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# Sex Dimorphism in the *Fgf21* Gene Expression in Liver and Adipose Tissues is Dependent on the Metabolic Condition

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**Abstract:** Fibroblast growth factor-21 (FGF21) beneficially affects carbohydrate and lipid metabolism. Previously, a sex-specific activation of *Fgf21* expression was observed in humans and animals with metabolic diseases. It is unknown whether the sex differences in the *Fgf21* expression are manifested in response to the natural physiological situations of fasting and refeeding. The aim of this work was to determine liver, White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT) expression of genes related to FGF21 signaling in response to 24 h fasting, 6 h refeeding (after 24 h fasting) and Diet-Induced Obesity (DIO) in C57Bl mice of both sexes. Obesity was induced by the consumption of palatable food for 10 weeks. mRNA levels of peroxisome proliferator-activated receptor-a and -*γ* (*Pparα*, *Pparγ*), FGF21 (*Fgf21*), coactivator of FGF receptors (*Klb*) and transcriptional coactivator (*Pgc-1α*) were measured by RT-PCR. The study showed that the fasting-induced increases in hepatic *Fgf21* gene expression and circulating FGF21 levels, as well as refeeding-induced increases in local WAT and BAT *Fgf21* gene expression, were biased toward females. DIO-induced increase in circulating FGF21 levels, as well as in *Fgf21* gene expression in the liver and BAT, were biased toward males. Considering that FGF21 is a novel metabolic regulator of energy homeostasis, sex differences in the responses to anabolic and catabolic stimulus could have translational implications for novel therapeutic outcomes.

**Keywords:** Sex-Specific FGF21 Signaling, Diet-Induced Obesity, Fasting, Refeeding, Mice

## Introduction

Fibroblast Growth Factor-21 (FGF21) is a circulating hepatokine that beneficially affects carbohydrate and lipid metabolism. FGF21 has been shown to enhance fatty acid β-oxidation during prolonged fasting, increased insulin sensitivity and stimulate glucose uptake in obese animals and promote fat browning in White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT) in response to cold exposure (Dutchak *et al*., 2012; Badman *et al*., 2007; Inagaki *et al*., 2007; Fisher *et al*., 2011). The liver is the main site of the production and release of FGF21 into the blood. Hepatic *Fgf21* expression is under the control of peroxisome proliferator–activated receptor α (PPARα) (Badman *et al*., 2007; Inagaki *et al*., 2007; Fisher and Maratos-Flier, 2016). In WAT and BAT, expression of the *Fgf21* gene is activated by PPARα and PPARγ (Hondares *et al*., 2011). Liver, WAT (Fisher *et al*., 2010) and BAT (Fisher *et al*., 2012) are known target tissues of FGF21 action.

FGF21 activates cell signaling by binding to the cell surface receptor complex composed of β-Klotho (KLB) and a conventional FGF receptor (FGFR) (Suzuki *et al*., 2008). In mice, KLB is expressed in all adipose tissues (Fisher *et al*., 2011) and in the liver (Xu *et al*., 2009). One potential candidate mediating the FGF21 response is PGC-1α, a transcriptional coactivator that regulates fatty acid oxidation by enhancing mitochondrial function and biogenesis. In mice, FGF21 reportedly regulates both Pgc-1α mRNA and protein levels in the liver (Fisher *et al*., 2011; Potthoff *et al*., 2009), protein PGC-1α levels in BAT and Pgc-1α mRNA levels in WAT (Fisher *et al*., 2012).

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FGF21 blood levels and hepatic expression increase in different stress conditions including various nutrient stresses such as starvation and in ketogenic, low protein and high fat diets (Cantó and Auwerx, 2012). Obesity, type 2 diabetes and Nonalcoholic Fatty Liver Disease (NAFLD) are associated with increased circulating FGF21. A variety of nutrient and hormonal factors regulate FGF21 synthesis in the liver, including glucagon, insulin, glucocorticoids and thyroid hormones (Erickson and Moreau, 2017; Adams *et al*., 2010). Apparently, sex steroids also may be involved in such regulation as a sex-specific activation of FGF21 expression was observed in humans and animals with various metabolic diseases. Wang *et al*. (2017) found that higher serum FGF21 levels were associated with an increased risk of type 2 diabetes in Chinese women but not in men. Diets that induce NAFLD also affected hepatic *Fgf21* expression in sex-specific manners: high-fat high-fructose diet increased *Fgf21* expression only in male rats (Chukijrungroat *et al*., 2017), methionine-choline deficient diet only increased expression in female mice (Lee *et al*., 2016) and cafeteria obesogenic diet only increased expression in male mice. It is unknown whether the sex differences in the expression of FGF21 are manifested only in metabolic diseases and, possibly, are a consequence of these diseases, or if the activation of *Fgf21* expression is sex-specific under natural physiological adaptations to nutritional stresses.

