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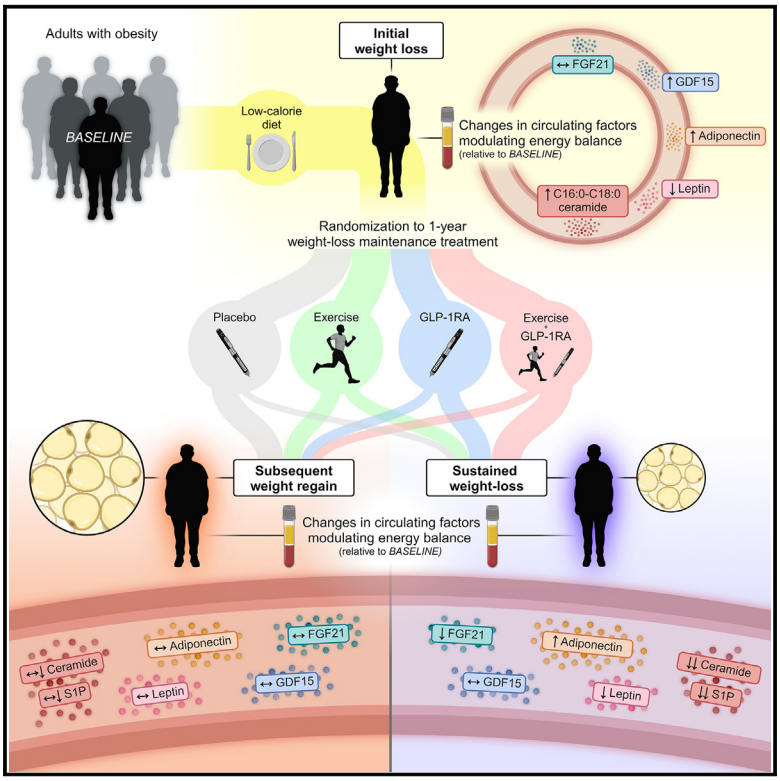
Weight-loss maintenance is accompanied by interconnected alterations in circulating FGF21-adiponectin-leptin and bioactive sphingolipids

Graphical abstract



Highlights

- Diet-induced weight loss (WL) transiently increases GDF15 and C16:0-C18:0 ceramides
- Sustained WL leads to reduced FGF21, leptin, ceramides, and S1P and increased adiponectin
- Weight maintainers and regainers exhibit distinct metabokine-sphingolipid alterations
- Clinically, these alterations associate with changes in cardiometabolic health outcomes

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In brief

Fiorenza et al. reveal selective and dynamic alterations in energy-balance-regulating metabokines and sphingolipids throughout weight loss, weight-loss maintenance, and weight regain in adults with obesity. Weight-loss maintenance elicits distinct remodeling patterns within the FGF21-adiponectin-leptin-sphingolipid axis as compared with weight regain, and these changes are associated with cardiometabolic health outcomes.

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Weight-loss maintenance is accompanied by interconnected alterations in circulating FGF21-adiponectin-leptin and bioactive sphingolipids

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SUMMARY

Weight loss is often followed by weight regain. Characterizing endocrine alterations accompanying weight reduction and regain may disentangle the complex biology of weight-loss maintenance. Here, we profile energy-balance-regulating metabokines and sphingolipids in adults with obesity undergoing an initial low-calorie diet-induced weight loss and a subsequent weight-loss maintenance phase with exercise, glucagon-like peptide-1 (GLP-1) analog therapy, both combined, or placebo. We show that circulating growth differentiation factor 15 (GDF15) and C16:0-C18:0 ceramides transiently increase upon initial diet-induced weight loss. Conversely, circulating fibroblast growth factor 21 (FGF21) is downregulated following weight-loss maintenance with combined exercise and GLP-1 analog therapy, coinciding with increased adiponectin, decreased leptin, and overall decrements in ceramide and sphingosine-1-phosphate levels. Subgroup analyses reveal differential alterations in FGF21-adiponectin-leptin-sphingolipids between weight maintainers and regainers. Clinically, cardiometabolic health outcomes associate with selective metabokine-sphingolipid remodeling signatures. Collectively, our findings indicate distinct FGF21, GDF15, and ceramide responses to diverse phases of weight change and suggest that weight-loss maintenance involves alterations within the metabokine-sphingolipid axis.

INTRODUCTION

Obesity management guidelines recommend a 5%–15% weight loss to improve cardiometabolic risk factors.[1] Although many individuals with obesity may successfully manage an initial large diet-induced weight loss, weight regain often occurs.[2,3] Hence, effective weight-loss maintenance strategies are crucial for reducing cardiometabolic risks associated with obesity. According to the energy balance model, energy expenditure and food intake are the key features of body weight regulation[4] and, thus, determinants of weight-loss maintenance. In addition to exercise, which sustains weight loss by increasing energy expenditure, pharmacological approaches effectively prevent weight regain by suppressing food intake. Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are emerging as first-line pharmacological treatments for sustained weight loss. Despite the well-established weight-lowering effects of these anti-obesity therapeutics, a highly heterogeneous response is frequently observed in the magnitude of weight loss,[5,6] aligning with the notion that sustained weight loss is mediated by intricate neuroendocrine mechanisms beyond glucagon-like peptide-1 signaling.[7] Thus, characterizing metabolic and endocrine alterations that accompany body weight regulation in response to exercise and GLP-1RA therapy may help disentangle the complex biology of sustained weight loss success.

Fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) are stress-responsive metabokines, defined as cytokines involved in the paracrine and endocrine regulation of systemic metabolism, deemed to modulate energy balance.[8,9] Pre-clinical data indicate that FGF21 is involved in GLP-1RA-induced weight loss[10] and is required to maximize the metabolic benefits of GLP-1RA.[11,12] Conversely, while GDF15 is unlikely to affect GLP-1RA action,[13,14] it may contribute to the magnitude of weight loss and fat mass reduction following bariatric surgery

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and exercise training, respectively.[15–17] Expression of FGF21 and GDF15 is regulated by the same molecular pathway, namely the integrated stress response, which is activated by a variety of cellular stresses.[18] Recent data indicate that the integrated stress response effector ATF4[19,20] is largely involved in weight regain after weight loss.[21] Thus, FGF21 and GDF15 may play a major role in weight-loss maintenance.

Paradoxically, circulating levels of FGF21 and GDF15 are chronically elevated in obesity,[22–25] leading to the assumption that obesity is a state of FGF21 and GDF15 resistance.[26,27] However, in contrast to obesity-related leptin resistance, exogenous administration of FGF21 or GDF15 analogs promotes beneficial metabolic responses in individuals with obesity and/or type 2 diabetes.[28–31] This indicates altered but retained responsiveness of FGF21 and GDF15 receptors in obesity and suggests that rewiring of the endogenous FGF21-GDF15 system may contribute to sustaining weight loss. Nevertheless, clinical data on FGF21 and GDF15 during and following anti-obesity treatments, such as exercise and GLP-1RA therapy, are inconsistent and limited to short-term and small-scale studies.

Dyslipidemia plays a major role in the pathogenesis of obesity-related cardiometabolic disorders. Sphingolipids such as ceramides are among the most harmful lipids for metabolic and cardiovascular health.[32] Emerging evidence indicates that ceramides are not only powerful biomarkers for cardiovascular risk[33–35] but also critical modulators of energy balance through a direct action on the central nervous system,[36–39] implying their potential contribution toward sustained weight loss. In this context, pre-clinical studies show an intertwined relationship between FGF21-adiponectin-leptin and ceramide metabolism, arguing in favor of a metabokine-ceramide axis modulating energy expenditure.[40–42] However, it remains unclear whether the circulating sphingolipid landscape is reconfigured during long-term sustained weight loss and whether specific sphingolipid remodeling signatures are linked to alterations in the circulating metabokine profile.

The overarching aim of this study was to characterize alterations in putative mediators of long-term weight-loss maintenance in humans with obesity. Through exploratory analyses of a four-arm randomized controlled trial, we first described adaptations within circulating metabokines and sphingolipids in response to an initial diet-induced weight loss and a subsequent weight-loss maintenance phase, including exercise and GLP-1RA treatment. Next, we profiled metabokine-sphingolipid alterations elicited by sustained weight-loss maintenance per se as compared with weight regain. We hypothesized that FGF21 and GDF15, along with the adipose-derived metabokines adiponectin and leptin, would be altered in response to the metabolic rewiring that accompanies sustained weight-loss maintenance and that these alterations would be interconnected with beneficial and selective remodeling signatures of the circulating sphingolipidome.

RESULTS

Overview of the study and changes in body weight, body composition, and appetite sensations

We collected data from adults with obesity and without diabetes who completed a randomized controlled trial for weight-loss maintenance.[43] Participants underwent an initial weight loss phase consisting of an 8-week low-calorie diet (800 kcal/day). Thereafter, participants were randomly assigned to a 1-year weight-loss maintenance phase including either an exercise program plus placebo, treatment with the GLP-1RA liraglutide, a combination of exercise and liraglutide, or placebo. Among the 195 participants who were randomized, 166 completed the trial (i.e., attended the visit 1 year after randomization irrespective of adherence to the assigned treatment) and were considered for the current analysis (Table 1 and Figure 1A). Supplementary analyses in the 130 participants who completed the trial according to the study protocol (per-protocol population; Table S1) are presented as supplemental information (Figures S1 and S2).

In the 166 participants who completed the trial, the low-calorie diet resulted in an average weight loss of 13.5 kg. After the 1-year weight-loss maintenance phase, the placebo group regained 5.9 kg, while the exercise (+1.7 kg; placebo-corrected difference: −4.2 kg; 95% confidence interval (CI), −8.0 to −0.3) and liraglutide (−1.4 kg; placebo-corrected difference: −7.3 kg; 95% CI, −11.1 to −3.5) groups maintained the weight loss, and the combined exercise and liraglutide group achieved a further reduction in body weight (−3.7 kg; placebo-corrected difference: −9.6 kg; 95% CI, −13.4 to −5.9). Changes in body composition, appetite sensations, and food preferences in the 166 participants who completed the trial are presented in Table S2.

