AdipoAtlas: A reference lipidome for human white adipose tissue

Graphical abstract

Highlights
- AdipoAtlas provides a reference lipidome of human white adipose tissue
- 1,636 and 737 lipids were identified and quantified by tissue tailored LC-MS lipidomics
- AdipoAtlas demonstrates prominent differences between subcutaneous and visceral tissue depots
- Obesity leads to the remodeling of sphingo-, ether-, and neutral lipid metabolism

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In brief
Lange et al. report a reference lipidome of human white adipose tissue by providing qualitative and quantitative analyses of lipid compositions in lean and obese states for subcutaneous and visceral depots. Several key events of lipidome remodeling in obesity are identified within the metabolism of sphingo-, ether-, and neutral lipids.
**AdipoAtlas: A reference lipidome for human white adipose tissue**

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**SUMMARY**

Obesity, characterized by expansion and metabolic dysregulation of white adipose tissue (WAT), has reached pandemic proportions and acts as a primer for a wide range of metabolic disorders. Remodeling of WAT lipidome in obesity and associated comorbidities can explain disease etiology and provide valuable diagnostic and prognostic markers. To support understanding of WAT lipidome remodeling at the molecular level, we provide in-depth lipidomics profiling of human subcutaneous and visceral WAT of lean and obese individuals. We generate a human WAT reference lipidome by performing tissue-tailored preanalytical and analytical workflows, which allow accurate identification and semi-absolute quantification of 1,636 and 737 lipid molecular species, respectively. Deep lipidomic profiling allows identification of main lipid (sub)classes undergoing depot-/phenotype-specific remodeling. Previously unanticipated diversity of WAT ceramides is now uncovered. AdipoAtlas reference lipidome serves as a data-rich resource for the development of WAT-specific high-throughput methods and as a scaffold for systems medicine data integration.

**INTRODUCTION**

The “industrial revolution” in modern omics technologies significantly enriched our understanding of human biology. Application of high-throughput transcriptomics and proteomics allowed to compile the Tissue Atlas within the Human Proteome Atlas project with expression levels of mRNA and proteins reported for 44 healthy human tissues, serving as a powerful resource for exploration of functional tissue specificities, future drug targets, and potential biomarkers.1 Lipidomics, an omics branch aiming to identify and quantify individual lipid species, is not yet as advanced in the characterization of cell-, tissue-, and organ-specific lipid compositions. The majority of lipidomics studies aim for high-throughput screening of large sample cohorts and clinical translation.2,3 Such analytical workflows, targeting robust applications, are optimized for bulk lipid extraction followed by a single analysis method and relative (disease versus control) quantification.

Considering the cooperative action of lipids in biological membranes and the tight coregulation of anabolic and catabolic pathways of lipid metabolism, identification of tissue- and cell-type-specific lipid signatures (reference lipidomes) is urgently required to facilitate deeper understanding of lipid biology in health and disease. Lipid cooperative actions are highly tissue/cell-type specific at all levels of their functional activities, including plasticity of cellular membranes, energy storage, redistribution, and coordinated signaling.4,5 Furthermore, capturing alterations in lipid metabolism might be as important as identifying static lipid signatures resistant to certain (patho)physiological stimuli.

Deep lipidome profiling cannot be performed in a high-throughput manner, as it requires tissue-specific optimization and application of several orthogonal analytical methods to ensure simultaneous coverage of lipid classes with different polarities, ionization properties, and a range of endogenous concentrations. By now, the best-characterized composition is available for the blood plasma lipidome, with around 600 lipid species described at lipid class and lipid molecular species levels.6–8 However, detailed quantitative inventory of peripheral tissue lipidomes is scarce. Currently, adipose tissue metabolism...
is attracting a lot of scientific attention. Obesity, characterized by white adipose tissue (WAT) expansion and metabolic dysregulation, has reached pandemic proportions in modern societies, with a prevalence of more than 20% of the population. Obesity is associated with an increased threat of premature death due to a significantly higher risk of developing type 2 diabetes mellitus (T2DM), hypertension, coronary heart disease, stroke, and several types of cancer. Remodeling of WAT metabolism in obesity and, importantly, in the development of metabolic complications, is a cornerstone in understanding disease etiology. So far, WAT metabolism has been studied from many different angles, including genetic predisposition to obesity via genomewide association studies and changes in transcriptomics, epigenetic, and proteomics patterns of WAT upon obesity development. However, studies reporting the detailed quantitative description of depot-specific (subcutaneous [SAT] versus visceral [VAT]) WAT lipidomes in lean and obese human individuals are limited.

Here, we present AdipoAtlas: a mass-spectrometry-based reference lipidome of human WAT reporting over 1600 and 700 lipid species on qualitative and quantitative levels, respectively. AdipoAtlas represents a data-rich resource freely available to all lipid researchers, will support further understanding of lipidomic alterations within human adipose tissue, and acts as a guideline to generate other tissue-specific lipidome maps.

RESULTS

WAT-tailored lipid extraction and fractionation

WAT acts as the main lipid storage organ, with triacylglycerol (TG) present at exceedingly high concentrations, masking other less abundant lipid classes (Figure 1A). For accurate molecular mapping of the WAT lipidome, both extraction and fractionation were optimized to ensure coverage of both highly abundant storage (TG) and less abundant membrane and signaling (phospholipids [PLs] and sphingolipids [SPLs]) lipids. To support in-depth lipidome profiling, we created tissue pools of WAT representing SAT and VAT depots of lean (n = 5; BMI = 23.1 ± 1.5 kg/m²; age = 68 ± 10.9 years; male/female = 3/2) and obese (n = 81; BMI = 45.1 ± 1.2 kg/m²; age = 45 ± 22 years; male/female = 26/55) individuals. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms. Indeed, pooling approaches are often used for time- and cost-intensive profiling of different levels of -ome organisms.

WAT lipidome profiling

To increase biological meaningfulness of lipidomics data, lipid annotations should provide information on the lipid class as well as on the discrete fatty acyl chain composition rather than on the total number of carbon atoms and double bond equivalents. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based lipidomics allows accurate identification of lipids at the molecular species level (e.g., PC 16:0_18:2), but in complex lipid mixtures with a high dynamic range of lipid concentrations, the coverage of the identified lipidome will depend on the total resolution of the analytical platform. For deep WAT lipidome profiling, we utilized three LC systems, each coupled on-line to high-resolution accurate mass (HRAM) MS/MS (Figure 2A).

Highly hydrophobic TG lipids represented by a large diversity of molecular species and a dynamic range of concentrations were separated using C30 reversed-phase LC (RPLC), whereas the more polar LLE fraction (PLs, sphingolipids [SLs], diglycerides [DGs]) was resolved using C18 RPC (Figure 2B). Highly polar acyl carnitines (CAs) were separated by hydrophilic interaction LC (HLIC) (Figure 2B), as they are not sufficiently retained on RP columns. Moreover, MS analysis using data-dependent acquisition (DDA) relied on different HRAM platforms and was specifically tailored to enhance identification depth for each targeted lipid class. To this end, C30 RPLC-separated TGs were analyzed in positive polarity on a Q Exactive Plus MS with traditional DDA and on an Orbitrap Fusion Lumos Trobody MS using AcquireX deep scan acquisition workflow for in-depth analysis by reducing overall subject-to-subject variation. Pooled samples were used to test three common extraction protocols (Folch, methyl-tert-butyl-ether [MBTE], and hexane/i-ProOH/HCOC [Hex/IPA]). The most efficient extraction method was chosen based on the recovery of unpolar and polar lipid classes assessed by quantitative high-performance thin-layer chromatography (qHPTLC) and 31P-NMR (Figures 1B, 1C, and S1). Overall, the Folch two-phase extraction protocol was shown to be the most efficient in recovering both unpolar and polar lipids in human WAT.

