Chapter 4

Calorie restriction and Roux-en-Y gastric bypass have opposing effects on circulating FGF21 in morbidly obese subjects

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ABSTRACT

Objective

To study the effect of different weight loss strategies on levels of the metabolic regulator FGF21 in morbidly obese females with normal glucose tolerance (NGT) or type 2 diabetes mellitus (T2DM).

Methods

In this observational intervention trial, weight reduction was achieved by Gastric Banding (GB, n=11) or Roux-en-Y Gastric Bypass (RYGB, n=16) in subjects with NGT, and by RYGB (n=15) or a very-low calorie diet (VLCD, n=12) in type 2 diabetics. Fasted and/or postprandial levels of FGF21, FGF19 (an FGF21-related postprandial hormone) and bile salts (implicated in regulation of FGF21 and FGF19 expression) were measured before, and 3 and 12 weeks after intervention.

Results

Fasted FGF21 levels were elevated in T2DM subjects. Calorie restriction by either GB or VLCD lowered bile salt and FGF21 levels. In contrast, RYGB surgery was associated with elevated bile salt and FGF21 levels.

Conclusions

Calorie restriction and RYGB have opposite effects on serum bile salt and FGF21 levels. Calorie restriction results in FGF21 approaching non-obese control levels, suggesting that this intervention is effective in reducing the “nutritional crisis” that appears to underly FGF21 elevation in obesity. FGF21 elevation after RYGB may contribute to the effect of this procedure.

INTRODUCTION

Fibroblast growth factors (FGF) 19 and 21 have been attributed diverse hormone-like metabolic functions (1;2). FGF21 has metabolic roles in both the fed and food-deprived state, with its expression regulated by both fasting (*e.g.* glucagon) and feeding (*e.g.* bile salts) signals. It is expressed mainly in the liver and white adipose tissue (WAT) where its transcription is regulated by peroxisome proliferator activating receptor (PPAR) alpha and PPARγ, respectively (3-7).In response to starvation, FGF21 mediates the effects of starvation-activated PPARα on hepatic lipid oxidation and ketogenesis, and inhibition of WAT lipolysis (3-7). In the fed state, FGF21 expression is induced in WAT where it regulates insulin-independent glucose uptake and mitochondrial oxidation (8). The regulation of FGF21 by fasting and feeding signals has led to the suggestion that FGF21 is a nutritional adaptation factor. FGF21 is elevated in obesity and type 2 diabetes mellitus (T2DM) (9;10)but there is no consensus as to whether FGF21 levels rise or decline with weight loss and/or normalization of insulin levels (11-13).

FGF19 is expressed in the distal small intestine after postprandial activation of the bile salt-activated transcription factor farnesoid-X receptor (FXR) (14-16). FGF19 stimulates hepatic protein and glycogen synthesis without inducing lipogenesis (14). Interestingly, FGF21 expression was recently demonstrated to be regulated by FXR as well (17), indicating that both endocrine FGFs may act as mediators of bile salt action. Circulating bile salts are correlated with insulin sensitivity (18)**,** accordingly, dysfunction of the bile salt-FXR-FGF19/FGF21 axis may contribute to hyperglycemia and hyperlipidemia.

Weight loss by calorie restriction or Roux-en-Y Gastric bypass surgery (RYGB) is a cornerstone in the current treatment for obesity and diabetes (19;20). The anatomical alterations after RYGB and peri-operative starvation likely affect endocrine FGFs in a direct manner, independent of the long-term effects of weight loss (11;21).The early effects of restrictive and metabolic weight loss strategies on (postprandial) bile salt, FGF19 and FGF21 levels have not been directly compared yet. Therefore we investigated the sub-acute effects of RYGB as opposed to calorie restriction by very-low calorie diet (VLCD) or gastric banding (GB) on these signaling molecules in obese subjects with normal glucose tolerance (NGT) or type 2 diabetes mellitus (T2DM). These different ways of calorie restriction (GB and VLCD) were chosen because of reluctance of surgeons to give T2DM subjects a gastric band, given the superior effects of RYGB (11;21). To elucidate whether RYGB exerts metabolic effects independent of weight loss, we examined subjects within 3 weeks after intervention when weight loss was

relatively small and similar among the treatment groups. To address the effect of more pronounced weight loss, we also studied subjects three months after the interventions.

SUBJECTS AND METHODS

Subjects and study design

The research design and methods have been described in detail elsewhere (22). In short, we included obese females eligible for dietary or surgical treatment. The subjects had normal fasting glucose (NGT) or T2DM (treated with oral medication only) according to WHO standards. Age-matched, healthy females with normal BMI served as a control group for pre-intervention comparisons. The protocol *(ClinicalTrials.gov: NTC01167959)* was approved by the medical ethics committee of the Leiden University Medical Center, and all subjects provided written informed consent before participation.