Circulating GDF15 transiently increases upon diet-induced weight loss whereas FGF21 decreases following weight-loss maintenance with combined exercise and GLP-1RA therapy

To characterize alterations in FGF21 and GDF15 in response to the initial weight loss and the subsequent weight-loss maintenance treatments, we measured their circulating levels before and after the low-calorie diet as well as during and after the weight-loss maintenance phase (Figure 1A). We found that FGF21 and GDF15 had distinct responses to the initial weight loss and to the subsequent weight-loss maintenance phase (Figures 1B and 1C), reinforcing the notion that these metabokines, albeit regulated by the same molecular pathway, play differential roles in metabolic adaptations.[44]

Specifically, serum FGF21 displayed highly heterogeneous responses to the 8-week low-calorie diet, resulting in a lack of significant changes (Figure 1B). This is partly aligned with prior data indicating that circulating FGF21 is either unaltered or reduced following moderate caloric restriction,[45–47] as opposed to the increases following severe caloric deficits.[48–50] Conversely, we found a marked increase in serum GDF15 in response to the low-calorie diet (Figure 1C), corroborating prior data showing slight but significant increments following short-term ($\leq$8 weeks) calorie restriction.[51,52] This, together with evidence indicating that GDF15 is unaffected by longer-term hypocaloric regimens,[53] supports the concept that GDF15, contrary to FGF21, acts as an endocrine signal of acute/short-term nutritional stress.

Exercise training and GLP-1RA therapy have been shown to elicit distinct as well as treatment duration-dependent alterations in circulating FGF21 and GDF15. Here, we report that after

Table 1. Demographic and clinical characteristics of study participants at baseline (week −8), at randomization (i.e., after the low-calorie diet; week 0), and after the weight-loss maintenance treatment period (week 52)

	All (*n* = 166)	Placebo (*n* = 40)	Exercise (*n* = 40)	Liraglutide (*n* = 41)	Ex + Lira (*n* = 45)
Male/female (*n*)	61/105	15/25	15/25	14/27	17/28
Age (years)	44 ± 12	44 ± 12	45 ± 12	46 ± 10	44 ± 13
Body weight (kg)					
wk −8	110 ± 15	–	–	–	–
wk 0	97 ± 13	97 ± 13	97 ± 13	94 ± 13	98 ± 12
wk 52	–	103 ± 14	98 ± 15	93 ± 18	95 ± 17
Body mass index (kg/m^2)					
wk −8	36.9 ± 2.9	–	–	–	–
wk 0	32.5 ± 2.8	32.3 ± 3.0	32.3 ± 2.9	32.4 ± 2.9	32.7 ± 2.4
wk 52	–	34.2 ± 3.1	32.9 ± 3.7	31.9 ± 4.7	31.4 ± 4.5
Fat mass (kg)					
wk −8	44.8 ± 6.9	–	–	–	–
wk 0	37.3 ± 6.9	36.7 ± 6.5	36.4 ± 8.5	37.4 ± 6.3	38.7 ± 6.2
wk 52	–	39.3 ± 6.7	34.4 ± 8.5	35.5 ± 9.1	34.0 ± 9.3
Fat percentage (%)					
wk −8	40.0 ± 6.1	–	–	–	–
wk 0	38.5 ± 6.8	37.8 ± 7.0	37.2 ± 7.0	39.5 ± 6.4	39.2 ± 6.9
wk 52	–	38.3 ± 6.6	35.0 ± 7.0	37.9 ± 7.2	35.7 ± 7.8
Fat-free mass (kg)					
wk −8	65.6 ± 13.3	–	–	–	–
wk 0	60.4 ± 11.9	61.4 ± 12.9	61.4 ± 10.6	58.1 ± 12.0	60.8 ± 11.9
wk 52	–	64.3 ± 13.3	64.0 ± 12.2	58.4 ± 13.7	61.5 ± 13.9
Fasting glucose (mmol/L)					
wk −8	5.3 ± 0.5	–	–	–	–
wk 0	4.9 ± 0.5	4.9 ± 0.5	5.0 ± 0.5	4.9 ± 0.4	4.9 ± 0.4
wk 52	–	5.2 ± 0.4	5.1 ± 0.5	4.8 ± 0.4	4.8 ± 0.4
Fasting insulin (ρmol/L)					
wk −8	98.3 ± 53.7	–	–	–	–
wk 0	46.4 ± 23.8	53.6 ± 31.5	39.8 ± 19.9	40.1 ± 16.7	51.5 ± 22.3
wk 52	–	79.5 ± 49.2	52.8 ± 27.0	60.0 ± 43.5	60.8 ± 25.0
Glucagon (ρmol/L)					
wk −8	10.2 ± 6.1	–	–	–	–
wk 0	7.7 ± 4.4	7.7 ± 3.1	7.5 ± 4.6	7.9 ± 4.2	7.8 ± 5.3
wk 52	–	8.1 ± 5.5	7.7 ± 4.9	7.9 ± 4.1	7.0 ± 5.3
Hemoglobin A1c (%)					
wk −8	36.3 ± 4.0	–	–	–	–
wk 0	34.0 ± 3.6	34.5 ± 3.7	33.8 ± 3.7	34.0 ± 3.5	33.6 ± 3.4
wk 52	–	35.3 ± 3.7	34.3 ± 3.2	32.7 ± 3.2	32.7 ± 3.2
HOMA-IR					
wk −8	3.9 ± 2.4	–	–	–	–
wk 0	1.7 ± 1.0	2.0 ± 1.4	1.5 ± 0.8	1.5 ± 0.7	1.9 ± 0.9
wk 52	–	3.1 ± 2.0	2.0 ± 1.2	2.2 ± 1.7	2.2 ± 1.0
Total cholesterol (mmol/L)					
wk −8	5.0 ± 1.0	–	–	–	–
wk 0	4.0 ± 0.9	4.1 ± 0.8	3.9 ± 0.9	4.3 ± 0.8	3.8 ± 0.9
wk 52	–	4.7 ± 1.0	4.6 ± 0.8	4.7 ± 0.9	4.4 ± 0.8

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Table 1. ***Continued***

	All (n = 166)	Placebo (n = 40)	Exercise (n = 40)	Liraglutide (n = 41)	Ex + Lira (n = 45)
HDL cholesterol (mmol/L)					
wk −8	1.3 ± 0.3	–	–	–	–
wk 0	1.1 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
wk 52	–	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.4	1.4 ± 0.4
LDL cholesterol (mmol/L)					
wk −8	3.1 ± 0.8	–	–	–	–
wk 0	2.4 ± 0.8	2.5 ± 0.7	2.3 ± 0.8	2.7 ± 0.7	2.2 ± 0.8
wk 52	–	2.8 ± 1.0	2.7 ± 0.7	2.9 ± 0.7	2.5 ± 0.7
VLDL cholesterol (mmol/L)					
wk −8	0.62 ± 0.28	–	–	–	–
wk 0	0.47 ± 0.17	0.49 ± 0.17	0.44 ± 0.13	0.45 ± 0.14	0.49 ± 0.23
wk 52	–	0.50 ± 0.22	0.51 ± 0.22	0.44 ± 0.18	0.54 ± 0.27
Triglycerides (mmol/L)					
wk −8	1.5 ± 1.0	–	–	–	–
wk 0	1.0 ± 0.4	1.1 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.5
wk 52	–	1.1 ± 0.5	1.1 ± 0.5	1.0 ± 0.4	1.2 ± 0.6
Alanine aminotransferase (U/L)					
wk −8	35 ± 22	–	–	–	–
wk 0	45 ± 46	49 ± 41	40 ± 27	36 ± 22	52 ± 71
wk 52	–	30 ± 14	24 ± 8	23 ± 10	25 ± 15
C-reactive protein (mg/L)					
wk −8	6.2 ± 7.2	–	–	–	–
wk 0	5.2 ± 7.2	6.1 ± 9.5	5.1 ± 6.4	3.7 ± 4.4	5.7 ± 7.5
wk 52	–	4.3 ± 5.5	3.7 ± 4.4	3.2 ± 4.0	3.3 ± 6.2
Systolic blood pressure (mmHg)					
wk −8	133 ± 16	–	–	–	–
wk 0	122 ± 13	122 ± 15	123 ± 14	121 ± 12	121 ± 12
wk 52	–	127 ± 16	127 ± 16	121 ± 16	122 ± 16
Diastolic blood pressure (mmHg)					
wk −8	86 ± 10	–	–	–	–
wk 0	79 ± 8	79 ± 7	78 ± 8	80 ± 8	78 ± 8
wk 52	–	82 ± 9	79 ± 10	80 ± 10	78 ± 10
Resting heart rate (bpm)					
wk −8	73 ± 10	–	–	–	–
wk 0	69 ± 11	70 ± 10	66 ± 12	68 ± 9	70 ± 13
wk 52	–	70 ± 9	64 ± 13	72 ± 9	71 ± 12
$\dot{V}O_2$max (L/min)					
wk −8	2.55 ± 0.62	–	–	–	–
wk 0	2.44 ± 0.66	2.48 ± 0.72	2.61 ± 0.74	2.32 ± 0.56	2.35 ± 0.57
wk 52	–	2.55 ± 0.81	2.96 ± 0.81	2.38 ± 0.71	2.69 ± 0.70

Data are means ± SD. HOMA-IR, homeostatic model assessment of insulin resistance; HDL/LDL/VLDL, high-/low-/very-low-density lipoprotein; $\dot{V}O_2$max, maximal oxygen uptake.

