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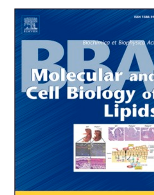

Sex-specific response of the human plasma lipidome to short-term cold exposure

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A B S T R A C T

Cold-induced lipolysis is widely studied as a potential therapeutic strategy to combat metabolic disease, but its effect on lipid homeostasis in humans remains largely unclear. Blood plasma comprises an enormous repertoire in lipids allowing insights into whole body lipid homeostasis. So far, reported results originate from studies carried out with small numbers of male participants. Here, the blood plasma's lipidome of 78 male and 93 female volunteers, who were exposed to cold below the shivering threshold for 2 h, was quantified by comprehensive lipidomics using high-resolution mass spectrometry. Short-term cold exposure increased the concentrations in 147 of 177 quantified circulating lipids and the response of the plasma's lipidome was sex-specific. In particular, the amounts of generated glycerophospholipid and sphingolipid species differed between the sexes. In women, the BMI could be related with the lipidome's response. A logistic regression model predicted with high sensitivity and specificity whether plasma samples were from male or female subjects based on the cold-induced response of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM) species.

In summary, cold exposure promotes lipid synthesis by supplying fatty acids generated after lipolysis for all lipid classes. The plasma lipidome, i.e. PC, LPC and SM, shows a sex-specific response, indicating a different regulation of its metabolism in men and women. This supports the need for sex-specific research and avoidance of sex bias in clinical trials.

Abbreviations

AGC	Automated gain control
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
Cer	Ceramide
CRP	C-reactive protein
DB	Double bond
DG	Diglyceride

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FA	Fatty acid
FAME	Fatty acid methyl ester
FC	Free cholesterol
FDR	False discovery rate
FFA	Free fatty acid
FN	False negatives
FP	False positives
fT3	Free triiodothyronine
GC–MS	Gas chromatograpy coupled to mass spectrometry
GGT	Gamma-glutamyl transferase
GL	Glycerolipid
GOT	Glutamic oxaloacetic transaminase
GPL	Glycerophospholipid
GPT	Glutamic pyruvic transaminase
HDL	High-density lipoprotein
HR-MS	High resolution mass spectrometry
HSL	Hormone sensitive lipases
IT	Injection time
LDL	Low-density lipoprotein
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MSX	Multiplexed acquisition
PC	Phosphatidylcholine
PC O	Phosphatidylcholine-ether
PE	Phosphatidylethanolamine
PE O	Phosphatidylethanolamine-ether
PI	Phosphatidylinositol
PLTP	Phospholipid transfer protein
PUFA	Polyunsaturated fatty acid
REE	Resting Energy Expenditure
ROC	Receiver operator characteristic
SL	Sphingolipid
SM	Sphingomyelin
TFA	Total fatty acid
TN	Thermoneutrality
TP	True positives
TG	Triglyceride
TSH	Thyroid-stimulating hormone
UCP1	Uncoupling protein 1
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

1. Introduction

Blood plasma is the most important matrix in clinical chemistry for analysis of biomarkers providing information about human physiology and disease. With hundreds of molecular species, it comprises an enormous repertoire in lipids such as fatty acids (FA), glycerophospholipids (GPL), glycerolipids (GL), sterols and sphingolipids (SL) that are not only outstanding in their vast number, but also in their wide structural and functional variety [1,2]. Imbalances in lipid metabolism and concentrations of circulating lipids provoked through lifestyle, i.e. excess intake of dietary fat in combination with a lack of physical activity, dramatically accelerate the development of metabolic disease, which is worldwide one of the leading causes of death [3,4].

For this reason, there has been a rapidly growing interest in recent years in restoring lipid homeostasis by increasing energy expenditure. This includes the exposure to cold stimulating the sympathetic nervous system to release the beta-adrenergic agonist norepinephrine, which in turn, activates adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). These enzymes release free fatty acids (FFA) from triglycerides (TG) in fat depots [5]. In brown adipose tissue (BAT), FFA fuel uncoupling protein (UCP) 1 located in the inner mitochondrial membrane, which dissipates the proton motive force by uncoupling ATP-synthesis [6]. In this process, called non-shivering thermogenesis, heat is generated, resulting in the consumption of significant amounts of energy. FFA transported with the blood stream to BAT after lipid droplet breakdown in white adipose tissue (WAT) may also fuel UCP1-mediated respiration [7,8]. Although the presence and function of human BAT positively correlates with energy expenditure, lower adiposity and a reduced risk for insulin resistance [9–12], its ultimate metabolic benefit is still under debate. In humans, physiologically relevant amounts of BAT are marginal relative to the body's total adipose tissue volume. In 20 young healthy men exposed to cold for 5 h, only 4.3 % of the total fat mass comprised BAT-containing fat depots [13]. Metabolically active BAT was detected solely in 6.8 % of 4842 individuals in a retrospective F-18-fluordesoxyglucose positron emission tomography-computer tomography scan study [14,15].

The question follows: What happens to FFA generated in human tissues after cold-induced lipolysis, which are not used to fuel UCP1, and how do they affect whole-body lipid metabolism? A recent study with ten male participants exposed for 30–120 min to cold indicated, that the majority of FFA is transported to the liver for metabolism to TG, before re-secretion via very low-density lipoproteins (VLDL) into the circulation [16]. Total plasma TG concentrations of 24 men were significantly elevated after being exposed to cold for 2 h at the shivering-threshold [17] and other studies with 6–39 probands exposed at 10–18 °C for 40–180 min suggest that ^{18}F-fluoro-thiaheptadecanoic acid is esterified into plasma TG [18–21]. In contrast, no changes in total plasma TG were observed in 6–25 probands and individuals with type 2 diabetes after a continuous stimulation at 18 °C for 120–300 min [16,22].

To shed some light on these heterogenous study results and on cold-stimulated whole body lipid metabolism, we investigated the plasma lipidome of a large and mixed-sex study cohort (171 individuals; male $n = 78$, female $n = 93$) exposed to cold for 2 h just below the shivering threshold. We used validated and quantitative lipidomics based on high-resolution mass spectrometry (HRMS) and applied machine learning to further evaluate the data. Our investigations included glycerophospholipids (GPL), glycerolipids (GL), sterols and sphingolipids (SL). As sex influences BAT activity, lipid and lipoprotein metabolism [15,23–25], we specifically asked, whether the plasma lipidome response to short-term cold exposure is sex-specific.

2. Materials and methods

2.1. Study design and participants

This study is a secondary analysis [26,27] including 93 female and 78 male volunteers. Sex was gathered as assigned by birth. Participants were between 18 and 51 years of age with a median age of 26.0 years and BMI of 24.7 kg/m^2. All attendees were without severe diseases (including diabetes) or acute infections (e.g. common cold), non-smokers, caucasian ancestry, without lactation or pregnancy (evaluation by self-report), stable body weight for the last three months (<3 kg body weight change) and performed <10 h of physical exercise per week. All volunteers were requested to refrain from food and caloric beverages for at least 10 h and from caffeine and exercise for at least 24 h before starting the intervention between 07:30 and 08:00 in the morning. Participants were asked to empty their bladder and wear only underwear before starting the measurement.

