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ORIGINAL ARTICLE

Opposite alterations in FGF21 and FGF19 levels and disturbed expression of the receptor machinery for endocrine FGFs in obese patients

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OBJECTIVE: Fibroblast growth factor (FGF)-21, and possibly FGF19, protect against type 2 diabetes mellitus (T2DM) and obesity in rodents. We investigated the circulating levels of FGF21 and FGF19 in obese patients with varying degrees of abnormal glucose homeostasis, and we determined gene expression for FGF receptors (FGFR1–4) and the co-receptor β-Klotho, in liver and adipose tissues.

SUBJECTS AND METHODS: We analyzed 35 lean healthy (71% men) and 61 obese patients (49% men, median body mass index (BMI): 40.5 kg m^{-2}, interquartile range: 34.7–46.2). Among obese patients, 36 were normoglycemic, 15 showed impaired glucose tolerance and 10 had T2DM. Biopsies from liver and visceral and subcutaneous fat from a subset of obese patients and controls were analyzed. FGF19 and FGF21 levels were measured using enzyme-linked immunosorbent assay, and tissue mRNA and protein levels by reverse transcription-polymerase chain reaction and immunoblotting.

RESULTS: FGF21 serum levels were significantly increased in obese patients compared with controls ($P < 0.001$), whereas FGF19 levels were decreased ($P < 0.001$). FGF21 levels were positively correlated with homeostasis model assessment of insulin resistance ($P = 0.0002$, $r = 0.37$) and insulin ($P = 0.001$, $r = 0.32$), whereas FGF19 levels were negatively correlated ($P = 0.007$, $r = -0.27$; $P = 0.003$, $r = -0.28$; respectively). After adjusting for BMI, the correlations of FGF21 and FGF19 levels with indicators of abnormal glucose homeostasis were not significant. In obese patients, the hepatic expression of FGF21 was increased. ($P = 0.04$). β-Klotho transcript levels in visceral fat ($P = 0.002$) and β-Klotho protein levels in subcutaneous ($P = 0.03$) and visceral fat ($P = 0.04$) were significantly reduced in obese patients, whereas hepatic levels for β-Klotho ($P = 0.03$), FGFR1 ($P = 0.04$) and FGFR3 ($P = 0.001$) transcripts were significantly increased.

CONCLUSIONS: Obesity is characterized by reciprocal alterations in FGF19 (decrease) and FGF21 (increase) levels. Although worsened in diabetic obese patients, obesity itself appears as the predominant determinant of the abnormalities in FGF21 and FGF19 levels. Opposite changes in β-Klotho expression in fat and liver indicate potential tissue-specific alterations in the responsiveness to endocrine FGFs in obesity.

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INTRODUCTION

Obesity is becoming one of the most important health problems of developed countries. It is a strong risk factor for the development of impaired glucose tolerance (IGT), which may lead to type 2 diabetes mellitus (T2DM). Increased body weight is also linked to dyslipidemia and hypertension, and the overall pattern of altered metabolic homeostasis that defines the metabolic syndrome. Complex metabolic and endocrine alterations, involving disturbed cross talk among the organs implicated in metabolic homeostasis, including adipose tissues, liver and/or pancreas, contribute to the development of the metabolic syndrome in obese patients. However, a comprehensive knowledge of the precise pathophysiological mechanisms by which obesity promotes the metabolic syndrome has not yet been achieved.

In recent years, the role of fibroblast growth factor-21 (FGF21) and FGF19, members of the so-called endocrine or atypical FGF sub-family, has been recognized as important for the control of glucose and lipid metabolism.[1,2] In rodents, FGF21 is mainly expressed and released by the liver, although other tissues can express substantially FGF21;[3] in humans a main hepatic origin of FGF21 is assumed.[4] Hepatic FGF21 is expressed and released in response to high fatty acid availability,[5] and FGF21 gene expression is regulated by PPARα-mediated transcriptional activation.[6] Obese rodents show high levels of circulating FGF21, but pharmacological administration or ectopic overexpression of FGF21 in obese and diabetic rodent models restores normal plasma glucose and triglycerides levels and improves insulin sensitivity regardless of body weight and adiposity.[1,7] Multiple studies indicate that FGF21 can even reduce body weight in rodent models of obesity.[8–10] Studies in humans have consistently shown a positive correlation between circulating FGF21 levels and body mass index (BMI), insulin levels, and glycemia;[11,12] on the basis of rodent studies and analysis of intracellular signaling pathways, some authors have proposed that obesity may be an FGF21-resistant state.[13] Moreover, FGF21 levels are usually found to correlate positively with insulin-resistant states in obese[11] and non-obese[14] patients. However, recent data indicated that

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treatment with a FGF21 analog lowers triglyceridemia and promotes body weight loss in obese patients.[15] It suggests that, as in obese rodents, the high basal levels of FGF21 in obese humans do not preclude responsiveness to exogenous FGF21, and the concept of FGF21 resistance in obesity remains controversial. It has recently been proposed that a rise in FGF21 serum levels could be a predictor of development of the metabolic syndrome and T2DM.[16] Several reports have also indicated that FGF21 levels are increased in patients with non-alcoholic fatty liver disease and correlate with markers of hepatic stress.[4] In contrast, FGF19 is secreted mainly by the ileum and its primary action appears to be the control of the hepatic biosynthesis of bile acids.[17] Apart from this, it has been reported that treatment with FGF19 or its transgenic overexpression in mice favors a healthy metabolic profile, with lowered serum glucose, triglycerides and cholesterol levels, and also improves insulin sensitivity and reduces body weight in high-fat diet-induced obese mice.[2,18] Some studies have reported that FGF19 has strong, insulin-like effects in the liver,[19,20] whereas other data suggested that most of the effects of FGF19 on metabolism occur through tissues other than the liver, probably adipose tissue.[21] Therefore, it appears that both FGF19 and FGF21 show an extensive overlap of metabolic actions. Data on FGF19 status in humans in relation to obesity, T2DM and the metabolic syndrome are scarce. Decreased FGF19 has been reported in obese and insulin-resistant patients,[22] and there are indications of a recovery of FGF19 levels to normality in obese patients after Roux-en-Y gastric bypass that could contribute to amelioration of diabetes.[23,24]