52 weeks of weight-loss maintenance treatment, serum FGF21 levels decreased with combined exercise and GLP-1RA therapy as compared with placebo or exercise alone. These results are consistent with previous studies showing unaltered FGF21 levels in response to either exercise training[54,55] or long-term treatment with the GLP-1RA liraglutide,[23] as opposed to the increased FGF21 reported following short-term treatment with liraglutide.[56] In contrast to FGF21, we observed that serum GDF15 increased following 26 weeks of exercise alone or in combination with GLP-1RA therapy, with no effect of GLP-1RA alone, as compared with placebo (Figure 1C); however, such increments were no longer apparent after 52 weeks. These findings agree with previous

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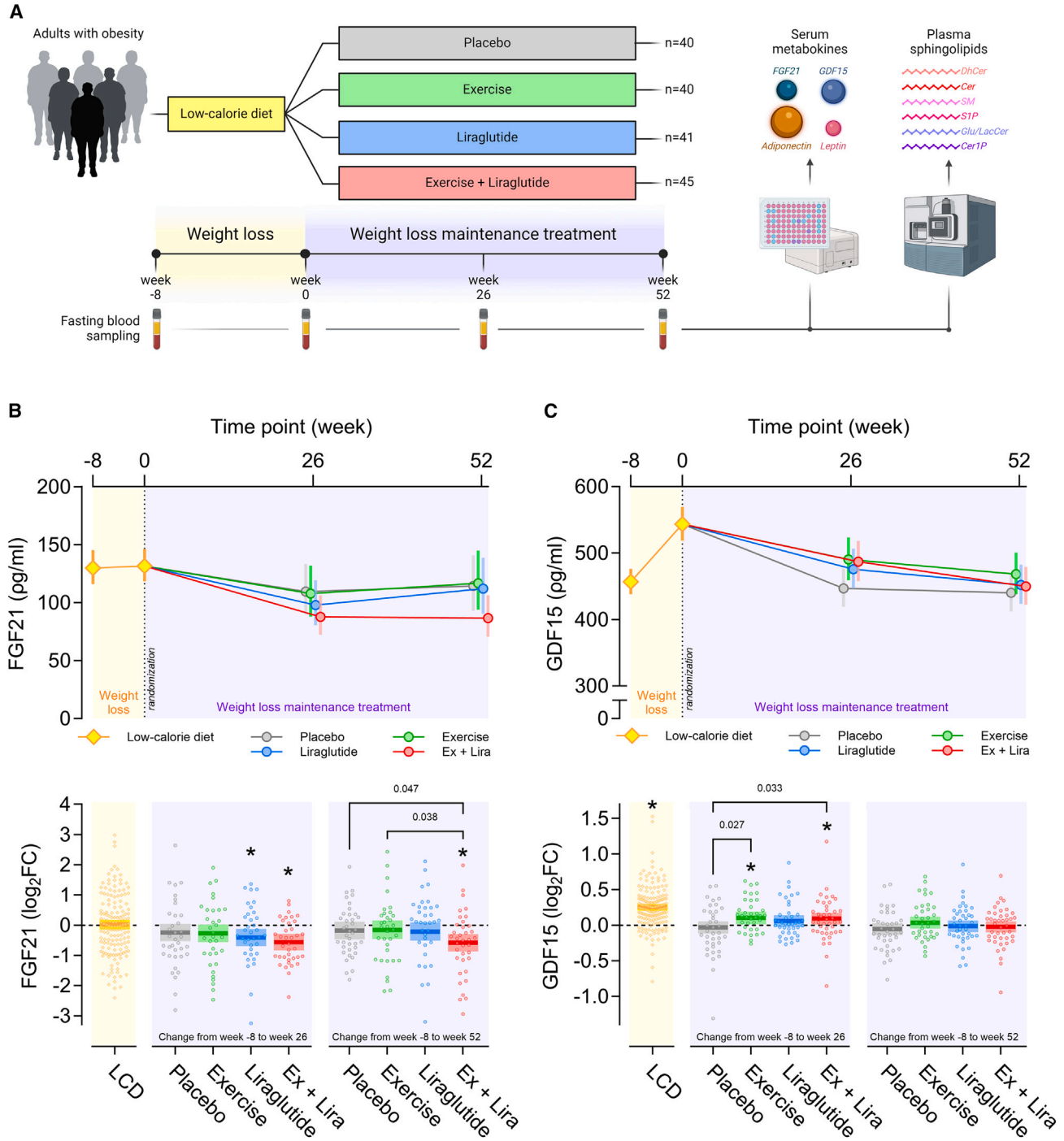

Figure 1. Circulating GDF15 transiently increases upon diet-induced weight loss whereas FGF21 decreases following weight-loss maintenance with combined exercise and GLP-1RA therapy

(A) Schematic overview of the study design.

(B and C) Levels of and changes in serum FGF21 and GDF15 as measured by ELISA in fasting blood samples. Time-course data are presented as estimated means ±95% confidence limits. Fold changes are expressed as the $\log_2$ fold change relative to baseline (week −8) and are presented as observed individual values with estimated means ±95% confidence limits. Constrained linear mixed models were used to estimate within- and between-treatment differences. *Significant within-treatment change ($p < 0.05$).

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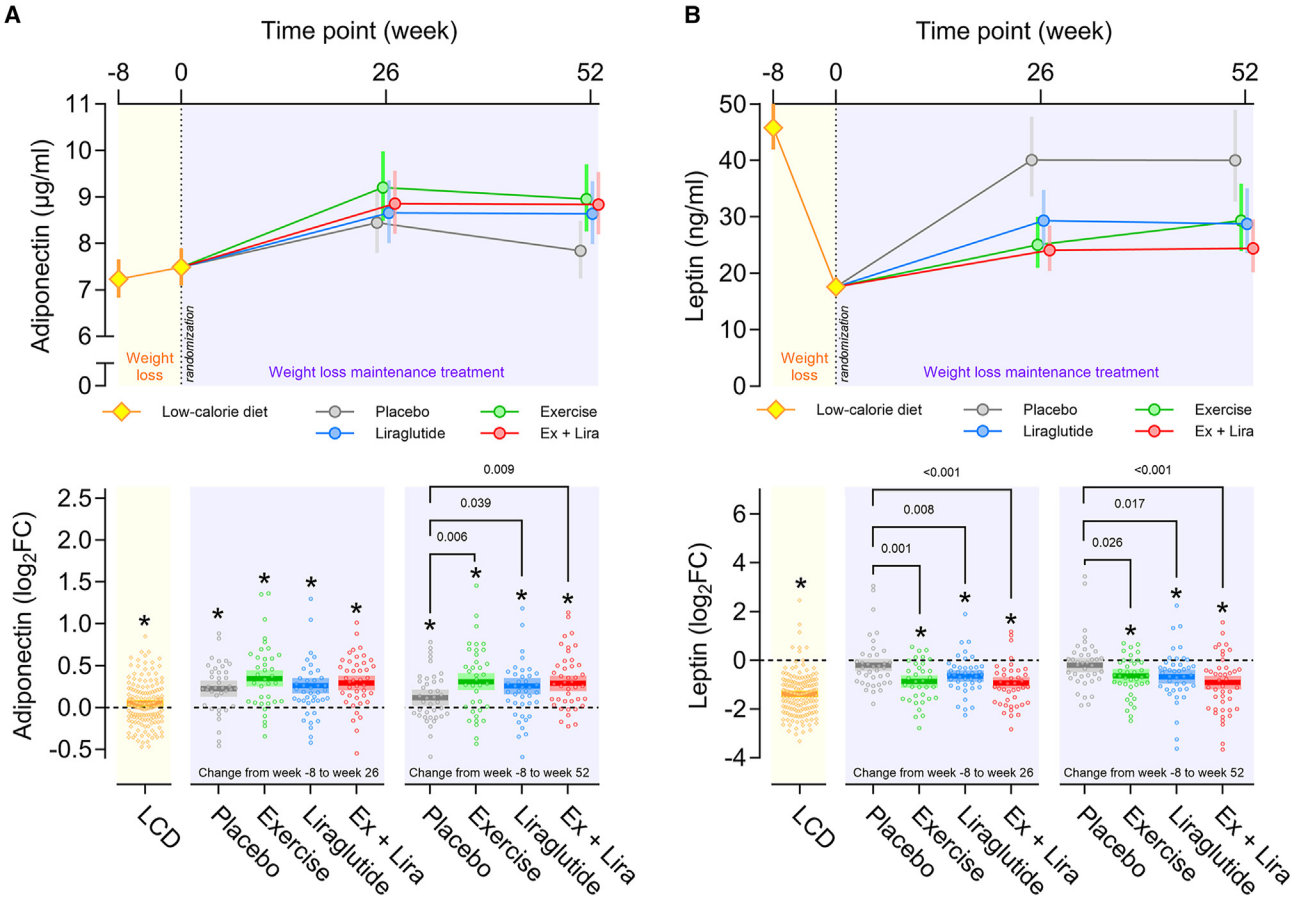

Figure 2. Circulating adiponectin and leptin profiles are ameliorated following weight-loss maintenance with exercise, GLP-1RA, and their combined treatment

Levels of and changes in serum adiponectin (A) and leptin (B) as measured by ELISA and radioimmunoassay (RIA), respectively, in fasting blood samples. Time-course data are presented as estimated means ±95% confidence limits. Fold changes are expressed as $\log_2$ fold change relative to baseline (week −8) and are presented as observed individual values with estimated means ±95% confidence limits. Constrained linear mixed models were used to estimate within- and between-treatment differences. *Significant within-treatment change ($p < 0.05$).

data suggesting that short-term but not long-term exercise affects circulating GDF15.[16,57] Likewise, the present results align with the documented lack of change in circulating GDF15 following either short- or long-term treatment with the GLP-1RA liraglutide alone.[23,58]

Taken together, the present findings indicate that the favorable body weight regulation elicited by combined exercise and GLP-1RA therapy was associated with a decline in circulating FGF21, suggesting that FGF21 signaling may be involved in sustained maintenance of weight loss.

Circulating adiponectin and leptin profiles are ameliorated following weight-loss maintenance with exercise, GLP-1RA, and their combined treatment

As the metabolic effects of FGF21 and GDF15 are partly mediated by adiponectin and leptin,[40,59–62] we measured the circulating levels of these adipose-derived metabokines. We found that adiponectin and leptin were upregulated and downregulated, respectively, in response to the low-calorie diet (Figure 2), a finding consistent with prior evidence[63] and in line with the observed concomitant reduction in fat mass.

It is well established that exercise and GLP-1RA therapy ameliorate circulating adiponectin and leptin profiles in individuals with obesity.[63–65] Here, we show that 1-year treatment with either exercise, GLP-1RA, or a combination of both increased adiponectin and decreased leptin levels as compared with placebo.

Taken together, these results indicate that after the initial weight loss, treatments promoting sustained maintenance of reduced body weight elicited further increases in plasma adiponectin and retained decrements in plasma leptin.