For short-term cold exposure, a personalized cooling protocol was applied. During the intervention, participants were invited to linger in a lying position between two water perfused blankets (Maxi-Therm Lite Blankets, Cincinnati Sub-Zero, OH, USA), which were connected to a cooling device (WiseCircu type WCR—P8, Witeg Labortechnik, Wertheim, Germany). The initial water temperature was set to 32 °C for blood sampling under thermoneutrality. Next, the cooling protocol was started by decreasing the water inlet temperature stepwise, as previously published by van der Lans et al. [28]. Participants were asked to report any reaction of the body caused by cold to assess the shivering threshold. As soon as shivering occurred, the current water inlet temperature was raised by 2 °C again. This temperature was kept for 120

min, unless participants started to shiver again. In that case, the temperature was increased until the shivering response stopped. After expiration of the time of cold exposure, blood samples were drawn again. The room temperature was 21–22 °C at thermoneutrality and 22–24 °C at cold exposure. The outside temperature data was obtained from the meteorological station in Freising which is run by DWD and LfL (Deutscher Wetterdienst and Bayerische Landesanstalt für Landwirtschaft). The skin temperature of the volunteers was monitored by attaching nine wireless thermosensors (iButtons DS-1921H, Thermochron; Maxim, Dallas, United States) to defined skin locations. One sensor was used to assess the temperature changes in the region of the supraclavicular fossae (BAT location) and eight sensors were used to assess the overall skin temperature as defined by ISO 9886 (ISO. ISO9886. Evaluation of thermal strain by physiological measurements. Geneva: International Standards Organization; 2004). Parameters shown in Table 1 and Tables S1–2 were measured as described previously [26,27]. FFA were determined using an enzymatic colorimetric method assay (Wako Chemicals, Neuss, Germany) and TG reported in Table 1 and S1–S2 were determined using an established commercial test kit by a certified laboratory service (SynLab Labordienstleistungen, Munich, Germany).

2.2. Ethics

All study participants provided written informed consent. The study protocol was reviewed and approved by the Ethics committee of the Faculty of Medicine of the Technical University of Munich (#236/16S). This study was registered at the German Clinical Register DRKS (accession number: DRKS00010489).

2.3. Lipid extraction

A volume of 10 μl plasma was subjected to lipid extraction as described by Bligh and Dyer [29]. The following non-naturally occurring lipid species were added as internal standards prior to lipid extraction: CE 17:0, CE 22:0, Cer 18:1;O2/14:0, Cer 18:1;O2[D7]/18:0, DG 14:0/14:0, DG 20:0/20:0, FC[D7], LPC 13:0, LPC 19:0, LPE 13:0, LPE 18:1[D7], PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0 (diphytanoyl), PI 18:1[D7]/15:0, SM 18:1;O2/12:0, SM 18:1;O2/18:1[D9], TG 17:0/17:0/17:0, and TG 19:0/19:0/19:0. The lipid containing chloroform phase was recovered by a pipetting robot (Tecan Genesis RSP 150) and vacuum dried. The residues were dissolved in chloroform/methanol/2-propanol (1:2:4 *v*/v/v) with 7.5 mM ammonium formate and subjected to mass spectrometric analysis.

Table 1
Participant characteristics.

	Stratified by Gender			
	Overall, n = 171	female, n = 93	male, n = 78	*p*-value
Age (years)	26.0 ± 4.8	25.6 ± 4.9	26.6 ± 4.7	0.072
BMI (kg/m^2)	24.7 ± 5.0	24.4 ± 5.7	25.1 ± 4.2	0.005
LDL (mg/dl)	103 ± 31	102 ± 28	105 ± 35	0.7
HDL (mg/dl)	57 ± 16	64 ± 15	50 ± 13	<0.001
TG (mg/dl)	87 ± 50	82 ± 38	94 ± 61	0.4
GOT (U/l)	20 ± 11	18 ± 11	22 ± 10	<0.001
GPT (U/l)	25 ± 14	20 ± 11	31 ± 15	<0.001
GGT (U/l)	16 ± 12	12 ± 8	20 ± 13	<0.001
TSH (μU/ml)	1.68 ± 0.75	1.58 ± 0.78	1.79 ± 0.71	0.023
fT3 (pg/ml)	3.29 ± 0.40	3.16 ± 0.36	3.44 ± 0.40	<0.001
CRP (mg/dl)	0.25 ± 0.43	0.36 ± 0.53	0.12 ± 0.21	<0.001

	Thermoneutrality vs. *Cold Exposure*			
	Cold, n = 171	TN, n = 171	Δ delta	p-value
FFA (μmol/l)	514 ± 225	294 ± 151	220	<0.001
TG	97 ± 50	87 ± 50	9.8	0.074
fT3 (pg/ml)	3.20 ± 0.41	3.29 ± 0.40	−0.09	0.034
CRP (mg/dl)	0.26 ± 0.45	0.24 ± 0.43	0.02	0.7

Values are presented as mean ± standard deviation. Unpaired statistical testing was used to test for/against differences between genders (Wilcoxon rank sum test). Pairwise testing was used to compare FFA levels at TN and CE (Welch Two Sample t-test). BMI (Body Mass Index), CRP (C-reactive protein), FFA (Free fatty acids), fT3 (free triiodothyronine), GGT (gamma-glutamyl transferase), GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvic transaminase), HDL (High Density Lipoprotein), LDL (Low density Lipoprotein), TN (Thermoneutrality), TSH (Thyroid-stimulating hormone), TG (Triglycerides).

2.4. Lipidomics

The analysis of lipids was performed by direct flow injection using a high-resolution hybrid quadrupole-Orbitrap mass spectrometer. A detailed description of the method was published previously by Höring et al [30]. Triglycerides (TG), diglycerides (DG) and cholesteryl ester (CE) were recorded in positive ion mode in *m/z* range 500–1000 for 1 min with a maximum injection time (IT) of 200 ms, an automated gain control (AGC) of $1{*}10^6$, three microscans and a target resolution of 140,000 (at 200 *m/z*). Lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) were analyzed in negative ion mode in *m/z* range 400–650 *m/z*. Phosphatidylcholine (PC), ether-PC (PC O), phosphatidylethanolamine (PE), ether-PE (PE O), phosphatidylinositol (PI), ceramide (Cer) and sphingomyelin (SM) were measured in *m/z* range 520–960 in negative ion mode [31]. Multiplexed acquisition (MSX) was applied for the $[M + NH_4]^+$ of free cholesterol (FC) (404.39 *m/z*) and FC [D7] (411.43 *m/z*) with 0.5 min acquisition time, with a normalized collision energy of 10 %, an IT of 100 ms, AGC of $1{*}10^5$, isolation window of 1 *m/z*, and a target resolution of 140,000 [32]. The quantification was performed by multiplication of the spiked IS amount with analyte-to-IS ratio. Lipid species were annotated according to the latest proposal for shorthand notation of lipid structures that are derived from mass spectrometry [33]. The lipidomics reporting checklist has been added as Supplementary Data 1 for further details [34].

