Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

The Effect of Short-Term Overfeeding on Serum Lipids in Healthy Humans

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Objectives: While chronic obesity is associated with alterations in circulating glycerolipids, sphingolipids and plasmalogens, the effects of short-term overfeeding in humans are unclear.

Design and Methods: Healthy individuals ($n = 40$) were overfed by 1,250 kcal day^{-1} for 28 days. Insulin sensitivity (hyperinsulinemic-euglycemic clamp), abdominal fat distribution and serum lipidomics (mass spectrometry) were assessed.

Results: Overfeeding increased liver fat, insulin resistance, serum C-reactive protein and urinary F2-isoprostanes. HDL increased (11% $\pm$ 2%, $P < 0.001$) while LDL, triglycerides and nonesterified fatty acids were unchanged. Three hundred and thirty three serum lipids were detected, of which 13% increased and 20% decreased with overfeeding. Total diacylglycerol and lysoalkylphosphatidylcholine (LPC(O)) concentrations decreased ($P < 0.01$), while total ceramide, Cer22:0 and Cer24:0 increased ($P \leq 0.01$). The most notable increases were observed in the HDL-associated phosphatidylethanolamine-based plasmalogens and their precursors alkylhosphatidylethanolamine (18 $\pm$ 5% and 38 $\pm$ 8% respectively, $P \leq 0.01$).

Conclusions: Overfeeding led to weight gain and changes in the serum lipid profile. Increases in ceramides were noted, which left unchecked may promote systemic insulin resistance. Uniform increases were observed in plasmalogens and their precursors. Because plasmalogens are powerful antioxidants, this may be an appropriate response against increased oxidative stress generated by over-nutrition. The metabolic consequences of changes in concentrations of many circulating lipid species with overfeeding require further study.

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Introduction

Chronic obesity is associated with impaired lipid homeostasis in muscle and liver. Circulating lipid species are a reflection of liver lipid composition (1,2) and associate with obesity and insulin resistance (2). Experimental over-nutrition and weight gain increase the deposition of fat in the liver and whole body insulin resistance (3,4). However, the circulating lipid(s) that potentially mediate this relationship are unclear.

Supra-physiological elevations of circulating nonesterified fatty acids (NEFA) by infusion of lipid emulsion induce insulin resistance in humans (5). However, short-term overfeeding (3-9 days) reduces fasting NEFA (6-9), despite reducing insulin sensitivity (3,9). Increases in circulating triacylglycerol, diacylglycerol (10) and ceramide species (11) are observed in obese compared with lean individuals and may contribute to insulin resistance. Obesity is also associated with decreased vinyl-ether linked phospholipids (plasmalogens) (12) and increased phosphatidylethanolamine (PE), phosphatidylcholine (PC), and alkylphosphatidylethanolamine (PE(O)) (10), with either increased (10,12) or decreased (13) lysophosphatidylcholine (LPC) species. Sexual dimorphism in lipid metabolism, including differences in concentrations of circulating lipid species (14) and in the

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metabolic response to changes in dietary fatty acid composition (15) have also been reported. In the fasted state, low density lipoprotein (LDL) is the major carrier of ceramide in the circulation, while the majority of plasmalogens are carried on high density lipoprotein (HDL) (18).

Caloric restriction and weight loss decreased serum triacylglycerol, phosphatidylethanolamine and phosphatidylcholine species (16). However, concentrations of sphingomyelin, ceramide and LPC species were unchanged (16). Notably, gastric bypass surgery with substantial weight loss decreased circulating ceramides in severely obese individuals (17). To our knowledge, there are no published serum lipidomics data from overfeeding studies in humans. In mice, high fat overfeeding increased triacylglycerol, diacylglycerol, and sphingolipid species, including sphingomyelin and ceramide species and decreased LPC species (13).

In the present study we evaluated the effect of 28 days of overfeeding on lipid species in serum in men and women. We hypothesized that overfeeding will increase diacylglycerol and ceramide species and decrease plasmalogens and that change(s) in serum lipid species with overfeeding would be correlated with changes in lipoprotein profile and insulin resistance.

Methods

Participants

The study cohort, intervention and metabolic tests have been described previously (4,9). The complete cohort described previously (4,9) was included in the present investigation. Briefly, sedentary, nonsmoking, nondiabetic men ($n = 21$) and women ($n = 20$) were recruited (36 Caucasian, 5 Asian). One man withdrew with a viral infection; hence, 40 subjects completed the study [age 37 ± 2 (range 21-65) years]. Five women were post-menopausal. Exclusion criteria were: weight instability (>2 kg change in the preceding 6 months), exercising more than 60 min per week, taking statins or medications known to affect insulin sensitivity or blood pressure, or a personal history of type 2 diabetes or cardiovascular disease. The study protocol was approved by the Human Research and Ethics Committee at St Vincent's Hospital, Sydney. Participants provided informed written consent before commencement. The study was registered at www.clinicaltrials.gov (NCT00562393).

Diets and physical activity

Dietary intervention was reported previously (4). Briefly, estimated energy requirement was calculated based on fat-free mass and fat mass, using equations previously generated by doubly labeled water and intake-balance techniques and a dietician planned individual menus for participants. Complete 3-day food intake was supplied before each metabolic study. From day 3 to day 0, all foods were provided at baseline energy requirements with a nutrient composition of 30% fat, 15% protein and 55% carbohydrates. On days 0-28 participants were instructed to increase their baseline energy intake by 1,250 kcal day^{-1} and the nutrient composition was targeted at 45% fat, 15% protein, and 40% carbohydrates. Towards this end, participants were provided with three high-energy, high-fat snacks per day, each providing ∼250 kcal (e.g., potato crisps, chocolate bars, cheesecake) and a liquid oil-based supplement (Benecalorie, Novartis, Basel, Switzerland, 330 kcal) mixed in a dairy dessert (∼200 kcal). Participants filled out checklists daily reporting snacks consumed. They completed 3-day diet diaries once before study commencement and twice during the overfeeding phase. Dietician counseling was provided weekly when the checklists were reviewed and any deviations from protocol were identified to provide alternative options and to improve adherence to the diet plan. Diets were analyzed for macronutrients and fatty acid composition using FoodWorks 2007 based on the Australian foods database (Xyris Software, Supporting Information Table S1). Thirty two of 40 participants returned the diet diaries of both study phases.