Individual ceramide and sphingosine-1-phosphate species are reduced following weight-loss maintenance with GLP-1RA alone or combined with exercise

Given the interplay between metabokines and ceramides,[40,66–69] we next explored whether the observed alterations in circulating metabokines were associated with changes in the plasma

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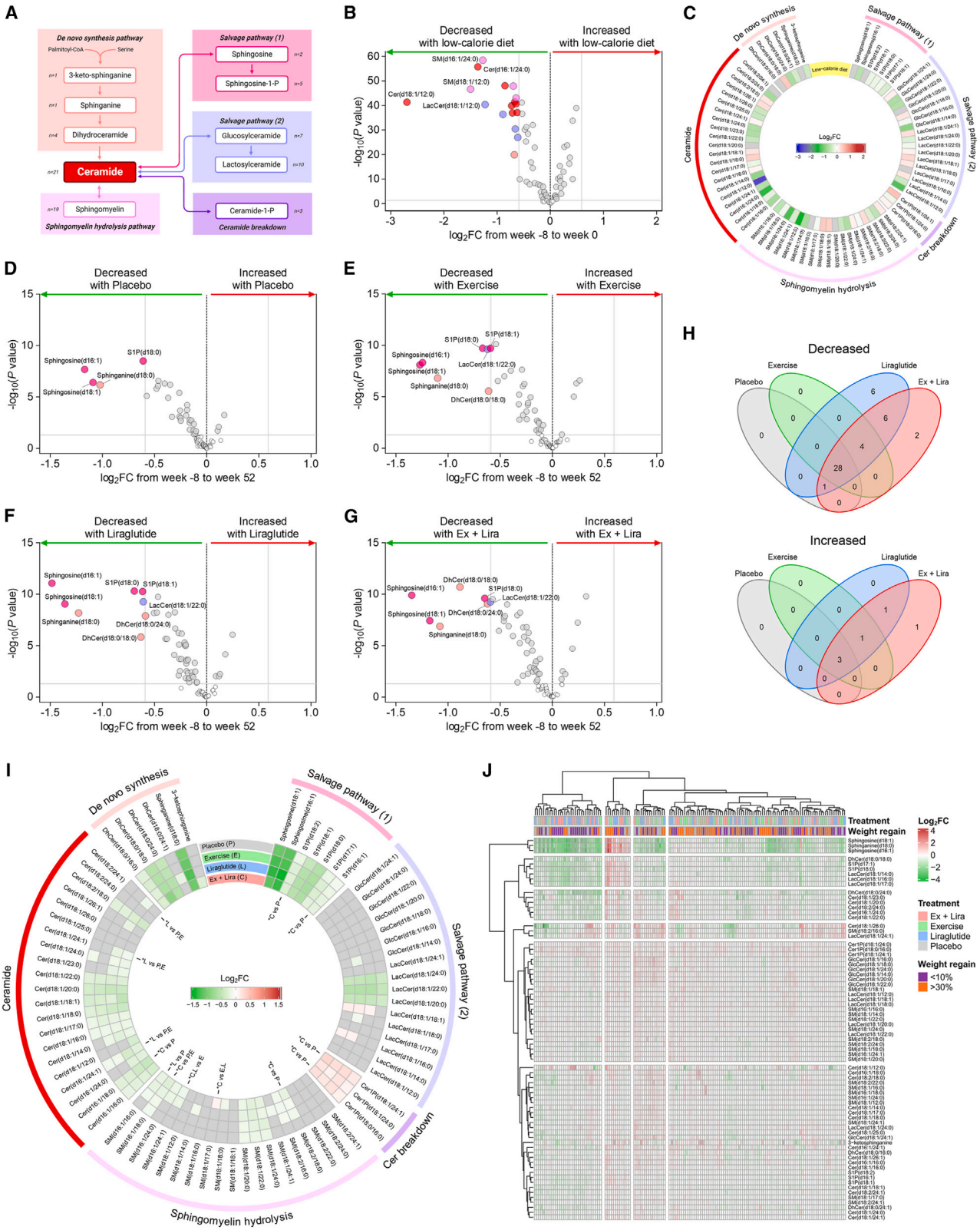

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sphingolipid profile. To this end, we employed a targeted sphingolipidomics approach, which enabled the quantification of 73 lipid species involved in the sphingolipid metabolic pathway (Figure 3A).

Our analysis revealed plasma sphingolipidome-wide remodeling in response to the low-calorie diet, with 42 and 21 sphingolipid species being downregulated and upregulated, respectively (Figures 3B and 3C). Interestingly, diet-induced weight loss was not associated with decrements in ceramide species causally linked to metabolic dysfunction, i.e., those containing the sphingoid base sphingosine (d18:1) and C16 or C18 acyl chain.[69,70] Instead, C16:0 and C18:0 ceramides were slightly but significantly increased following the low-calorie diet, likely due to the lipolytic stimulus elicited by caloric restriction, which ultimately led to ceramide release from adipose and skeletal muscle tissue.

In addition to ceramides, other bioactive sphingolipids, such as sphingosine-1-phosphate (S1P), are associated with obesity-related metabolic dysfunction.[71] While hypothalamic S1P exerts anorexigenic effects, pre-clinical models of obesity display higher circulating levels of S1P, possibly because of a compensatory mechanism for the lower hypothalamic expression of S1P receptors.[37] Here, we observed small but significant decrements in plasma S1P following the low-calorie diet (Figure 3C), which may indicate improved hypothalamic S1P sensitivity.

Despite the emerging clinical significance of ceramides for cardiometabolic health,[34] few human data are available on the effects of anti-obesity treatments on the circulating sphingolipidome. In the current study, we observed an overall decrease in plasma ceramide levels following GLP-1RA therapy alone or in combination with exercise (Figure 3I). Notably, ceramide species containing the C24:1 acyl chain were reduced to a greater extent in response to GLP-1RA therapy as compared with placebo and exercise (Figure 3I). The present decline in long and very-long acyl chain (C16–C22) ceramides following exercise treatment partly aligns with a prior study reporting exercise-induced decrements in a wider range of ceramide species in humans with obesity.[72] Likewise, the lower plasma ceramide levels observed following liraglutide treatment corroborate previous findings[73] and further extend the pool of ceramide species responsive to GLP-1RA therapy.

Interestingly, we found that plasma sphingoid base levels (i.e., sphinganine, sphingosine, and S1P) were overall reduced in a treatment-independent fashion following the weight-loss maintenance phase and that sphingosine was the sphingolipid metabolite most markedly decreased (Figures 3D–3G and 3J). This trend was also apparent for dihydroceramides (Figures 3I and 3J), suggesting that sphingolipid metabolites involved in the "salvage" or "*de novo* synthesis" pathway were markedly downregulated during weight-loss maintenance.

Taken together, the present results indicate that the circulating sphingolipidome underwent substantial remodeling in response to diet-induced weight loss as well as to weight-loss maintenance treatments. Remarkably, the initial weight loss and the subsequent weight-loss maintenance phase evoked divergent alterations in a subset of ceramides causally linked to obesity.

Differential alterations in FGF21, adiponectin, leptin, and individual sphingolipid species in weight maintainers and weight regainers

Next, to interrogate the putative contribution of metabokines and sphingolipids to sustained weight loss per se independent of the weight-loss maintenance treatment, we performed subgroup analyses of participants who either maintained or regained weight during the weight-loss maintenance phase. Participants were assigned to the "weight maintainers" and "weight regainers" subgroups if, at the end of the weight-loss maintenance phase, they regained either <10% or >30%, respectively, of the weight lost in response to the initial low-calorie diet (Figure 4A).

We observed that circulating FGF21 and leptin were downregulated, whereas adiponectin was upregulated in weight maintainers as compared with weight regainers (Figures 4B–4E). In addition, a multitude of sphingolipid species were differentially regulated in weight maintainers versus regainers, most notably dihydroceramides, ceramides, sphingosines, and S1P (Figure 4F).

Taken together, these findings further support the concept of an interconnected regulation of metabokines and sphingolipids during sustained weight-loss maintenance and suggest that remodeling of the circulating metabokine-sphingolipid profile depends on weight-loss maintenance per se rather than on the specific weight-loss maintenance treatment.

Wide range of metabokine-sphingolipid associations

In light of pre-clinical data advocating for an FGF21-adiponectin-ceramide axis modulating energy balance,[40–42] we next sought to explore the translational significance of this mechanism by assessing the relationship between circulating metabokine and sphingolipid levels. At baseline, we observed that FGF21 and

Figure 3. Individual ceramide and S1P species are reduced following weight-loss maintenance with GLP-1RA alone or combined with exercise

(A) Schematics of sphingolipid metabolism pathway. Italic numbers indicate the individual lipid species identified for each class of sphingolipids.

(B and C) Volcano plot and heatmap showing changes, expressed as the $\log_2$ fold change relative to baseline (week −8), in plasma levels of individual sphingolipid species in response to the 8-week low-calorie diet. Gray color in heatmap denotes non-significant changes.

(D–G) Volcano plots showing within-treatment changes, expressed as the $\log_2$ fold change relative to baseline (week −8), in plasma levels of individual sphingolipid species in response to the 52-week weight-loss maintenance treatments.

(H) Venn diagrams showing the overlap between treatment-induced significant changes in plasma sphingolipids.

(I) Heatmap showing changes in plasma levels of individual sphingolipid species, expressed as $\log_2$-transformed fold changes relative to baseline (week −8), in response to the 52-week treatment with placebo (P), exercise (E), liraglutide (L), and combined exercise and liraglutide (C). Constrained linear mixed models were used to estimate within-treatment changes and between-treatment differences. Gray color denotes non-significant within-treatment changes. *Significant between-treatment difference ($p < 0.05$).

(J) Hierarchical clustering of changes in individual sphingolipid species in response to the weight-loss maintenance phase. Each column represents a study participant.