2.5. Total fatty acid analysis

Total fatty acids (TFA) were quantified as described previously [35]. Briefly, fatty acid methyl esters (FAME) were generated by acetyl chloride and methanol treatment, before extraction with hexan. TFA analysis was carried out using a Shimadzu 2010 GC–MS system. FAME were separated on a BPX70 column (10-m length, 0.10-mm diameter, 0.20-μm film thickness) from SGE using helium as the carrier gas. The initial oven temperature was 50 °C and was programed to increase at 40 °C/min to 155 °C, 6 °C/min to 210 °C, and finally 15 °C/min to 250 °C. The FA species were quantified by single ion monitoring, to detect specific fragments of saturated and unsaturated FA (saturated, 74 *m/z*; mono-unsaturated, 55 *m/z*; di-unsaturated, 67 *m/z*; poly-unsaturated, 79 *m/z*). The internal standard was non-naturally-occurring C21:0 iso. Plasma aliquots for TFA analysis were available from 165 of 171 study participants (Male: $n = 75$; Female: $n = 90$).

2.6. Statistical analysis of lipidomic data

Lipidomic data were analyzed according to the principles described previously [36,37]. For generation of volcano plots, all data were $\log_2$ transformed to ensure that they were normally distributed. Lipid species were excluded if they were undetectable in >50 % of the samples. A standard two-sided, paired *t*-test assuming unequal variances was used to test for significantly different abundances in the conditions. The Benjamini–Hochberg method was used to calculate the false discovery rate (FDR) and to account for multiple testing ($p_{adj} < 0.01$). Fold changes were calculated as the difference between mean of the $\log_2$-transferred values at TN and cold exposure. To compare mean lipid

concentrations between groups a standard two-sided, unpaired t-test assuming unequal variances was applied.

2.7. Classification and prediction model

The prediction model was built using Chat GPT Plus (https://openai.com/blog/chatgpt-plus). Logistic regression was applied as a supervised decision tree learning procedure to classify the target variables using all 177 detected lipid species in plasma as input [38]. A repeated internal cross-validation, which is very well suited for the size of the dataset investigated here [39], was applied to understand and validate the predictiveness of our models. Therefore, we ran the following procedure 50 times for each variable: (I) The sample set (n = 342) was split randomly into a 75 % training set and a 25 % test set; (II) The ROC curve and AUC were computed; (III) The top 10 lipid species for prediction were calculated. To evaluate the model performance we calculated the average of the 50 iterations for the following parameters: ROC-AUC, accuracy (proportion of correct predictions over total predictions), precision (proportion of correct positive prediction; "true positives [TP]/(TP + false positives [FP])"), recall (proportion of relevant predictions; "TP/(TP+ false negatives [FN]"); F1 score (harmonic mean of the precision) and recall representing both precision and recall in one metric. The highest possible value of an F1-score is 1.0, indicating perfect precision and recall. The coefficient values displayed in the figures showing the top 10 most important input variables are based on the average coefficient values over the 50 iterations of classification. A higher absolute value of the coefficient indicates greater importance in predicting the target variable.

2.8. Code availability

Computer codes can be made available from the corresponding author on request.

3. Results

3.1. Cold exposure increases concentrations in the vast majority of circulating complex lipids and total fatty acids

This study is based on samples obtained from a previous cross-sectional trial, where 78 male and 93 female human volunteers (n = 171) with a median age of 26.0 years and a median BMI of 24.7 kg/m^2 were exposed to cold for 120 min. Blood samples were drawn at thermoneutrality and after cold exposure (Fig. S1A) [27]. Relevant characteristics of study participants can be found in Table 1 and Tables S1–S2. Those include markers for hepatic function (GOT, GPT, GGT), adipose tissue status (adiponectin, leptin) and thyroid hormones (TSH, fT3), which were all in the normal reference ranges for men and women.

To investigate the plasma lipidome, an untargeted, direct infusion lipidomic profile was determined using electrospray ionization coupled to HRMS comprising: (1) Glycerophospholipids (GPL): phosphatidylcholine (PC), phosphatidylcholine-ether (PC O), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylethanolamine-ether (PE O), lysophosphatidylethanolamine (LPE) and phosphatidylinositol (PI). (2) Glycerolipids (GL): diglycerides (DG) and triglycerides (TG). (3) Sterols: free cholesterol (FC) and cholesteryl ester (CE). (4) Sphingolipids (SL): sphingomyelin (SM), and ceramide (Cer). Plasma samples were randomly analyzed in three batches, including quality controls. Quantification was based on lipid class-specific internal standards that do not occur naturally.

In total, 177 lipid species were quantified. With approximately 40 % CE was the major lipid class, followed by PC (20 %), FC (18 %) and TG (17 %) (Fig. S1B). SM (4 %) was the dominating SL lipid class, LPC (3 %) the primarily occurring lyso-lipid (Fig. S1C). To ask whether short term cold exposure alters the lipidome, lipid concentrations in plasma samples before and after cold exposure were compared. After correcting for multiple testing by controlling the false discovery rate at 0.01, the levels of 154 (87 % of all detected lipids) lipid species were found significantly different, of those 147 lipids (83 % of all detected ones) were increased after cold exposure, mostly by 10–30 % (Fig. 1A). Most significant alterations were observed in CE 22:6, PC 38:6, TG 56:7 and FC of the major lipid classes, as well as Cer 42:2;O2, DG 36:3, LPC 22:6, LPE 22:6, PC O 38:5, PE 38:6, PE O 38:6, PI 38:4 and SM 36:2;O2 of the minor lipid classes. Of note, all of them (except for FC and SM) are poly-unsaturated. CE 22:6, LPC 22:6 and LPE 22:6 contain docosahexaenoic acid (FA 22:6; n-3); PC 38:6, PE 38:6 and PE O 38:6 very likely contain FA 22:6; n-3 in their acyl chains. While percental plasma lipid class composition remained largely unchanged (Fig. 1B-C), the total lipid concentrations, as well as those of each analyzed lipid class, significantly increased after 2 h of cold exposure (Fig. 1D-F).

To provide further evidence, that cold exposure affects circulating PUFA levels, we analyzed total FA (TFA) using by gas chromatography coupled to mass spectrometry (GC–MS). TFA are all FA occurring in plasma, including those bound to complex lipids, such as to GPL, GL, SL and sterols. In total, 30 TFA species were detected and quantified. Of those, the concentrations of 29 TFA species were elevated at colder temperatures by 5–23 % including the major TFA: FA 16:0, FA 16:1 n-7, FA 18:0, FA 18:1 n-9, FA 18:2 n-6 and FA 20:4 n-6 (Fig. 2A). As observed for complex lipids, the most significantly increased candidates were polyunsaturated including FA 22:6 n-3, FA 22:5 n-3, FA 22:4 n-6 and FA 20:3 n-6. The percental distribution of the TFA's double bond (DB) numbers was largely similar before and after cold exposure, while the overall TFA levels were higher by 12 % in plasma after cold treatment (Fig. 2B-E).