Subjects were studied (after ≥10 hrs fasting overnight) within a month before, 3 weeks after, and 3 months after intervention. Fasting and multiple postprandial (after 266 milliliters Nutridrink®, 400 kcal; 49 calorie% carbohydrate,35 calorie% lipids, 16 calorie% protein) blood samples were taken before and 3 weeks after intervention. Blood was collected in a SST® Gel and Clot Activator tube (Becton and Dickinson) and a vacutainer on EDTA with added Aprotinin or Dipeptidyl-Peptidase-IV (DPP-IV) inhibitor as appropriate (22).

Logistic reasons precluded the study of participants the day prior to operation, however, we ensured that no changes in weight and diet had occurred between the first test occasion and the actual intervention.

Interventions

Standard operating procedures were followed for GB and RYGB and patients were prescribed a staged meal plan after surgery (22). T2DM subjects undergoing dietary intervention (VLCD) were prescribed commercially available Prodimed® (Prodimed Benelux BV, Valkenswaard, The Netherlands), a high-protein, low-calorie meal replacement plan (22).

Assays

All samples were analyzed after one freeze-thaw cycle. Serum cholesterol, high-density-lipoprotein (HDL), triglycerides (TG), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and C-Reactive Protein (CRP) were measured on a Modular Analytics P-800 system (Roche Diagnostics, Mannheim, Germany). Low-density-

lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation. Commercial kits were employed for the assay of non-esterified fatty acids (Wako) and total bile salts (Diazyme). Serum FGF19 and FGF21 levels were measured with in-house developedELISAs as described elsewhere (23;24). The interassay coefficients of variation of the FGF19 and FGF21 ELISA were 8.3% and 6.7%, respectively.The lower limit of detection of both ELISAs is 0.01 ng/milliliters, and the assays are linear up to 0.30 and 0.50 ng/milliliters for FGF19 and FGF21, resp.

Statistical analysis

Data were analyzed using SPSS 17.0. Data are presented as means ± standard error of the mean. Non-normally distributed data were log transformed. Differences between subject groups (NGT vs. T2DM) and lean controls at baseline were compared by one-way ANOVA. The effect of the meal challenge, at baseline and 3 weeks after intervention was analyzed by repeated measures ANOVA. Within-group treatment effects at three weeks and three months as compared to baseline were analyzed by paired t-tests. Between-group treatment effects at three weeks and three months as compared to baseline were compared with a mixed-effects model with the patient groups and diabetes as fixed effects and the subject-specific deviances modeled with random intercepts. Bonferoni correction was applied to correct for multiple comparisons. A *P*-value <0.05 was considered statistically significant.

RESULTS

Subject characteristics

Baseline characteristics of the study groups are presented in table 1. All obese subjects and healthy controls were Caucasian females with a mean age of 49.4±0.6 yrs. We included 32 subjects with T2DM and 30 NGT obese individuals. Eight subjects dropped out during the course of the study either because they were unable to comply with the VLCD (n=2), or because of mild postoperative complications after RYGB (n=3, of which 2 had T2DM) or logistical issues (n=3).

Prior to intervention, 50% of T2DM patients used antihypertensives against 33% in NGT patients (P=0.15, supplemental table 1). Pre-intervention use of oral antidiabetics was comparable between both groups of diabetic subjects. At the day of intervention, all blood glucose lowering agents were discontinued to avoid hypoglycaemia. Only Metformin treatment was reinstalled if fasting blood glucose levels remained above 7 mmol/L after intervention (27% of subjects after RYGB vs. 17 % of subjects after VLCD, P=0.32).

Table 1 - Baseline characteristics of the study groups.