Participants were sedentary and were asked to maintain the same low level of physical activity throughout the study. Physical activity was assessed by questionnaires and reported levels at baseline and overfeeding were not significantly different (231 ± 2 and 230 ± 1 metabolic equivalent of task (METs) hours/week, $P = 0.4$).

Metabolic testing

As reported previously (4,9,19), participants attended the Clinical Research Facility at 8 am after a 12-h fast at baseline and at days 3 and 28 of overfeeding. Baseline and +28-day visits were identical; weight, height and blood pressure were measured and fasting blood samples were drawn. Insulin sensitivity was measured by a 2-hr hyperinsulinemic (60 mU m^{-2} min^{-1})-euglycemic (5.0 mmol L^{-1}) clamp. Clamp insulin sensitivity was calculated as the total glucose infusion rate (GIR) between 90 and 120 min of the clamp normalized to fat free mass (FFM).

Body composition

Body composition and abdominal fat distribution were estimated at baseline and 28 days of overfeeding, as described previously (4). Briefly, fat mass and fat free mass were assessed by dual energy X-ray absorptiometry (DXA, Lunar DPX-Lunar Radiation, Madison, WI). Three cross-sectional computed tomography (CT) scans (Phillips Gemini GXL), 1-cm width, centered on the L2-L3 and L4-L5 disc space, and the T12-L1 disc space were also performed to assess abdominal adipose tissue distribution and hepatic fat content. CT attenuation [Hounsfield units (HU)] was determined in three regions of interest (ROI) for liver and spleen, each ROI of ∼120 mm^2. ROIs were placed manually to avoid major vessels and were given similar placement on reassessment. Abdominal areas of adipose tissue were defined by attenuation values of −50 to 150 HU. The spleen was not visualized in two participants. CT images were analyzed using Gemini software (GXL Host System).

Biochemical analysis

Blood glucose was analyzed using a glucose oxidase electrode (YSI Life Sciences) and serum insulin by radioimmunoassay (Linco Research, St Charles, USA). HOMA-IR was calculated as fasting insulin (mU L^{-1}) × fasting glucose (mmol L^{-1})/22.5. Serum HDL cholesterol and triglycerides were evaluated by enzymatic colorimetry (Roche, IN). LDL cholesterol was calculated by the Friedewald equation. NEFA concentrations were measured by enzymatic colorimetric assay (Wako, Osaka, Japan). Urinary-F2-isoprostane was analyzed using gas chromatography-mass spectrometry in spot urine samples that were centrifuged at 4°C, snap-frozen with butylated hydroxytoluene (BHT, 0.005%) and stored at −80°C. The results

were normalized to creatinine content, as previously described (9). High sensitivity C-reactive protein (CRP) was measured using a Beckman Coulter Synchron LX system Chemistry Analyzer, with reagents and calibrators supplied by Beckman Coulter (Sydney, Australia), as previously described (19).

Sample preparation and lipid extraction

Fasting blood samples were drawn and serum collected and stored in −80°C until extraction. Quality control (QC) serum samples were included at a ratio of 1:20 throughout the assay. Lipids were extracted from 10-μL serum with a single phase chloroform:methanol (2:1 vol/vol; 20 volumes) extraction method following the addition of a 10 μL internal standard mixture (containing 20 internal standards) (20).

High performance liquid chromatography-mass spectrometry analysis

Lipid analysis was performed by liquid chromatography, electrospray ionization-tandem mass spectrometry using an Agilent 1200 liquid chromatography system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ionspray source (350°C) and Analyst 1.5 data system (20). The predominant lipid species of the following lipid classes were measured: sphingosine (Sph), dihydroceramide (dhCer), ceramide (Cer), monohexosylceramide (MHC), dihexosylceramide (DHC), trihexosylcermide (THC), G_{M3} ganglioside (GM3), sphingomyelin (SM), phosphatidylcholine (PC), alkylphosphatidylcholine (PC(O)), alkenylphosphatidylcholine (plasmalogen, PC(P)), lysophosphatidylcholine (LPC), lysoalkylphosphatidylcholine (lysoplatelet activating factor, LPC(O)), phosphatidylethanolamine (PE), alkylphosphatidylethanolamine (PE(O)), alkenylphosphatidylethanolamine (plasmalogen, PE(P)), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), cholesterol ester (CE), free cholesterol (COH), diacylglycerol (DAG) and triacylglycerol (TAG) (20-22). In addition, neutral loss (141 Da) scanning in positive mode and product ion scanning in negative mode, was employed to identify and characterize six lysophosphatidylethanolamine (LPE) species, 13 alkylphosphatidylethanolamine (PE(O)) species, and 10 alkenylphosphatidylethanolamine (plasmalogen, PE(P)) species which were combined with the existing measures.

For a number of the lipids which contain two fatty acid chains the MRM-based measurements here do not directly determine the constituent fatty acids but rather the sum of the number of carbons and the sum of the number of double bonds across both fatty acids. Accordingly, we denote these species as the combined length and number of double bonds, e.g., PC 36:4. A total of 71 diacylglycerol and triacylglycerol species and 262 other lipid species were analyzed in two separate experiments. Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding stable isotope or non-physiological internal standard. Results were expressed as μmol L^{-1} of serum. Total lipid classes were calculated from the sum of the individual lipid species within each class (20).

Statistical analysis

Data are presented as mean ± SEM. Repeated measures ANOVA was used to detect the effect of overfeeding on body composition and metabolic measures with gender as the between subject factor. Significance threshold of the metabolic and clinical data was set at $P = 0.05$. Lipidomics data were log10-transformed to ensure normal distribution prior to statistical analyses. Analyses were undertaken to produce a mixed-model ANOVA for the data based on a single between-subjects factor and a single within-subjects (repeated measures) factor. The between-subjects factor considered was gender. Tukey adjustment was made for multiple comparisons. Normal probability plots of the P values versus their rank were then examined to determine statistical significance. Relationships that were found to be nonlinear were indicative of statistically significant results. The data below P of 0.05 were in the nonlinear regions, however a more stringent statistical significance threshold of $P = 0.01$ was then applied. The percentage changes in serum lipids with 28 days overfeeding were calculated using the difference in the original (non-logged) data relative to the baseline level. Pearson's correlations were calculated between lipids detected in serum and clinical and metabolic variables, and presented as a heat map with the color indicating the pairwise linear correlation coefficient for those pairs with P values <0.01. Stepwise regression was used to determine whether adjustment for age, BMI and serum HDL, LDL and triglycerides could explain the concentrations of lipids at baseline and whether age, change (day 28—baseline, Δ) in BMI, Δ HDL cholesterol, Δ LDL cholesterol, Δ triglycerides could explain the change in the concentrations of lipids that changed significantly with overfeeding. Any missing data was removed from the analysis and not imputed. Statistical analyses were performed using SPSS 20 statistical package and custom scripts in Matlab R2011a (Mathworks).