3.2. The lipidomic response to cold is sex-specific in human plasma

Sex has an influence on the plasma lipidome [2] and whole-body lipid metabolism [24,25]. Consequently, we asked whether the response to cold is sex-specific for the major plasma lipid classes. We analyzed the datasets from male and female participants separately and compared the changes in lipid concentrations (log2(Fold Change)) as well as the deltas of lipid concentrations originating after cold exposure (referred to as "cold-induced concentration deltas"). At thermoneutrality, total PC concentrations were significantly lower by 13 % in men compared to women, whereas the lipid concentrations of total TG, CE and FC were similar between the sexes in our cohort (Fig. 3 A, H; Fig. S2A, F). Also, the lipid species composition was comparable, with mono-, di-, and tri-unsaturated species dominating the major lipid classes. These include PC 34:1, PC 34:2, PC 36:2, TG 50:2, TG 52:2, TG 53:3, CE 16:0, CE 18:1 and CE 18:2 (Fig. 3B, I; Fig. S2B).

After cold treatment, lipid profile comparison between the male and female participants revealed obvious differences. Women showed a stronger response to cold in PC than men. The changes (log2(Fold Change)) of nearly all detected PC species were higher in plasma samples from female individuals (Fig. 3C). The levels of the major PC species PC 34:1, PC 34:2, PC 36:2 were increased by 18 %, 11 % and 8 %, while in male participants only by 13 %, 7 % and 4 %. The cold-induced concentration deltas of total PC, PC 34:1, PC 34:2 and PC 36:2 concentrations were approximately 2-times higher in women than in men (Fig. 3D-E). For TG an opposite scenario was observed. The major species TG 52:2, TG 52:3 and TG 52:4 had elevated changes in male participants (Fig. 3 J). The cold-induced concentration deltas of total TG including TG 52:4 were higher in men (20 nmol) compared to women (10 nmol) (Fig. 3 K-L). In contrast to PC and TG, CE and FC showed a largely similar response in both sex groups (Fig. S2C-E, G-H). To test whether the changes in PC and TG can be linked with fatty acid length or saturation, we calculated the cold-induced concentrations deltas according to acyl chain length and the number of double bonds (DB). We found that specifically mono- and di-unsaturated PC species with 34–36 carbons (Fig. 3F-G), and di-, tri- and tetra-unsaturated TG species with 52 carbons showed differential responses in male and female individuals

Fig. 1. Cold exposure increases concentrations in 83 % of all quantified lipid species in plasma of human volunteers
(A) Volcano plot displaying lipids whose concentrations are significantly changed after 2 h of cold exposure (Cold). $p < 0.01$ after correcting for multiple testing by controlling the false discovery rate is indicated. (B) Distribution of the major and (C) minor lipid classes in % at thermoneutrality (TN) and Cold. (D) Concentrations of total, (E) major and (F) minor lipid classes at TN and Cold. *p < 0.01 indicates a significant difference relative to TN, determined using a two-sided Student's *t*-test. Shown are means +SD from $n = 171$ volunteers.

(Fig. 3 M-N).

Analysis of TFA revealed comparable overall TFA levels in men and women, but slightly increased concentrations of FA 18:2 n-6 and some minor TFA like FA 18:3 n-6, FA 22:6 n-3 and 24:1 n-9 in female participants at thermoneutrality (Fig. S3A-C). However, the response (log2 (Fold Change)) to cold exposure was largely comparable for most analyzed species in both sexes (Fig. S3D), potentially with a tendency towards stronger responses in men, supported by increased cold-induced concentration deltas for FA 18:3 n-6 and FA 22:5 n-3 (Fig. S3E-G).

3.3. The BMI correlates with the plasma lipidome response to cold in women

Metabolic flexibility, the ability to alter metabolism in response to stimuli, depends on body composition, [40] and the thermogenic response depends on the BMI [27]. Hence, we asked whether circulating lipids can be associated with the BMI after cold exposure.

As our study included predominantly healthy individuals with a BMI in the ranges between 20 and 25 kg/m^2 ($n = 51$, equals 52 %) for female and 22–25 kg/m^2 ($n = 46$, equals 63 %) for male participants, but less participants with BMIs >25 kg/m^2, and < 20 kg/m^2 or 22 kg/m^2, we chose to bin our study population into four subgroups with BMI <20, 20–25, 25–30, >30 kg/m^2 (women) and < 22, 22–25, 25–30, >30 kg/m^2 (men) to balance the BMI distribution. In female subjects, the means of the BMI subgroups correlated negatively with the log2 fold changes of the dominating lipid classes PC, TG and CE including their major species with $R^2 > (-)0.95$ (Fig. 4A, C, E) and the minor lipid classes Cer, DG, PC O, PE, PE O, PI and SM with $R^2 > (-)0.86$, but not with FC, LPC and LPE