Most likely, some of the overlapping metabolic effects of FGF21 and FGF19 are attributable to their largely common mechanisms of action. FGF receptors (FGFRs), mainly FGFR4, which is expressed in liver, and FGFR1, which is predominantly expressed in adipose tissues,[25,26] are considered the primary mediators of the metabolic effects of FGF21 and FGF19, although it has been reported that FGFR2 and FGFR3 can also mediate FGF19 and FGF21 effects.[21,27] However, for both endocrine FGFs, the interaction of the FGFRs with the protein β-Klotho, as an obligatory co-receptor, is absolutely required for the induction of cellular responses.[27–29] Some studies have proposed that hepatic FGFR4 is the major mediator of FGF19, but not FGF21, effects, whereas FGFR1 has been reported to show a higher affinity for FGF21.[27] However, there is no general agreement that such preferential FGFR isoform-mediated effects for FGF21 and FGF19 exists, and several reports indicate that the liver, despite expressing preferentially FGFR4, may be a target of FGF21 action.[30,31] Moreover, data indicating complex alterations in the responsiveness of transgenic mice with adipose-specific knockout of FGFR1c to FGF21 and FGF19[32] suggest that some of the differences in the effects of FGF21 and FGF19 could be attributed to different sites of action as well as to different receptor affinities.

To gain insight into the specific role of dysregulation of the FGF21/FGF19 endocrine system in relation to obesity and/or the development of T2DM, we analyzed the FGF21 and FGF19 levels and the expression of molecular actors for FGF21/19 responsiveness (FGFRs and β-Klotho) in hepatic and adipose tissue from obese patients exhibiting distinct alterations in glucose homeostasis (NG, glucose intolerance, overt T2DM) and compared them with those of healthy controls.

MATERIALS AND METHODS

A total of 96 Caucasian subjects were recruited from lean healthy volunteers ($n = 35$, 25 male, 10 female) and obese patients ($n = 61$, 30 male, 31 female) seen by the Departments of Endocrinology and Surgery at the Clínica Universidad de Navarra (Pamplona, Spain) and Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The study was approved, from an ethical and scientific standpoint, by the hospital's ethical committees responsible for research, and written informed consent of participants was obtained. Patients underwent a clinical assessment, including a medical history, physical examination, body composition analysis and comorbidity evaluation. Obesity was classified as BMI $\geqslant 30\ kg\ m^{-2}$, whereas lean controls were $\leqslant 25\ kg\ m^{-2}$. Body fat was estimated by air-displacement plethysmography (Bod-Pod; Life Measurements, Concord, CA, USA).[33] Obese patients were further subclassified according to three established diagnostic thresholds for diabetes: (1) normoglycemia (NG; $n = 36$), defined as fasting plasma glucose $\leqslant 100\ mg\ dl^{-1}$ and plasma glucose $\leqslant 140\ mg\ dl^{-1}$ 2 h after an oral glucose tolerance test; (2) IGT; $n = 15$, defined as fasting plasma glucose between 100 and $125\ mg\ dl^{-1}$ or plasma glucose between 140 and $199\ mg\ dl^{-1}$ 2 h after an oral glucose tolerance test; and (3) T2DM ($n = 10$), defined as fasting plasma glucose $\geqslant 126\ mg\ dl^{-1}$ or plasma glucose $\geqslant 200\ mg\ dl^{-1}$ 2 h after an oral glucose tolerance test.[34] T2DM subjects were not receiving insulin therapy, or PPARγ agonists or other