Even with the optimal extraction, polar lipids represented a minor fraction of WAT lipidome (Figures 1A and 1C). To facilitate deep lipidomic profiling, we performed fractionation of WAT lipid extracts. Lipid fractionation methods utilize differences in molecular motifs or polarity of lipid classes, and here, we compared three orthogonal protocols based on (1) hydrophobicity and ionization state (amino propyl solid phase extraction, SPE; hereafter LipFrac); (2) the presence of phosphodiester groups (zirconia-oxide-based solid phase extraction [Zr-SPE]); and (3) polarity-dependent partitioning between a two-phase solvent system (using liquid-liquid extraction [LLE]). All three protocols showed similar recovery of phosphatidylcholines (PCs), phosphatidylethanoamines (PEs), and sphingomyelines (SMs) lipids (Figure 1D). LipFrac resulted in the highest recovery of free fatty acids (FFAs) but discriminated acidic PLs (e.g., phosphatidylinositol [PI]) due to their strong binding to the stationary phase. Zr-SPE effectively enriched phosphate-group-containing lipid classes but increased content of lysoPL and FFA due to PL alkaline hydrolysis during the elution step. LLE, based on a partitioning between a hexane and an ethanol/water phase, delivered sufficient enrichment efficiency for all polar lipid classes and was the most time efficient protocol (Figure 1D).

Here, we performed WAT-specific optimization of lipid extraction and fractionation protocols and identified the most efficient sample preparation strategy based on Folch lipid extraction followed by polarity-based LLE, allowing non-discriminative recovery and enrichment of lipids of different classes (Figures 1E and 1F).

Overall, the Folch two-phase extraction protocol was shown to be the most efficient in recovering both unpolar and polar lipids in human WAT.
identification of TG molecular species. Less abundant amphiphilic lipids, on the other hand, were detected by DDA on a Q Exactive platform and a Orbitrap Fusion Lumos instrument, both in the positive and the negative modes. As we originally failed to detect any cholesteryl esters (CEs), retinol esters, desmosterol esters, or cardiolipins in WAT total extracts, we set up targeted parallel reaction monitoring (PRM) for the detection of most prominent species. That allowed us to detect seven CEs otherwise masked by highly abundant TGs. However, both retinol and desmosterol esters, as well as cardiolipins, remained undetected in human WAT.

To ensure high-confidence accurate lipid identification, we used three software tools: LipidHunter,\textsuperscript{21} LipidSearch, and Lipostar.\textsuperscript{22} Obtained results were cross-matched, and the list of putative lipid identifications was manually curated to exclude false-positive identifications. We further validated manually curated lipid annotations by plotting the retention time of a given lipid species against its Kendrick mass defect (KMD) to...
the hydrogen base (Figure 2C). Detailed description of manual MS/MS curation and retention time mapping for lipids of different subclasses can be found at https://github.com/SysMedOs/AdipoAtlasScripts/blob/main/LipidIdentification/AdipoAtlas_Lipid_Identification_Manual.pdf. In some cases (MG, short acyl chain TG, CAR), coelution with lipid standards was used to validate their identity (Figure S2). Such rigorous curation of lipid annotations allowed us to resolve lipid classes, which are often not discriminated. For instance, using a defined set of specific fragment ions as well as retention time mapping, unambiguous identification of acyl-, alkyl-, and alkenyl-PL, as well as lysoPL, became possible.

Overall, we obtained a list of 1636 lipids representing 23 lipid subclasses (Figure 2D; Table S1). TGs display the highest lipidome complexity, making up 63.2% of all identified lipids followed by PL (16.8%), SP (9.8%), DG (5.4%), lysoPL (2.8%), acylcarnitines (1.7%), and CEs (0.4%). Eventually, we achieved a three-dimensionally curated (RT-MS-MS/MS), high-confidence lipid inventory of human WAT covering all major lipid classes including glycerolipids, PLs, SPs, CEs, and acylcarnitines, representing the most detailed description of human WAT lipidome to date.

Quantitative analysis of human WAT lipidome
The significance of accurate quantification for harmonization of lipidomics data was recently underlined by the lipidomics community. However, several analytical challenges need to be accounted for when aiming for accurate lipid quantification. MS signal intensity is molecular structure dependent and, as such, requires application of internal standards (ISTDs) to support accurate quantification. The highest possible quantitative accuracy can be obtained by using isotopically labeled ISTDs for each molecular species in the sample, which is, unfortunately, still not feasible at the whole-lipidome level. Here, we performed semi-absolute quantification of identified WAT lipids using lipid-subclass-specific ISTDs at concentrations close to the endogenous analytes (Figure 3A). WAT-specific ISTD mixture was designed to cover the whole range of identified lipid classes (Table S2). Next, 6-point calibration curves were generated for each ISTD spiked in the adipose tissue matrix to determine the linear response range (Figures S3 and S4). Final ISTD amounts in the mixture were chosen to represent intensity close to the native lipids of the corresponding lipid class while still displaying a linear behavior of ISTDs in the concentration-response relationship. Pooled WAT samples were spiked with the designed ISTD mixture prior to lipid extraction and fractionation and analyzed using full-scan LC-MS or PRM platforms utilizing three types of LC separations as described above.

All obtained data were corrected for lipid isotopic patterns (type 1), incomplete isotopic enrichment of deuterated ISTDs (Figure 3A), and abundances of different ionization adducts (Table S3). Since not only the lipid class, but also the fatty acyl chain composition, in a given lipid will determine its MS response, we defined acyl-chain-specific response factors for the most abundant and diverse WAT lipid class, TG, that were used to increase the quantitative accuracy of TG (Figure S5; Table S4). Thus, using a comprehensive separation strategy coupled to HRAM MS detection and an in-house-designed ISTD mixture customized to WAT, we performed semi-absolute quantification of the human WAT lipidome covering 522 lipid molecular species as well as 215 TGs quantified at the lipid class level, providing the most detailed semi-quantitative mapping of the human adipose tissue lipidome to date (Figure 3B; Table S5).

Adipose tissue lipids span a wide concentration range and display lipid-class-specific fatty acyl signatures
Quantitative analysis of pooled samples of the assessed cohort enables the investigation of the global human adipose tissue lipidome across lean and obese WAT and, thus, provides a general view of the composition of the WAT lipidome. Quantified WAT lipids displayed a huge dynamic range of concentrations from 12 amol/µg protein (CAR 20:5) up to 8 nmol/µg protein (TG 52:2), spanning over eight orders of magnitude (Table S5). Total TG concentration (96.2 nmol/µg protein) within WAT is overarching other lipid classes by two orders of magnitude, resembling their role as energy storage lipids. Importantly, concentrations of individual TG species ranged over five orders of magnitude, showing the molecular-species-dependent abundance, with just the 20 most abundant TGs representing over 71% of the total TG amount. Those top 20 TGs contained primarily saturated and monounsaturated fatty acyl chains, with an average of two double bonds per three acyl chains (Figure 3C). CEs were the second most abundant lipid class, of which CE 20:4 was the most concentrated (Figure 3F).

Unpolar lipids were followed by PCs, PEs, and SMs. Interestingly, PC and PE lipid species showed inverted distribution of the corresponding subclasses (Figure 3D). Thus, diacyl-PCs were ≈ 4.5 times more abundant than ether-PCs, whereas ether-PEs were ≈ 3 times more abundant than diacyl-PEs. Specifically, plasmalogen PEs were the most abundant lipid subclass within PE lipids. Closer inspection of the fatty acyl chain distribution revealed class-/subclass-specific differences. For PC lipids, the fatty acyl chain abundance was largely similar among acyl-, alkyl-, and alkenyl-species, whereas for PEs, a higher concentration of polyunsaturated fatty acids (PUFA)-containing alkenyl-PEs (plasmalogens) was observed over diacyl-PEs. Interestingly, plasmalogen lipids were previously reported to be
Figure 3. Quantitative representation of human WAT lipidome and description of analytical strategy used
(A) Schematic depiction of the quantitative lipidomics workflow.
(B) Quantitative distribution of lipid class and corresponding lipid molecular species within subclasses of human WAT. Total lipid class concentration is represented by bold lines (SUM), and each single lipid molecular species is represented by thin lines.