	Controls (12)	NGT (27)	T2DM (27)	P_{ANOVA}	$P_{controls\ vs.\ NGT}$	$P_{controls\ vs.\ T2DM}$	$P_{NGT\ vs.\ T2DM}$
Age (yrs)	49.2 ± 1.8	47.7 ± 1.3	51.0 ± 1.4	0.180			
Weight (kg)	64.4 ± 2.1	124.3 ± 2.3	117.2 ± 3.3	0.000	0.000	0.000	0.059
BMI (kg/m²)	21.7 ± 0.5	43.8 ± 0.6	42.0 ± 1.1	0.000	0.000	0.000	0.125
Waist (cm)	78.0 ± 1.7	122.2 ± 1.7	123.2 ± 2.1	0.000	0.000	0.000	0.706
Fat mass (%)	35.5 ± 0.7	56.3 ± 0.4	55.8 ± 0.8	0.000	0.000	0.000	0.565
CRP (mg/L)	1.9 ± 0.4	7.3 ± 1.2	7.6 ± 1.2	0.017	0.010	0.007	0.859
Total cholesterol (mmol/L)	4.4 ± 0.1	5.0 ± 0.3	4.6 ± 0.2	0.144			
HDL-Cholesterol (mmol/L)	1.7 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.000	0.000	0.000	0.493
LDL-Cholesterol (mmol/L)	2.9 ± 0.3	2.9 ± 0.2	2.5 ± 0.1	0.121			
Triglycerides (mmol/L)	1.0 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	0.001	0.079	0.000	0.012
AST (U/L)	15.6 ±1.0	18.8 ± 1.3	26.2 ± 3.7	0.041	0.486	0.025	0.047
ALT (U/L)	8.6 ± 0.7	15.0 ± 1.1	21.2 ± 3.6	0.027	0.175	0.009	0.098
HOMA-IR	0.3 ± 0.0	2.4 ± 0.4	4.6 ± 0.6	0.000	0.012	0.000	0.001
FFA (mmol/L)	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	0.024	0.243	0.010	0.062
Total bile salts (µmol/L)	3.9 ± 1.0	3.4 ± 0.3	4.7 ± 0.6	0.188			
FGF19 (ng/mL)	0.12 ± 0.02	0.067 ± 0.008	0.10 ± 0.01	0.017	0.013	0.475	0.022
FGF21 (ng/mL)	0.14 ± 0.02	0.25 ± 0.04	0.47 ± 0.06	0.000	0.155	0.000	0.001

Values are presented as means ± SEM. Differences between lean controls and patient groups at baseline were evaluated with one-way ANOVA. The Bonferroniposthoc test was used to correct for multiple testing. *A P-value <0.05* was considered statistically significant. Abbreviations: BMI:body massindex; CRP: c-reactive protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FFA: free fatty acids; NGT: normal glucose tolerant; T2DM: type 2 diabetes mellitus.

Effects of intervention on weight loss and glucose homeostasis

Effects of dietary or surgical intervention on weight loss and glucose homeostasis in this cohort were recently reported (22). In brief, relative weight loss after three weeks was similar (4.8-7.3%) in NGT and T2DM groups with patients remaining markedly obese (BMI 37.7-40.9 kg/m^2). This was accompanied by decreased fasting glycemia and postprandial glucose excursion in T2DM patients. After three months, GB was less effective (10.2%) in inducing weight loss than RYGB or dietary intervention (14.6-16.9%). Fasting hyperglycemia and hyperinsulinemia were largely normalized or reversed at this later timepoint, indicating restored insulin sensitivity (22).