Table 1. Demographic, anthropometric and metabolic parameters

		OBESE (OB) (n=*61*)									
	Lean Control (C) (n = *35*)	*NG* (n = *36*)	P *vs* C	*IGT* (n = *15*)	P *vs* C	P *vs NG*	*T2DM* (n = *10*)	P *vs* C	P *vs NG*	P *vs IGT*	P C vs *OB*
Sex (*n* of men (%))	25 (71.4)	18 (50)	0.089	7 (46.6)	0.117	0.989	5 (50)	0.263	0.979	0.998	0.063
Age	42.9+1.1	37.6+2.3	0.079	47.5+2.9	0.081	0.053	42.2+2.4	0.757	0.332	0.232	0.427
BMI	23.9+0.5	39.2+1.2	< **0.001**	42.9+1.9	< **0.001**	0.100	46.4+3.6	< **0.001**	**0.028**	0.351	< **0.001**
WHR	0.90+0.01	0.97+0.02	< **0.001**	1.05+0.02	< **0.001**	0.495	1.01+0.02	**0.001**	0.738	0.783	< **0.001**
Total body fat (%)	24.7+0.8	47.2+1.3	< **0.001**	50.3+2.4	< **0.001**	0.236	51.2+2.4	< **0.001**	0.179	0.801	< **0.001**
Total cholesterol ($mg\ dl^{-1}$)	189.5+6.1	184.4+7.8	0.384	211.4+9.1	0.090	0.062	189.4+11.4	0.796	0.893	0.169	0.930
Triglycerides ($mg\ dl^{-1}$)	85.5+6.9	106.3+7.1	**0.003**	136.8+24.4	**0.002**	0.120	132.4+16.6	< **0.001**	0.142	0.909	**0.001**
HDL cholesterol ($mg\ dl^{-1}$)	54.2+2.4	50.1+3.3	0.314	49.5+3.1	0.259	0.908	46.3+3.7	0.162	0.614	0.546	0.161
LDL cholesterol ($mg\ dl^{-1}$)	177.3+6.7	112.6+6	0.418	129.6+9	0.429	0.132	117.4+9.8	0.866	0.735	0.417	0.755
Glucose ($mg\ dl^{-1}$)	87.1+1.2	90.2+1.6	0.061	104.3+3.8	< **0.001**	< **0.001**	119.9+9.3	< **0.001**	< **0.001**	0.080	< **0.001**
Insulin ($\mu U\ ml^{-1}$)	5.8+0.6	15.7+1.6	**0.003**	18.8+1.5	< **0.001**	0.255	21.7+3	< **0.001**	0.156	0.347	< **0.001**
HOMA-R	1.5+0.2	3.3+0.3	**0.004**	4.9+0.5	< **0.001**	**0.028**	6+0.8	< **0.001**	**0.004**	0.220	< **0.001**
SBP (mmHg)	118.8+2.6	120.6+2	0.585	133+4.4	**0.006**	**0.005**	134.8+6.4	**0.013**	**0.008**	0.819	**0.036**
DBP (mmHg)	70.3+1.8	77.1+1.4	**0.004**	82.1+2.3	< **0.001**	0.059	85.1+3.6	< **0.001**	**0.018**	0.463	< **0.001**
AST ($U\ L^{-1}$)	14.3+7.7	17.6+2.3	0.197	18.3+2.7	0.086	0.870	16.7+1.6	0.386	0.581	0.409	0.387
ALT ($U\ L^{-1}$)	16.6+8.5	25.9+3.3	0.114	27.5+4.4	0.314	0.784	25.7+5.5	0.334	0.976	0.809	0.078
GGT ($U\ L^{-1}$)	13.8+6.3	21.8+2.4	0.592	33.1+12.1	**0.024**	0.155	32.8+8	< **0.001**	0.169	0.523	**0.001**

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure.GGT, y-glutamyl transferase; HOMA-R, homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; LDL, low-density lipoprotein; NG, normoglycemic; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; WHR, waist-to-hip ratio . Parameters are expressed as mean+s.e.m. unless especified. *P*-values were calculated using one-way analysis of variance or Kruskal–Wallis tests, with Tukey *post hoc* adjustments. Bold lettering is shown when $P < 0.05$.

medication likely to influence endogenous insulin levels. Patient demographics and anthropometric and metabolic data are shown in Table 1.

Serum was obtained from blood drawn from seated patients after a 12-h overnight fast and at least 15 min after the placement of a peripheral intravenous catheter. Glucose, insulin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase (GGT), were measured as previously described.[35–38] Serum FGF19 and FGF21 levels were determined using non-cross-reactive enzyme-linked immunosorbent assays specific for the corresponding human proteins (Biovendor, Brno, Czech Republic). Serum FGF21 levels showed a skewed distribution in obese patients and lean healthy control population, and were thus log-transformed before analysis, as described previously.[39]

Biopsy samples of liver, subcutaneous fat from the abdominal area and omental fat from lean healthy controls (liver, $n=5$; subcutaneous fat, $n=7$; and visceral fat, $n=6$) and obese individuals (liver, $n=61$; subcutaneous fat, $n=16$; visceral fat, $n=61$) were collected from patients undergoing either liver biopsies (when hepatic tumor was suspected and the result was negative, in the case of lean controls), Nissen fundoplication (for hiatal hernia repair; also for lean volunteers) or prior to Roux-en-Y gastric bypass (for treatment of morbidly obese subjects). Tissue samples were immediately frozen and stored at −80 °C. Total RNA was isolated using a column-affinity-based methodology that included on-column DNA digestion (RNeasy; Qiagen, Hilden, Germany). One microgram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers (Applied Biosystems, Foster City, CA, USA). TaqMan reverse transcription-polymerase chain reaction was performed using the ABI PRISM-7700HT sequence detection system (Applied Biosystems). The reverse transcription-polymerase chain reactions were performed in a final volume of 25 µl using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent, and primer pair/probe sets specific for FGF21 (Hs00173927_m1), FGF19 (Hs00192780_m1), FGFR1 (Hs00222484_m1), FGFR2 (Hs01552918_m1), FGFR3 (Hs00179828_m1), β-Klotho (Hs00545621_m1), FGFR4 (Hs01106908_m1), CEBPα (Hs00269972_m1) and 18 S rRNA (Hs99999901). Controls with no RNA, primers or RT were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA levels for the genes of interest. Expression levels of gene transcripts were considered negligible when, under the above reverse transcription-polymerase chain reaction conditions, cycle threshold was $\geqslant 40$. Values were normalized to that of the reference control (18 S ribosomal RNA)[40] using the comparative $2^{-\Delta CT}$ method, following the manufacturer's instructions. Parallel calculations performed using the reference gene PPIA (Hs99999904) yielded essentially the same results.