(legend continued on next page)
enriched in brain and heart tissues. Here, we demonstrate that in human WAT, plasmalogen PEs represent the fourth most abundant lipid class with a total concentration of 11.3 pmol/μg protein, and the most abundant molecular species of plasmalogen PEs are rich in PUFA. LysOPE and lysoPE lipids were one order of magnitude less abundant than PCs and PEs, with acyl chain composition similar to the corresponding diacyl-species, indicating active lipid remodeling within these PL classes via the Lands cycle (Figure 3D). Other PLs were detected only as diacyl species, with PS being the next most abundant class, followed by PIs and PGs. All minor PL lipids showed fatty acyl chain distribution characteristic of THs classes.24,25 Thus, the most abundant PS molecular species were PS 18:0_18:1 and PS 18:0_18:2, whereas PIs were rich in FA 20:4. The most abundant PG was PG 18:1_18:1 (Figure 3D).

DGs and acylICARs displayed quantities in the medium abundance range, illustrating their role as intermediates within lipid metabolism. Indeed, DG acyl chain distribution was quite similar to PLs, indicating the role of DGs as structural precursors of membrane lipids (Figure 3E). The most abundant acylcarnitine was CAR 2:0, in line with its proposed function as a sink for acetyl equivalents that accumulates due to the constant energy surplus to prevent complete coenzyme A (CoA) consumption, especially in highly metabolically active tissues such as WAT (Figure 3E). Other abundant species, CAR 3:0 and CAR 5:0, possibly originate from branched-chain amino acid oxidation.29,30 Additionally, relatively high concentrations of CAR 16:0, 16:1, 18:0, 18:1, and 18:2 were observed, indicating acylICARs shuttling medium-chain FAs to mitochondrial β-oxidation.26 This quantitative assessment of CAR species within human WAT further supports its role as a highly metabolically active organ.

Another lipid class of high metabolic importance often implicated in the development of obesity-related pathologies is ceramides (Cers) (Figure 3G). We found preferential incorporation of saturated or monounsaturated long- and very-long-chain FAs in Cer lipids, typical for this lipid class. The most abundant (203 fmol/μg protein) Cer in human WAT was Cer 34:1, represented by two isomeric species: Cer 18:1_2/16:0 and Cer 16:1_2/18:0. Cer 18:1_2/16:0 and corresponding Cer synthase (CerS6) were previously associated with weight gain and glucose intolerance.31 On the other hand, Cers with sphingoid bases (SPBs) other than SPB 18:1_2 are rarely monitored and, thus, usually not reported.31,32 Overall, we demonstrated previously unanticipated diversity of Cer subclasses in human WAT. Cers were represented by species with varying lengths of SPBs, of which SPB 18:1_2 and SPB 16:1_2 were the most abundant. Dihydroceramides (dHCer), precursors in de novo Cer biosynthesis, were concentrated in one order of magnitude lower than Cers themselves. The next most abundant Cer subclass was hexosylated Cer derivatives, closely followed by sphingadienine-Cer, a class of lipids only recently discovered and monitored in human blood plasma.33 Finally, to our surprise, human WAT was enriched in deoxy-Cer lipids. This potentially cytotoxic Cer subclass was detected in human blood plasma, where it represents a minor (0.1%–0.3%) fraction of total SPBs. Here, WAT contained significant amounts of deoxy-Cer lipids, corresponding to 12.6% of all Cer subclasses. Considering the close interconnection of SP metabolic pathways and already established functional differences of structurally diverse Cer lipids,30,34 AdipoAtlas significantly enriches the current knowledge of the human WAT lipid composition.

**PUFA-containing TGs are specifically upregulated in obese WAT**

Having a detailed, semi-quantitative map of human WAT in hand, we compared global lipidome compositions of SAT and VAT from lean and obese individuals. As expected, we found a statistically significant upregulation of TGs in obese adipose tissue (Figure 4A). Interestingly, all obesity-upregulated TGs contained at least one PUFA residue, with FA 20:4, FA 20:5, FA 22:5, and FA 22:6 acyl chains being the most upregulated PUFA-esters in that respect (Figure 4B). Conversely, TG species containing mostly saturated fatty acid and monounsaturated fatty acid residues were markedly decreased in the adipose tissue of obese individuals (Figure 4A). Correlation analysis further confirmed strong co-regulation of PUFA-TG (Figure 4C), indicating that the observed trend is not limited to single lipid species but is a general feature of TGs in obesity. Previously, obesity-driven global accumulation of long-chain PUFA-containing TGs was demonstrated in murine models and human biopsies.35–38 Increased activity of the FA elongase Elov6 was proposed to play a role as a common phenomenon in rodent and human adipose tissue in response to excessive nutrient consumption.38 This suggests that besides upregulation of total enzymatic FA elongation/desaturation machinery, the generation of specific TG molecular species is regulated during obesity development and opens up the questions of how and why specific lipogenic enzymes generate distinct TG species and what their role is in obesity development.

**Increase in sphingadienine-containing Cers is a hallmark of obese adipose tissue**

Obesity is one of the main risk factors for the development of T2DM, precluded by organ-specific or systemic insulin resistance.39 Cers are well-known mediators of insulin resistance, with Cer levels in different organs reflecting insulin sensitivity34. Here, in the obese adipose tissue, we found a marked upregulation of Cers with FA 24:1 acyl chains and Cers with the unusual SPB sphingadienine (SPB 18:2;O2) (Figures 4A and 4D). Sphingadienine (SPB 18:2;O2) Cers are a (so far) functionally undescribed class, the synthesis of which has been proposed in adipose tissue but not confirmed until now.38 Upregulated sphingadienine Cers contained a diverse range of esterified acyl chains (from FA 14:0 up to FA 24:0) (Figure 4D). Correlation analysis showed that there is a strong co-regulation between all upregulated SPB-18:2;O2-containing Cers (Figure 4B), indicating an increased biosynthesis of this unusual SPB or/and an increased acylation rate of the sphingadienine base.
Plasmalogen PLs are a depot-specific signature in acquired obesity

Encouraged by the fact that AdipoAtlas provided new insights in the lipidomics signature of obese versus lean adipose tissue, we next looked at the possible difference in lipid compositions of obese SAT and VAT depots. Interestingly, a clear discrimination of SAT and VAT depots was possible based on their respective plasmalogen PL signatures (Figure 4E). Thus, higher amounts of plasmalogen PC with long-chain PUFA (e.g., FA 20:4, FA 20:5, FA 22:6) were characteristic of obese SAT, whereas plasmalogen PEs accumulated in obese VAT. The majority of VAT-upregulated plasmalogen PEs carried 18-carbon-long fatty acyl chains (Figure 5B). Differential regulation of plasmalogen PEs was already indicated in previous studies. Here, we further demonstrated the involvement of plasmalogen PCs and, importantly, fatty acyl specificity within the regulated lipid species.

DISCUSSION

Deep profiling of tissue-specific lipidomes is essential to support our understanding of human biology by elucidating not only tissue-/organ-specific lipid remodeling mechanisms, but also the cross-talk between different tissues, its impact on systemic regulation of the lipid metabolism, and genome-lipid associations. In contrast to robust screening applications necessary for the analysis of large sample cohorts and potential clinical translation, deep lipidomics profiling of a particular tissue, organ, or cell type cannot be performed in a high-throughput manner and requires rigorous tissue-tailored optimizations and application of multiple orthogonal analytical workflows. Here, we provide the example of an analytical strategy targeting deep lipidomic profiling of human WAT, which can be transferred and adapted for the generation of reference lipidomes from any other human tissue. The workflow included three main steps to ensure non-discriminative deep lipidome coverage, providing a qualitative and quantitative inventory of human WAT lipidome:

(1) Tissue-tailored lipid extraction and fractionation to ensure non-discriminative coverage of all lipid (sub)classes was optimized by testing several protocols using HPTLC as a robust readout method, allowing fast quantitative assessment of extraction and fractionation efficiencies.

(2) Rigorous identification of lipid molecular species was performed by employing multiple separation (C18 RPC, C30 RPC, HILIC) and MS analysis platforms (+ DDA, AcquireX, PRM). LC-MS/MS analysis of four sample pools (SAT and VAT from lean and obese individuals) resulted in over 110 datasets used for lipid identification. Importantly, identification was performed using three independent software tools and was followed up by manual curation of MS/MS spectra and retention time mapping. Such a meticulous strategy was rewarded by accurate identification of over 1600 lipid molecular species, including rarely resolved ones. For instance, using specific sets of fragmentation ions and subclass-specific elution order, unambiguous identification of diacyl-, alkyl-, and alkenyl-PC and PE lipids was achieved. Furthermore, three-dimensional (LC-MS-MS/MS) curation including control for in-source fragmentation artifacts allowed us to uncover the unexpected diversity of the WAT sphingolipidome. To support accurate lipid identification at the molecular species level, we provide here a summary of lipid fragmentation patterns and retention time maps that can be used by other researchers aiming for deep lipidome mapping.