Results

The effect of overfeeding on body composition and metabolic markers

At baseline, women had less abdominal visceral adipose tissue, lower blood glucose and higher NEFA, HDL cholesterol and CRP concentrations compared with men (Table 1). In response to overfeeding, body weight, total and abdominal fat mass depots increased and liver density (inversely proportional to liver fat) decreased, independently of gender. Fasting serum glucose, insulin, and HOMA-IR increased and clamp GIR decreased, independently of gender. Circulating NEFA, triglycerides and LDL cholesterol concentrations were unchanged, whilst CRP, HDL and total cholesterol and urinary F2-isoprostanes increased significantly with overfeeding, independently of gender (Table 1).

The effect of overfeeding on serum lipids

Few baseline differences in lipid species were detected between men and women (Supporting Information Table S2). Three hundred and thirty three lipid species were detected in serum and 110 changed with overfeeding, of which 43 (13%) increased and 67 (20%) decreased (Table 2), all were independent of gender.

We first examined the data by class of lipids and observed that total alkenylphosphatidylethanolamine (phospahtidylethanolamine-based plasmalogens, PE(P)) and their precursor alkylphosphatidylethanolamine (PE(O)) increased significantly with overfeeding ($P \leq 0.01$, Figure 1a). An increase in total ceramide concentration was also observed with overfeeding ($P = 0.01$, Figure 1a). Lysoalkylphosphatidylcholine

TABLE 1 The effect of overfeeding on adiposity and metabolic markers

	Whole cohort (n = 40)		Men (n = 20)		Women (n = 20)		RM-ANOVA P	
	Baseline	Overfeeding	Baseline	Overfeeding	Baseline	Overfeeding	Time	Group
Weight (kg)	75.3 ± 1.9	78.1 ± 1.9	81.2 ± 2.3	84.3 ± 2.3	69.4 ± 2.3**	71.9 ± 2.4	<0.001	0.3
Body mass index (kg m^{-2})	25.6 ± 0.6	26.6 ± 0.6	25.4 ± 0.7	26.4 ± 0.7	25.8 ± 0.9	26.7 ± 1.0	<0.001	0.9
Body fat mass (kg)	25 ± 1	27 ± 1	23 ± 2	25 ± 2	27 ± 2	29 ± 2	<0.001	0.4
Visceral fat (cm^2, at L4/5)	77 ± 8	86 ± 8	93 ± 14	104 ± 13	62 ± 7*	70 ± 8	<0.001	0.6
Subcutaneous fat (cm^2, at L4/5)	256 ± 17	276 ± 17	220 ± 19	240 ± 19	286 ± 26	307 ± 25	<0.001	0.8
Liver density (Hu)	55 ± 2	53 ± 2	53 ± 3	50 ± 4	56 ± 2	55 ± 2	<0.001	0.08
Liver to spleen attenuation ratio	1.25 ± 0.04	1.22 ± 0.05	1.20 ± 0.08	1.14 ± 0.08	1.30 ± 0.05	1.28 ± 0.04	0.06	0.2
Fasting glucose (mmol L^{-1})	4.5 ± 0.1	4.6 ± 0.0	4.6 ± 0.1	4.6 ± 0.0	4.4 ± 0.1*	4.5 ± 0.1	0.02	0.08
Fasting insulin (pmol L^{-1})	69 ± 4	79 ± 4	69 ± 6	80 ± 5	70 ± 5	77 ± 5	0.008	0.5
NEFA (mmol L^{-1})	0.30 ± 0.02	0.28 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	0.38 ± 0.03**	0.37 ± 0.03	0.4	0.9
Triglycerides (mmol L^{-1})	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.9	0.02
LDL-cholesterol (mmol L^{-1})	2.8 ± 0.1	2.8 ± 0.1	3.0 ± 0.2	3.0 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	0.9	0.9
HDL-cholesterol (mmol L^{-1})	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1*	1.6 ± 0.1	<0.001	0.6
Total-cholesterol (mmol L^{-1})	4.6 ± 0.2	4.8 ± 0.2	4.7 ± 0.2	5.0 ± 0.2	4.5 ± 0.2	4.6 ± 0.2	0.008	0.2
HOMA-IR	1.9 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.3 ± 0.2	1.9 ± 0.1	2.2 ± 0.2	0.005	0.9
GIR (μmol min^{-1} kg^{-1} FFM)	54.8 ± 2.8	50.3 ± 2.5	53.6 ± 4.7	47.5 ± 4.0	56.0 ± 3.2	53.2 ± 2.8	0.03	0.4
CRP (mg/L)	1.1 ± 0.2	1.5 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	1.6 ± 0.2**	2.0 ± 0.4	0.05	0.9
Urinary F2-Isoprostanes (pmol $mmol^{-1}$ creatinine)	367 ± 33	493 ± 59	316 ± 35	404 ± 33	416 ± 54	578 ± 108	0.03	0.5

Data presented as mean ± SEM.
Difference from men at baseline *$P < 0.05$ and **$P < 0.01$.

(LPC(O)) and diacylglycerol species decreased significantly ($P < 0.01$, Figure 1a). No other changes in total concentrations of lipid classes were observed.