For quantification of protein levels, adipose tissue samples were homogenized in cold buffer (10 mM HEPES pH 7.5, 5 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$) and a cocktail of protease inhibitors (Complete-mini; Roche, Basel, Switzerland). For western blot analysis, homogenates containing 40 µg of protein were mixed with equal volumes of 2x sodium dodecyl sulfate-loading buffer, incubated at 90 °C for 5 min, and electrophoresed on sodium dodecyl sulfate/polyacrylamide gels. After transferring to Immobilon-P membranes (Millipore, Billerica, MA, USA), proteins were probed using antibodies against β-Klotho (Abcam, Cambridge, UK), Phospho-ERK1/2 and total ERK1/2 (Cell Signaling Technology, Danvers, MA, USA). Goat anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescence (ECL) reagents (Immobilon; Millipore) were used to detect immunoreactive proteins. Membranes were stained with Coomassie blue (Sigma-Aldrich, St Louis, MO, USA) to normalize the amount of protein loaded. Multi-Gauge software (Fujifilm, Japan) was used for densitometric analyses.

Data were expressed as means ± s.e.m. or percentages. The normality of parameter distributions was determined using a Kolmogorov–Smirnov analysis. Where appropriate, statistical analyses were performed using Kruskal–Wallis test or one-way analysis of variance with pairwise Tukey *post hoc* adjustements. Correlation analysis was used to determine the linear relationships between anthropometric, and metabolic variables and serum FGF19 and FGF21 quantitative parameters. Statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, IL, USA) and the SAS version 9.1.3 software (SAS Institute, Cary, NC, USA), *P*-values < 0.05 (determined by two-sided tests) were considered significant.

RESULTS

A total of 96 individuals were analyzed in this study; 35 were healthy lean individuals and 61 were obese patients subclassified according to their glycemic status, as described in Materials and Methods. Table 1 summarizes anthropometric and biochemical characteristics of the studied population. In accord with the study design, obese individuals had higher levels of obesity descriptors, such as BMI, waist-to-hip ratio and percentage of total body fat than lean individuals ($P < 0.001$ in all cases). No significant differences were found for sex, age, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, aspartate aminotransferase or alanine aminotransferase among different groups. A comparison of the overall obese patient population with lean healthy controls showed an elevation of the usual metabolic syndrome descriptors, including triglycerides ($P=0.001$), glucose ($P < 0.001$), the homeostatic model assessment of insulin resistance (HOMA-R; $P < 0.001$) and insulin ($P < 0.001$), in the obese group. Also in accord with the study design, obese patients with impaired insulin tolerance or T2DM showed higher glucose ($P < 0.001$ both cases) and HOMA-R levels ($P=0.028$ and $P=0.004$, respectively) than NG obese individuals. In the overall obese population, systolic blood pressure and diastolic blood pressure were significantly elevated relative to healthy controls ($P=0.036$ and $P < 0.001$, respectively).

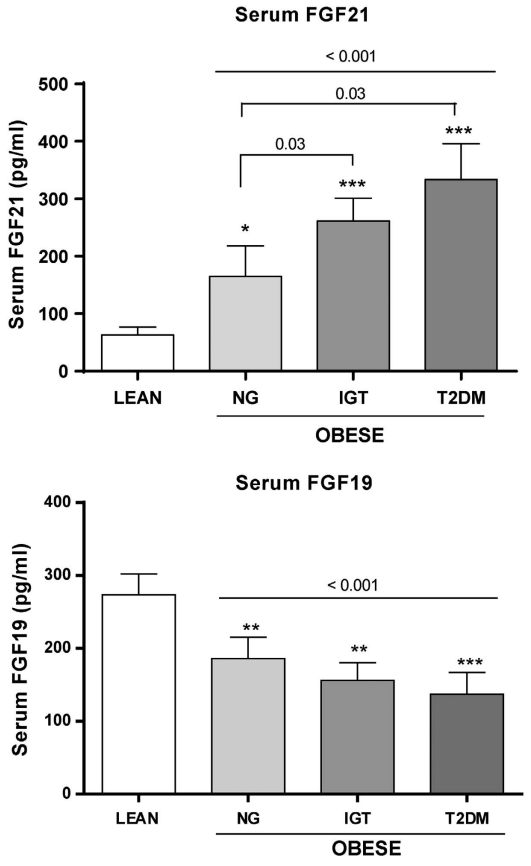

Figure 1. Serum levels of FGF19 and FGF21 in lean healthy controls and obese individuals grouped according to their glycemic state. NG, normoglycemic; IGT impaired glucose tolerance; and T2DM, type 2 diabetes mellitus. Serum levels (pg ml^{-1}) of FGF21 are log-transformed. Data are shown as means ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparison between obese sub-groups and controls. *P*-values are shown in cases where comparisons between the overall obese population and lean controls or between different obese sub-groups are significant.

Among parameters unrelated to glycemic status, GGT levels were higher in the overall group of obese patients compared with those in healthy controls ($P = 0.001$); among patient sub-groups, IGT and T2DM patients had significantly higher GGT levels than controls ($P = 0.024$ and $P < 0.001$, respectively) (Table 1).

In the total study population, including obese patients and lean individuals, serum FGF21 levels ranged from 10 to 633 pg ml^{-1} and serum FGF19 levels ranged from 29 to 427 pg ml^{-1}. There were no significant differences in serum FGF19 ($P = 0.68$) or serum FGF21 ($P = 0.81$) levels between men and women. Serum FGF21 levels were significantly higher in the overall obese patient population than in lean healthy controls ($P < 0.001$). All obese patient sub-groups had significantly higher FGF21 levels than controls regardless of their glycemic status; however, obese patients with compromised glycaemia (IGT and T2DM) showed a significant increase in serum FGF21 compared with obese NG patients ($P = 0.03$ for both comparisons) (Figure 1). Regardless of glycemic status, serum FGF19 levels were decreased in all obese patient groups compared with lean healthy controls ($P < 0.001$) (Figure 1).