No previous studies have addressed the possible sex differences in the transcriptional response of FGF21-signaling system to anabolic and catabolic states in liver and adipose tissues. The aim of this work was to determine the expression of genes related to FGF21 signaling in liver and adipose tissues in response to states of different nutrient availability in mice of both sexes. The study showed that upregulation of *Fgf21* gene expression in liver and adipose tissues was biased toward different sexes in different metabolic situations: toward females at adaptation to fasting (liver) and refeeding (WAT, BAT), but toward males under adaptation to diet induced obesity (liver, BAT). Considering that FGF21 is a novel metabolic regulator of glucose, lipid and energy homeostasis, gender differences in the response to anabolic and catabolic stimuli could have translational implications for novel therapeutic outcomes in type 2 diabetes treatment.

## Materials and Methods

All experiments were performed according to 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and Russian national instructions for the care and use of laboratory animals. The protocols were approved by the Independent Ethics Committee of the Institute of Cytology and Genetics (Siberian Division, Russian Academy of Sciences).

C57BL mice were bred in the vivarium of the Institute of Cytology and Genetics. Two separate experiments were performed. The first experiment was aimed to investigate the influence of food deprivation and the followed refeeding on FGF21 signaling. The mice were housed individually for 3 weeks before the start of the experiment under a 12:12-h light-dark regime at an ambient temperature of 22°C. Fifteen-week old female and male mice weighing 26.8±0.3g (males) and 22.5±0.5g (females) were used. Animals were deprived from food at 9.00 am. Some animals were killed by decapitation after 24 h of food deprivation and others received standard food and were decapitated after 6 h of refeeding. The mice from the control groups were decapitated simultaneously with the food-deprived mice. There were 6-7 mice in every experimental group.

The second experiment was aimed to investigate the influence of diet-induced obesity on FGF21 signaling. For this experiment, male and female mice were housed in group (3 mice per cage) and were fed with standard laboratory chow purchased from Assortiment Agro (Moscow region, Turacovo, Russia) from the weaning until the age of 10 weeks. Ten-week old male and female mice were devided into Control Diet (CD) group and group, which was stimulated to develop obesity (DIO). To induce obesity, mice were fed during 10 weeks with mixed diet, which consisted of standard laboratory chow supplemented with sweet cookies, sunflower seeds and lard – sweet and fat diet. Previously we have shown that this sweet and fat diet rapidly induced obesity in C57Bl mice (Makarova *et al*., 2013). Mice of control group consumed for 10 weeks standard laboratory chow. At 20-week age, CD animals weighted 29.3±0.5 g (males) and 24.4±0.7 g (females) and mice with diet-induced obesity weighted 40.7±1.9 g (males) and 34.2±1.5 g (females). There were 6 mice in every experimental group.

In both experiments, trunk blood was collected after decapitation to measure FGF21 concentrations and samples of liver, brown and white abdominal tissues were collected to measure gene expression. Plasma concentrations of FGF21 were measured with Rat/Mouse Fibroblast Growth Factor ELISA Kit (EMD Millipore Corporation, Billerica, MA, USA).

### *Relative-Quantitation Real-Time PCR*

Total RNA was isolated from tissue samples with ExtractRNA (Evrogen, Moscow, Russia) according to the manufacturer's instructions. The first-strand cDNA was synthesized with Moloney Murine Leukemia Virus

(MMLV) reverse transcriptase (Evrogen, Moscow, Russia) and oligo(dT) as a primer. Applied Biosystems TaqMan gene expression assays (*Fgf21*, Mm00840165_g1; KLB, Mm00473122_m1; Pparg, Mm00440940_m1; PPARa, Mm0040939_m1; Ppargc1, Mm01208835_m1) with β-actin as an endogenous control (TaqMan endogenous controls with FAM dye label and MGB mouse β-actin (ACTB)) and qPCRmix-HS LowROX Master Mix (Evrogen, Moscow, Russia) were used for relative quantitation real-time PCR. Sequence amplification and fluorescence detection were performed with the Applied Biosystems ViiA™ 7 Real-Time PCR System. Relative quantitation was performed by the comparative CT method, where CT the is threshold cycle.