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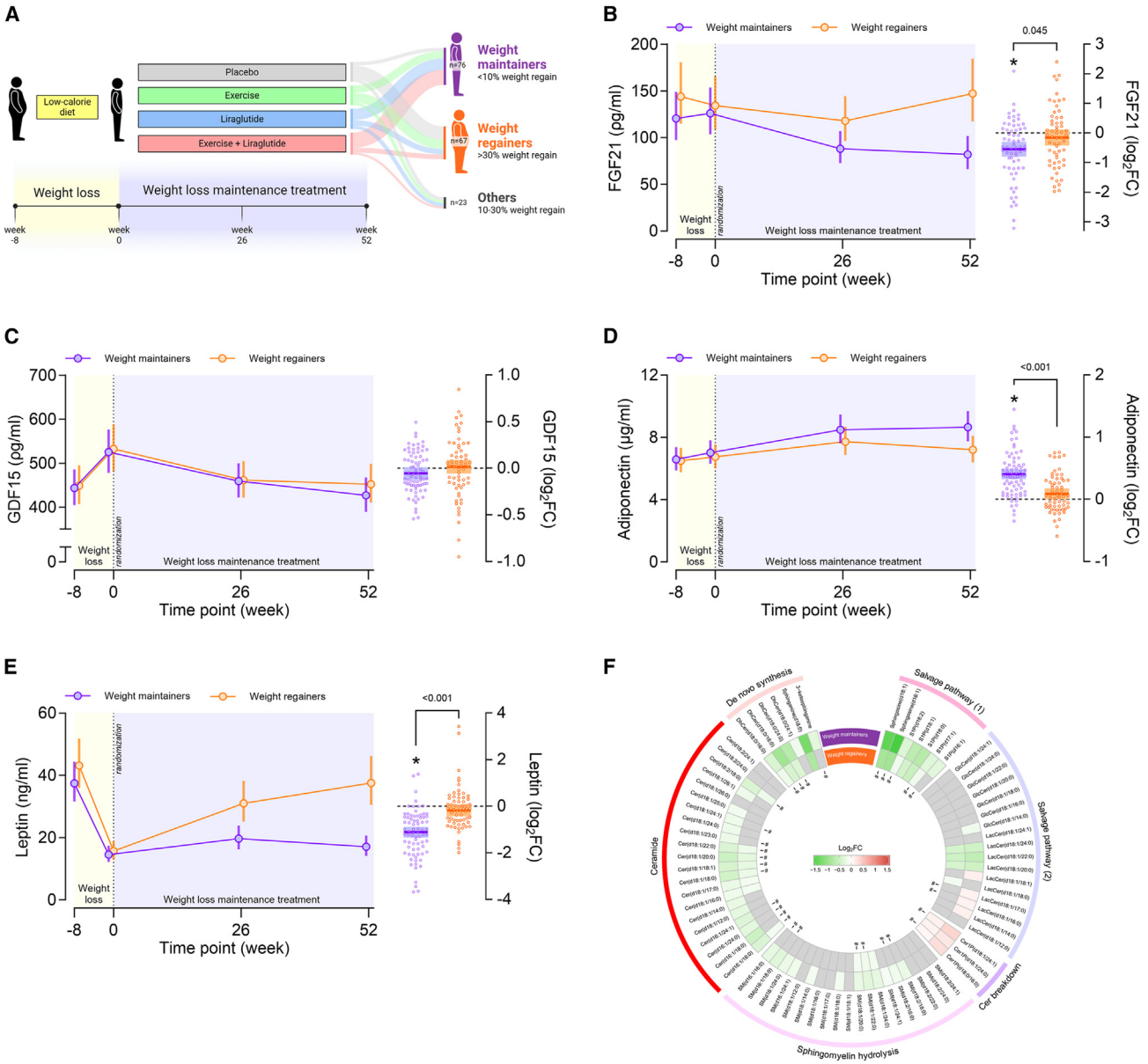

Figure 4. Differential alterations in FGF21, adiponectin, leptin, and individual sphingolipid species in weight maintainers and weight regainers

(A) Schematics of the weight "maintainers" and "regainers" subgroup analysis.

(B–E) Levels of and changes in circulating metabokines in weight maintainers and weight regainers. Time-course data are presented as estimated means ±95% confidence limits. Changes were computed as $\log_2$-transformed fold changes (Log_2FC) at the end of treatment (week 52) relative to baseline (week −8) and are presented as observed individual values with estimated means ±95% confidence limits.

(F) Changes in circulating sphingolipids in weight maintainers and weight regainers. Changes were computed as $\log_2$-transformed fold changes (Log_2FC) at the end of treatment (week 52) relative to baseline (week −8). Linear mixed models were used to estimate within-group changes and between-group differences. *Significant within-group change ($p < 0.05$). #Significant between-group difference ($p < 0.05$).

adiponectin levels were inversely related and that these metabokines were associated with a multitude of sphingolipid species, most notably dihydroceramides and C18:0-C20:0 ceramides (Figure 5). Next, we tested whether these associations were also apparent in the adaptive response to the weight-loss maintenance phase and found an inverse relationship between the magnitude of change in adiponectin and C18:0-C20:0 ceramides, whereas positive associations were found between alterations in FGF21 and S1P species. These findings not only point toward the translational significance of the FGF21-adiponectin-ceramide axis in humans but also extend this mechanism to a larger pool of sphingolipid species.

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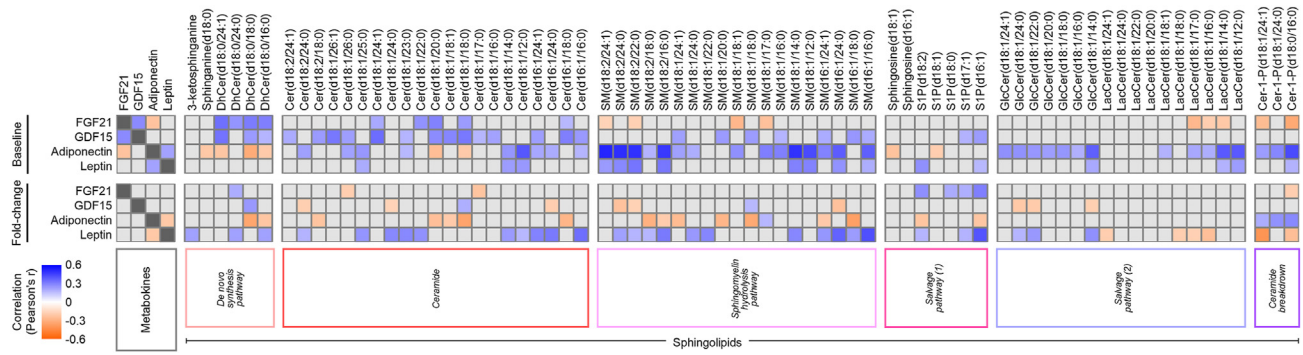

Figure 5. Wide range of metabokine-sphingolipid associations

Correlations between baseline (week −8) levels of and changes (log_2-transformed fold change at the end of treatment (week 52) relative to baseline (week −8) in circulating metabokines and sphingolipids. For this analysis, all four weight-maintenance treatment groups were pooled together. Only significant correlations ($p < 0.05$) are shown (gray color denotes non-significant correlations).

Besides FGF21 and adiponectin, leptin has also been proposed to interact with ceramide metabolism.[66] Here, we report positive associations between baseline leptin levels and a subset of S1P species. This suggests a concomitant regulation of leptin and S1P sensitivity and aligns with pre-clinical data indicating that leptin signaling mediates the anorexigenic effects of hypothalamic S1P.[37] Interestingly, we also found that the magnitude of change in leptin following the weight-loss maintenance phase was positively associated with changes in a multitude of sphingolipid species, most notably dihydroceramides, ceramides, sphingomyelins, and glucosylceramides.

Altogether, the present findings indicate an intertwined relationship between metabokines and bioactive sphingolipids, suggesting a conserved role for a metabokine-sphingolipid axis in the regulation of energy balance in humans with obesity.

Remodeling of the circulating metabokine-sphingolipid profile is associated with changes in cardiometabolic health outcomes

Circulating levels of FGF21, GDF15, adiponectin, and leptin are closely related to cardiometabolic health.[74–76] Likewise, individual sphingolipid species are directly linked to cardiometabolic risk.[33,77,78] To interrogate the clinical relevance of the observed alterations in circulating metabokines and sphingolipids, we examined whether such alterations were associated with changes in cardiometabolic health outcomes (Figure 6).

Among the metabokines, we found that alterations in FGF21, adiponectin, and leptin, but not GDF15, were associated with changes in body weight and fat mass. Interestingly, despite the purported insulin-sensitizing action of FGF21,[60] no significant associations were found with indices of insulin resistance and glycemic control, which, however, were associated with alterations in adiponectin levels. Among the bioactive sphingolipids, alterations in a subset of dihydroceramide, ceramide, and S1P species were positively associated with changes in body weight and fat mass. Conversely, alterations in ceramide-1-phosphate species were negatively associated with changes in body composition and glycemic control indices. Unsurprisingly, we found a large number of positive associations between changes in plasma ceramide and cholesterol levels, supporting the mounting body of evidence indicating that these molecules share common regulatory mechanisms.[34]

Taken together, these data indicate that the overall improvements in cardiometabolic health elicited by the weight-loss maintenance phase were associated with selective alterations in circulating metabokines and sphingolipids.