We next examined the effects of overfeeding on each individual lipid species (Figure 1b). Fifty percent of the PE(P) plasmalogen species detected and 92% of their precursor species PE(O) increased significantly with overfeeding and none were decreased (Table 2 and Figure 1b). We observed the largest increase in PE(O-36:2) with a 93 ± 15% increase from baseline with overfeeding (Figure 1b). Whilst total concentration of the phosphatidylinositol (PI) class was unchanged, several species were significantly increased in response to overfeeding. Conversely, 67% of the LPC(O) species were significantly reduced with overfeeding (Table 2). Some species of sphingomyelin, alkenylphosphatidylcholine (PC(P)), and lyso-phosphatidylethanolamine (LPE) lipid species were also decreased significantly (Figure 1b), although total concentrations of these classes were not altered (Figure 1a).

In the remainder of lipid classes, the response to overfeeding was mixed, with some species decreasing and other species increasing significantly. Total triacylglycerol concentration was unchanged with overfeeding, but within the 44 triacylglycerol species detected, 6 decreased and 2 increased significantly (Table 2, Figure 1b). However, these were all low abundant triacylglycerol species (ranging from 0.1 to 3.4% of total class, Figure 1b). Total diacylglycerol concentration was reduced with overfeeding, and within the 27 species detected, 7 decreased and 1 increased. Three of the 7 species that decreased were of relatively high abundance, namely 16:0/18:2, 16:1/18:1, and 18:1/18:2 (ranging from 7 – 20% of total class, Figure 1b). An increase in total ceramide was also observed (P = 0.01) with significant increases in the most abundant lignoceric (Cer24:0) and less abundant behenic (Cer22:0)-containing ceramide species, whilst stearic acid (Cer18:0) and nervonic acid (Cer24:1)-containing ceramide species were reduced with overfeeding (Figure 1b). While there was no change in the total concentrations of the dihydroceramide, G_{M3} ganglioside, cholesterol ester or LPC, an increase in the species containing C22:0 and C24:0 was observed in each of these classes (Figure 1b). In contrast, C18:0 containing dihydroceramide and C24:1 containing monohexosylceramide (MHC) and G_{M3} ganglioside species significantly decreased (Figure 1b). While 67 and 70% of phosphatidylcholine (PC) and phospahtidylethanolamine (PE) species did not change with overfeeding (Table 2), there was a commonality in the structure of those species that did change (e.g., C38:6, C40:6 and C40:7 decreased and C36:1 increased in both classes, Figure 1b) and some changes in alkylphosphatidylcholine (PC(O)) species followed those in PC (C32:0 and C40:7) and PE (C36:2).

Relationships between serum lipids and metabolic markers at baseline and in response to overfeeding

Relationships between individual lipid species and age, lipoprotein and NEFA concentrations, BMI, liver density, serum CRP, urinary F2-isoprostanes and insulin resistance at baseline are provided in Figure 2a. Briefly, the majority of LPC species correlated positively with LDL cholesterol and some also correlated positively with liver density and inversely with CRP. Interestingly LPC 22:6 correlated

TABLE 2 Lipid classes detected in serum and the direction of the effect of overfeeding

Class	Abbreviation	[Class] at baseline; Mean ± SEM μmol L^{-1}	Species in class	Increased	Decreased
Alkylphosphatidylethanolamine (1 ether linked acyl chain)	PE(O)	4.1 ± 0.3	13	12	-
Ceramide	Cer	5.3 ± 0.3	6	2	2
GM3 ganglioside	GM3	2.7 ± 0.1	6	2	2
Lysoalkylphosphatidylcholine	LPC(O)	0.59 ± 0.03	9	-	6
Dihydroceramide	dhCer	0.55 ± 0.03	6	2	1
Alkenylphosphatidylethanolamine (plasmalogen 1 vinyl ether linked acyl chain)	PE(P)	3.8 ± 0.2	10	5	-
Sphingomyelin	SM	372 ± 15	19	-	8
Lysophosphatidylcholine	LPC	178 ± 8	22	2	7
Alkylphosphatidylcholine	PC(O)	45 ± 1	18	4	3
Monohexosylceramide	MHC	9.0 ± 0.5	6	1	1
Phosphatidylcholine	PC	1398 ± 46	46	2	13
Phosphatidylethanolamine	PE	29 ± 2	20	2	4
Diacylglycerol	DAG	36 ± 3	27	1	7
Alkenylphosphatidylcholine	PC(P)	20 ± 1	8	-	2
Phosphatidylinositol	PI	93 ± 4	17	4	-
Cholesterol ester	CE	2747 ± 119	26	2	4
Triacylglycerol	TAG	527 ± 44[a]	44	2	6
Lysophospahtidylethanolamine	LPE	1.5 ± 0.1	6	-	1
Sphingosine	Sph	(5.0 ± 0.4)′10^{-3}	1	-	-
Dihexosylceramide	DHC	6.0 ± 0.3	6	-	-
Trihexosylceramide	THC	1.7 ± 0.1	6	-	-
Free cholesterol	COH	820 ± 41	1	-	-
Phosphatidylglycerol	PG	0.26 ± 0.02	3	-	-
Phosphatidylserine	PS	0.47 ± 0.04	6	-	-
Bis-monoacylglycerophosphate	BMP	(21 ± 1)′10^{-3}	1	-	-
Total lipids detected			333	43	67

Classes are sorted on per cent of species within class that changed with overfeeding.
[a]TAG concentrations were not divided by a correction factor of 0.47 to adjust for the difference between response factors of the internal standard and the individual TAG species.

positively with insulin sensitivity (positively with GIR and inversely with HOMA-IR). LPC(O-16:0) and LPC(O-22:1) correlated inversely with CRP. Sphingolipids correlated mostly with LDL cholesterol and many of the ceramide and sphingomyelin species also correlated positively with age. Most triacylglycerol species correlated positively with triglycerides, whereas diacylglycerol species correlated positively with LDL and triglycerides. Many triacylglycerol and diacylglycerol species were also positively correlated with BMI and HOMA-IR. Many PE(O) and PE(P) species correlated positively with age, HDL cholesterol, liver density and GIR. We also performed linear regression with the total lipid class as the dependent variable and age, BMI and serum lipoprotein concentration as the explanatory variables for PE(P), PE(O), LPC(O), ceramide or DAG that all changed significantly with overfeeding (Table 3). At baseline, 33.4 % of PE(P) concentration was explained by HDL cholesterol and age, 37.4% of PE(O) by HDL and LDL cholesterol, 31.1% of LPC(O) by LDL cholesterol and BMI and 77.8% of diacylglycerol concentration by triglycerides and age.