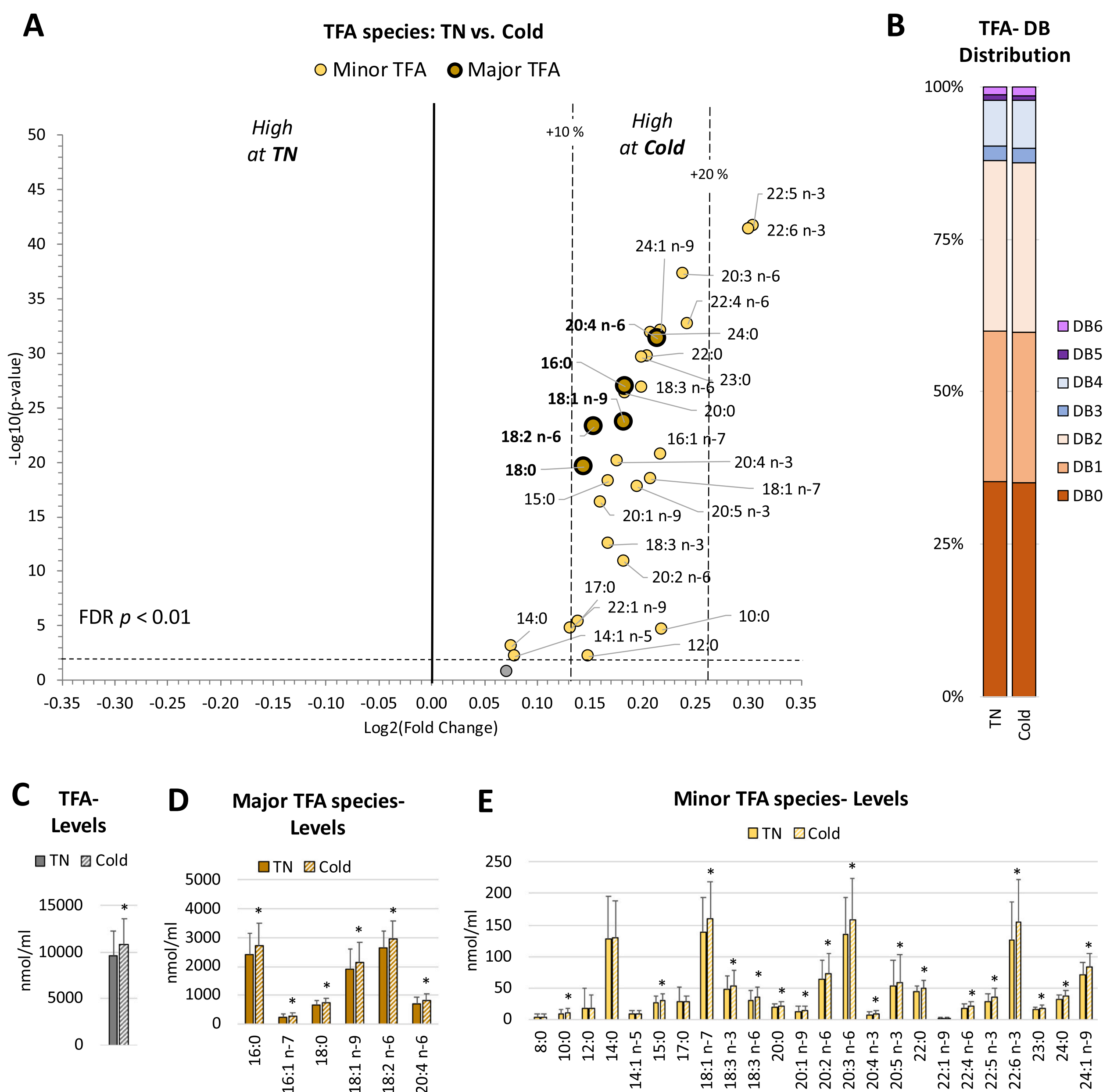

Fig. 2. Cold exposure increases concentrations in almost all quantified total fatty acid species in plasma of human volunteers
(A) Volcano plot displaying total fatty acids (TFA) whose concentrations are significantly changed after 2 h of cold exposure (Cold). $p < 0.01$ after correcting for multiple testing by controlling the false discovery rate is indicated. (B) Distribution of TFA grouped according to their number of double bonds (DB) in % at thermoneutrality (TN) and Cold. (C) Concentrations of overall, (D) major and (E) minor TFA species at TN and Cold. *$p < 0.01$ indicates a significant difference relative to TN, determined using a two-sided Student's *t*-test. Shown are means +SD from $n = 165$ volunteers.

(Fig. S4). Also, in women the log2 fold changes of 15 of the 30 measured TFA species, i.e. containing 18–24 carbons like FA 18:2 n-6, FA 20:4 n-6 and FA 24:1 n-9, showed negative correlations with the BMI subgroups with $R^2 > (-)0.80$ (Fig. S5). Surprisingly, we did not find such strong relations between BMI and the lipidome response to cold in men (Fig. 4B, D, F; Fig. S4-S5).

In essence, these results suggest that, in contrast to men, there is an enhanced lipidomic response to short-term cold exposure in women with a lower BMI.

3.4. The lipidomic response allows prediction of cold exposure and sex

To further strengthen our findings and to investigate our dataset with an independent approach, machine learning was applied. We ran models based on logistic regression using all 177 detected major and minor species as input variables to classify the target variables: (A) whether plasma samples originated from individuals at thermoneutrality or cold exposure or (B) whether samples originated from male or female volunteers based on their lipidomic responses to cold exposure. Logistic regression is a supervised machine learning algorithm that predicts the likelihood of something by fitting a sigmoid function to the data [38], in this case, lipid species. It allows a binary or multi-class classification, here into (A) or (B). A repeated internal cross validation was applied to understand and validate the predictivity of our models [39]. Therefore, we ran the following procedure 50 times for each variable: (I) The sample set ($n = 342$) was randomly split into a 75 % training set and a 25 % test set; (II) The receiver operator characteristic (ROC) curve and the area under the curve (AUC) were computed; (III) The top 10 lipid species for prediction were calculated.