Baseline fasting bile salt, FGF19 and FGF21 levels

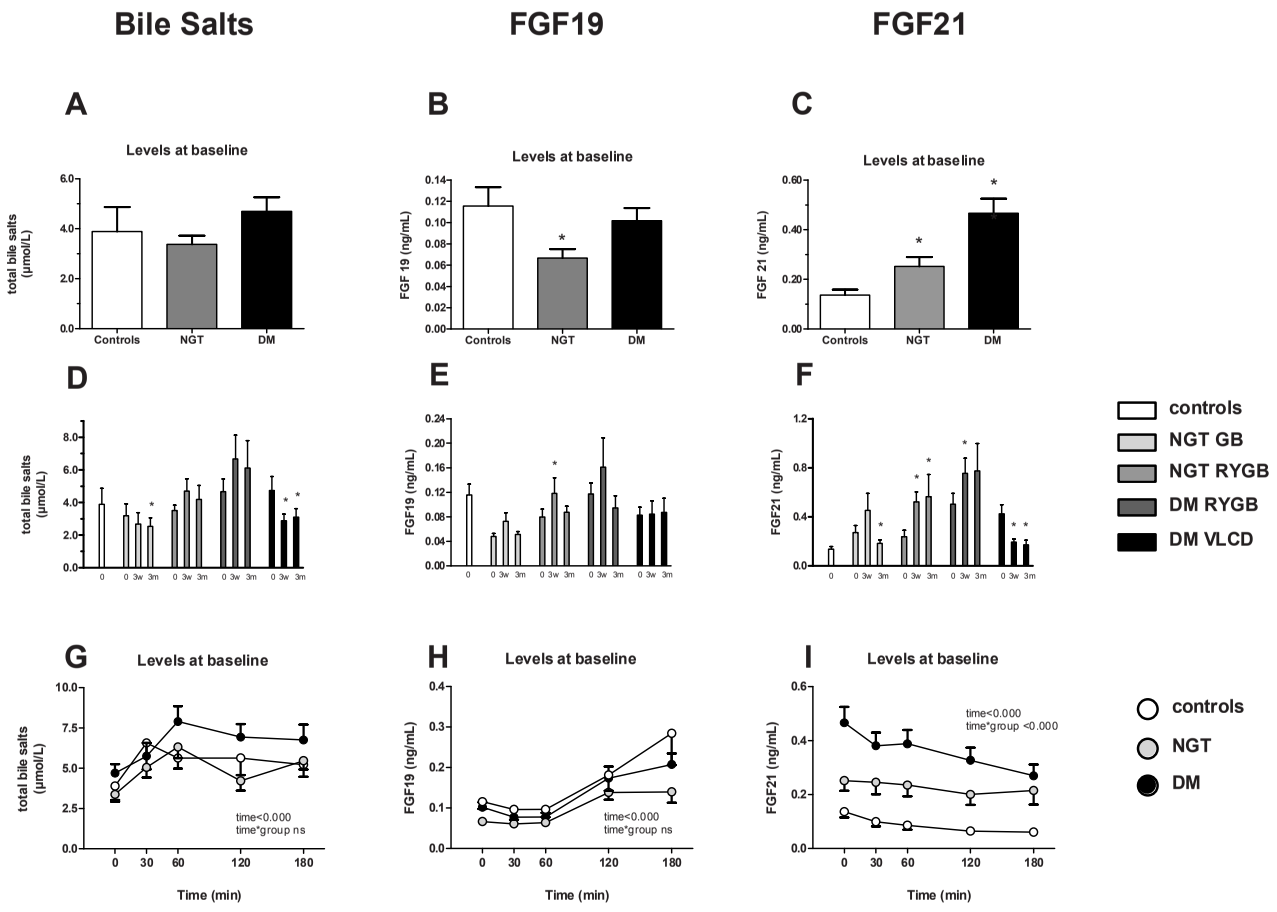
Total bile salt levels prior to intervention were not different between lean controls and NGT or T2DM obese subjects (table 1, figure 1A). Baseline FGF19 levels were reduced in NGT obese subjects as compared to lean controls and obese T2DM subjects who had similar levels (table 1, figure 1B). Baseline fasted FGF21 levels were higher in T2DM obese subjects compared to healthy lean controls or NGT obese subjects (table 1, figure 1C).

Postprandial bile salt, FGF19 and FGF21 response

Bile salt levels increased after intake of a mixed-meal in all groups with levels peaking at 30-60 min (figure 1G). The postprandial bile salt response at baseline was similar in all groups (time*group effect: *P*=0.07). As expected, the postprandial increase in FGF19 levels lagged behind the postprandial rise of bile salts, and was first apparent after 2 hrs (figure 1H). The postprandial FGF19 response at baseline was similar in all groups (*P*=0.12). A standard mixed-meal test resulted in a significant postprandial decline of FGF21 level in all groups, which was more pronounced in T2DM obese subjects as compared to NGT obese subjects (group*time: *P*=0.042) and lean controls (group*time: *P*<0.001) (figure 1I).

Effects of intervention on fasting bile salt, FGF19 and FGF21 levels

Bile salt levels were not significantly changed three weeks after GB, but after 3 months a reduction of serum bile salts was seen (-20%, *P*=0.034; table 2, figure 1D). Likewise, VLCD resulted in decreased bile salt levels that reached significance at the three weeks and three months timepoint (respectively -39%, P=0.03 and -35%, *P*=0.046; table 2, figure 1D). Fasting bile salt levels were not affected by RYGB in either NGT or T2DM subjects (table 2, figure 1D). Analysis of pooled data of all subjects undergoing RYGB (n=31), however, showed a significant increase in bile salt levels after three weeks (4.1±0.4 to 5.7±0.8 µmol/L; *P=0.049*).


Bile Salts
FGF19
FGF21
A
B
C
Levels at baseline
total bile salts (µmol/L)
FGF 19 (ng/mL)
FGF 21 (ng/mL)
Controls
NGT
DM
D
E
F
FGF19 (ng/mL)
FGF21 (ng/mL)
0 3w 3m
controls
NGT GB
NGT RYGB
DM RYGB
DM VLCD
G
H
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time<0.000
time*group ns
time<0.000
time*group <0.000
Time (min)
controls
NGT
DM

<< Figure 1 - Baseline levels of total bile salts, FGF19 and FGF21 in lean controls and obese subjects with normal glucose tolerance (NGT) or diabetes (DM) in fasted state.
(A-C) Baseline fasted levels of total bile salts, FGF19 and FGF21 in lean controls (n=12) and obese subjects with normal glucose tolerance (NGT, n=27) or diabetes (DM, n=27). **(D-F)** Fasted levels of afore mentioned analytes at baseline, and at three weeks (3w) and 3 months (3m) after intervention. **(G-I)** Prandial responses in lean controls (open symbols), and obese subjects with normal glucose tolerance (NGT, grey symbols) or diabetes (DM, black symbols) at baseline. Asterisk denotes a significant difference (P<0.05) between groups at baseline, or within groups after intervention. Abbreviations: n.s.,non-significant; NGT,normal glucose tolerance; DM, diabetes; GB, gastric banding; RYGB,Roux-en-Y-gastric bypass; VLCD, very low calorie diet; min, minutes.