### *Statistical Analysis*

The results are presented as the means ± SE from the indicated number of mice. Two-way ANOVA was used with the factors "sex" and "experimental group" (control, fasting, refeeding or SD and DIO) with multiple comparisons using *post hoc* Fisher's LSD test. Significance was determined as P<0.05.

## Results

### *Fasting and Refeeding Induced Changes in the Expression of Genes Related to FGF21 Signaling*

The fasting-induced increase in circulating FGF21 levels was more pronounced in females (Fig. 1A), which was reversed by refeeding in the mice of both sexes. Large statistical error observed in plasma FGF21 levels in females under fasting conditions was not caused by small sampling size. Perhaps increased plasma FGF21 level heterogeneity was due to the influence of physiological factors, such as the estrous cycle, which was not taken into account in this study.

In the liver, fasting increased and refeeding normalized the expression of all genes related to FGF21 signaling regardless of sex (Fig. 2A, 2C, 2E, 2G). The sex influenced only the expression of hepatic Pparα and *Fgf21* genes (Fig. 2A, 2C) and the expression was higher in females, than in males. The fasting-induced upregulation in hepatic *Fgf21* mRNA levels was biased toward females (Fig. 2C). In WAT, fasting and refeeding did not change the expression of the Pparα, Pgc-1α and Klb genes in mice of either sex (Fig. 3A, 3G, 3I). WAT Pparγ mRNA levels were lower in females than in males and fasting decreased and refeeding reversed them to normal in the mice of both sexes. Refeeding sharply upregulated WAT *Fgf21* gene expression only in females (Fig. 3E). In BAT, fasting did not change and refeeding differentially changed the expression *Pparα* and *Fgf21* genes: *Pparα* mRNA level was downregulated (Fig. 4A) and *Fgf21* mRNA level – upregulated in refed mice of both sexes (Fig. 4E). The refeeding-induced increase in the BAT *Fgf21* gene expression was more pronounced in females (Fig. 4E). Fasting increased and refeeding normalized the BAT *Pgc*-1α gene expression equally in male and female mice (Fig. 4I).

Together, these results demonstrated the impact of sex on the transcriptional response of metabolic organs to fasting/refeeding. Fasting-induced increases in hepatic *Fgf21* gene expression and circulating FGF21 levels, as well as refeeding-induced increases in local *Fgf21* gene expression in adipose tissues, were biased toward females.

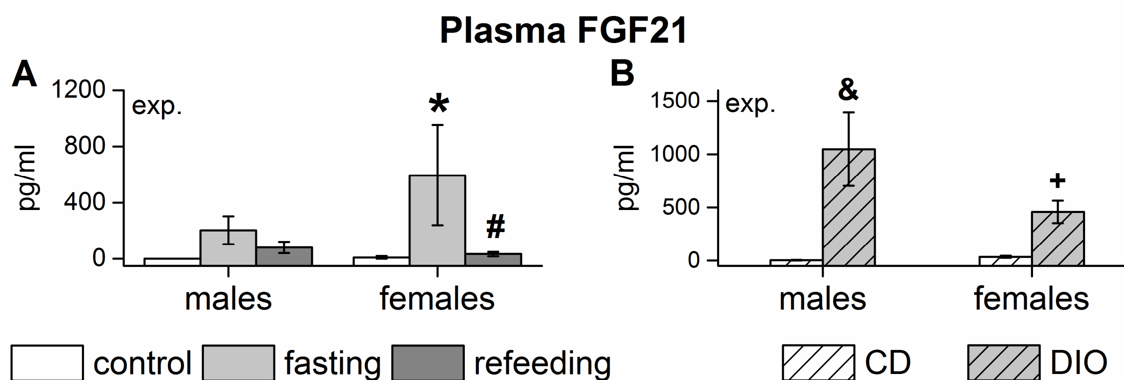


**Fig. 1:** Plasma FGF21 levels in mice under different feeding conditions: *ad libitum* feeding state, 24 h fasting, 6 h refeeding following 24 h fasting and in mice that consumed for 10 weeks a standard show diet (CD) or palatable food which caused Diet-Induced Obesity (DIO). The results are presented as the means ± SE (n = 6-7 mice per group). Significance was determined as P<0.05. Exp, experimental group effect. *P<0.05 – versus control group, #P<0.05 – versus fasting group, &P <0.05 - versus CD group, +P<0.05 - versus males in the same group