We investigated the relationship between serum FGF21 and FGF19 levels and several anthropometric and biochemical parameters using correlation analysis (Table 2). When the overall population was considered, including obese and lean individuals, FGF21 levels positively correlated with BMI, waist-to-hip ratio, percentage of total body fat, triglycerides, glucose, insulin, HOMA-R, systolic blood pressure, diastolic blood pressure and GGT. These positive correlations disappeared after adjusting for BMI. When adjusted by HOMA-R, a significant, positive, correlation between FGF21 and percentage of total body fat ($P = 0.03$, $r = 0.24$) and BMI ($P = 0.004$, $r = 0.34$) in the overall population remained. In contrast, FGF19 serum levels negatively correlated with BMI, percentage of total body fat, insulin and HOMA-R in the overall population (Table 2.). These negative correlations for FGF19 disappeared after adjusting for BMI. After adjusting for HOMA-R, the percentage of total body fat remained significantly and negatively correlated with FGF19 serum levels ($P = 0.02$, $r = -0.24$). When the analysis was restricted to the obese population, FGF21 serum levels were still positively correlated with BMI, percentage of total body fat, glucose, insulin and HOMA-R. In the same restricted obese population, FGF19 maintained its negative correlation with BMI and insulin. After adjusting for BMI, all statistically significant correlations in the obese population disappeared for FGF21 and FGF19, whereas the positive correlation of BMI with FGF21 serum levels remained significant after adjusting for HOMA-R ($P = 0.032$, $r = 0.306$).

Given the profound and opposite alterations in endocrine FGF19 levels (decreased) and FGF21 levels (increased) in obese patients, we analyzed the expression of the genes encoding these endocrine FGFs and their receptors and co-receptor in adipose tissues (omental and subcutaneous) and liver. FGF21 mRNA expression was practically undetectable in visceral and subcutaneous adipose tissues from all patient groups. The FGF19 mRNA expression was also undetectable in both adipose tissues and in liver for all groups, as expected, given the known intestine-specific expression of FGF19.[41,42] FGF21 mRNA was expressed in the liver, and the overall obese patient group of patients showed a significant increase in hepatic FGF21 mRNA expression ($P = 0.04$) (Figure 2). Hepatic FGF21 mRNA levels and serum FGF21 levels showed a positive, significant, correlation in the obese patient population analyzed ($P = 0.026$; $r = 0.41$). A comparison of obese individuals with differing glycemic status showed no significant differences among sub-groups, although all sub-groups maintained significantly increased expression of hepatic FGF21 mRNA relative to healthy controls (NG, $P = 0.02$; IGT, $P = 0.04$; T2DM, $P = 0.02$).

An examination of components of the endocrine FGF responsive machinery showed that the hepatic expression of mRNA for the co-receptor β-Klotho was significantly higher in obese individuals than in lean healthy controls ($P = 0.03$). In contrast, the mRNA expression of the main endocrine FGFs receptor in liver, FGFR4, was not significantly changed compared with lean healthy individuals, although it tended to be higher in obese patients (Figure 2). Interestingly, both FGFR1 and FGFR3 expression levels were higher in obese patients groups than in healthy controls ($P = 0.04$ and $P = 0.001$, respectively), regardless of glycemic status. Considering the tendency toward lower gene expression of FGF21 and the endocrine FGFs receptor machinery in lean individuals relative to the obese patient groups, we assessed the hepatic expression of C/EBPα, a master transcriptional regulator

Table 2. Correlation analysis of circulating FGF21 and FGF19 levels with demographic, anthropometric and metabolic parameters

	Total individuals				*Obese patients*			
	Serum FGF21		*Serum FGF19*		*Serum FGF21*		*Serum FGF19*	
	r	P	r	P	r	P	r	P
Age	0.03	0.758	0.10	0.299	0.06	0.592	0.10	0.389
BMI	0.49	**< 0.001**	−0.31	**0.001**	0.43	**< 0.001**	−0.24	**0.034**
Waist-to-hip ratio	0.23	**0.021**	−0.11	0.284	0.12	0.318	−0.11	0.331
Total body fat	0.39	**< 0.001**	−0.30	**0.001**	0.32	**0.006**	−0.13	0.243
Total cholesterol	−0.04	0.698	0.17	0.087	−0.05	0.688	−0.18	0.134
Triglycerides	0.24	**0.020**	−0.18	0.080	0.14	0.273	−0.16	0.183
Glucose	0.37	**< 0.001**	−0.11	0.273	0.31	**0.016**	−0.06	0.503
Insulin	0.32	**0.001**	−0.28	**0.003**	0.21	**0.041**	−0.22	**0.047**
HOMA-R	0.37	**< 0.001**	−0.27	**0.007**	0.23	**0.039**	−0.16	0.203
SBP	0.25	**0.016**	−0.08	0.449	0.23	0.076	−0.02	0.852
DBP	0.31	**0.002**	−0.16	0.124	0.24	0.067	−0.11	0.371
AST	0.08	0.148	−0.16	0.116	0.03	0.782	−0.12	0.332
ALT	0.10	0.331	−0.02	0.846	0.04	0.739	−0.05	0.637
GGT	0.27	**0.025**	−0.04	0.714	0.19	0.122	−0.09	0.463

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; FGF, fibroblast growth factor; GGT, y-glutamyl transferase; HOMA-R, homeostasis model assessment of insulin resistance; SBP, systolic blood pressure. Correlations were calculated for both, overall population and just for obese individuals. Units and abbreviations are as in Table 1. Statistical significance is from Pearson correlation test. Values in bold represent $P \leqslant 0.05$.

