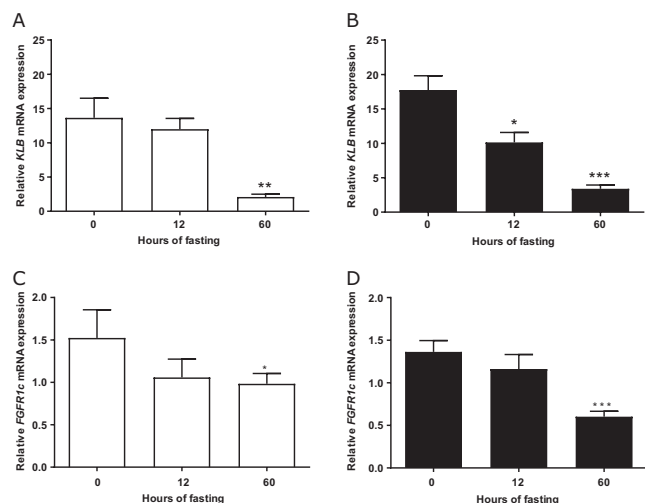
Changes in plasma FGF21 in lean and obese subjects during fasting. Plasma FGF21 in lean (open bars) and obese subjects (closed bars) at time point 0h (A). Changes in plasma FGF21 during fasting in lean (B) and in obese (C) subjects. Individual plasma levels of FGF21 during fasting in lean (D) and obese (E) subjects. Results (A, B, and C) are mean  $\pm$  s.e.m., analyzed by repeated-measures analysis,  $n=7$ . \* denotes statistically significant differences at  $**P<0.01$ .

at the end of the fast are unchanged in the lean subjects. This study only included seven subjects in each group and no statistically significant correlations between FGF21 and FFA were observed at any time points. Additional studies are required to address if a potential correlation between FGF21 and FFA is altered by fasting in lean and obese subjects.

From this study, it was not possible to determine which tissue was responsible for the circulating FGF21, as liver biopsies were not obtained, but the liver has been shown to be the major contributor to plasma FGF21 (Markan *et al.* 2014). However, the increased fat mass, which has been shown to express FGF21 in obese subjects (Muise *et al.* 2008) may also have contributed to the decrease in plasma FGF21 levels, but like other studies (Vienberg *et al.* 2012), we were unable to detect any FGF21 mRNA expression in human WAT in the basal state (non-insulin stimulated), and this was independent of the time of sampling and BMI.

During starvation the human body primarily burns FFAs from body fat stores, along with small amounts of muscle tissue to provide energy for the brain. FGF21 has been suggested to be part of a negative feedback loop where FFA stimulates hepatic FGF21 expression and

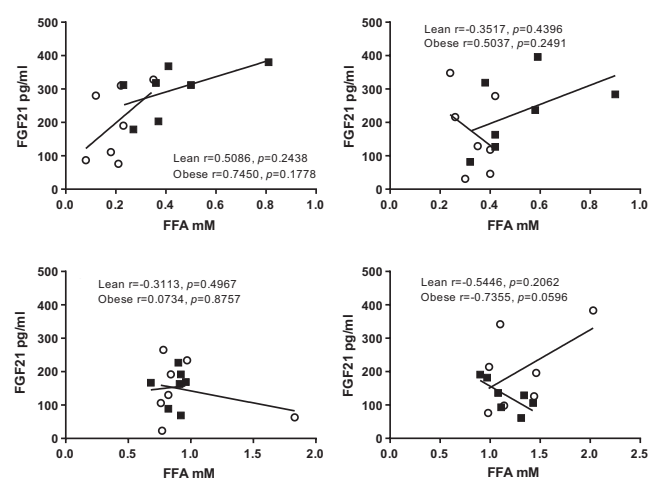
secondary FGF21 decreases lipolysis in adipose tissue (Chen *et al.* 2011); interestingly, a downregulation of *KLB* has been reported in fasting northern elephant seals (Suzuki *et al.* 2015) potentially diminishing the inhibitory effect of FGF21 on lipolysis. In this study, a significant downregulation of *KLB* was observed in response to 60h of fasting independent of BMI indicating that regulation of FGF21 receptor BKL in WAT could play an important adaptive role in response to fasting in humans. Similar findings have previously been described in humans fasted for 10 days where a three-fold decrease in *BKL* was observed in WAT (Fazeli *et al.* 2015). In agreement with the down regulation of *BKL*, a decrease in thermogenesis and plasma adiponectin, pharmacological effects of FGF21, were observed (Fazeli *et al.* 2015). Fazeli *et al.* also showed that PGC-1 $\alpha$ , a classical FGF21 target gene in WAT (Fisher *et al.* 2012), is downregulated in humans fasted for 10 days. A weakness of our study is the lack of FGF21 target genes measurements in the adipose tissue during fasting as this would have indicated whether the significant and time-dependent decrease in *KLB* had caused a decrease in FGF21 activity in the adipose tissue as observed in the study by Fazeli *et al.* (2015). Another weakness is lack of BKL protein determination in WAT, however, Markan

**Figure 2**

Expression of *BKL* is downregulated in adipose tissue upon fasting. mRNA expression of Beta-klotho (*KLB*) in lean (A) and obese (B) subjects during fasting. Changes in fibroblast growth factor receptor-1c (*FGFR1c*) in lean (C) and obese (D) subjects during fasting. Results are mean  $\pm$  s.e.m., analyzed by repeated-measures analysis,  $n=5-7$ , \* denotes statistically significant differences at \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$ .

*et al.* has previously shown that the mRNA expression of *KLB* correlated well with the protein level (Markan *et al.* 2017).

Collectively, downregulation of *KLB* during fasting might be seen as an adaptive mechanism adjusting energy homeostasis e.g. lipolysis to the fasting condition. The decrease in plasma FGF21 observed in the obese subjects during the 60h of fasting may correlate to a decrease

**Figure 3**

Plasma FGF21 and FFA correlation during fasting. Plasma FGF21 and FFA correlation at time 0h (A), time 12h (B), time 36h (C) and time 60h (D). Lean subjects (open circles) and obese subjects (close squares),  $n=7$ . Data are analyzed with Pearson's correlations test.

in plasma TG reflecting an improvement in hepatic TG content, but the exact mechanism for why short-term fasting decreases plasma FGF21 in obese subjects needs to be further elucidated.

**Declaration of interest**

B A is an employee and minor stock holder of Novo Nordisk A/S.

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**Author contribution statement**

E B N performed the measurements, analyzed the data and wrote the manuscript. C Ø, T A and H V contributed to the design of the study and reviewed the manuscript. B A conceived the idea, performed the measurements, analyzed the data and wrote the manuscript.

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