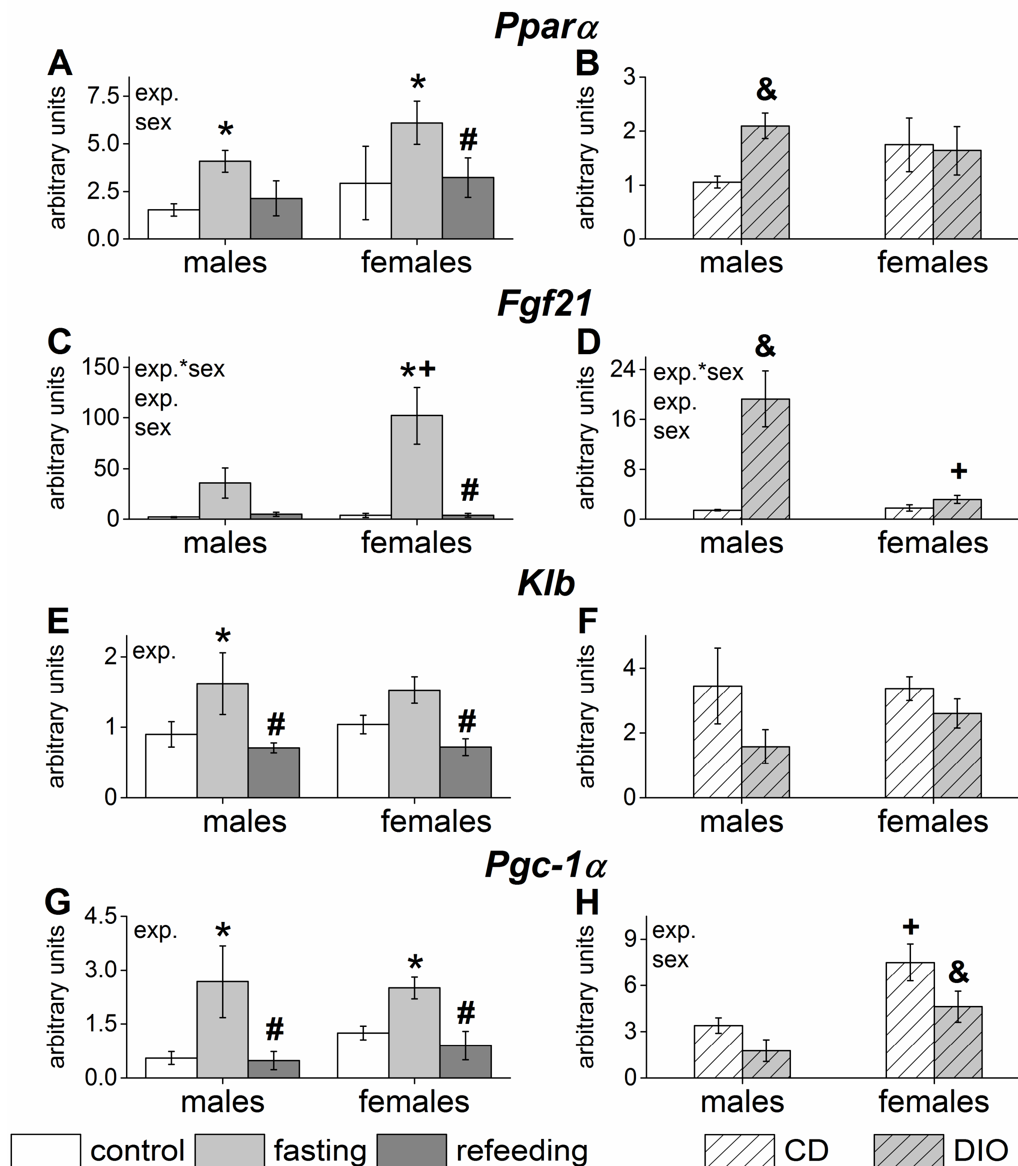


**Fig. 2:** Expression of genes related to FGF21 signaling in the liver of mice under different feeding conditions: *ad libitum* feeding state, 24 h fasting, 6 h refeeding following 24 h fasting and in mice that consumed for 10 weeks a standard show diet (CD) or palatable food which caused Diet-Induced Obesity (DIO). The results are presented as the means ± SE (n = 6-7 mice per group). Two-way ANOVA was used with the factors "sex" and "experimental group" (control, fasting, refeeding, or CD and DIO) with multiple comparisons using *post hoc* Fisher's LSD test. Significance was determined as P<0.05. Sex, sex effect; Exp, experimental group effect; and Sex*Exp, sex and experimental group interactive effect. *P<0.05 – versus control group, #P<0.05 – versus fasting group, $^{\&}$P <0.05 - versus CD group, $^{\&s}$P<0.05 – versus CD group by Student's t-test, +P <0.05 - versus