We next examined the associations between changes (Δ) in serum lipids and change (Δ) in lipoproteins, metabolic, inflammation and oxidative stress markers with overfeeding (Figure 2b). Briefly, changes in some LPC species correlated inversely with change in BMI and positively with change in liver density. Changes in many dihydroceramide species correlated positively with change in HDL cholesterol whereas the change in dhCer24:1 and Cer18:0, Cer20:0, Cer24:1, were correlated positively with change in LDL cholesterol. Changes in the majority of triacylglycerol and diacylglycerol species correlated with change in triglycerides, and some species also correlated inversely with change in BMI. Changes in PE(O) and PE(P) species correlated positively with change in HDL whereas changes in LPC(O) species correlated inversely with change in BMI. Changes in PC(O) and PE(O) 34:2 correlated positively with change in urinary F2-isoprostanes. Linear regression of the change in total lipid class PE(P), PE(O), LPC(O), DAG or ceramide with explanatory variables age, Δ BMI and Δ serum lipoprotein concentrations revealed that 12-14% of the change in PE(P) and PE(O) was explained by change in HDL cholesterol. Whereas 10% of the change in LPC(O) was explained by change in LDL cholesterol, 57% of the change in diacylglycerol was explained by triglycerides and age, and 21% of the change in ceramide was explained by change in LDL and HDL cholesterol.

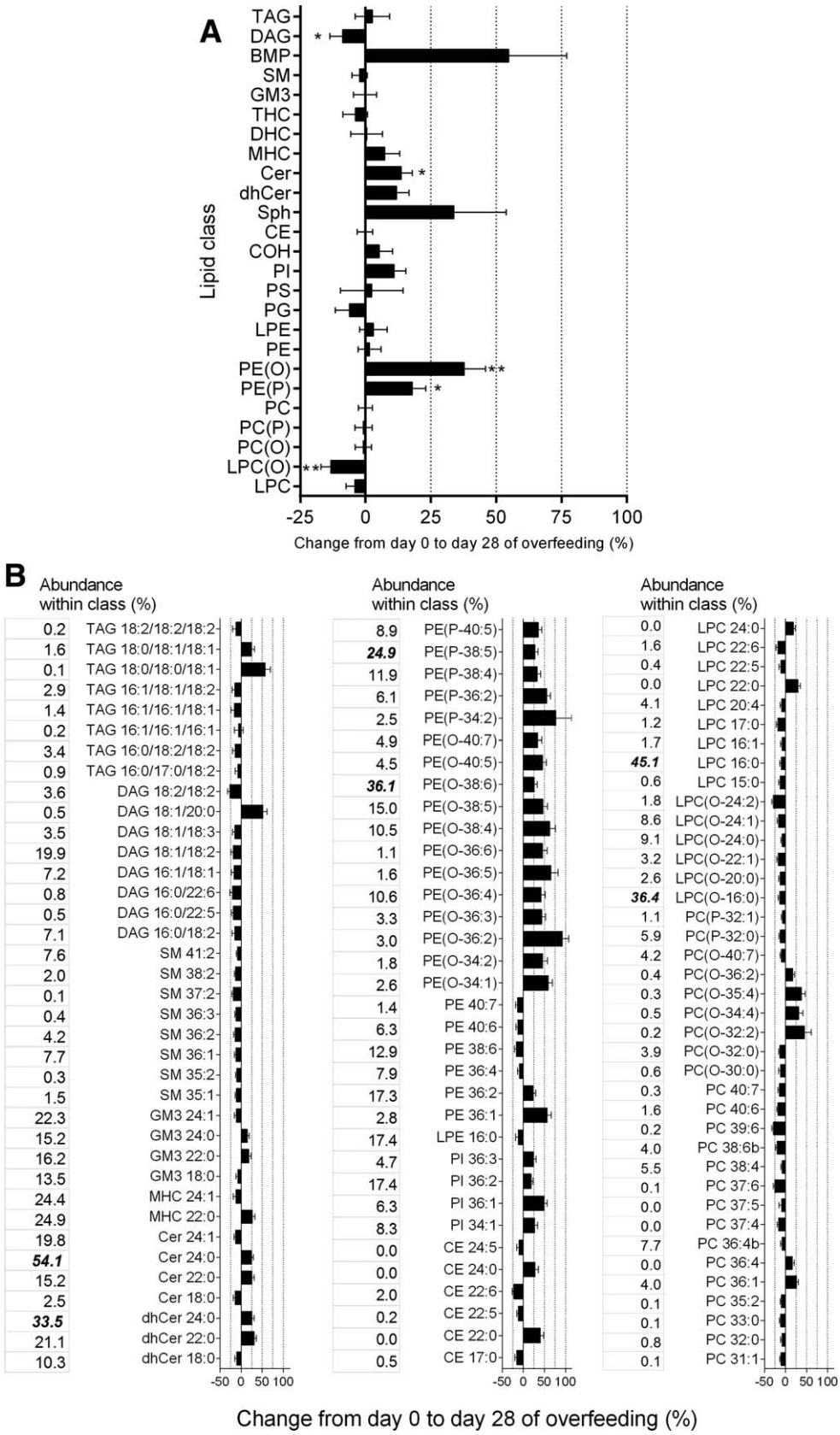

FIGURE 1 The effect of overfeeding on serum lipid classes (**a**) and individual lipid species that were significantly changed $^{*}P \leq 0.01$ and $^{**}P < 0.001$. (b) most abundant species within class are in bold. For abbreviations of lipids please refer to Table 2.