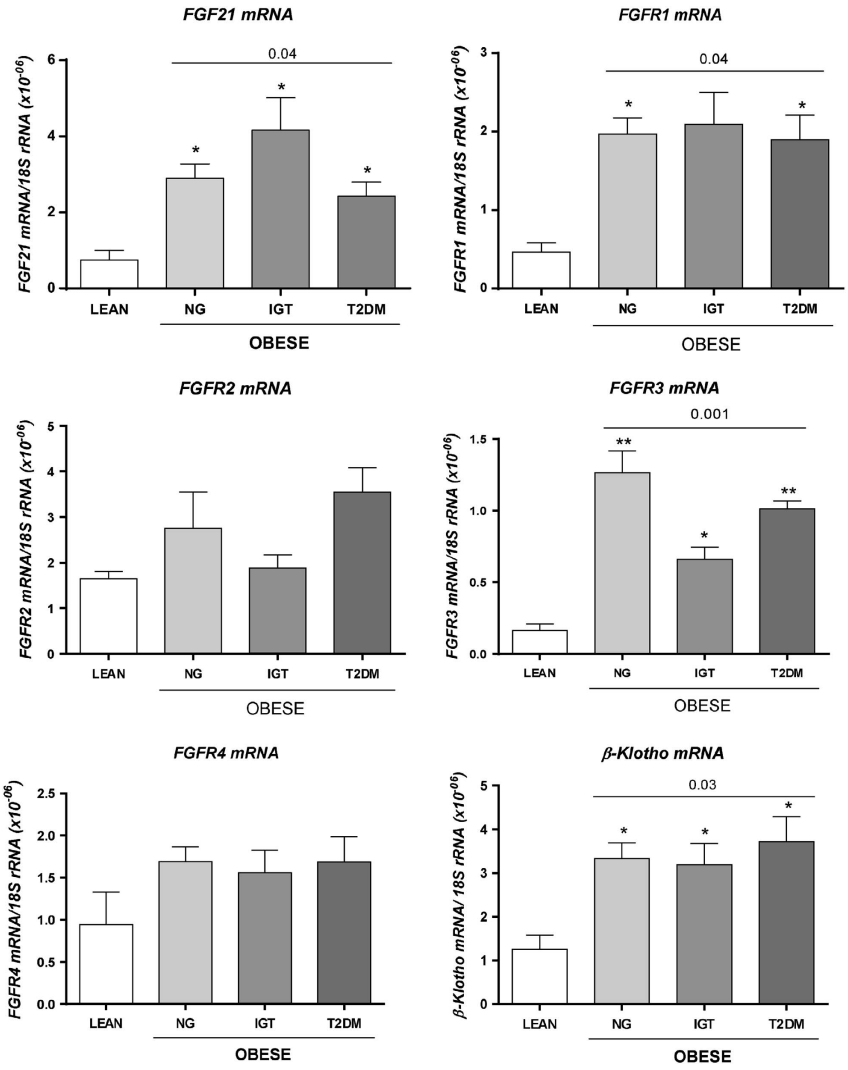

Figure 2. FGF21, FGFR1–4 and β-Klotho mRNA expression in livers of lean healthy controls ($n = 5$) and obese individuals ($n = 61$) grouped according to their glycemic state. NG, normoglycemic ($n = 36$); IGT, impaired glucose tolerance ($n = 15$); and T2DM, type 2 diabetes mellitus ($n = 10$). Values are expressed relative to 18S rRNA (means ± s.e.m.). *$P < 0.05$ and **$P < 0.01$, for comparison between obese sub-groups and controls. *P*-values are shown in cases where comparisons between the overall obese population and lean controls are significant.

of hepatic metabolism and differentiation not expected to be upregulated in obesity.[43] In fact, the C/EBPα mRNA expression was significantly lowered in obese patients compared with lean controls (fivefold decrease, $P < 0.01$, data not shown), a profile opposite to that of FGF-related genes. This finding confirms the specificity of the changes reported for FGF21 and FGF receptor/co-receptor expression.

We also analyzed gene expression in omental and subcutaneous adipose tissues of patients. In this case, sample availability prevented us from establishing an IGT patient group among obese patients; patients could be divided only into NG and T2DM sub-groups. The expression of transcripts for the main receptor for endocrine FGFs in adipose tissue, FGFR1, was not significantly changed among groups in subcutaneous adipose tissue or visceral adipose tissue, although there was a non-significant tendency toward higher visceral fat from obese individuals compared with healthy controls (Figure 3a.) The expression of β-Klotho mRNA in adipose tissues was significantly lower than in visceral adipose tissue from obese individuals ($P = 0.002$) regardless of their glycemic status ($P = 0.0034$ for NG patients; $P = 0.0032$ for T2DM patients). β-Klotho expression also tended to be lower in subcutaneous fat from patients, especially in T2DM individuals, but the difference did not reach statistical significance (Figure 3b, left). To confirm β-Klotho gene expression results, we analyzed β-Klotho protein levels in lean healthy controls and obese patients (sample availability restricted this analysis to NG obese patients). This analysis showed that β-Klotho protein levels were significantly lower in obese individuals than in controls in both subcutaneous ($P = 0.03$) and visceral ($P = 0.04$) fat depots (Figure 3b, middle). An examination of the levels of phosphorylated ERK1/2 in visceral adipose tissue samples indicated a significant reduction in obese patients vs healthy controls (Figure 3c). At the mRNA level, there were no significant differences in FGFR3 and FGFR4 between obese and healthy individuals. In contrast, expression of FGFR2 transcript, which was significantly lower in visceral than in subcutaneous, was increased in obese patients ($P = 0.004$), in both NG ($P = 0.01$) and T2DM ($P = 0.003$) sub-groups (Figure 3a).