(3) Semi-absolute quantification of human WAT was based on lipid-class-specific ISTDs that were carefully selected to represent the diversity of endogenous lipids. To this end, a tissue-tailored ISTD mixture was designed and validated. Quantitative data were processed using several types of isotopic corrections, adducts, and in-source fragments. Additionally, response factors accounting for the distribution of diverse acyl chains in TG lipids were calculated and applied to enhance the accuracy of the quantification results. That allowed us to provide semi-quantitative values for 23 lipid subclasses and to appreciate the extraordinarily dynamic range of lipid concentrations within and between different lipid subclasses in human WAT.

Human WAT reference lipidome was reconstructed using pooled samples representative of SAT and VAT depots from lean and obese individuals. Although the application of pooled samples limits a detailed assessment of disease-specific lipid alterations, several depot- and phenotype-specific lipid features became apparent. Accumulation of TG lipids in WAT is a known hallmark of obesity. AdipoAtlas demonstrated diverse WAT TGs, represented by 1029 molecular species. Interestingly, TGs were also characterized by the largest dynamic range of concentrations, with only 20 TGs covering over 70% of the total TG concentration. Thus, the most abundant TG, 52:2, corresponded to 6.8 μg/μg of AT proteins. Although the top 20 most abundant WAT TGs were mostly saturated (two double bonds per three acyl chains on average), PUFA-rich TG species were significantly upregulated in obese WAT. These results are in line with previous studies illustrating the enrichment of PUFA TG in obese adipose tissue. However, mechanistic understanding of such
Figure 5. Complexity of the human WAT sphingolipidome

(A) Sankey plot displays the concentration of Cer subclasses, its corresponding esterified SPBs, and FAs. Depicted concentrations were calculated by averaging concentrations of WAT from SAT and VAT depots of lean and obese patients in order to reflect the general WAT sphingolipidome. Length of boxes corresponds to the determined concentrations.

(B) Differential regulation of sphingosine and sphingadienine SPBs over Cer subclasses in obese (ob) and lean tissues from VAT and SAT depots.

(C) Differential regulation of deoxy-sphingosine and deoxy-sphingadienine SPBs over Cer subclasses in obese (ob) and lean tissues from VAT and SAT depots. Statistical significance was calculated by ANOVA. *p \leq 0.05; **p \leq 0.01; ***p \leq 0.005.
specificity remains limited. Using in vitro reconstituted artificial lipid droplets (LDs), we recently demonstrated that lipid composition of both the neutral core TGs and the PL monolayer dictates the size of artificial LDs. Interestingly, higher amounts of PUFA-TG lead to the formation of much larger LDs, whereas PUFA-PC had the opposite effect. Thus, it is intriguing to speculate that elevated levels of PUFA-TGs observed in obese WAT might correlate with the larger size of the LDs in hypertrophic adipocytes.

To verify that WAT sphingolipidome is more complex, with SPB 18:2;O2 Cer representing only 69.2% of all classical Cers (Figure 5), we performed targeted metabolomics analysis of WAT tissue samples. Our data indicated that Cer levels in obesity and obesity-associated diseases including T2DM, fatty liver diseases, and metabolic syndrome. Details on Cer tissue depot specificity (SAT versus VAT) remain less obvious. The majority of the studies reporting Cer levels in human WAT utilized targeted mass spectrometry detection methods that cover only “classical” species (Cer, d3H Cer, and sometimes their glycosylated derivatives), in which SPB 18:1:O2 (sphingosine) is acylated with different fatty acyl chains. Using our advanced analytical workflow, we demonstrated that WAT sphingolipidome is more complex, with SPB 18:1:O2 Cer representing only 69.2% of all classical Cers (Figure 5). *AdipoAtlas* facilitated the identification of four additional bases: SPB 16:1:O2 (26.1%), SPB 19:1:O2 (2.6%), SPB 17:1:O2 (2.1%), and SPB 20:1:O2 (0.7%). Both SPB 18:1:O2 and SPB 16:1:O2 were elevated in obese WAT, and their levels were higher in VAT compared to SAT of lean and obese origin (Figure 5B). Overall, “classical” Cers represent only 40% of total Cer species quantified in WAT. The next most abundant subclass was glycosylated Cer (25%) containing up to three hexoses (Hex(n))Cer.

Interestingly, we identified two more highly abundant “atypical” Cers in human WAT: deoxyCer and sphigadienineCer. DeoxyCers are acylated derivatives of 1-deoxy-sphingosine (SPB 18:0;O), synthesized by SPT from palmitate and alanine instead of serine. They are typically considered as toxic by-products in Cer metabolism, as they can neither be degraded via classical Cer catabolic pathways nor be converted to SM and glycoCer species. DeoxyCers were only recently identified in human adipose tissue, with higher levels in VAT relative to serum, particularly in obese individuals with T2DM. Plasma levels of deoxyCer were positively associated with age, BMI, and waist-to-hip ratio, proposing them as a hallmark of metabolic complications. Interestingly, in plasma, SPB 18:1:O2 represents only a minor (0.1%–0.3%) fraction, whereas *AdipoAtlas* revealed 10-times-higher values in human WAT (12.6%) (Figure 5A). This suggests a significant enrichment of these potentially toxic species in WAT, although the exact role of deoxyCers remains to be uncovered. Previously believed to be mainly of hepatic origin, deoxyCers were shown to be directly synthesized by adipocytes during differentiation. Thus, *AdipoAtlas*, as well as recently reported data, allows us to propose human WAT as an important reservoir and a source of potentially toxic deoxyCer.

SphigadienineCer represented another abundant Cer subclass in human WAT (Figure 5). In comparison to “classical” Cer, sphigadienineCers contain one more double bond at the position Δ14Z (SPB 18:2:O2). Previously, SPB 18:2:O2 Cers were shown to reflect metabolic fitness due to an inverse association with homeostatic model assessment for insulin resistance, BMI, and incidence of cardiovascular events. Although the existence of SPB 18:2:O2 has long been known, the enzyme (FA desaturase 3; FADS3) responsible for the introduction of Δ14Z double bond was discovered only recently. According to gene expression data from GTEx portal (https://gtexportal.org/home/index.html), the highest FADS3 expression levels were found in peripheral nerve, aorta, and WAT. Gender-specific expression analysis further unraveled the highest expression of FADS3 in female WAT. So far, sphigadienineCers were not characterized in adipose tissue. Here, we report that they represent 19% of all Cer subclasses within human WAT (Figure 5A). Moreover, 18:2:O2 Cers were elevated in both SAT and VAT obese depots with significant enrichment of this lipid class in VAT versus SAT. Importantly, elevated levels of 18:2:O2 Cers represented a specific signature of obese WAT, showing statistical significance even for the pooled samples utilized in this study (Figure 4D). Although depot- and phenotype-specific increase in sphigadienineCer displayed the trend similar to Cer, their fatty acyl chain distribution was somewhat different, with a lower portion of FA 18:0 acylated into 18:2:O2 Cer in comparison to 18:1:O2 Cer. Our results strongly suggest that sphigadienineCer accumulation is a hallmark of obesity. Previously published data showed significant gender specificity for deoxy- and sphigadienineCer subclasses, with SPB 18:2:O2 being more abundant in men and SPB 18:2:O2 Cer in women. Interestingly, we observed an inverse correlation between those lipid classes in lean and obese WAT (Figure 5B), which might be explained by 2/3 prevalence of female WAT donors in our sample pools. Further analysis of individual samples based on the *AdipoAtlas* list will provide deeper insights into gender- and disease-specific signatures of Cer lipids.