males in the same group

### *DIO-Induced Changes in the Expression of Genes Related to FGF21 Signaling*

In males, DIO caused a significant 1000-fold increase in the FGF21 blood level (Fig. 1B), which could reflect a 16-fold increase in hepatic *Fgf21* mRNA levels (Fig. 2D). In the liver, expression of only two genes was dependent on sex: *Fgf21* mRNA levels were lower and *Pgc*-1α mRNA levels were higher in females than in males (Fig. 2D, 2H). In males, unlike females, DIO upregulated *Fgf21* and *Pparα* gene expressions (Fig. 2B, 2D). In females, DIO only reduced hepatic *Pgc-1α* gene expression (Fig. 2H). In WAT, DIO increased *Fgf21* gene expression (Fig. 3F), decreased *Pparγ* gene expression (Fig. 3D) and did not change the expression of other genes related to FGF21 signaling (Fig. 3B, 3H, 3J) in mice of both sexes.

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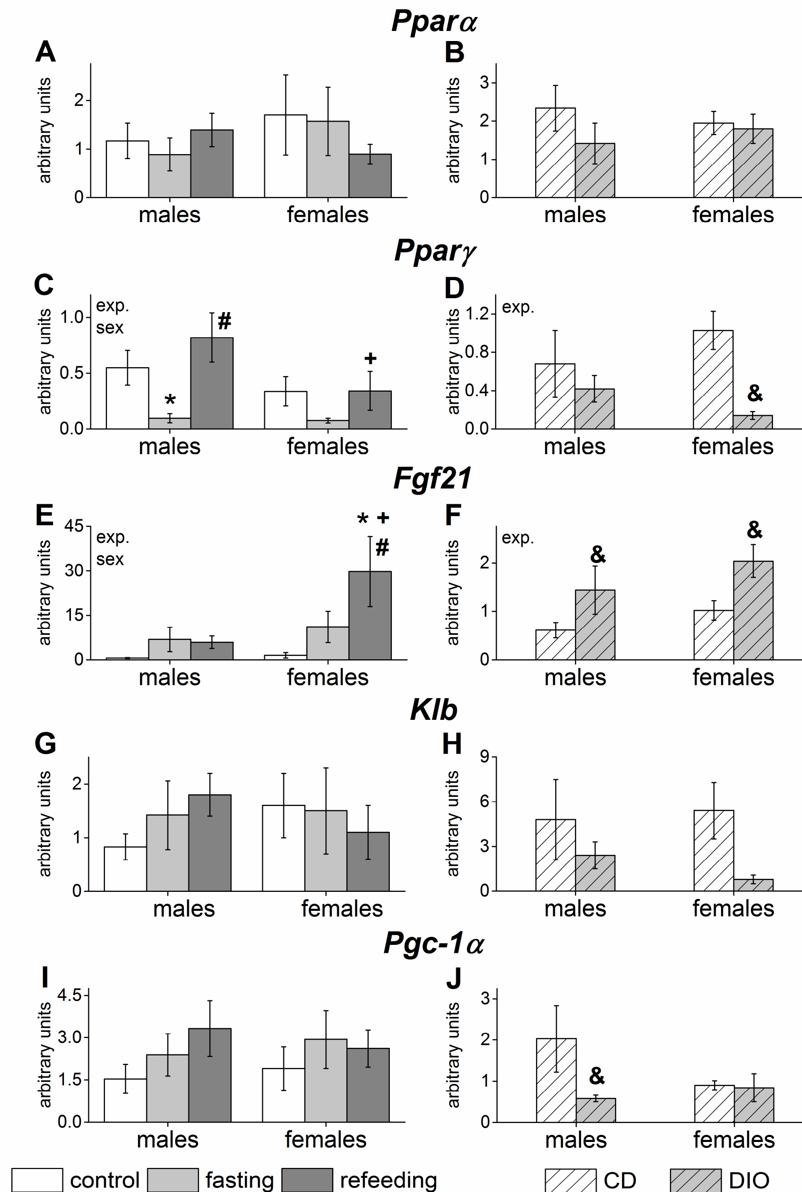