Only RYGB in NGT subjects caused a slight increase in fasting FGF19 levels (+52%, *P=0.045*) after three weeks (figure 1E, table 2). None of the interventions, however, caused a lasting change in fasting FGF19 levels. When data from RYGB subjects was pooled, there was a trend towards an increase in FGF19 levels after three weeks (0.10±0.01 to 0.14±0.03 ng/milliliters,+41%, P=0.063).

GB did not affect FGF21 levels after three weeks (P=0.15), but resulted in a decrease after three months (-33%, P=0.012; table 2, figure 1F). The other restrictive intervention (VLCD) led to sustained decline (2.2-2.5 fold) of FGF21 levels after three weeks (P=0.002) and three months (P=0.004, table 2, figure 1F). In contrast, RYGB induced an increase in FGF21 level after three weeks in both NGT (P=0.002) and T2DM (P=0.007) subjects with levels remaining significantly elevated after three months in NGT subjects (P=0.004, table 2, figure 1F). Analysis of the entire group of subjects undergoing RYGB, revealed increased FGF21 levels three weeks (P<0.001) and three months (P=0.052) after surgery (data not shown). Between-group analysis showed a significant different effect of VLCD as compared to RYGB at both timepoints and of GB as compared to RYGB after three months on FGF21 levels (P<0.001).

Effects of intervention on postprandial bile salt, FGF19 and FGF21 response

None of the interventions had an effect on the postprandial bile salt response ($P_{time*occasion}$=n.s., figure 2A-D). Nevertheless, the altered small intestinal anatomy after RYGB appears to underlie a decline in time to peak in the T2DM subjects (NGT: 78±13 to 84±14 min; P=0.74 and T2DM: 110±11 to 72±14 min; P=0.034, figure 2C).

The restrictive interventions had no effect on the postprandial FGF19 response ($P_{time*occasion}$=n.s., figure 2E,H). RYGB, however, led to a leftward shift (*i.e.* an earlier

Table 2 - Effects of intervention on serum biochemistry in NGT (upper half) and T2DM (lower half) subjects.

	1. NGT-GB (n=11)			2. NGT-RYGB (n=16)		
	Before	**After 3 weeks**	**After 3 months**	**Before**	**After 3 weeks**	**After 3 months**
FFA (mmol/L)	0.9 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.6 ± 0.1 **	1.2 ± 0.7
Total bile salts (µmol/L)	3.1 ± 0.7	2.7 ± 0.7	2.5 ± 0.5*	3.5 ± 0.3	4.7 ± 0.8	4.2 ± 0.9
FGF19 (ng/mL)	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.08 ± 0.01	0.12 ± 0.03*	0.09 ± 0.01
FGF21 (ng/mL)	0.27 ± 0.06	0.45 ± 0.14	0.18 ± 0.03*	0.24 ± 0.05	0.52 ± 0.08*	0.56 ± 0.18*#

	3. T2DM-RYGB (n=15)			4. T2DM-VLCD (n=12)		
	Before	**After 3 weeks**	**After 3 months**	**Before**	**After 3 weeks**	**After 3 months**
FFA (mmol/L)	1.2 ± 0.1	1.8 ± 0.1 **	1.1 ± 0.1	1.2 ± 0.1	1.5 ± 0.1 *	1.3 ± 0.2
Total bile salts (µmol/L)	4.7 ± 0.8	6.7 ± 1.4	6.1 ± 1.7	4.7 ± 0.9	2.9 ± 0.4*	3.1 ± 0.5*
FGF19 (ng/mL)	0.12 ± 0.02	0.16 ± 0.05	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.09 ± 0.02
FGF21 (ng/mL)	0.50 ± 0.09	0.75 ± 0.12*	0.78 ± 0.22	0.42 ± 0.08	0.19 ± 0.03*	0.17 ± 0.04*

Values are presented as means ± SEM. The effect of intervention after three weeks and three months was compared with a mixed-effects model with the patient groups and diabetes as fixed effects and the subject specific deviances modelled with random intercepts. The Bonferroniposthoc test was used to correct for multiple testing. Asterisk denotes a significant effect of intervention within groups as compared to baseline (*P<0.05, ** P< 0.001). # and& denote a significant different effect of interventions in the NGT groups (#) and T2DM groups (&). Abbreviations: BMI: body mass index; CRP: c-reactive protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FFA: free fatty acids; AUC: area under the curve; NGT: normal glucose tolerant; T2DM: type 2 diabetes mellitus; GB: gastric banding; RYGB: roux-en-y-gastric bypass; VLCD: very low calorie diet.