Another emerging class of lipids potentially involved in the regulation of cellular and systemic lipid homeostasis is etherPL (ePL), including plasmalogens (pPL). Previously, brain and heart were identified to be rich in pPL. *AdipoAtlas* showed that ePC
and ePE lipids composed 41% of total PCs and PEs. Specifically, we demonstrated that PUFA-rich ePE (40 pmol/μg protein) represented the fourth most abundant lipid class in human WAT, closely following the most abundant PC PLs (62 pmol/μg protein). We identified depot-specific signatures of ePLs with higher levels of PUFA pPCs in SAT and enrichment of C18 fatty acyl chain containing pPLs in VAT. Ether lipids, and plasmalogens especially, play a central role in lipid quality control, adaptive responses to the change in lipid saturation levels, and maintenance of membrane fluidity. pPEs compose over 20% of inner leaflets of the plasma membrane, making them important players in membrane remodeling during adipocyte hypertrophic growth. Moreover, levels of circulating plasmalogens were inversely associated with hypertension, prediabetes, T2DM, cardiovascular diseases, and obesity. Interestingly, inverse co-regulation of ether lipids and SPs was recently demonstrated, with depletion of ePL leading to the Cer accumulation and vice versa. AdipoAtlas provides an inventory of ePL molecular species including resolved diacyl-, alkyl-, and alkenyl-PLs, which can be used as a resource for close, targeted follow up of this inverse correlation in larger sample cohorts. However, one should note that many of the observed changes might reflect adaptive, rather than maladaptive, mechanisms employed by WAT to escape lipotoxicity. Indeed, it is likely that many toxic, maladaptive effects observed in obesity and other metabolic disorders might be a result of the initially adaptive responses failed under the chronic metabolic burden. For instance, reported lipidotoxic effects of Cers should be seen in the perspective of metabolic malfunction, deriving from dysregulation of Cer metabolism rather than original Cer function. Indeed, in conditions of energy oversupply and constantly increasing flux of FFAs, Cers play an important role in preventing uncontrolled distribution of lipotoxic FFAs characterized by detergent-like (membrane lysis) activities. Cers were reported to promote safe FFA translocation via CD36 and to increase FFA incorporation into TGs by increasing TG synthesis and decreasing lipolysis rates. Cers are also linked with decrease in mitochondrial efficiency, possibly via induction of mitochondrial fission, leading to higher consumption of FA substrates per unit of ATP production and thus allowing cells to utilize higher amounts of FAs at the condition of excess energy supply. The role of Cers in inhibition of insulin signaling is usually seen as a negative effect. However, it is interesting to speculate that the initial effect of Cers on the inhibition of Akt signaling via protein kinase C zeta and protein phosphatase 2A, leading to “selective” insulin resistance in response to glucose, is an adaptive mechanism further promoting FFA utilization. The adaptive role of Cer synthesis in the condition of FFA oversupply is additionally supported by the presence of regulatory feedback loops. Thus, controlled activation of lipolysis via β-adrenergic stimulation and adiponectin signaling leads to the inhibition of Cer synthesis and increases their degradation, respectively. However, in conditions of continuous oversupply of FFAs (energy-rich diet and increased de novo lipogenesis), adaptive Cer signaling and its feedback regulation will fail, leading to “full” insulin resistance, pro-inflammatory signaling, and, ultimately, apoptosis as the last option of defense and safe disposal of overwhelming amounts of detergent-like FFA.

Overall, deep lipidomic profiling allowed us to reconstruct human WAT reference lipidome. AdipoAtlas provides an inventory of over 1600 lipid molecular species from 23 lipid (sub)classes fortified by their semi-quantitative values in two WAT depots (SAT and VAT) from lean and obese individuals. That allowed us to demonstrate an amazing diversity of adipose tissue lipids together with an assessment of their quantities between and within lipid classes. Several important lipid signatures characteristic of obesity were discovered or reproduced, pointing out the qualitative and quantitative accuracy of the applied methodology. AdipoAtlas is freely available for all researchers interested in WAT biology and can be further used to design human-WAT-specific high-throughput experiments targeting quantification of any given lipid (sub)class in large sample cohorts. Moreover, AdipoAtlas will provide currently missing scaffolding for systems biology integration of lipidomics data via reconstruction of lipid-centric genome scale metabolic models, linking big omics data with identification of disease characteristic metabolic, and signaling pathways.

Limitations of the study
Tissue pools used in the study proved to be useful for in-depth characterization of human WAT lipidome. However, this reference lipidome does not account for the age and gender variability within human populations. Furthermore, data on the inter- and even intra- (longitudinal) individual variability need to be provided in the future to reflect WAT remodeling upon obesity development. Measurement of WAT lipidomes in individual samples using large clinical cohorts has to be performed to address these limitations. Further integration of lipidomics data with transcriptomics and/or proteomics results will amplify the utility of AdipoAtlas as a resource.

STAR★METHODS
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at [https://doi.org/10.1016/j.xcrm.2021.100407](https://doi.org/10.1016/j.xcrm.2021.100407).

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AUTHOR CONTRIBUTIONS

M.F. conceived the project, guided the research, assisted with the experiments and data interpretation, and wrote the manuscript. M.L. designed and performed most of the experiments, analyzed and interpreted data, and wrote the manuscript. G.A. performed lipid identification, including manual annotation and retention time mapping. Z.N. performed lipid identification and data interpretation, and wrote the manuscript. M.B. provided human WAT samples. All authors edited and approved the manuscript.

DECLARATION OF INTERESTS

M.B. received honoraria as a consultant and speaker from Amgen, AstraZeneca, Bayer, Boehringer-Ingelheim, Lilly, Novo Nordisk, Novartis, and Sanofi. All other authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Lipids**          |        |            |
| Cer/Sph Mixture I   | Avanti Polar Lipids Inc. | LM6002-1EA |
| Cer 18:0;O3/16:0    | Avanti Polar Lipids Inc. | 860617P    |
| Cer 18:0;O3/8:0     | Avanti Polar Lipids Inc. | 860609P    |
| Cer 18:0;O2/8:0     | Sigma Aldrich          | C8605      |
| Cer 18:0;O2/12:0    | Avanti Polar Lipids Inc. | 860635     |
| Cer 18:1;O2/17:0;O[2R-OH] | Avanti Polar Lipids Inc. | 860817P |
| Cer 18:1;O6/6:0     | Cayman Chemical      | Cay25493-500 |
| SPLASH® LIPIDOMIX® | Avanti Polar Lipids Inc. | 330707-1EA |
| PC 16:0/18:1        | Avanti Polar Lipids Inc. | 850457    |
| PE 16:0/18:1        | Avanti Polar Lipids Inc. | 850757    |
| LPC 18:1            | Avanti Polar Lipids Inc. | 845875    |
| LPE 18:1            | Avanti Polar Lipids Inc. | 846725    |
| PA 16:0/18:1        | Avanti Polar Lipids Inc. | 840857    |
| PS 16:0/18:1        | Avanti Polar Lipids Inc. | 840034    |
| SM 18:1;O2/18:1     | Avanti Polar Lipids Inc. | 860587    |
| Deuterated Acylcarnitine Mix | EURISO-TOP GmbH | NSK-B-1 |
| FA 18:1             | Sigma Aldrich       | O1008      |
| FA 18:0 ([13]C1) (99 atom% 13C) | Sigma Aldrich | 299162 |
| FC                  | Sigma Aldrich       | C8667      |
| CE18:0              | Sigma Aldrich       | C79409     |
| MAG Mix – 1         | Larodan Inc.        | 90-3001    |
| DG 18:1/18:1/0:0 ([13]C3) | Larodan Inc. | 78-1892-7 |
| DG 16:0/16:0/0:0    | Sigma Aldrich       | D9135      |
| TAG Mix – 10        | Larodan Inc.        | 90-3010    |
| TG 18:1/18:1/18:1 ([13]C3) | Larodan Inc. | 78-1891-7 |
| TG 16:0/16:0/16:0 ([13]C3) | Larodan Inc. | 79-1600-7 |
| TG Standard Mix – GLC 768 | Nu-Chek Prep Inc. | GLC-768 |
| TG Standard Mix 2 – GLC 406 | Nu-Chek Prep Inc. | GLC-406 |
| TG 20:1/20:1/20:1   | Nu-Chek Prep Inc.   | T-270      |
| TG 20:2/20:2/20:2   | Nu-Chek Prep Inc.   | T-280      |
| TG 20:3/20:3/20:3   | Nu-Chek Prep Inc.   | T-290      |
| TG 20:4/20:4/20:4   | Nu-Chek Prep Inc.   | T-295      |
| TG 20:5/20:5/20:5   | Nu-Chek Prep Inc.   | T-325      |
| TG 14:1/14:1/14:1   | Nu-Chek Prep Inc.   | T-205      |
| TG 16:1/16:1/16:1   | Nu-Chek Prep Inc.   | T-215      |
| TG 16:0/16:0/18:1   | Larodan Inc.        | 34-1602    |