**Fig. 3:** Expression of genes related to the FGF21 signaling in white adipose tissue of mice under different feeding conditions: *ad libitum* feeding state, 24 h fasting, 6 h refeeding following 24 h fasting and in mice that consumed for 10 weeks a standard show diet (CD) or palatable food which caused Diet-Induced Obesity (DIO). The results are presented as the means ± SE (n = 6-7 mice per group). Two-way ANOVA was used with the factors “sex” and “experimental group” (control, fasting and refeeding or CD and DIO) with multiple comparisons using *post hoc* Fisher’s LSD test. Significance was determined as P<0.05. Sex, sex effect; Exp, experimental group effect. *P<0.05 – versus control group, #P<0.05 – versus fasting group, &P <0.05 – versus CD group, +P <0.05 – versus males in the same group.

In BAT, DIO had differently and sex specific effects on Pparγ and *Fgf21* gene expressions: Pparγ expression was downregulated (Fig. 4D) in only males and *Fgf21* gene expression was upregulated (Fig. 4F) in males more pronouncedly than in females. As a result, *Pparγ* mRNA levels were higher and *Fgf21* mRNA levels were lower in DIO females than in DIO males.DIO downregulated *Klb* gene expression regardless of sex (Fig. 4H). In addition, BAT *Pgc-1α* mRNA level was higher in females than males (Fig. 4J).

Together, these results demonstrated the impact of sex on the transcriptional response of the liver and BAT to obesity. DIO-induced increases in circulating FGF21 levels, as well as in *Fgf21* gene expression, in the liver and BAT were more pronounced in males than in females.