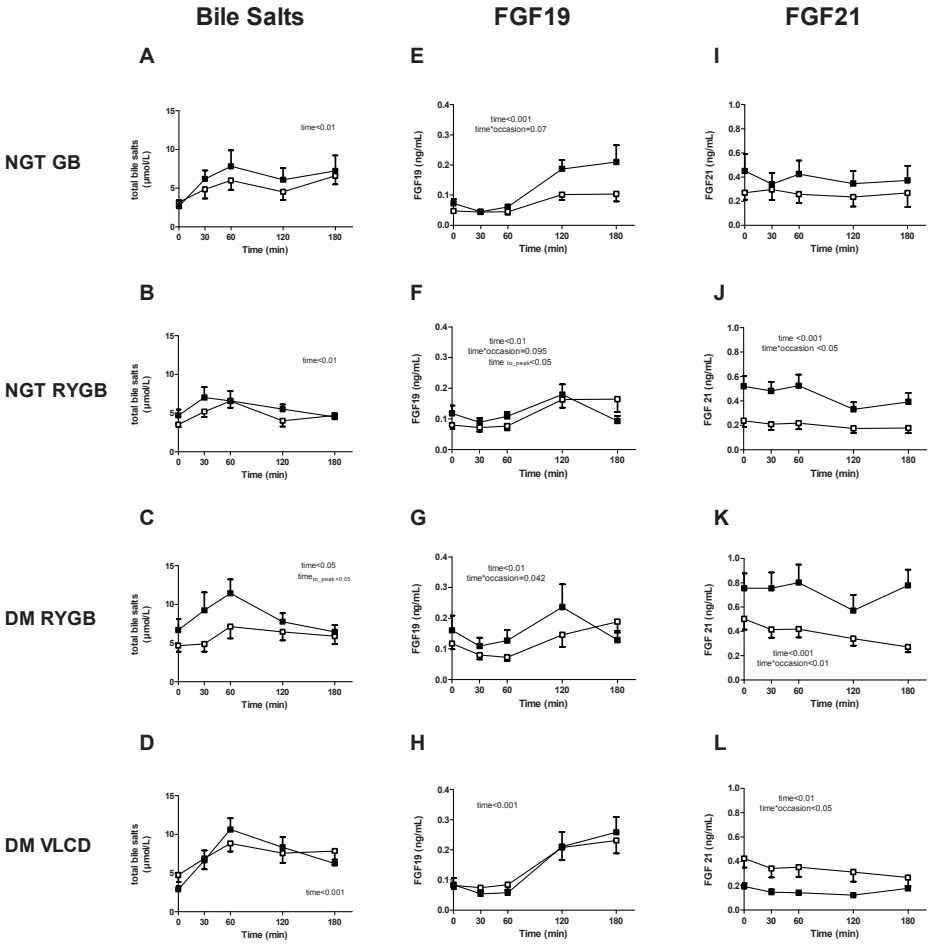

Figure 2 - Prandial responses of bile salts, FGF19 and FGF21 at baseline and three weeks after intervention.

Prandial responses of bile salts (A-D), FGF19 (E-H) and FGF21 (I-L) at baseline (open symbols) and three weeks after intervention (black symbols). Values are mean ± SEM. Open symbols reflect baseline data, closed symbols reflect 3 weeks data. Significant effects (P<0.05) of time (during meal tolerance test) or occasion (difference in response between occasions) as analyzed by repeated measures ANOVA are described in the figures. Abbreviations: NGT, normal glucose tolerance; DM, diabetes; GB,gastric banding; RYGB, Roux-en-Y-gastric bypass; VLCD, very low calorie diet; min, minutes.

time to peak: 145±17 to 128±11 min; *P*=0.41) in the FGF19 response curve in T2DM subjects ($P_{time*occasion}$=0.042, figure 2G). A similar trend was observed in NGT subjects ($P_{time*occasion}$=0.095,time to peak: 139±8 to 92±10 min, *P*=0.002, figure 2F).

GB did not affect the postprandial FGF21 response following a mixed-meal test ($P_{time*occasion}$=0.21, figure 2I). The postprandial decline of FGF21 levels was, however, less pronounced after VLCD ($P_{time*occasion}$ =0.008, figure 2L). RYGB resulted in an altered postprandial FGF21 response in both NGT and T2DM subjects. In both subject groups, a transient drop in FGF21 level was apparent after RYGB at the 2 hrstimepoint. Moreover, both NGT and T2DM subjects showed a less pronounced postprandial FGF21 response after RYGB (NGT: $P_{time*occasion}$=0.041, T2DM: $P_{time*occasion}$<0.01, figure 2J, K).