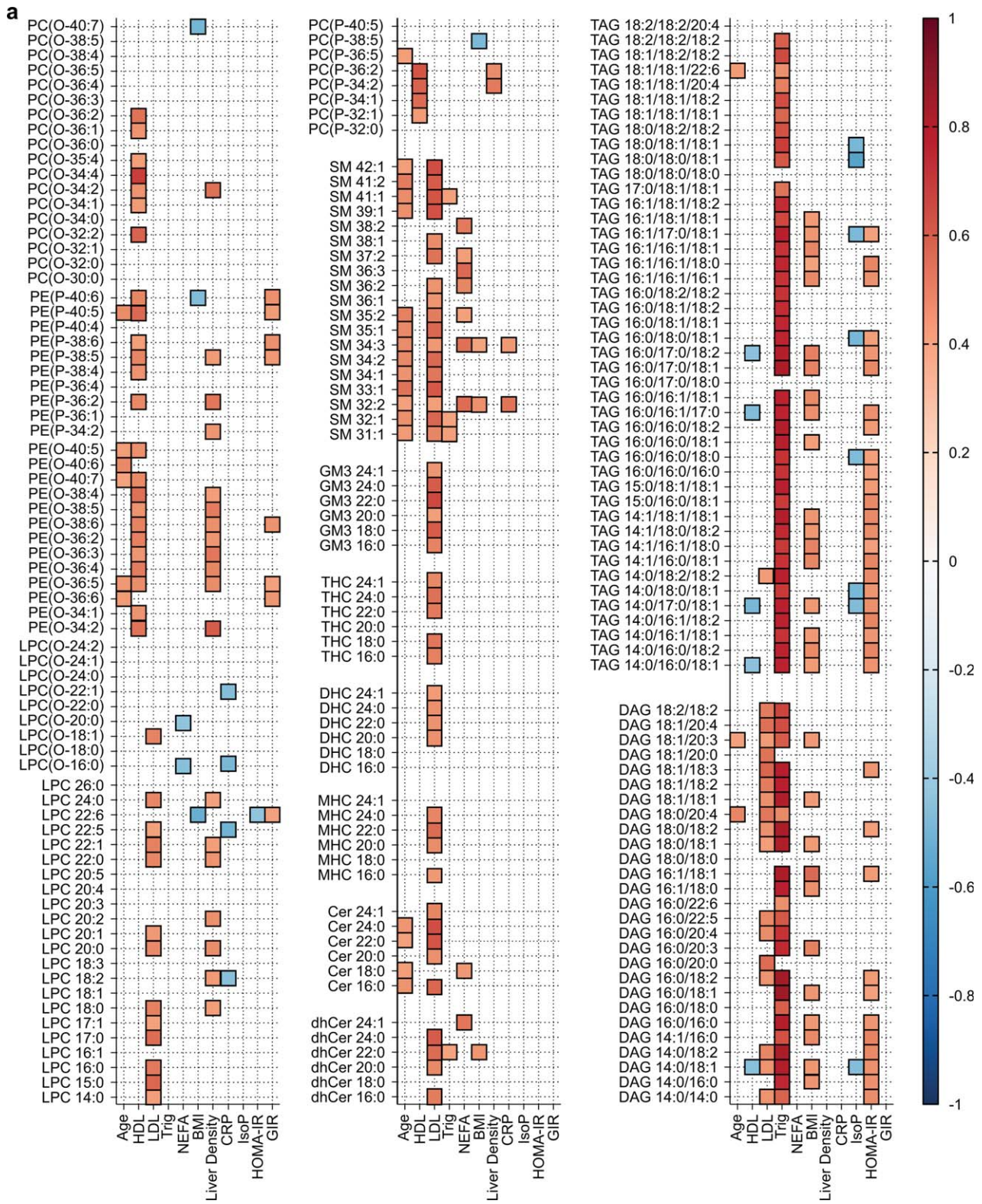

FIGURE 2 Heat maps based on Pearson's correlations between serum lipid species and lipoprotein concentrations and metabolic markers at baseline (**a**) and the change with overfeeding (b). The *R* value of the correlation is shown in the color, all $P < 0.01$. For abbreviations of lipids please refer to Table 2.