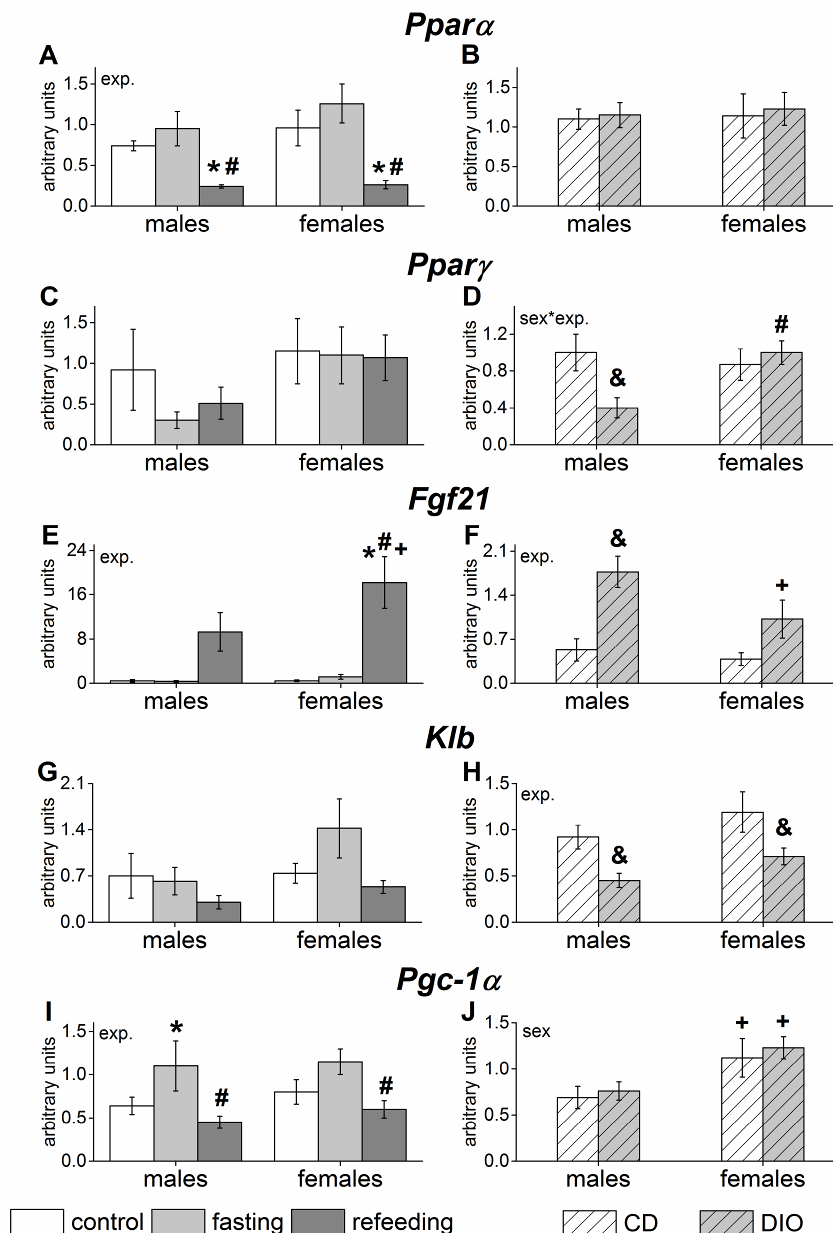


**Fig. 4:** Expression of genes related to the FGF21 signaling in brown adipose tissue of mice under different feeding conditions: *ad libitum* feeding state, 24 h fasting, 6 h refeeding following 24 h fasting and in mice that consumed for 10 weeks a standard show diet (CD) or palatable food which caused Diet-Induced Obesity (DIO). The results are presented as the means ± SE (n = 6-7 mice per group). Two-way ANOVA was used with the factors "sex" and "experimental group" (control, fasting, refeeding or CD and DIO) with multiple comparisons using *post hoc* Fisher's LSD test. Significance was determined as P<0.05. Sex, sex effect; Exp, experimental group effect; and Sex*Exp, sex and experimental group interactive effect. *P<0.05 – versus control group, #P<0.05 – versus fasting group, $^{\&}$P<0.05 – versus CD group, $^{+}$P<0.05 – versus males in the same group

## Discussion

Previously, a sex-specific activation of *Fgf21* expression was observed in humans and animals with various metabolic diseases (Wang *et al*., 2017; Chukijrungroat *et al*., 2017; Lee *et al*., 2016; Gasparin *et al*., 2018). Our results are in line with data from Gasparin and coauthors (Gasparin *et al*., 2018), who found that DIO increased *Fgf21* mRNA expression in the liver, WAT and BAT in male, but not female mice. The new finding of our study is demonstration of a female-specific activation of FGF21 signaling in response to the natural

physiological states fasting and refeeding. Hence, manifestation of sex dimorphism in the *Fgf21* expression depended on the metabolic conditions.

Both fasting and obesity are characterized by increased levels of circulating free fatty acids, which can activate nuclear PPARα in the liver, leading to upregulation of *Fgf21* (Mai *et al*., 2009). It can be assumed that one of the reasons for sex-specific hepatic *Fgf21* upregulation under fasting and obesity was sex-specific *Pparα* gene transcription. In the fasting/refeeding experiment, hepatic *Pparα* mRNA levels were higher in females than in males. This is in accordance with a study showing a higher expression of hepatic PPARα protein in female than in male mice (Lu *et al*., 2013). In the DIO experiment, *Pparα* expression was activated only in obese males. We speculate that the molecular mechanism of the sex-specific expression of *Pparα* gene in the liver may include the effects of estrogens. There is evidence that the effect of estrogens on hepatic *Pparα* gene expression differed in obese and lean rats. In young 8-week-old rats, estrogen deficiency associated with ovariectomy decreased and estrogen administration increased Pparα gene expression in the liver (Paquette *et al*., 2013). This can explain the fact that in the fasting/refeeding experiment, the hepatic *Pparα* expression in females was higher than in males. In contrast, in obese mice, estrogen administration downregulated hepatic *Pparα* gene expression (Jeong and Yoon, 2007). We propose that the lack of DIO-activating influence on hepatic *Pparα* gene expression was due to estrogen actions in female mice.