DISCUSSION

This is the first study to compare the time course of the metabolic effects of RYGB with that of restrictive weight loss strategies on fasting and postprandial bile salt, FGF19 and FGF21 levels in obese subjects with NGT and T2DM. The major novel finding of this study is that calorie restriction (by VLCD and GB) and RYGB have a different impact on fasting levels of bile salts and FGF21. Calorie restriction resulted in lowering of FGF21 and bile salt levels whereas RYGB surgery resulted in elevated FGF21 and bile salts levels, both in the context of a decline in glucose levels in T2DM subjects.

Replicating earlier findings (9-11) we observed elevated levels of FGF21 in obese subjects at baseline, coinciding with elevated insulin levels in all obese subjects and elevated glucose levels in T2DM subjects. FGF21 transcription is induced by fasting (glucagon and PPARα) and feeding (glucose and bile salts) signals, allowing adaptation to the individual's metabolic state. It has been postulated that during non-ketotic conditions, glucose and insulin maintain basal levels of FGF21 (17). Moreover, recent evidence suggests that postprandial elevation of bile salts influences FGF21 levels through transcriptional induction of FGF21 by hepatic FXR, and FGF19-mediated stabilization of FGF21 protein (17).

The metabolic state in obesity likely reflects a continuous "fed" state in the face of elevated circulating insulin, free fatty acids (FFA) and bile salts. In this context, elevated FGF21 levels may be an attempt to overcome insulin resistance by inducing glucose uptake in adipose tissue (5-7;25) and through elevated expression of glucose transporter-1 in skeletal muscle (26). Paradoxically, the more severely disturbed metabolic state in

T2DM is characterized by elevated glucagon (27) and FFA levels (table 1) relative to the existing hyperglycemia, thereby further activating hepatic PPARα with concomitant FGF21 induction (28). More recent data suggest that glucagon directly stimulates FGF21 secretion from hepatocytes and adipocytes and moreover, that several effects of glucagon (i.e. lipolysis) were attenuated after reduction of FGF21 secretion (29;30). As such, FGF21 seems a metabolic regulator of the beneficial effects of glucagon.

So far, no consensus has been reached as to whether human FGF21 levels rise or decline with weight loss or nutritional interventions. No alterations in FGF21 were observed after moderate weight loss over a 6 months period in obese subjects (12). Other studies found an increase in FGF21 after 3 weeks of VLCD in obese NGT subjects (13). Several reports, though, have shown increased bile salts and FGF19 after RYGB. (11;21;31;32) In the present study we show that weight loss *per se* (for instance through calorie restriction) reduces bile salt and FGF21 levels. Thus, after RYGB, a restrictive and metabolic procedure, one looks at the outcome of two apparently counteracting processes, with the effect of weight loss apparently being overruled by a yet unknown metabolic stimulus that results in elevation of both bile salts and FGF21. The consistent decrease in FGF21 levels we observed during calorie restriction, recently also noted in an animal model of calorie restriction and related to reduced hepatic FGF21 expression in rats, suggests the relative absence of fasting or feeding signals. Apparently, a VLCD counteracts the disturbed metabolic status in obesity, but does not induce the nutritional deregulation with ketogenesis responsible for FGF21 expression in severe fasting. VLCDs rapidly reduce hepatic endogenous glucose production in obese diabetic patients (33), which reduces the “glucose trigger” that may be responsible for enhanced hepatic FGF21 expression before intervention. Indeed, VLCD reduced fasting and postprandial glucose and insulin levels in this cohort of T2DM subjects, suggesting an improved glucose homeostasis (22). Interestingly, we also observed a consistent decrease of fasted bile salt levels following calorie restriction by either GB or VLCD. Although the mechanism behind this decrease is unknown, it is possible that alterations in gut microbiome after GB or VLCD affect bile salt reabsorption in gut and liver.

Lowering of FGF21 levels by VLCD may also be due to the relative high protein content of the diet, which is higher compared to generally used low calorie diets. This may prevent activation of the amino acid-deprivation signaling pathway that was recently implicated in FGF21 gene induction (34;35). RYGB increased FGF21 levels, coinciding with a rise in postprandial insulin in the face of decreased glucose levels but elevated FFA levels