**Liquid and thin-layer chromatography equipment**

| HybridSPE® – Phospholipid, 30 mg/1 ml | Merck KGaA | 55261-U |
| Strata® NH2, 55 µm, 70 Å, 200 mg/3 ml | Phenomenex Inc. | 8B-S009-FBJ |
| Accucore C30 column (150 × 2.1 mm; 2.6 µm, 150 Å) | Thermo Fisher Scientific | 27826-152130 |
| Accucore C18 column (150 × 2.1 mm; 2.6 µm, 150 Å) | Thermo Fisher Scientific | 16126-152130 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Acquity UPLC BEH HILIC SI column (100 x 1.0 mm; 1.7 μm, 130 Å) | Waters Corp. | 186003458 |
| HPTLC silica gel plates 60, 20x10 cm | Merck KGaA | 1.05633.0001 |
| **Solvants and additives** | | |
| Acetonitrile (ULC/MS-CC/SFC grade) | Biosolve | 0001204102BS |
| 2-Propanol (i-PrOH) (ULC/MS-CC/SFC grade) | Biosolve | 0016264102BS |
| Methanol (ULC/MS-CC/SFC grade) | Biosolve | 0013684102BS |
| Formic acid (ULC/MS-CC/SFC grade) | Biosolve | 00069141ABBS |
| Chloroform (Emure®) | Sigma Aldrich | 1024451000 |
| Methyl-tert-butyl-ether (≥99%) | Sigma Aldrich | 34875 |
| Ammonium formate (MS grade) | Sigma Aldrich | 70221 |
| Ammonium acetate (MS grade) | Sigma Aldrich | 73594 |
| Ethanol (Rotisolv®) | Carl Roth GmbH+Co. KG | P076.1 |
| Acetone (≥99.9%) | Carl Roth GmbH+Co. KG | KK40.1 |
| n-hexane (Rotisolv®, HPLC) | Carl Roth GmbH+Co. KG | 7339.2 |
| Acetic acid (100%, p.a.) | Carl Roth GmbH+Co. KG | 3738.2 |
| **Software and algorithms** | | |
| LipidHunter | 21 | https://github.com/SysMedOs/lipidhunter |
| LipoStar | 22 | https://moldiscovery.com/software/lipostar/ |
| LipidSearch™ | Thermo Fisher Inc. | IQLAAEGBSFAPMBFK |
| Merging identification lists from various software tools | This paper | https://github.com/SysMedOs/AdipoAtlasScripts |
| OriginPro 2017 | OriginLab Corp. | https://www.originlab.com/index.aspx?go=Products/Origin2017&pid=3240 |
| Graphpad Prism Version 5.02 | GraphPad Software | https://graphpad.com |
| Metaboanalyst | 60 | https://www.metaboanalyst.ca/ |
| **Deposited data** | | |
| Raw and analyzed data | Raw data | https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp (MSV000086729); https://metabolomicsworkbench.org (http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Project&ProjectID=PR001112) |
| **Biological samples** | | |
| Visceral and subcutaneous white adipose tissue biopsies of lean and obese patients | Leipzig Obesity BioBank | N/A |

### RESOURCE AVAILABILITY

#### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Maria Fedorova (maria.fedorova@tu-dresden.de)

#### Materials availability
This study did not generate new unique reagents

#### Data and code availability
All raw lipidomics data have been deposited at https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp and https://metabolomicsworkbench.org are publicly available as of the date of publication. Accession numbers and DOI are listed in the Key resources table.

All original code has been deposited at GitHub repository and is publicly available as of the date of publication. Accession links are listed in the Key resources table.
Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Samples of human white adipose tissue from a total of 86 donors were kindly provided by Matthias Blüher as a part of Leipzig Obesity BioBank. Tissue collection was approved by the Ethics committee of the University of Leipzig (approval number: 159-12-21052012) and all subjects gave written informed consent before taking part in the study. Removed tissue samples were flash frozen in liquid nitrogen and stored at −80°C until further analysis. For the purpose of this study, we included adipose tissue samples from abdominal visceral (VAT) and subcutaneous (SAT) fat depots of lean (n = 5; BMI = 23.1 ± 1.5 kg/m²; age = 68 ± 10.9 y; male/female = 3/2) and obese (n = 81; BMI = 45.1 ± 1.2 kg/m²; age = 45 ± 2.2 y; male/female = 26/55) individuals from Caucasian population. Representative tissue pools were generated according to depot and phenotype specificity (Figure 1A).

METHOD DETAILS

Sample preparation
75 mg (for workflow optimization) or 50 mg (for LC-MS analysis) of frozen adipose tissue (AT) were cut on ice and collected into Lysing Matrix tubes containing ceramic beads (lysing matrix D, 1/8’’, 2 ml, MP Biomedicals, Eschwege, Germany). All the applied solvents were supplemented with 0.1% (w/v) BHT and extraction was performed on ice. Extraction ratio AT [mg] / Extraction solvent [mL] was 10. For LC-MS analysis in-house designed WAT Lipid Standards Mixture (100 μL, in CHCl₃/MeOH (2:1, v/v); Table S2) was spiked before homogenization.

Lipid extraction

Folch method
AT was homogenized in 1 mL of MeOH by FastPrep24™ 5G (3x30s, Lysing Matrix D) with cooling on ice after each homogenization round. Homogenate were transferred into glass tubes (11.5 mL, round bottom culture tubes, VWR) using glass Pasteur pipettes. Beads and lysing tubes were washed with MeOH (400 μL) and CHCl₃ (1000 μL), solution was transfer into glass tube. CHCl₃ (1.8 mL) was added (to reconstitute ratio CHCl₃/MeOH/H₂O = 2:1, v/v), mixture was incubated on the roller mixer (4°C, 1 h, 210 rpm), and H₂O (840 μL) was added (to reconstitute ratio CHCl₃/MeOH/H₂O = 8:4:3, v/v), incubated on roller mixer (4°C, 10 min, 210 rpm), and centrifuged to achieve phase separation (4°C, 10 min, 2000 x g). The lower phase was collected. Extracts were dried in vacuo (Eppendorf concentrator 5301, 1 mbar).

Methyl tert-butyl ether (MTBE) method
AT was homogenized in 1 mL of MeOH by FastPrep24™ 5G (3x30s, Lysing Matrix D mode) with cooling on ice after each homogenization round. Homogenate were transferred into glass tubes (11.5 mL, round bottom culture tubes, VWR) using glass Pasteur pipettes. Beads and lysing tubes were washed with MTBE (1000 μL), and the solution was transferred to a glass tube. MTBE (3.95 mL) was added (to reconstitute ratio MTBE/MeOH = 3.3:1, v/v), mixture was incubated on roller mixer (4°C, 1 h, 210 rpm), and H₂O (1240 μL) was added (to reconstitute ratio MTBE/MeOH/H₂O = 3.3:1:0.8, v/v), incubated on roller mixer (4°C, 10 min, 210 rpm), and centrifuged to achieve phase separation (4°C, 10 min, 2000 x g). The lower phase was collected. Extracts were dried in vacuo (Eppendorf concentrator 5301, 1 mbar).