Sex-dependent regulation of *Fgf21* expression in different metabolic conditions seems not to be restricted to the liver. Previously, upregulation of *Fgf21* expression in response to refeeding was shown in the WAT in male mice (Dutchak *et al*., 2010). We found that refeeding also induced *Fgf21* expression in BAT and we revealed for the first time that the refeeding-induced activation of *Fgf21* gene expression was more pronounced in females than in males in both WAT and BAT. Mechanisms of the refeeding-induced upregulation of WAT and BAT *Fgf21* gene expression in females obviously were not associated with PPARs. In WAT, refeeding did not influence the *Pparα* and *Pparγ* mRNA levels. In BAT, refeeding reduced the control and fasting Pparα mRNA levels and did not change Pparγ mRNA levels. Adipose tissues are tightly controlled by adrenergic hormones, primarily through the direct sympathetic-neuronal release of norepinephrine and epinephrine onto brown (Cannon and Nedergaard, 2004) and white adipocytes (Wang *et al*., 2008). Refeeding increases norepinephrine turnover in WAT and BAT (Young *et al*., 1982). Hondares and coauthors (Hondares *et al*., 2008) demonstrated that norepinephrine acts through β-adrenergic receptors to increase *Fgf21* gene expression and PPARα and PPARγ are not required for this action. We propose that in female mice, the predominant activation of β adrenergic receptors could lead to the upregulation of local WAT and BAT *Fgf21* expression at refeeding. Exact mechanisms underlying the sex differences in the WAT and BAT transcriptional responses to refeeding and the physiological significance of the increased WAT and BAT *Fgf21* expression deserve further investigation.

The DIO-induced change in BAT *Fgf21* mRNA expression was similar to that found in the liver. In contrast to refeeding, obesity upregulated BAT *Fgf21* expression in males more than in females. These results are in agreement with the data reported by Gasparin and coauthors (Gasparin *et al*., 2018), who observed that obesity increased the BAT and WAT *Fgf21* mRNA expression in male but not female mice. The mechanisms of the predominant increase of BAT *Fgf21* mRNA expression in the DIO males obviously were not connected with PPARs; DIO did not alter BAT *Pparα* gene expression and reduced BAT *Pparγ* gene expression in male mice. At present, the mechanisms underlying the activation of BAT *Fgf21* gene expression in obesity and the role of sex hormones in this regulation are not well known.

There are some limitations in our study. First, FGF21 realizes its beneficial effect by regulating the expression of many target genes in the liver and adipose tissues and only expression of genes related to FGF21-signaling were measured in this study. Second, our observations are solely based on mice models. Since there is a difference in carbohydrate-lipid metabolism between rodents and humans, the physiological relevance of our findings remains to be confirmed in human studies.

## Conclusion

The study was addressed to the impact of sex in expression of gene encoding FGF21. The new finding of our study is demonstration of a female-specific activation of FGF21 signaling in response to the natural physiological states fasting and refeeding. The study showed that the fasting-induced increases in hepatic *Fgf21* gene expression and circulating FGF21 levels, as well as refeeding-induced increases in local WAT and BAT *Fgf21* gene expression, were biased toward females, while obesity-induced increase in circulating FGF21 levels, as well as in *Fgf21* gene expression in the liver and BAT, were biased toward males. Hence, sex dimorphism in the *Fgf21* expression depended on the metabolic conditions. Considering that *FGF21* is a novel metabolic regulator of energy homeostasis, sex differences in the responses to anabolic and catabolic stimulus could have translational implications for novel therapeutic approaches for type 2 diabetes treatment.

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## Author's Contributions

**Nadezhda Bazhan:** Contributed considerably to conception and design of research, to the writing of the manuscript.

**Tatiana Jakovleva:** Contributed to acquisition, analysis and interpretation of data.

**Natalia Balyibina:** Contributed to acquisition of data.

**Anastasia Dubinina:** Contributed in drafting the article, prepared figures.

**Elena Denisova:** Contributed to acquisition of data.

**Natalia Feofanova:** Contributed to acquisition of and analysis of data.

**Elena Makarova:** Contributed to conception and design of research to analysis and interpretation of data.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved final version of manuscript and there are no ethical issues involved.

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