DISCUSSION

Here we report that obesity results in opposite alterations in FGF21 and FGF19 in serum: a rise in FGF21, but a reduction in FGF19. This finding confirms previous observations of a rise in

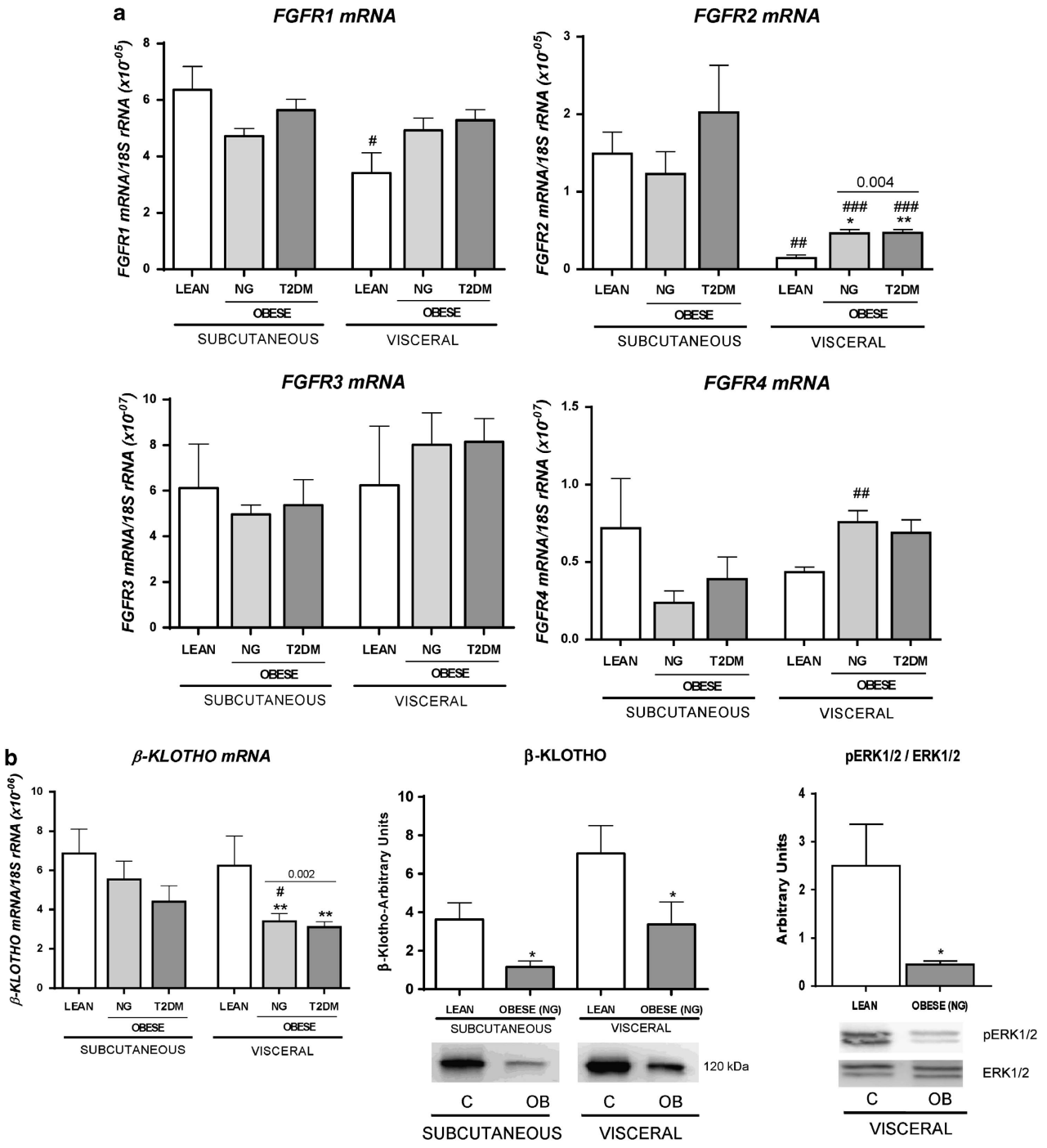

Figure 3. (**a**) FGFR1–4 mRNA expression in subcutaneous and visceral adipose tissues from lean healthy controls ($n = 7$ and $n = 6$, respectively) and obese individuals grouped according to their glycemic state. NG, normoglycemic ($n = 8$ and $n = 36$, respectively); and T2DM, type 2 diabetes mellitus ($n = 8$ and $n = 10$, respectively). Values are expressed relative to 18S rRNA (means ± s.e.m.). $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison between obese sub-groups and controls in each fat depot. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ for comparisons between expression levels in the same group in different fat depots. *P*-values are shown in cases where comparisons between the overall obese population and lean controls are significant. (**b**) β-Klotho mRNA expression (left), β-Klotho protein levels (middle) in subcutaneous and visceral adipose tissues, and levels of ERK1/2 phosphorylation in visceral adipose tissue (right) from lean healthy controls and obese individuals. Sample number for immunoblot assays was four for every sample and patient group. mRNA expression values are expressed relative to 18S rRNA (means ± s.e.m.). Protein levels were determined by densitometric quantitation of protein bands in immunoblot images. *P*-ERK1/2 levels were expressed relative to total ERK1/2. Data are expressed as means ± s.e.m. for each group (in arbitrary units). $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison between obese sub-groups and controls in each fat depot. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ for comparisons between expression levels in the same group in different fat depots. *P*-values are shown in cases where comparisons between the overall obese population and lean controls are significant.