Hexane/i-PrOH/HOAc method
AT was homogenized in 1 mL of MeOH by FastPrep24™ 5G (3x30s, Lysing Matrix D mode) with cooling on ice after each homogenization round. Homogenate were transferred into glass tubes (11.5 mL, round bottom culture tubes, VWR) using glass Pasteur pipettes. Beads and lysing tubes were washed with hexane (Hex; 100%, Roth), and i-PrOH (350 μL), HOAc (105 μL; 100%, Roth), and H₂O (1.125 mL) were added to glass tube (to reconstitute ratio HOAc/i-PrOH/Hex = 2.20:30:30, v/v; Mix/H₂O = 2.5:1, v/v), mixture was incubated on a roller mixer (4°C, 1 h, 210 rpm), Hex (3.75 mL) was added, incubate on the roller mixer (4°C, 10 min, 210 rpm), and centrifuged to achieve phase separation (4°C, 10 min, 2000 x g). The lower phase was collected with Pasteur pipette. Re-extraction was done by adding MTBE/MeOH/H₂O (1.95 mL; 10:3:3:2.5, v/v) with subsequent incubation on the roller mixer (4°C, 10 min, 210 rpm), and centrifugation (4°C, 10 min, 2000 x g) to achieve phase separation. Organic phases were combined and dried in vacuo (Eppendorf concentrator 5301, 1 mbar).

Protein concentration determination
Aqueous phases after lipid extraction were dried in vacuo (Eppendorf concentrator 5301, 1 mbar), redissolved in the buffer containing 7 M urea, 2 M thiourea, 1% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5 and protein concentration was determined by Bradford assay.61
Lipid fractionation

Liquid-liquid extraction (LLE)\textsuperscript{62}

AT lipid extract corresponding to 20 mg of AT dissolved in CHCl\textsubscript{3}/MeOH (150 µL; 2:1, v/v). Formic acid (0.1% v/v) acidified MeCN (900 µL) was added. Solution was loaded onto dry SPE cartridge (HybridSPE\textsuperscript{®} – Phospholipid, 30 mg/1 ml, Lot: 4800102, Supelco), washed subsequently with formic acid (0.1% v/v) acidified MeCN (1 mL), MeCN (1 mL), and eluted with MeCN containing 5% (w/v) NH\textsubscript{4}OH (2 × 1 mL). Eluates were dried in vacuo (Eppendorf concentrator 5301, 1 mbar).

Zr-SPE\textsuperscript{63}

Dry AT lipid extract corresponding to 20 mg of AT dissolved in CHCl\textsubscript{3}/MeOH (150 µL; 2:1, v/v). Formic acid (0.1% v/v) acidified MeCN (900 µL) was added. Solution was loaded onto dry SPE cartridge (Strata\textsuperscript{®} NH\textsubscript{2}, 55 µm, 70 Å, 200 mg/3 mL, Lot: S17-003383, Phenomenex) was conditioned with Hexane (6 mL), and equilibrated with CHCl\textsubscript{3} (6 mL). AT lipids were loaded, and eluted subsequently with CHCl\textsubscript{3}/i-PrOH (2:1, v/v, 15 mL, unpolar fraction), Et\textsubscript{2}O/HOAc (100:2, v/v, 5 mL, fatty acids fraction), MeOH (5 mL, neutral phospholipids fraction) and H2O/PrOH/EtOH/H2O (1:1:5:1, v/v). Dried TLC plates were immersed in acetone/H2O (8:2, v/v) containing primuline (0.05%, w/v) for 5 s (Camag Chromatogramm Immersion Device III). Images were acquired with a CCD camera (Bio-Rad ChemiDoc MP, Bio-Rad) using the primuline fluorescence (Ex: Blue Epi light illumination; Em: Filter 530/28). Densitometric analysis was performed with Image Lab (Version 5.2.1, Bio-Rad).

Phospholipid quantification by \textsuperscript{31}P-NMR spectroscopy\textsuperscript{65}

Dried AT lipid extracts were resuspended in buffer containing 200 mM sodium cholate, 5 mM EDTA, 50 mM Tris-HCl, pH 7.65 (500 µL) by vigorous vortexing for 2 min. Samples were placed in 5 mm NMR tubes and \textsuperscript{31}P-NMR spectra were recorded on a Bruker DRX-600 spectrometer operating at 242.88 MHz. All measurements were performed using a selective \textsuperscript{31}P/\textsuperscript{1}H NMR probe at 37°C with composite pulse decoupling (Waltz-16) to eliminate \textsuperscript{31}P–\textsuperscript{1}H coupling. Pulse intervals of the order of T\textsubscript{1} were used to allow quantitative analysis of phospholipid integral intensities. Other NMR parameters were as follows: acquisition time 1 s, data size 8–16 k, 60° pulse, pulse delay 2 s and a line-broadening (LB) of 1 Hz. Chemical shifts were referenced to the resonance of di-lauroyl-phosphatidic acid that was added as concentration and frequency standard. Further details are available in.\textsuperscript{65}

Quantitative high performance thin layer chromatography (qHPTLC)

Unpolar lipids

AT lipid extracts were dissolved in CHCl\textsubscript{3}/MeOH (2:1, v/v), and an amount corresponding to 1-10 µg AT wet weight were loaded using Camag Linomat 5 (Camag, Switzerland) on TLC plates (HPTLC silica gel 60, 20x10 cm, Merck). On each plate 7 dilutions of unpolar lipid TCL standards were loaded for quantitative lipid class specific calibration (Table 1). Plates were developed using hexane/CHCl\textsubscript{3} (8:2, v/v). Dried TLC plates were immersed in acetone/H\textsubscript{2}O (8:2, v/v) containing primuline (0.05%, w/v) for 5 s (Camag Chromatogramm Immersion Device III). Images were acquired with a CCD camera (Bio-Rad ChemiDoc MP, Bio-Rad) using the primuline fluorescence (Ex: Blue Epi light illumination; Em: Filter 530/28). Densitometric analysis was performed with Image Lab (Version 5.2.1, Bio-Rad).

Polar lipids

AT lipid extract polar fractions were dissolved in CHCl\textsubscript{3}/MeOH (2:1, v/v), and amount corresponding to 1-5 mg AT wet weight were loaded using Camag Linomat 5 (Camag, Switzerland) on TLC plates (HPTLC silica gel 60, 20x10 cm, Merck). On each plate 7 dilutions of polar lipid TCL standards were loaded for quantitative lipid class specific calibration (Table 1). Plates were developed using CHCl\textsubscript{3}/EtOH/TEA/H\textsubscript{2}O (5:5:1:1, v/v). Dried TLC plates were immersed in acetone/H\textsubscript{2}O (8:2, v/v) containing primuline (0.05%, w/v) for 5 s (Camag Chromatogramm Immersion Device III). Images were acquired with a CCD camera (Bio-Rad ChemiDoc MP, Bio-Rad) using the primuline fluorescence (Ex: Blue Epi light illumination; Em: Filter 530/28). Densitometric analysis was performed with Image Lab (Version 5.2.1, Bio-Rad).

Liquid-extraction static acquisition mass spectrometry

TLC plates were analyzed by using a TLC spot extraction system (Plate Express, Advion) coupled online to electrospray (ESI) Ion-Trap (IT) MS (amaZon SL, Bruker). TLC spots were extracted using pure MeOH as extraction solvent. ESI-IT parameter were as follows: electrospray voltage: 5.5 kV, end plate offset: 500 V; nebulizer gas: 7 psi; dry gas: 3 L/min; capillary temperature: 240° C; sheath gas (He) flow rate: 25 arbitrary units. Spectra were acquired in enhanced resolution mode and recorded in either positive or negative polarity. A maximum ionization time of 50 ms was applied. Data were subsequently analyzed using DataAnalysis (Bruker Daltonics, Bremen, Germany).
Chromatography

Unpolar lipid separation (C30 RPC)
Total lipid extracts (represented mostly by triacylglycerols) were reconstituted in CHCl₃/MeOH (2:1, v/v), required amount was transferred into HPLC vials and dried in vacuo. The dried lipids were reconstituted in i-PrOH/CHCl₃ (1:1, v/v) to a concentration of 2.5 mg/tissue/mL i-PrOH and 5 μL (= 12.5 mg/tissue) were loaded onto the column. Triacylglycerols were separated by reversed phase liquid chromatography (RPLC) on a Thermo Scientific Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with an Thermo Scientific Accucore C30 column (150 x 2.1 mm; 2.6 μm, 150 Å, Thermo Fisher Scientific, Sunnyvale, USA). Lipids were separated by gradient elution with solvent A (MeCN/H₂O, 1:1, v/v) and B (i-PrOH/MeCN/H₂O, 85:10:5, v/v) both containing 5 mM NH₄HCO₃ and 0.1% (v/v) formic acid. Separation was performed at 50°C with a flow rate of 0.3 mL/min using the following gradient: 0-5 min – 50 to 80% B (curve 5), 5-22 min – 80 to 95% B (curve 4), 22-26 min – 95% isocratic, 26-26.1 min – 95 to 100% B (curve 5), 26-47 min – 100% B isocratic, 47-47.1 min – 100 to 50% B followed by 8 min re-equilibration at 50% B.