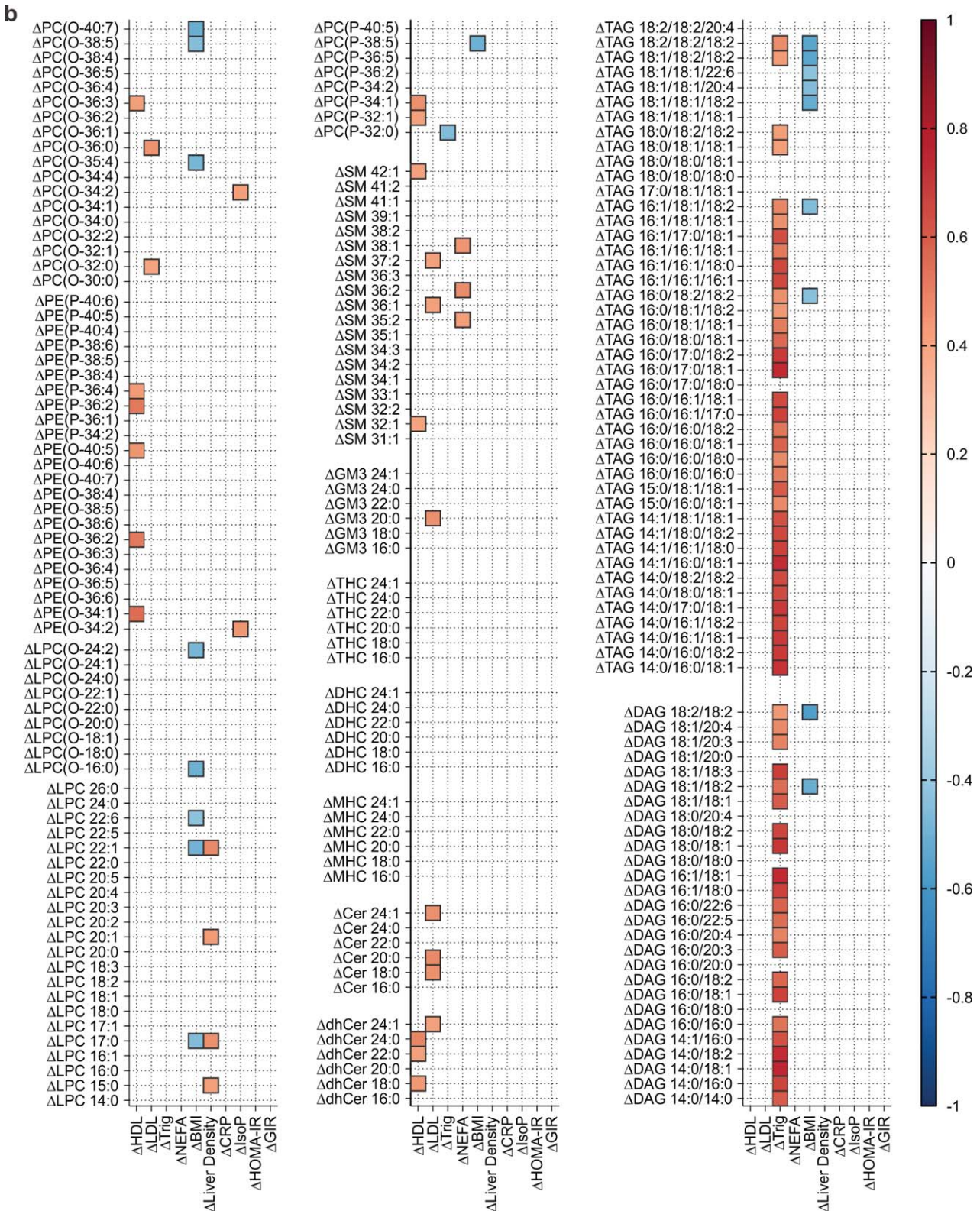

FIGURE 2 Continued

TABLE 3 Linear regression to explain the variability in lipid classes at baseline and overfeeding

Dependent variable	Model	Standardized β	P	Adjusted R^2
Baseline PE(P)	HDL	0.472	0.001	0.245
	HDL, age	0.325	<0.001	0.334
Baseline PE(O)	HDL	0.575	<0.001	0.282
	HDL, LDL	0.327	<0.001	0.374
Baseline LPC(O)	LDL	0.569	0.002	0.215
	LDL, BMI	−0.343	<0.001	0.311
Baseline Diacylglycerol	TG	0.857	<0.001	0.752
	TG, age	0.178	<0.001	0.778
Baseline Ceramide	LDL	0.63	<0.001	0.381
Δ PE(P)	Δ HDL	0.378	0.018	0.120
Δ PE(O)	Δ HDL	0.40	0.012	0.137
Δ LPC(O)	Δ LDL	0.346	0.031	0.096
Δ Diacylglycerol	Δ TG	0.730	<0.001	0.499
	Δ TG, age	−0.279	<0.001	0.566
Δ Ceramide	Δ LDL	0.406	0.02	0.114
	Δ LDL, Δ HDL	0.341	0.005	0.211

Classes that changed significantly with overfeeding were tested at baseline and in response to overfeeding. Variables were calculated by a linear regression model with baseline lipid concentration or change in lipid concentration (day 28—baseline, Δ) as outcome and adjusted for: age, baseline BMI, baseline HDL, baseline LDL and baseline triglycerides for baseline lipid concentration and age, Δ BMI, Δ HDL, Δ LDL and Δ triglycerides for Δ lipid. β = beta-estimate of linear regression model; R^2 = explained variance; PE(P), phosphatidylethanolamine-based plasmalogens; PE(O), alkylphosphatidylethanolamine; LPC(O), lysoalkylphosphatidylcholine; BMI, body mass index.

Only 8/333 lipid species correlated with reported dietary fat intakes at baseline, and the change in 12/333 lipid species were correlated with the change in dietary fat intake with overfeeding (Supporting Information Table S3).

Discussion

Overfeeding resulted in weight gain, increased fat deposition in adipose tissue and liver, increases in systemic markers of inflammation and oxidative stress and reduced insulin sensitivity. These changes occurred without significant changes in serum NEFA, LDL cholesterol or triglycerides, but with an 11% increase in HDL cholesterol. Marked changes in circulating lipids were detected in response to overfeeding, and more lipids were decreased (20%) than increased (13%). Total diacylglycerol and LPC(O) concentrations were reduced and total ceramide concentrations increased with overfeeding, although this was not a uniform response within this class. Large uniform increases were noted in PE(P) and PE(O) species with overfeeding. This, together with the more modest increase in HDL, suggests that overfeeding may modulate HDL particle composition.

HDL cholesterol is now recognized as having a modulating effect on glucose homeostasis by enhancing glucose uptake in muscle and stimulating insulin secretion from the pancreas (23). These effects may be mediated by removal of lipids from peripheral cells via reverse cholesterol transport and direct signaling events mediated by HDL transport proteins or receptors (23). As we and others reported previously, HDL cholesterol increased significantly with overfeeding (4,6). We now report significant increases in the phosphatidylethanolamine-based plasmalogen (PE(P)) and PE(O) species with overfeeding. PE(P) species are alkenylphospholipids with a vinyl-ether bond in the *sn*1 position. PE(P) are antioxidants (24) and thus may provide a counter mechanism to cope with the increased systemic oxidative stress observed in this study. Notably, the change in PE(P) species did not correlate with the change in the systemic oxidative stress marker urinary F2-isoprostanes measured here, but PE(O-34:2) increased significantly with overfeeding and correlated positively with the change in oxidative stress. PE(P) species are abundant in HDL (18), and consistent with this, we observed strong positive correlations between HDL cholesterol and PE(P) species at baseline. Interestingly, the increase in species of PE(P) and their precursors PE(O) with overfeeding was at least twice that observed in HDL cholesterol and regression analysis to explain the variance in the change in PE(P) and PE(O) classes revealed that only 12 and 14% could be attributed to the change in HDL respectively, and none to LDL. This suggests a change in the composition of HDL particles may have occurred in response to overfeeding, and may reflect peripheral tissue lipid composition (25) and modify HDL structure and function (26). However, we did not perform lipidomics of isolated HDL and therefore this requires further study.