DISCUSSION

Through exploratory analyses of blood samples from a cohort of adults with obesity enrolled in a randomized controlled trial for weight-loss maintenance, we performed longitudinal profiling of metabolic cytokines and bioactive sphingolipids deemed to affect energy balance and, thereby, potentially contributing to long-term weight-loss maintenance. Owing to the design of the trial, including an initial diet-induced weight loss followed by long-term treatment with exercise, GLP-1RA, or both combined as interventions to prevent weight regain, we demonstrated that FGF21 and GDF15 respond differently to diverse weight regulation phases. Specifically, serum GDF15 levels, but not FGF21, increased upon the initial diet-induced weight loss. In contrast, serum FGF21 levels, but not GDF15, decreased in response to the subsequent weight-loss maintenance phase with combined exercise and GLP-1RA therapy, and this coincided with increased adiponectin and decreased leptin levels. While we observed plasma sphingolipidome-wide remodeling throughout the different phases of the study, ceramide species linked to obesity displayed divergent alteration patterns in response to diet-induced weight loss as compared with the subsequent weight-loss maintenance phase. Subgroup analyses in participants who either maintained or regained the weight lost demonstrated that weight-loss maintenance (independent of the treatment) elicited a decline in circulating FGF21 and leptin, an increase in adiponectin, and more marked decrements in ceramide and S1P species as compared with weight regain. Lastly, we reveal several associations between circulating metabokines and sphingolipids, whose alterations in response to the weight-loss maintenance phase were in turn associated with favorable changes in markers of cardiometabolic health. From a clinical

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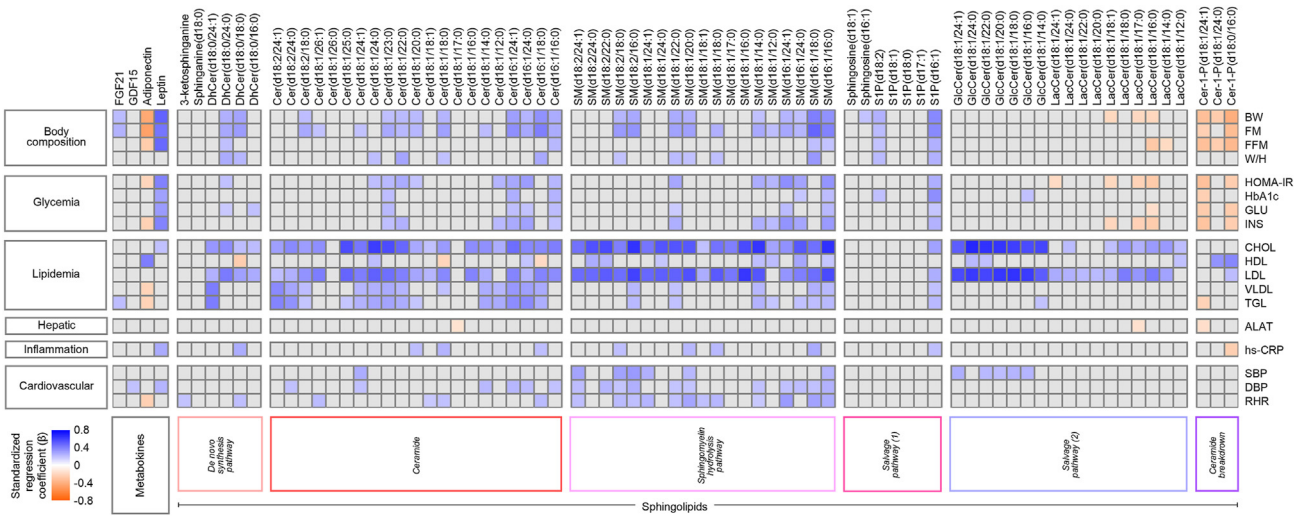

Figure 6. Remodeling of the circulating metabokine-sphingolipid profile is associated with changes in cardiometabolic health outcomes

Associations between changes in circulating metabokine-sphingolipid levels and markers of cardiometabolic health. Changes were computed as the $\log_2$-transformed fold change at the end of treatment (week 52) relative to baseline (week −8). For this analysis, all four weight-maintenance treatment groups were pooled together. Linear regression models adjusted for treatment group, age, gender, and baseline value of the outcome variable were used to calculate standardized β regression coefficients. Only significant associations ($p < 0.05$) are shown (gray color denotes non-significant associations). BW, body weight; FM, fat mass; FFM, fat-free mass; W/H, waist-to-hip ratio; GLU, fasting glucose; INS, fasting insulin; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; VLDL, very-low-density lipoprotein cholesterol; TGL, triglycerides; ALAT, alanine aminotransferase; hs-CRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure; DBP, diastolic blood pressure; RHR, resting heart rate.

standpoint, these associations, although not necessarily indicative of causation, underscore the significance of alterations within the circulating metabokine-sphingolipid profile for healthy weight-loss maintenance.

Despite emerging data supporting the metabolic benefits of exogenous FGF21 or GDF15 analogs, it remains elusive whether and how these metabokines respond to or mediate the metabolic rewiring that accompanies sustained weight loss. In this context, the vast majority of clinical evidence is limited to short-term and/or small-scale studies.[54–56,58] In a few long-term large-scale clinical studies, circulating FGF21 and GDF15 levels were unaltered following 1-year treatment with the GLP-1RA liraglutide.[23] Likewise, circulating GDF15 levels remained unchanged after 26 weeks of exercise.[57] However, these studies described responses specifically associated with weight loss rather than weight-loss maintenance. Given the emerging dichotomous view of weight loss and weight-loss maintenance, i.e., distinct processes characterized by different determinants and different responses to treatments,[79] it remains unclear whether the endogenous FGF21-GDF15 system is further remodeled with sustained weight-loss maintenance. The present study addresses this gap by profiling changes in circulating FGF21 and GDF15 during an initial weight loss and a subsequent weight-loss maintenance phase, thus enabling the discernment of alterations that occur in response to promotion versus maintenance of weight loss.

The observation that FGF21 levels were lowered after 1-year treatment with combined exercise and GLP-1RA therapy (i.e., the most effective intervention in preventing weight regain) as compared with placebo suggests a link between FGF21 signaling and sustained weight-loss maintenance. This is further corroborated by subgroup analyses in weight maintainers and regainers, showing that FGF21 decreased to a greater extent in response to weight maintenance as compared with weight regain. These findings align with data from obesity surgery studies showing a trend toward a decrease in circulating FGF21 levels 12 months after bariatric surgery,[23,46,80–82] as opposed to the apparent increase occurring 3–6 months after surgery.[49,80,83–85] Thus, while surgery-induced weight loss is mediated by neural pathways beyond those targeted by GLP-1RA therapy or exercise,[86] data from obesity surgery studies suggest a decrease in FGF21 following long-term weight-loss maintenance. Mechanistically, the observed decline in circulating FGF21 may stem from ameliorated FGF21 sensitivity due to increased expression of its receptors. Indeed, pre-clinical data indicate that exercise stimulates the expression of fibroblast growth factor receptor 1 (FGFR1) in adipose tissue,[87] whereas GLP-1RA therapy attenuates obesity-related reductions in hepatic levels of FGFR1 and the co-receptor β-klotho.[12] Conversely, the absence of GDF15 alterations in response to weight-loss maintenance treatments may indicate a secondary role for GDF15 signaling in long-term weight regulation with exercise and/or GLP-1RA therapy. In partial support of this, the GDF15-GFRAL pathway has been shown to be independent of the GLP1 pathway.[88–90] Notably, the marked increase in GDF15 along with the decline in leptin observed in response to the initial diet-induced weight loss agrees with prior data indicating extensive crosstalk between GDF15 and leptin signaling in mediating weight loss.[91]

Taken together, these findings indicate that FGF21 and GDF15 respond and possibly contribute to distinct phases of body weight regulation in human obesity, ultimately supporting the dichotomous view of weight loss and weight-loss

maintenance as distinct processes affected by differential mechanisms.

Here, we show that, in concert with FGF21-adiponectin-leptin, the circulating sphingolipidome was also remodeled during weight-loss maintenance. The finding that ceramide species associated with metabolic dysfunction (C16:0 and C18:0 ceramides) increased upon initial diet-induced weight loss, but decreased following the subsequent weight-loss maintenance phase, further underlines the importance of discriminating the factors potentially involved in either of these weight regulation phases. In this direction, the present study provides insight into the prognostic utility of ceramide scores.[35,92,93] Indeed, according to the ceramide risk score,[92,94] the observed increase in C16:0 and C18:0 ceramides, along with the decrease in C24:0 ceramide upon the diet-induced weight loss, albeit transient, indicates an increased risk for cardiovascular disease. This contrasts with the overall amelioration of conventional biomarkers of cardiovascular disease risk (i.e., cholesterol and triglycerides) and indicates that plasma ceramide and cholesterol levels are differentially regulated in response to a short-term caloric deficit; an important factor to account for in the interpretation of ceramide risk scores in clinical practice.

Conclusions

The present study describes dynamic alterations in circulating metabokines and bioactive sphingolipids across different phases of body weight regulation, with specific emphasis on diet-induced weight loss and exercise- and/or GLP-1RA-mediated maintenance of weight loss. Overall, our findings indicate that weight-loss maintenance was accompanied by interconnected alterations in FGF21-adiponectin-leptin and bioactive sphingolipids, supporting the translational relevance of a metabokine-sphingolipid axis modulating energy balance and potentially involved in the maintenance of reduced body weight. Furthermore, the changes in cardiometabolic health outcomes observed in response to the weight-loss maintenance phase were associated with selective alterations of the circulating metabokine and sphingolipid profiles, pointing to clinical relevance of changes in individual metabokines and sphingolipids for healthy weight-loss maintenance.

Limitations of the study

Here, we report exploratory analyses of a randomized controlled trial designed with changes in body weight as the primary endpoint.[43] The rather heterogeneous changes in body weight among participants undergoing the same weight-loss maintenance treatment may limit the inferences that can be drawn on the association between weight-loss maintenance and metabokine-sphingolipid alterations. To address this, we conducted subgroup analyses of weight ''maintainers'' and ''regainers'' demonstrating that, independent of the treatment, weight-loss maintenance and weight regain elicited distinct alterations in the circulating FGF21-adiponectin-leptin-sphingolipid profile.

The present findings describe changes in systemic metabokine and sphingolipid levels, thus providing insights into their potential endocrine action in sustaining a reduced body weight. However, FGF21 and GDF15 may also act in an autocrine/paracrine manner,[75,95] implying that tissue-specific analyses would be necessary to fully elucidate their potential role in mediating weight-loss maintenance. Furthermore, additional research is warranted to ascertain whether the observed changes in fasting FGF21 and GDF15 levels are similarly evident in the fed state. Lastly, although we report a wide range of associations between circulating metabokines and sphingolipids during body weight regulation, these associations are not proof of causation. In this direction, mechanistic human studies including selective manipulation of endogenous metabokine and/or sphingolipid levels would clarify how these factors influence each other and ultimately affect energy balance in humans with obesity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101629.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.F., A.C., C.E.W., and S.S.T.; clinical study design, B.M.S., J.J.H., S.M., and S.S.T.; clinical investigation and sample collection, R.M.S., S.B.K.J., C.R.J., J.R.L., and C.J.; sample analysis, M.F., A.C., and

M.H.N.; formal analysis, M.F.; visualization, M.F. and N.P.B.; writing – original draft, M.F. and R.M.S.; writing – review and editing, M.F. and S.S.T.; funding acquisition, C.E.W. and S.S.T. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript.