FGF21 levels in distinct obese populations[11,12,16,44–46] and, by simultaneously evaluating FGF19 levels, establishes for the first time that this endocrine FGF is oppositely altered. Moreover, despite our initial indications that abnormally high FGF21 levels were associated with decreased insulin sensitivity and T2DM in the obese patient population, our statistical analysis revealed that obesity itself appears to be the primary determinant of the rise in FGF21 levels. Thus, a significant increase in FGF21 levels was already observed in NG obese patients. Moreover, despite significant associations of FGF21 levels with indicators of insulin resistance and altered glucose homeostasis, adjustment for BMI variations eliminated most of this statistically significant associations in both the overall study population and among obese patients.

Recent data indicate that increase in FGF21 may be predictive of the development of T2DM in obese patients.[16,46] It is possible that the rise in FGF21 in our NG obese population is an early disturbance indicating a higher risk of developing T2DM. Prospective and longitudinal analyses would be required to confirm this possibility. On the other hand, our current data indicating enhanced FGF21 expression in the liver of obese patients relative to lean controls, regardless of glycemic status, are consistent with a mostly hepatic origin of the abnormal increase in FGF21 in the blood of obese patients. In fact, we found virtually no expression of the FGF21 gene in white adipose tissue from either lean or obese patients. This is consistent with previous reports[4,47,48] indicating that, in contrast with rodent models, FGF21 expression is more restricted to the liver in humans and blood FGF21 levels are not likely to be influenced by expression in white adipose tissue. Also in accord with other reports,[4,49,50] we found a significant association of FGF21 levels with indicators of hepatic stress that commonly occur in obesity. Further research will be needed to establish whether a rise in FGF21 is associated with early signs of hepatic damage that accompany the development of obesity.

For FGF19, our current data are in agreement with a previous report of reduced FGF19 in obese patients,[22] and reports of a similar tendency in obese patients before bariatric surgery.[23,24] The direct association of an abnormal reduction in FGF19 levels with obesity itself, regardless of glycemic status, is even more marked, as we found that similarly low FGF19 levels occurred in obese patients regardless of the extent to which glycemic homeostasis was impaired.

On the other hand, FGF19 acts as a postprandial hormone[51] and there are evidences of differential response of FGF21 to carbohydrate ingestion in individuals according to their diabetes pathology condition.[52] The current study was performed in overnight-fasted individuals, and further research will be needed to confirm whether our findings occur similarly at the postprandial state.

Another relevant contribution of the present study is the observation that protein levels of β-Klotho, the key co-receptor mediating tissue responsiveness to FGF21 and FGF19, were reduced in white adipose tissue from obese patients. Moreover, levels of phospho-ERK1/2, considered a major intracellular mediator of β-Klotho/FGFRs signaling, were reduced in visceral fat from obese patients. In this sense, our findings in human obese patients relative to lean healthy controls are very similar to previous observations in rodents. Fisher *et al.*[13] reported that, in obese rodent models, FGF21 responsiveness is impaired and ERK1/2 phosphorylation is reduced in association with abnormally reduced β-Klotho expression in white adipose tissue, but not in liver. This reduction in β-Klotho expression in white adipose tissue from obese rodents has been subsequently confirmed.[53] Moreover, it has been recently reported that non-human primates show reduced levels of β-Klotho in white adipose tissue in association with high-fat-induced obesity.[54] Our current findings indicate a similar scenario in humans. It is likely that, in humans as in rodents, impaired expression of components of the cellular FGF21 (and FGF19)-responsive machinery in obesity are involved in the paradoxical outcome of increased FGF21 levels without the expected beneficial metabolic effects. In both cultured rodent and human adipocytes, exposure to pro-inflammatory signals, as is known to occur in adipose tissue of obese patients, represses β-Klotho expression.[53] Several experimental models have also shown that impaired β-Klotho expression is sufficient to impair the ability of FGF21[55] and of FGF19[56] to promote a healthy metabolic profile. On the other hand, the expression of β-Klotho and some FGFRs is increased in liver of obese patients. There is growing evidence that FGF21 can also act on the liver[57] and, therefore, it cannot be excluded that enhanced hepatic responsiveness to FGF21 occurs as an adaptive response to the obese condition. Moreover, given the well-established hepatic action of FGF19, perhaps enhanced β-Klotho expression could be a compensatory response to the decrease in FGF19 that serves to ensure the canonical effects of this endocrine FGF on hepatic bile acid metabolism.

Concerning the alterations in the expression of FGFRs themselves, obesity is characterized by enhanced expression of most FGFR isoforms in the liver. In this case, reasoning similar to that put forward to explain changes in β-Klotho expression may apply, specifically, a compensatory induction that maintains hepatic function in conditions of FGF19 reduction. However, it must be kept in mind that FGFRs can mediate autocrine/paracrine cellular responses to FGFs other than FGF19 and FGF21.

In summary, despite the present study has obvious limitations, especially in relation to the availability of tissue samples from human patients and healthy individuals, consistent conclusions could be obtained. We have found that the levels of FGF21 are increased whereas those of FGF19 are decreased in obese patients. Moreover, because these changes are associated with an abnormal decrease in the expression of β-Klotho, the sensitivity to FGF19 and FGF21 is most likely reduced in adipose tissue from obese patients. However, a reduced sensitivity to endocrine FGFs cannot be deduced from the data in the liver, but the opposite. Further research will be necessary to determine to what extent changes in adipose tissue and liver in obese patients can affect the metabolically beneficial actions of endocrine FGFs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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