Consistent with the fact that most glycerolipids are carried on VLDL, we found that the majority of diacylglycerol and triacylglycerol species in the circulation were correlated with serum triglycerides levels (at fasting, a reflection of VLDL) and linear regression analysis supported this result. Triacylglycerols and diacylglycerols are the primary source of fatty acids utilized in energy production, although cholesterol esters can also contribute significantly to this pool. In this study, moderate overfeeding had no significant effect on triglycerides and total serum triacylglycerol concentrations. Whilst there are no previous studies in humans investigating serum lipidomics with overfeeding, a caloric restriction study also reported no change in serum triglycerides concentration (16). However, in that study the short chain saturated fatty acids-containing triacylglycerol species were decreased

(16). Interestingly, triacylglycerol 18:0/18:0/18:1 which decreased following weight loss in that study (16), was increased with overfeeding in this study. Similarly, the DHA-containing triacylglycerol 18:1/18:1/22:6 was not changed by caloric restriction in that study (16) or overfeeding in this study. This suggests that some, but not all triacylglycerol species are sensitive to changes in dietary intake, although we did not find any correlations between these measures. Hepatic diacylglycerol may impair hepatic insulin sensitivity in obese individuals (27,28) and comparative lipidomics studies of liver and serum suggested that serum lipids reflect liver lipid composition (1). Overfeeding increased liver fat content as assessed by CT. Gold standard measures of liver fat were not conducted in the present study. However, findings by magnetic resonance spectroscopy (MRS) and CT measurement of liver fat content are closely correlated (29) and others have also reported increased liver lipid by overfeeding using MRS (30). Whilst we did not directly assess hepatic insulin sensitivity we observed positive relationships between serum diacylglycerol species and HOMA-IR, which is considered a marker of hepatic insulin resistance. We therefore predicted that diacylglycerol species would increase with overfeeding. However, total diacylglycerol was reduced in this study, with reductions in the abundant 18:1/18:2, 16:1/18:1, and 16:0/18:2 species. These results are not consistent with data generated from cross-sectional studies showing increased diacylglycerol levels in obese compared with lean individuals (10). The reason for this discrepancy is not clear and may be explained by the dynamic nature of our short-term overfeeding versus chronic obesity, and the healthy nonobese cohort studied here. Our results also contrast a recent rodent study that showed increases in diacylglycerol and triacylglycerol following 12-weeks high fat diet. However, the diet implemented in that study resulted in an almost global increase in circulating lipid species and the metabolic consequences were more extreme, since HOMA-IR increased 7.5-fold in mice (13) versus 1.2-fold in this study in humans.

Ceramide is the "hub" lipid in sphingolipid biosynthesis, synthesized *de novo* from dihydroceramide and generated from breakdown of sphingomyelin. Ceramide also serves as substrate for sphingomyelin and higher order glycosphingolipids, including monohexosylceramide and G_{M3}-ganglioside (31). Ceramide is well established as an important mediator of insulin resistance (32) and pharmacological inhibition of enzymes involved in ceramide generation improves insulin sensitivity in rodents (33). Further, substantial weight loss by gastric bypass in severely obese individuals improved insulin sensitivity which correlated with reductions in ceramide species in the circulation (17). However, in this study we did not observe correlations between any of the sphingolipids and insulin resistance at baseline or in response to overfeeding. LDL cholesterol is the main carrier of ceramide in the circulation (18) and a recent study revealed that LDL ceramide content was significantly increased in insulin-resistant compared with insulin-sensitive individuals (34). Consistent with this, we found that at baseline 38% of total ceramide concentration was explained by LDL, and HDL cholesterol and triglycerides concentrations did not contribute significantly to the model. With overfeeding, total ceramide increased significantly, with 11% of the increase explained by the change in LDL cholesterol and a further 10% by the change in HDL cholesterol. Interestingly, LDL aggregates from atherosclerotic lesions isolated from humans are 10- to 50-fold more ceramide enriched compared with plasma LDL cholesterol (35) and the increase in ceramide may suggest a role in the deleterious effects of chronic overfeeding and obesity on vascular health. In human plasma, Cer18:0, Cer20:0 and Cer24:1 are increased in obese patients with type 2 diabetes compared with lean individuals (11) and other sphingolipid species are not altered (10). The ceramide species that increased with overfeeding in this study were Cer22:0 and Cer24:0, the latter is the most abundant in plasma in humans (18,34) and infusion of LDL-Cer24:0 to lean mice caused insulin resistance in parallel with a tendency to increase ceramide content in the plasma membrane fraction of skeletal muscle (34). The changes observed here in ceramide were mirrored in dihydroceramide species (i.e., increases in dhCer 22:0 and 24:0 and decrease in 18:0), and suggest that the increases in ceramide species with overfeeding were at least in part due to *de novo* synthesis. However, regulation of ceramide synthases specific to long chain fatty acids in liver may also play a role, but this cannot be confirmed in this study.

Lysophosphatidylcholine (LPC) species are products of phosphatidylcholine hydrolysis by secretory phospholipases and lecitin:cholesterol acyltransferase (LCAT) and are carried on HDL (18,25), LDL and albumin (18). LPC may be pro-inflammatory and atherogenic (36) and were increased in serum of obese (37) and type 2 diabetic patients (38). However, this is not consistently reported with some studies showing decreased serum LPC species in individuals with impaired glucose tolerance (39) and obesity (13). In this study, LPC species were not correlated with measures of adiposity or insulin resistance at baseline, except for the DHA-containing LPC (22:6) which was associated with insulin sensitivity. In response to overfeeding, LPC 22:6 decreased as did 6 other LPC species, although the majority of LPC species were unchanged. This result contrasts with a recent report in the high fat fed rodent model where LPC species were almost universally decreased by overfeeding (13). In our study, LPC(O) concentrations were universally decreased by overfeeding. The relationship between LPC(O) and insulin sensitivity have not been studied to date. LPC(O) is also known as lysoplatelet activating factor, and is not only the precursor for but opposes the actions of platelet activating factor (PAF), thus inhibiting activation and aggregation of neutrophils and platelets (40). In the present study, LPC(O) species correlated inversely with the inflammation marker CRP at baseline, but their change with overfeeding did not correlate with change in any metabolic or inflammatory marker. Thus, the metabolic consequences of reductions in LPC(O) with overfeeding, if any, remain to be investigated.

In conclusion, a moderate increase in fat and energy intake resulted in changes in serum lipids in healthy nonobese humans. Increases were noted in circulating ceramides which left unchecked may promote systemic insulin resistance. The modest increase observed in HDL, together with larger increases in PE(P) and PE(O) suggest a change in HDL lipid composition with overfeeding. Because PE(P) act as antioxidants, this may provide an appropriate response to deal with potentially deleterious effects of overfeeding in healthy humans. The metabolic consequences of changes in concentrations of many circulating lipid species with overfeeding require further study. O

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