DECLARATION OF INTERESTS

A family member of R.M.S. holds Novo Nordisk stocks. S.M. is on advisory boards of AstraZeneca, Boehringer Ingelheim, Eli Lilly, Merck Sharp & Dohme, Novo Nordisk, and Sanofi Aventis; receives lecture fees from AstraZeneca, Boehringer Ingelheim, Merck Sharp & Dohme, Novo Nordisk, and Sanofi Aventis; and is a research grant recipient from Novo Nordisk and Boehringer Ingelheim. J.J.H. is on the advisory board of Novo Nordisk. S.S.T. is a research grant recipient and receives lecture fees from Novo Nordisk.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Methanol Optima™ LC-MS	Fisher Chemicals	A454-212
Acetonitrile Optima™ LC-MS	Fisher Chemicals	A955-212
Isopropanol Optima™ LC-MS	Fisher Chemicals	A461-212
Formic acid Optima™ 99%LC-MS	Fisher Chemicals	A117-50
Ammonium Formate LC-MS >99%	Sigma-Aldrich	70221
Cer(d16:1/16:0)	Cayman Chemicals	24426
Cer(d18:1/02:0)	Avanti Polar Lipids	860502
Cer(d18:1/04:0)	Avanti Polar Lipids	860504
Cer(d18:1/08:0)	Avanti Polar Lipids	860508
Cer(d18:1/10:0)	Avanti Polar Lipids	860510
Cer(d18:1/12:0)	Avanti Polar Lipids	860512
Cer(d18:1/14:0)	Avanti Polar Lipids	860514
Cer(d18:1/16:0)	Avanti Polar Lipids	860516
Cer(d18:1/17:0)	Avanti Polar Lipids	860517
Cer(d18:1/18:0)	Avanti Polar Lipids	860518
Cer(d18:1/18:1)	Avanti Polar Lipids	860519
Cer(d18:1/20:0)	Avanti Polar Lipids	860520
Cer(d18:1/22:0)	Avanti Polar Lipids	860501
Cer(d18:1/24:0)	Avanti Polar Lipids	860524
Cer(d18:1/24:1)	Avanti Polar Lipids	860525
CerP(d18:1/16:0)	Avanti Polar Lipids	860533
CerP(d18:1/24:0)	Avanti Polar Lipids	860527
DhCer(d18:0/16:0)	Avanti Polar Lipids	860634
DhCer(d18:0/18:0)	Avanti Polar Lipids	860627
DhCer(d18:0/24:0)	Avanti Polar Lipids	860628
DhCer(d18:0/24:1)	Avanti Polar Lipids	860629
GlcCer(d18:1/12:0)	Avanti Polar Lipids	860543
GlcCer(d18:1/16:0)	Avanti Polar Lipids	860539
GlcCer(d18:1/18:0)	Avanti Polar Lipids	860547
GlcCer(d18:1/18:1)	Avanti Polar Lipids	860548
GlcCer(d18:1/24:1)	Avanti Polar Lipids	860549
GlucosylSph(d18:1)	Avanti Polar Lipids	860535
LacCer(d18:1/12:0)	Avanti Polar Lipids	860545
LacCer(d18:1/16:0)	Avanti Polar Lipids	860576
LacCer(d18:1/17:0)	Avanti Polar Lipids	860595
LacCer(d18:1/18:0)	Avanti Polar Lipids	860598
LacCer(d18:1/18:1)	Avanti Polar Lipids	860590
LacCer(d18:1/24:0)	Avanti Polar Lipids	860577
LacCer(d18:1/24:1)	Avanti Polar Lipids	860597
S1P(d17:1)	Avanti Polar Lipids	860641
S1P(d18:1)	Avanti Polar Lipids	860492
SM(d18:1/12:0)	Avanti Polar Lipids	860583
SM(d18:1/16:0)	Avanti Polar Lipids	860584

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SM(d18:1/17:0)	Avanti Polar Lipids	860585
SM(d18:1/18:0)	Avanti Polar Lipids	860586
SM(d18:1/18:1)	Avanti Polar Lipids	860587
SM(d18:1/24:0)	Avanti Polar Lipids	860592
SM(d18:1/24:1)	Avanti Polar Lipids	860593
Spa(d18:0)	Avanti Polar Lipids	860498
Spa1P(d18:0)	Avanti Polar Lipids	860536
Sph(d18:1)	Avanti Polar Lipids	860490
d_7-Cer(d18:1/16:0)	Avanti Polar Lipids	860676P
d_7-Cer(d18:1/18:0)	Avanti Polar Lipids	860677P
d_7-Cer(d18:1/24:1)	Avanti Polar Lipids	860679P
d_9-SM(d18:1/18:1)	Avanti Polar Lipids	791649C
d_7-Sph(d18:1)	Avanti Polar Lipids	860657P
d_7-S1P(d18:1)	Avanti Polar Lipids	860659P
d_3-GlcCer(d18:1/16:0)	Matreya LLC	1533
d_5-GlcCer(d18:1/18:0)	Avanti Polar Lipids	860638P
d_3-LacCer(d18:1/16:0)	Matreya LLC	1534
Critical commercial assays		
Human FGF-21 ELISA Kit	BioVendor R&D	RD191108200R
Human GDF-15 Quantikine ELISA Kit	R&D Systems	DGD150
Human Adiponectin ELISA Kit	BioVendor R&D	RD195023100
Human Leptin RIA Kit	Millipore	HL-81K
Software and algorithms		
GraphPad Prism v.9.3.0	GraphPad	N/A
SAS v.9.4	SAS Institute	N/A
Masslynx (version v4.1)	Waters Corporation	N/A
Targetlynx (version v4.1)	Waters Corporation	N/A
Other		
Liraglutide 6 mg/mL, Saxenda®	Novo Nordisk A/S	N/A
Placebo	Novo Nordisk A/S	N/A
Meal replacement products	Cambridge Weight Plan	N/A
Waters Acquity Binary Solvent Manager i-Class	Waters	N/A
Waters Acquity Sample Manager	Waters	N/A
Waters Xevo TQ-S mass spectrometer	Waters	N/A
Zorbax Eclipse Plus C18, RRHD (100 mm × 2.1 mm, 1.8 μm)	Agilent	959758–902
Zorbax Eclipse Plus C18 guard column (5 mm × 2.1 mm, 1.8 μm)	Agilent	821725–901
Amber LC-MS vials with pre-slit caps	Waters	600000669CV
LC-MS vial 150 μL inserts	Waters	WAT094171

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Signe S. Torekov (torekov@sund.ku.dk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Individual participant data reported in this paper will be made available, after anonymization, under the European Union's General Data Protection Regulation (EU GDPR) and the Danish Data Protection Agency regulations from the lead contact upon request and will require a signed data sharing agreement.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

A total of 215 participants were enrolled in the trial, of which 195 completed the low-calorie diet and were randomized (at week 0) in a 1:1:1:1 ratio stratified by gender (male/female as defined by the gender assigned at birth as per the civil registration number in Denmark) and age (</≥ 40 years) to placebo (n = 49); exercise (n = 48); liraglutide (n = 49); combined exercise and liraglutide (n = 49) treatment for one year. At the end of the trial, 166 participants attended final assessments. Among the 195 participants who were randomized, 166 participants completed the trial (i.e., attended the visit one year after randomization irrespective of adherence to the assigned treatment) and were considered in the present study. Among the 166 participants who completed the trial, 130 participants adhered to their assigned treatment (per-protocol population).

Inclusion criteria were: i) age 18–65 years old, ii) body mass index 32 to 43 kg/m^2, and iii) usage of safe contraceptive methods or being in a post-menopausal state. Exclusion criteria were: i) presence of any known severe chronic disease, such as type 1 or 2 diabetes, angina pectoris, coronary heart disease, congestive heart failure, severe renal or hepatic impairment, inflammatory bowel disease, gastroparesis, cancer, chronic obstructive lung disease, psychiatric disorders, including a history of major depressive or other severe psychiatric conditions, ii) usage of medications known to affect body weight, iii) prior bariatric surgery, iv) a history of idiopathic acute pancreatitis, v) familial or personal history of multiple endocrine neoplasia type 2 or familial medullary thyroid carcinoma, vi) osteoarthritis deemed too severe for full participation in the exercise program, vii) pregnancy, planned pregnancy, or breastfeeding, viii) allergies to any of the study medication components, and ix) habitual high-intensity exercise exceeding 2 h per week.

Study participants were provided with comprehensive oral and written information and subsequently signed a written consent form. The trial was approved by the Ethical Committee in the Central Danish Region (H-16027082) and the Danish Medicines Agency (EudraCT: 2015-005585-32). The trial adhered to ICH Good Clinical Practice guidelines and the principles outlined in the Declaration of Helsinki.

METHOD DETAILS

Study design

The present study is part of a double-blind, randomized, placebo-controlled trial conducted at Hvidovre Hospital and the University of Copenhagen, Denmark, from August 2016 to November 2019. The trial protocol and primary outcome results (body weight) have been previously reported.[43,96] Here, we present exploratory analyses from blood samples taken before and after an 8-week low-calorie diet, as well as after 26 and 52 weeks of treatment with either exercise, GLP-1RA therapy, combined exercise and GLP-1RA therapy, or placebo plus usual activity.

Low-calorie diet

All study participants followed a low-calorie diet (800 kcal/day; Cambridge Weight Plan) for 8 weeks, with weekly meetings and weighing with trial staff. Participants who lost ≥5% of their initial body weight during the low-calorie diet were randomized to 1 year of weight-loss maintenance treatment.