For both types of target variables (A, B), a very effective classification

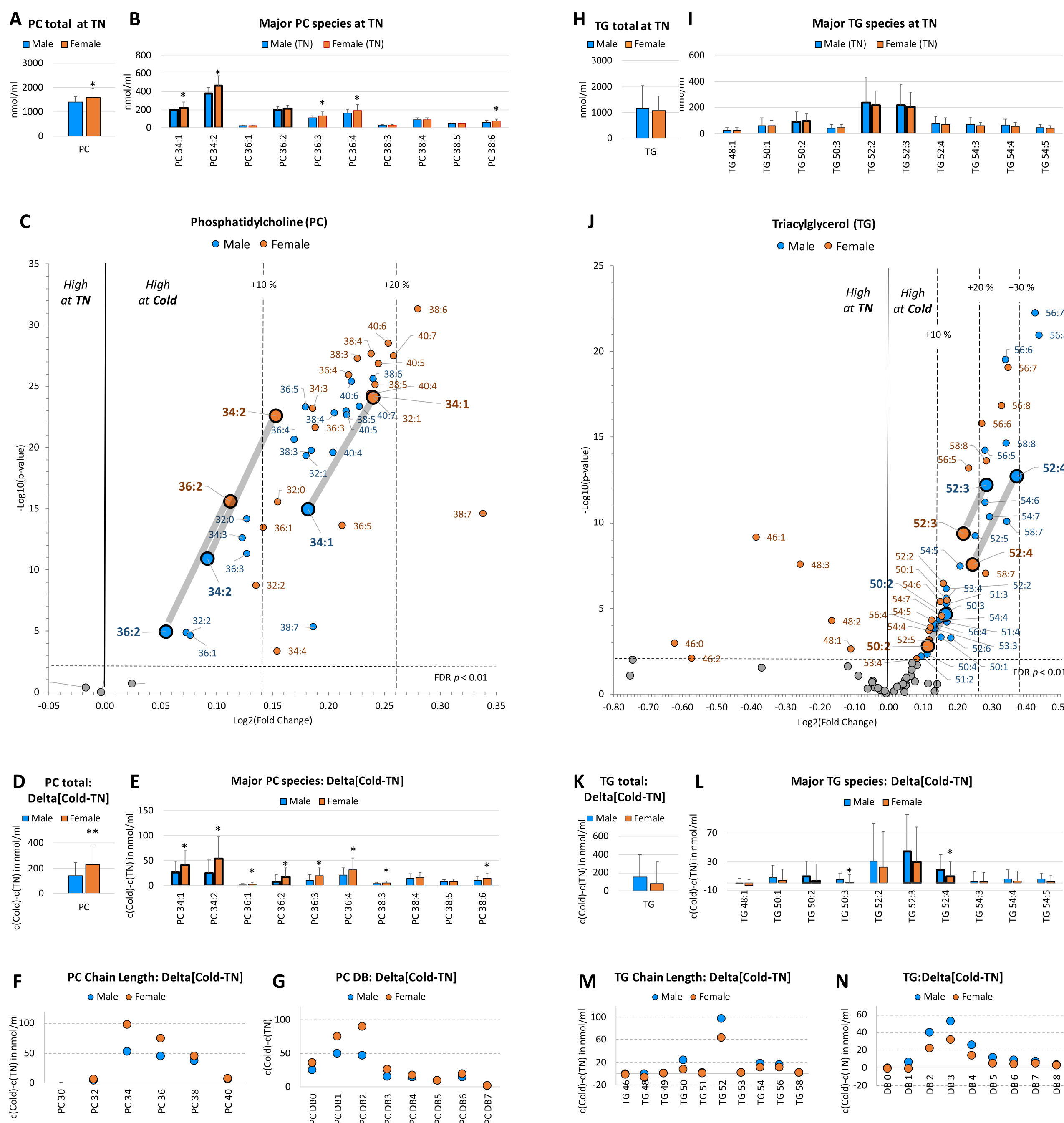

Fig. 3. Alterations in circulating PC levels are higher in women ($n = 93$), while TG shows stronger concentration changes in men ($n = 78$) in response to cold (A) Circulating concentrations of total and (B) the 10 main PC species at TN in male and female subjects. (C) Volcano plot showing the Log2 Fold Changes (Log2(Fold Change)) of PC species in plasma obtained from male and female subjects after 2 h exposure to cold. (D) Deltas of total PC and (E) the 10 main PC species concentrations originating after cold exposure (calculated: c[TN]-c[Cold]; referred to as "cold-induced concentration deltas" in the results section). (F) Cold-induced concentrations deltas according to acyl chain length and (G) the number of double bonds (DB) in PC. (H) Circulating concentrations of total and (I) the 10 main TG species at TN in male and female subjects. (J) Volcano plot showing the Log2 Fold Changes of TG species in plasma obtained from male and female subjects after 2 h exposure to cold. (K) Deltas of total TG and (L) the 10 main TG species concentrations originating after cold exposure. (M) Cold-induced concentrations deltas according to acyl chain length and (N) the number of DB in TG. The major 3 lipid species with highest concentrations per lipid class are highlighted with bold edges or depicted enlarged. Shown are means +SD from n = 171 volunteers. *p < 0.01 indicates a significant difference, determined using a two-sided Student's t-test in bar plots. In volcano plots, p < 0.01 after correcting for multiple testing by controlling the false discovery rate is indicated.

and prediction with a high sensitivity and specificity (average ROC-AUC >0.9) was possible (Fig. 5A, D). Among the top 10 most important input variables in model (A) allowing a separation of plasma samples of individuals at thermoneutrality from those of cold exposure (Fig. 5A), were six polyunsaturated lipid species (Fig. 5C). These included four lyso-GPL species (LPE 20:4, LPE 22:6, LPE 18:2, LPC 22:5), PE O 36:3 and CE 20:4. LPE 22:6, which ranked as second most important candidate, was also among the most significant different candidates in our initial data analysis (Fig. 1A). These results strengthen that lipids, particularly those containing PUFA, increase upon cold exposure in males and females. The 10 most important lipid species in model (B) allowing prediction of whether samples were from male or female