(table 2). The multiple mechanistic effects of the gastrointestinal rearrangements of the RYGB procedure (19) likely entail both "feeding signals" (*i.e.* exaggerated postprandial insulin (22)), and "fasting signals" (*i.e.* elevated FFA and glucagon (25;27)) thereby activating PPARα and PPARγ. Exogenously administered FFAs elevate FGF21 levels in healthy subjects (25). RYGB surgery resulted in a transient elevation of circulating FFAs after three weeks and may thereby contribute to elevated FGF21 levels shortly after RYGB surgery (table 2). However, a similar transient rise in FFA levels was apparent after VLCD in the context of reduced FGF21 levels. This underlines the complexity of (nutritional) regulation of FGF21. Nevertheless, given the multiple beneficial metabolic actions of FGF21, the elevation of FGF21 may contribute to the beneficial metabolic effects of RYGB (3;6;8;26;36;37), for example by stimulation of glucagon induced lipolysis. Indeed, it was recently shown that short-term treatment with LY2405319, an FGF21 analog, induces improvements in dyslipidemia (38), which makes it a promising new treatment option for selected metabolic disorders in obesity.

FGF21 levels display a circadian rhythm which peaks during the fasting state. In the postprandial state FGF21 is negatively regulated through insulin-responsive up-regulation of E4 binding protein 4. Recent data suggest a suppressive effect of an oral fat load on FGF21 levels, which may depend on the employed stimulus or the population studied (39). The significant decline we observed in all groups after food intake, suggests a suppressive effect of mixed-meal intake as well. We observed the most pronounced decline in the postprandial FGF21 excursion, including a transient drop after 2 hrs, in NGT and T2DM subjects after RYGB. This is presumably mediated by a suppressive effect of the expedited rise in insulin levels after RYGB in both NGT and T2DM subjects (22). Further studies will have to determine if expedited suppression of FGF21 after RYGB can be confirmed.

Unlike earlier findings of our group and others (11;31), RYGB only modestly elevated fasting bile salt levels and had no effect on fasting FGF19 levels in the current cohort. We have no ready explanation for this discrepancy. The present study cohort consisted entirely of obese females, at risk (female, obese, forty's) to develop gallstone disease, whereas both genders were represented in the earlier studies (11;31). To address whether prior cholecystectomy had an effect on post-RYGB bile salt or FGF19 levels, we performed a subgroup analysis. At baseline, subjects with prior cholecystectomy (n=10) tended to have slightly higher bile salt levels than subjects with intact gallbladder (n=21) (5.4±1.3 vs. 4.6±0.5 μmol/L, *P=0.20*). Whereas subjects with intact gallbladder showed increased post-RYGB fasted bile salt levels (4.6±0.5 to 7.0±1.2 μmol/L; *P=0.02*), this was

not the case in subjects with prior cholecystectomy (5.4±1.3 to 5.8±1.3 µmol/L; *P=0.66*). Although the exact effect of prior cholecystectomy on post-RYGB bile salts/FGF19 levels needs to be established in larger cohorts, the above notions indicate that this may (in part) underlie the discrepancy between earlier and present findings. Further insight into the exact mechanism by which RYGB increases bile salt and FGF19 levels, *e.g.* via increased gut mucosal villi length or an altered gut microbiome (40), may shed further light on this issue.

Interestingly, we did observe an earlier postprandial FGF19 peak after RYGB, suggesting an earlier induction of ileal FGF19 expression by reabsorbed bile salts. This may be explained by delivery of bile at a site closer to the ileal site of bile salt reclamation, and shortened contact time between ingested nutrients and bile after RYGB. A leftward shift in the postprandial bile salt excursion in NGT subjects and an earlier (30 min) postprandial increase of bile salt levels in T2DM subjects, are in support of such notion.

This study has some limitations. First of all, to minimize heterogeneity in the subjects group only female subjects, of which 80% were post-menopausal, were included. This implicates that the results of this study apply only to female subjects. Unfortunately, due to the large impact of GB and RYGB procedures, randomization of interventions was not allowed in our protocol. Moreover, two different interventions have been used in the calorie restriction group, which may have introduced a difference in effect. Although self-reported calorie intake was comparable between GB and VLCD subjects (data not shown), the calorie composition (carbohydrates, protein, fat) might have been slightly different. We can not determine whether or not this may have influenced our results in fasting condition. Postprandial responses, however, were obtained after exactly the same test meal.

In conclusion, we present evidence for opposing short-term effects of weight loss induced by either RYGB or VLCD on FGF21 levels in obese subjects. Whereas calorie restriction by VLCD appears to effectively reduce the “nutritional crisis” that apparently underlies elevated FGF21 expression in obesity, RYGB results in elevated FGF21 levels. In view of the beneficial metabolic effects of FGF21, this may in fact contribute to the effect of the RYGB procedure in ameliorating glucose homeostasis. Our study adds to the notion that bile salts may elevate FGF21 levels via intestinal (FGF19-dependent) and hepatic FXR activation. Thus it would be of interest to study the effect of FXR agonists on surgical weight loss and improvement of metabolic parameters in obese patients with T2DM.

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