Following randomization, all participants engaged in 12 individual consultations aimed at supporting weight-loss maintenance. These consultations included body weight measurements and dietary guidance aligned with the dietary recommendations established by the Danish Authorities. The guidelines provided comprehensive dietary advice, including recommendations to consume a diverse range of foods, reduce fat content, incorporate whole grains and vegetables, limit salt and sugar intake, and prioritize hydration through water consumption. Participants were provided with informational pamphlets outlining these guidelines, and their contents were thoroughly reviewed. Additionally, participants were encouraged to seek inspiration from websites and digital applications that facilitate activities such as calorie counting and meal planning, promoting the adoption of a healthier lifestyle. For a detailed account of the weight-loss maintenance support provided during the trial, including specific dietary advice, see the study protocol.[43]

Exercise program

The exercise intervention was based on the World Health Organization (WHO) recommendations of minimum 150 min of moderate intensity exercise per week, 75 min of vigorous intensity exercise per week, or a combination of both.[97] Participants were encouraged to take part in group exercise sessions twice a week and in solo exercise twice a week as well. The group sessions lasted for 45 min, comprising 30 min of high-intensity interval cycling exercise, followed by 15 min of circuit training combining vigorous aerobic

exercise and resistance-based exercises. During individual sessions, participants had the freedom to choose their preferred form of exercise, as long as it fell within the moderate-to-vigorous intensity range. Exercise intensity was monitored by pulse watches with heart rate monitors (Polar A300, Polar Electro, Finland). No exercise was permitted 24 h prior to examinations. The exercise intervention is described in detail elsewhere.[43] Participants not randomized to exercise, were instructed to maintain their habitual physical activity level.

GLP-1RA treatment and placebo

Liraglutide (Saxenda) at a concentration of 6 mg/mL and a volume-matched placebo were self-administered by the participants through subcutaneous injections using injector pens. The initial dose was 0.6 mg/day, with weekly increments of 0.6 mg/day following consultations, up to a maximum of 3.0 mg/day. In cases where participants experienced adverse events that were deemed unacceptable at the intended dose, they were given the highest dose that they could tolerate without such adverse events occurring. Enrollment of participants was not affected by the discontinuation of medication.

Adherence to the interventions

Within the 166 participants who completed the trial, those in the exercise group were engaged in 118 ± 74 min/week of exercise (125 ± 66 and 112 ± 85 min/week from week 7 to week 26 and from week 27 to week 52, respectively) at an average intensity corresponding to 78 ± 4% of their maximum heart rate (HR_{max}) (78 ± 4 and 78 ± 4 %HR_{max} from week 7 to week 26 and from week 27 to week 52, respectively). Similarly, participants in the combined exercise and liraglutide group participated in 112 ± 73 min/week of exercise (117 ± 67 and 108 ± 86 min/week from week 7 to week 26 and from week 27 to week 52, respectively) at an average intensity of 79 ± 5 %HR_{max} (79 ± 4 and 78 ± 5 %HR_{max} from week 7 to week 26 and from week 27 to week 52, respectively). Across the treatment groups receiving liraglutide therapy, participants adhered to an average dose of 2.8 mg/day.

The per-protocol population was defined as participants who completed the interventions as prescribed, which required them to engage in a minimum of 75% of the physical activity recommendations outlined by the WHO (150 min/week of moderate-intensity, or 75 min/week of vigorous-intensity aerobic physical activity, or an equivalent combination of both), and consistently take 2.4 or 3.0 mg/day of liraglutide or placebo for at least 75% of the intervention duration. Within the per-protocol population, the exercise group was engaged in 156 ± 54 min/week of exercise (157 ± 47 and 155 ± 64 min/week from week 7 to week 26 and from week 27 to week 52, respectively) at an average intensity corresponding to 78 ± 4% of their maximum heart rate (HR_{max}) (79 ± 4 and 78 ± 4 %HR_{max} from week 7 to week 26 and from week 27 to week 52, respectively). Similarly, the combined exercise and liraglutide group participated in 144 ± 67 min/week of exercise (152 ± 63 and 149 ± 88 min/week from week 7 to week 26 and from week 27 to week 52, respectively) at an average intensity of 78 ± 5 %HR_{max} of (79 ± 4 and 79 ± 5 %HR_{max} from week 7 to week 26 and from week 27 to week 52, respectively). Across the treatment groups receiving liraglutide therapy, the per-protocol population consistently adhered to a dose of at least 2.9 mg/day.

Blood sample collection, processing, and storage

Venous blood samples were collected in the fasted state (≥ 10 h) at week −8 (before the low-calorie diet, baseline), week 0 (after the low-calorie diet, at randomization), week 26 (mid-visit), and week 52 (end-of-treatment). Vacuette EDTA and serum tubes were centrifuged at 2000 g for 10 min to collect plasma and serum, which were stored at −80°C until analysis.

Serum metabokines

Serum FGF21, GDF15, and adiponectin levels were measured by ELISA according to the manufacturer's instructions. Serum leptin levels were measured by radioimmunoassay.

Plasma sphingolipidomics

Sample extraction

For the extraction, samples were divided into 16 extraction batches consisting of 45 samples, 2 QC of Extraction (QCExt) and 1 Blank of Extraction each. On the analysis day, samples and QCs were thawed at 4°C in a refrigerator. Samples were then vortexed for 30 s and 25 μL of each sample were transferred to an Eppendorf tube. Next, 10 μL of an internal standard solution containing deuterium labeled sphingolipids (at least one per class) was added to each sample. After 10 s vortexing, 250 μL of LC-MS methanol was added to each Eppendorf tube. Eppendorf tubes were then closed and vortexed for 10 s. Afterward, samples were sonicated for 15 min on an ice bath to facilitate protein precipitation and avoid the temperature increasing above 20°C. Samples were then centrifuged at 12000 g for 15 min. An aliquot of 100 μL was finally transferred to two LC-MS vials equipped with a 150 μL insert. To prepare the QC of injection (QCInj), 50 μL of each sample from the first four extraction batches was pooled into a 4 mL glass vial. The extract mixture was then pooled and aliquoted back in LC-MS vials as the samples to control for the instrumental quantification reproducibility. Finally, all samples and QCs were stored at −20°C until LC-MS quantification, within two weeks of extraction.

LC-MS quantification of sphingolipids

Relative quantification of sphingolipids was performed as previously described.[73] Chromatographic separation was carried out on an ACQUITY UPLC System with a sample manager cooled to 8°C (Waters Corporation, Milford, MA, USA). Sphingolipids were separated on a Zorbax Rapid Resolution RRHD C18 Column, 80 Å, 1.8 μm, 2.1 mm × 100 mm (Agilent Technologies; 758700-902) using

a guard column (Agilent Technologies, 821725-901) (5 × 2 mm, 1.8 μm particle size). Mobiles phases A and B consisted of 5mM ammonium formate (Sigma; 70221)/0.2% formic acid (Optima, Fisher-Scientific, 10596814) in water and in methanol (VWR, 34966), respectively. Separation was carried out at a 450 μL/min flow rate and at a column temperature of 40°C. The following chromatographic gradient was used: 0 min, 75% B; time range 0 → 1 min, 75% B (constant); time range 1 → 5 min, 85 → 100% B (linear increase); time range 5–15.2 min, 100% B (isocratic range); time range 15.2 → 15.3 min, 100 → 75% B (linear decrease); time range 15.3 → 16 min, 75% B (isocratic column conditioning).

Samples were then analyzed on a Waters Xevo TQ-S system equipped with an Electrospray Ion Source (ESI) and ScanWave collision cell technology operating in the positive mode. A class specific single reaction monitoring (SRM) transition for each sphingolipid and internal standard was used. For compounds with no commercially available standard, predicted retention time was estimated based on number of carbons and unsaturations from class analogue sphingolipids. The method does not distinguish glucosylated species (GlcCer) from galactosylated species (GalCer), and Glc sphingolipids are therefore potentially a mixture of the two species. However, GlcCer represents more than 90% of the total hexosylceramide mixture.

QUANTIFICATION AND STATISTICAL ANALYSIS

To test the hypothesis that weight loss and weight-loss maintenance treatments elicit alterations (relative to baseline (week −8)) in circulating levels of metabokines and sphingolipids and to investigate whether these alterations differ between weight-loss maintenance treatments, we used constrained longitudinal data analysis via linear mixed models,[98] i.e., all treatment groups were assumed to be equal before randomization (Figures 1, 2, and 3). Constrained linear mixed models included treatment, time (week), age, gender, and treatment-time interaction as fixed factors, an unstructured covariance pattern, and a repeated effect for time at the participant level. Next, to test the hypothesis that weight-loss maintenance and weight regain per se (i.e., irrespective of the treatment) elicit differential alterations (relative to baseline (week −8)) in circulating levels of metabokines and sphingolipids, we conducted comparative analyses of two subgroups based on the magnitude of weight regain (Figure 4). Specifically, participants were assigned to "maintainers" and "regainers" subgroups if percent weight regain from initial lost weight was <10% or >30%, respectively. For these analyses, within- and between-group differences were estimated using linear mixed models including subgroup, time (week), age, gender, subgroup-time interaction, treatment, and treatment-time interaction as fixed factors, an unstructured covariance pattern, and a repeated effect for time at the participant level. In case of heteroscedasticity (i.e., unequal variance), $\log_2$ transformation was applied before analysis.

To test the hypothesis of an intertwined relationship between circulating metabokines and sphingolipids, correlation analyses were performed by Pearson correlation between baseline levels of and changes in metabokine and sphingolipid levels (Figure 5). For these analyses, all four weight-maintenance treatment groups were pooled together. Lastly, linear regression models were used to explore the association between changes in metabokine-sphingolipid levels and markers of cardiometabolic health (Figure 6). For these analyses, all four weight-maintenance treatment groups were pooled together, and the models adjusted for treatment group, age, gender, and baseline value of the outcome variable.

All the analyses were exploratory and unadjusted for multiplicity. The level of significance for all analyses was set at $p < 0.05$. p values were evaluated using Kenward-Roger approximation of the degrees of freedom. Analyses were performed using SAS Enterprise Guide version 7.15.

Data are graphically presented as observed individual values with model-based estimated means ±95% confidence limits, unless otherwise stated.

ADDITIONAL RESOURCES

The trial is registered under the identifier EudraCT: 2015-005585-32 and ClinicalTrials.gov: NCT04122716.