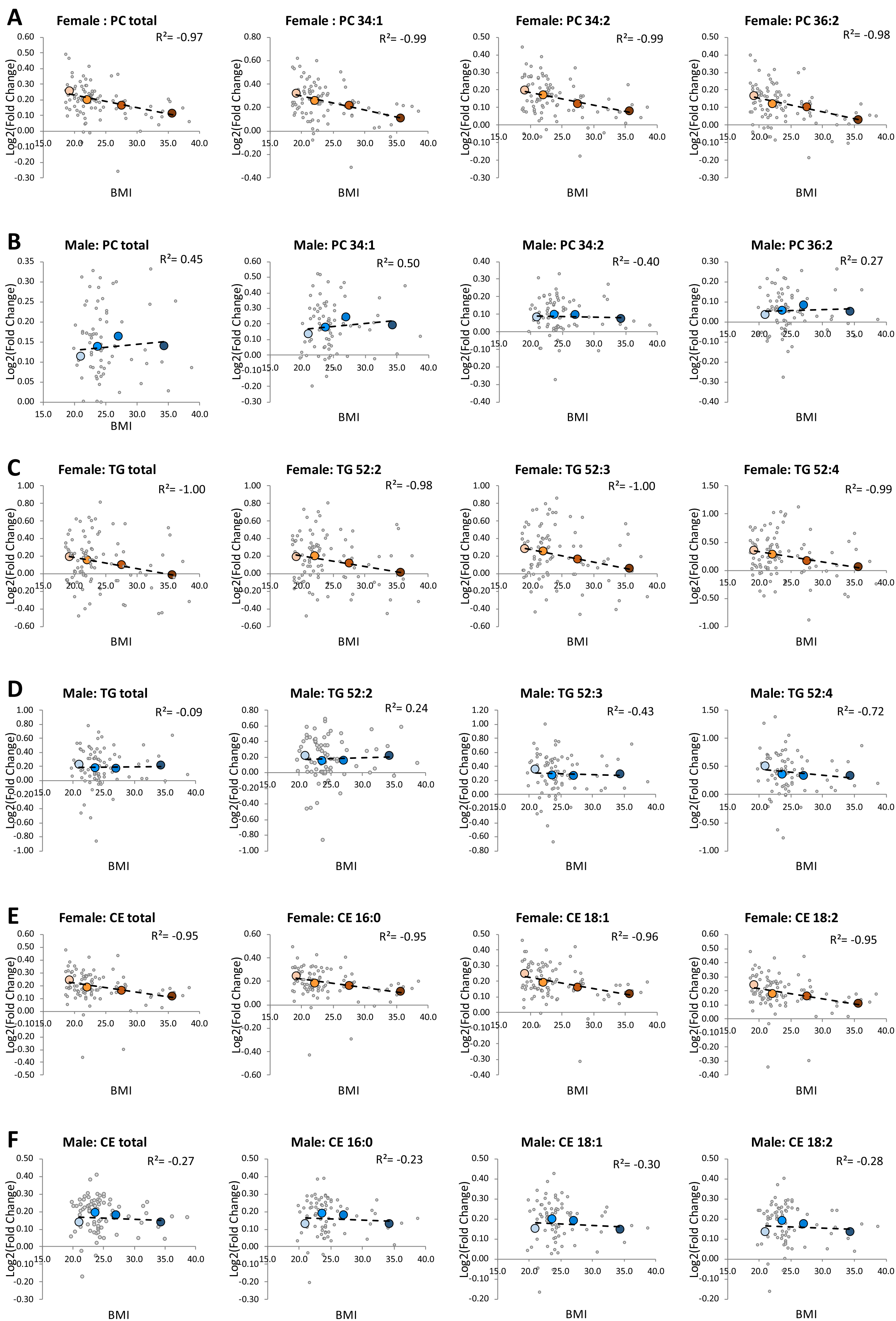

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Fig. 4. BMI subgroups correlate with the responses of PC, TG and CE to cold in women, but not in men
(A) Correlation of total PC levels including those of its 3 major species with BMI subgroups in women and (B) men. (C) Correlation of total TG levels including those of its 3 major species with BMI subgroups in women and (D) men. (E) Correlation of total CE levels including those of its 3 major species with BMI subgroups in women and (F) men. The study population was binned into four subgroups with BMI <20 (n = 15), 20–25 (n = 51), 25–30 (n = 13), >30 (n = 14) (woman) and < 22 (n = 13), 22–25 (n = 46), 25–30 (n = 9), >30 (n = 10) (men). Shown are means per subgroup as colored dots and the individual datapoints of all participants included in the correlation as grey dots. R^2 indicate Pearson's correlation coefficients calculated from the subgroup means.

subjects based on their responses to cold exposure (Fig. 5D) consisted of six choline-containing GPL (PC 32:0, PC O-38:5, PC O-40:5, PC O-36:2, LPC 20:3, LPC 20:4) and four SM species (SM 37:1;O2, SM 42:1;O2, SM 32:2;O2, SM 39:2;O2) (Fig. 5F). With 0.99, the average ROC-AUC was even higher compared to model (A) (Fig. 5D). Model performance parameters (accuracy, precision, recall, F1 score) were between 0.95 and 0.97 (Fig. 5E), and also higher compared to model (A) with parameters between 0.84 and 0.86 (Fig. 5B).

Together, these results demonstrate that changes in the plasma lipidome, i.e. GPL and SL species, can predict sex and thus, support that the plasma lipidome responds differently to short-term cold exposure in male and female participants.

4. Discussion

Analyzing plasma samples from a cohort of 171 individuals using quantitative lipidomics, we found that short-term cold exposure elevates the concentrations in 83 % of the quantified lipid species. Until now, the effects of cold on the plasma lipidome were reported with mixed, if not contradictory, results, as outlined in the introduction. Translation of results obtained from mouse studies to humans is limited due to substantial differences in lipoprotein metabolism. For example, mice lack cholesterol ester transfer protein (CETP) which exchanges CE from HDL particles with TG in VLDL and LDL [41–43]. Further, mice have a different adipose tissue composition than humans, with a significantly higher proportion of brown adipocytes (up to 50 %) relative to the total amount of adipocytes [44].

To the best of our knowledge, all previous human studies have specifically analyzed TG, except for a detailed study by Straat and colleagues, in which comprehensive lipidomic profiling was applied to serum samples obtained from ten lean young men, who were exposed to cold for 30–120 min [16]. They found major changes in TG including a decrease of total levels after 30 min of cold exposure, followed by a significant increase after 1 h, and a return to baseline levels after 2 h. Only the levels of specific TG species containing >5 DB remained increased until the end of the cold stimulus. The authors discussed that cold exposure induces intracellular lipolysis in white adipose tissues, which fuels the liver with FFA in order to synthesize TG and release VLDL particles after 60 min. Subsequently, cold-activated thermogenic tissues, i.e. BAT and skeletal muscle, might take up TG-derived FA from VLDL for oxidation. In our study, the majority of lipid species from all lipid classes, including TG and PC were raised after 2 h of cold exposure. VLDL contains not only TG, but also significant amounts of PC supporting the hypothesis of an activation of liver lipid synthesis after a cold

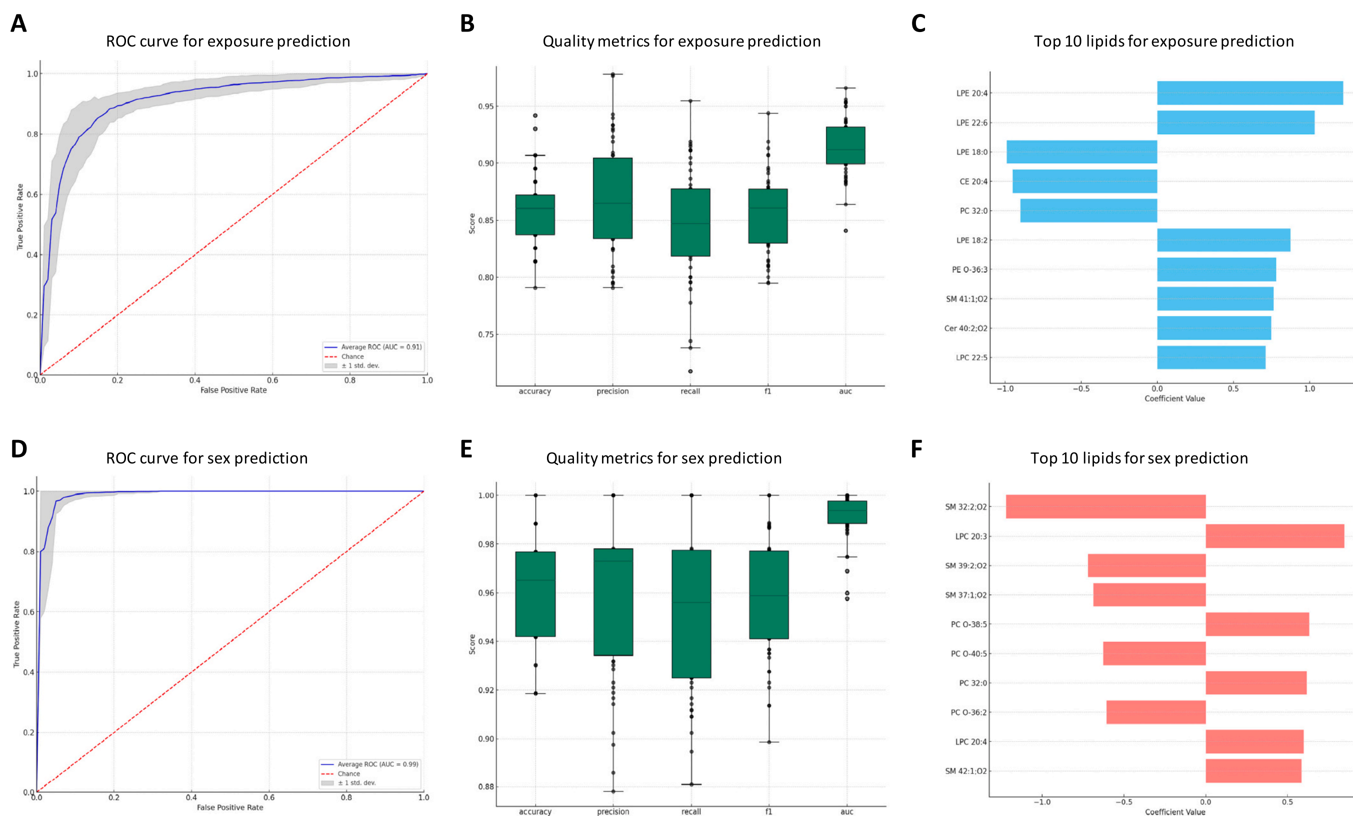

Fig. 5. The lipidomic response allows prediction of cold treatment and sex using a logistic regression model
(A-C) Models based on logistic regression were built to classify whether plasma samples originate from individuals at thermoneutrality or cold exposure or (D-F) whether samples originate from male or female subjects based on its lipidomic response to cold exposure. For repeated internal cross validation the sample set (n = 342) was split randomly into a 75 % training and 25 % test set. The model was run 50 times. (A, D) Average ROC curves based on a logistic regression model using all 177 quantified lipid species as input variables; (B, E) Average model performance parameters including accuracy, precision, recall, F1 score and ROC-AUC, as explained in the methods section; (C, F) Top 10 most important variables allowing classification, calculated from all 50 iterations.

stimulus [45]. Our findings show that the percental lipid composition in plasma remains unchanged after 2 h. This indicates that FFA originating from cold-induced lipolysis are incorporated to a fractionally similar extent into the major and minor lipid classes.

Among all lipids analyzed, complex lipids containing polyunsaturated PUFA as well as polyunsaturated TFA showed the most significant increases. This implies that PUFAs were released as precursors for metabolism, e.g. in the liver. As TG in human adipose tissue contains primarily saturated and mono-unsaturated acyl chains [46], PUFAs might derive from other lipid classes, i.e. GPL. In white adipose tissue, in addition to ATGL and HSL hydrolyzing TG, there are substantial amounts of phospholipases, releasing FA from poly-unsaturated PC and PE, present [46,47]. These phospholipases are also induced by beta-adrenergic stimulation, such as cold [47,48]. GPL sources for lipolysis may also include non-adipose tissues such as skeletal muscle, which is highly metabolically active in healthy men after a 3-h exposure to 18 °C [49]. Recently, increased LPC and LPE contents were reported in skeletal muscle of mice after a 3-day cold exposure [50].

The foremost new finding in our study is the sex-specific response of the plasma lipidome to a 120-min short-term cold exposure just below the shivering threshold. Sex-related differences in the (unstimulated) human circulating lipidome, with higher contents of PC, PE and SM, but lower amounts of LPC and LPE in women, as detected in our cohort (Fig. 3A, Fig. S2I), were already reported previously [2,51–54]. Applying an established knowledge-based data analysis strategy, we found that especially the generation of PC is higher in female study participants in response to cold. Advanced data analyses using machine learning revealed that also several SM species have a high potential to differentiate between plasma obtained from cold-exposed men and women. SM is a main component of VLDL particles, whose kinetics of secretion by the liver and clearance from the blood are known to differ between men and women [24,25,45]. Although predicting sex based on the lipidomic response to cold may have limited practical benefit, this independent and unbiased approach for data analysis clearly supports the hypothesis of a sex-specific lipid metabolism in humans upon cold exposure, particularly of PC and SM. It is worth noting that in our study, there was no difference in the amounts of total FFA generated after 2 h of cold exposure between men and women (Table S1). Thus, this cannot account for the observed results.

The reasons explaining the lipidome's differential responses in men and women could be multifaceted: (1) BAT activity might play a role. Although the overall metabolic relevance of BAT might be quite small (as discussed in the introduction), it is clear that women have significantly larger and more active brown adipose tissue depots than men [55]. Also, in women BMI subgroups negatively correlate here with the response of all lipid classes (except for LPC and LPE) as well as with BAT activity and white adipose tissue mass, which was reported previously [56]. (2) Sex and thyroid hormones might regulate lipid metabolism. Plasma levels of estrogens like 17- β-estradiol were associated with thermogenesis in healthy adults [57] and some evidence suggests that VLDL metabolism might depend on estrogen signaling [58,59]. In diabetic mice, estradiol treatment suppresses liver lipogenesis by maintaining acetyl-CoA carboxylase (ACC) phosphorylation [60], which might explain the lower increase in TG concentrations in female individuals found in our study. Interestingly, Sales and colleagues could demonstrate that the sex-associated differences in the unstimulated plasma lipidome are enhanced by the intake of hormonal contraceptives in women [2]. We exclude major effects due to (the lack of) estrogen cycles within the female group, because only 2 of 93 female participants of our cohort were > 40 years old (48 and 52 years of age). In addition to sex hormones, thyroid hormones are well-known regulators of hepatic lipid metabolism. In our cohort, basal thyroid-stimulating hormone (TSH) units and free triiodothyronine (fT3) levels were higher in men by 13 % and 8 % compared to women (Table 1). Additionally, after 2 h cold treatment fT3 concentrations dropped less severely in male (by 0.05 pg/ml) than in female participants (by 0.13 pg/ml). (Table S1; TSH was not measured after cold exposure). In rats, thyroid hormones were reported to stimulate the transcription of several key genes involved in de novo lipogenesis (including ACC and fatty acid synthase, which might contribute to the lower increase in TG concentrations in females found here), but reduce apolipoprotein B100 (Apo B100) levels decreasing VLDL production in the liver [61,62]. However, although sex and thyroid hormones may play critical roles in regulating lipid and lipoprotein metabolism, it is important to note, that their metabolic consequences are diverse, indicating the presence of other cofounding modulators. (3) These could include sex-biased gene expression in tissues [63], sex-specific differences in insulin sensitivity and adipokines, like adiponectin, regulating lipid metabolism [26,64]. The selection of our study volunteers was controlled in the best way possible, but obvious physical and physiological differences like unequal lifestyle, diet and body composition between men and women cannot be neglected as potential cofounders.

Together, we show that short-term cold exposure elevates concentrations of almost all lipids in human plasma indicating an increased lipid synthesis and whole-body lipid metabolism. The lipidomic response to cold is sex-specific. Especially PC and SM species showed differential changes, proposing a different regulation of their metabolism in men and women. Our findings substantiate the demand for sex-specific research and avoidance of a sex bias in clinical trials [65]. In particular, this applies to studies that focus on lipid homeostasis. To fully understand human lipid metabolism and related diseases, investigations must consider sex-specific differences, which is a prerequisite for personalized medicine and patient treatment.

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CRediT authorship contribution statement

Marcus Höring: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation. **Sarah Brunner:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Julius Honecker:** Writing – review & editing, Investigation. **Gerhard Liebisch:** Writing – review & editing, Methodology, Investigation. **Claudine Seeliger:** Investigation. **Laura Schinhammer:** Investigation. **Melina Claussnitzer:** Investigation. **Ralph Burkhardt:** Writing – review & editing, Resources, Investigation. **Hans Hauner:** Writing – review & editing, Writing – original draft, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Josef Ecker:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Lipidomic data can be found as Supplementary Data 2

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