Polar lipid separation (C18 RPC)
Polar lipid fraction was reconstituted in CHCl₃/MeOH (2:1, v/v), required amount was transferred into HPLC vials and dried in vacuo. The dried lipids were reconstituted in pure i-PrOH to a concentration of 0.5 mg/tissue/mL i-PrOH and 5 μL (= 2.5 mg/tissue) were loaded onto the column. Polar lipid fractions (lyso-/phospholipids, sphingolipids, diacylglycerols) were separated by reversed phase liquid chromatography (RPLC) on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with an Thermo Scientific Accucore C18 column (150 x 2.1 mm; 2.6 μm, 150 Å, Thermo Fisher Scientific, Sunnyvale, USA). Lipids were separated by gradient elution with solvent A (MeCN/H₂O, 1:1, v/v) and B (i-PrOH/MeCN/H₂O, 85:10:5, v/v) both containing 5 mM NH₄HCO₃ and 0.1% (v/v) formic acid. Separation was performed at 50°C with a flow rate of 0.3 mL/min using the following gradient: 0-20 min – 10 to 86% B (curve 4), 20-22 min – 86 to 95% B (curve 5), 22-26 min – 95% isocratic, 26-26.1 min – 95 to 10% B (curve 5) followed by 5 min re-equilibration at 10% B.

Acylcarnitine separation (Si HILIC)
Polar lipid fraction was reconstituted in CHCl₃/MeOH (2:1, v/v), required amount was transferred into HPLC vials and dried in vacuo. The dried lipids were reconstituted in pure i-PrOH to a concentration of 0.5 mg/tissue/mL i-PrOH and 5 μL (= 2.5 mg/tissue) were loaded onto the column. Acylcarnitines were separated by hydrophilic interaction chromatography (HILIC) on a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with an Acquity UPLC BEH HILIC Si column (100 x 1.0 mm; 1.7 μm, 130 Å, Waters Corp.). Lipids were separated as described previously⁶⁷ by gradient elution with solvent A (MeCN/H₂O, 96:4, v/v) and B (H₂O) both containing 7 mM NH₄OAc. Separation was performed at 40°C with a flow rate of 0.15 mL/min using following gradient: 0-20 min – 10 to 86% B (curve 4), 20-22 min – 86 to 95% B (curve 5), 22-26 min – 95% isocratic, 26-26.1 min – 95 to 10% B (curve 5) followed by 5 min re-equilibration at 10% B.

Mass spectrometry
Each sample was analyzed on different LC-MS platforms. High resolution accurate mass (HRAM) orbitrap based MS was employed in combination with C18 and C30 RPC, or HILIC chromatographic separation. Overall, 111 LC-MS/MS analysis were performed for WAT lipids identification.

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Table 1. Unpolar lipid TLC standards used for qHPTLC lipid-class-specific quantification

| Lipid       | Calibration range [nmol] |
|-------------|--------------------------|
| TG 16:0/16:0/18:1 | 36.00 18.00 7.20 3.60 1.80 0.36 0.18 |
| FC          | 77.59 38.79 15.52 7.76 3.88 0.78 0.39 |
| DG 16:0/16:0/0 | 52.73 26.37 10.55 5.27 2.64 0.53 0.26 |
| CE 18:0     | 45.79 22.90 9.16 4.58 2.29 0.46 0.23 |
| FA 18:1     | 106.21 53.10 21.24 10.62 5.31 1.06 0.53 |

Polar lipid TLC - ISTD

| Lipid       | Calibration range [nmol] |
|-------------|--------------------------|
| TG 16:0/16:0/18:1 | 36.00 18.00 7.20 3.60 1.80 0.36 0.18 |
| PC 16:0/18:1 | 39.47 19.73 7.89 3.95 1.97 0.39 0.20 |
| PA 16:0/18:1 | 43.05 21.52 8.61 4.30 2.15 0.43 0.22 |
| FA 18:1     | 106.21 53.10 21.24 10.62 5.31 1.06 0.53 |
| LPC 18:1    | 57.51 28.75 11.50 5.75 2.88 0.58 0.29 |
| LPE 18:1    | 62.55 31.28 12.51 6.26 3.13 0.63 0.31 |
| PE 16:0/18:1| 41.78 20.89 8.36 4.18 2.09 0.42 0.21 |
| PS 16:0/18:1| 37.98 18.99 7.60 3.80 1.90 0.38 0.19 |
| SM 18:1:02/18:1 | 41.15 20.57 8.23 4.11 2.06 0.41 0.21 |
Data dependent acquisition on Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer

C18, C30 RPC and HILIC were coupled on-line to Thermo Scientific Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a HESI probe. Mass spectra were acquired in positive and negative modes with the following ESI parameters: sheath gas – 40 a.u., auxiliary gas – 10 L/min, spray voltage – 3.5 kV (positive ion mode); –2.5 kV (negative ion mode), ion transfer temperature – 300°C, S-lens RF level – 35% and aux gas heater temperature – 370°C. For polar, unpolar lipids and acylcarnitines identification data were acquired in data dependent acquisition (DDA) modes with survey scan resolution of 140 000 (at m/z 200), AGC target 1e6 Maximum IT 100 ms in a scan range of m/z 350-1200 (380-1200 for unpolar lipids, 150-1200 for acyl carnitines). Data dependent MS2 were acquired with a resolution settings of 17 500 at 200 m/z, AGC target 1e5 counts, Maximum IT 60 ms, loop count 15, isolation window 1.2 m/z and stepped normalized collision energies of 10, 20 and 30% (15, 20 and 30% for unpolar lipids). A data dependent MS2 was triggered when an AGC target of 2e2 (2e3 for unpolar lipids, 2e1 for acyl carnitines) was reached followed by a Dynamic Exclusion for 10 s. All isotopes and charge states > 1 were excluded. All data was acquired in profile mode.

Data dependent acquisition on Orbitrap Fusion Lumos Tribrid mass spectrometer

C30 RPC were coupled on-line to Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with a HESI probe. Mass spectra were acquired in positive and negative modes with the following ESI parameters: sheath gas – 40 L/min, auxiliary gas – 10 L/min, spray voltage – +3.5 kV and –2.8 kV, capillary temperature – 250°C, S-lens RF level – 25 and aux gas heater temperature – 320°C. Data was acquired in data dependent acquisition mode with survey scan resolution of 120 000 (at m/z 200), AGC target 4e5, Maximum IT 100 ms in a scan range of m/z 500-950. Data dependent MS2 spectra were acquired with the following settings: automatic gain control target: 4e5 counts; max. injection time: 100 ms. The filters used were MIPS (small molecule), precursor selection range (500-950), charge state (1), dynamic exclusion (exclusion duration 8 s, exclude isotopes, mass tolerance ± 5 ppm) and target exclusion (polarity specific). MS/MS spectra were acquired in the Ion Trap mass analyzer at rapid scan rate (HCD, stepped collision energy: 10, 20, 30%; Isolation Window: 1.2 m/z; automatic gain control target: 2e4 counts; max. injection time: 35 ms, centroid data mode).

Acquire X on Orbitrap Fusion Lumos Tribrid mass spectrometer for in-depth triacylglycerol identification

Deep scan AcquireX Intelligent Data Acquisition Technology method was used to ensure in-depth identification of AT triacylglycerols. Two instrument methods were used – Full MS and ddMS3. The exclusion override factor was set to 10 and [M+H]+, [M+NH4]+, [M+Na]+ were selected as preferred ions. Three solvents blank replicate were analyzed before sample injection and the latest was used as exclusion reference. Injection volume was always 1 µL. A Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, San Jose, USA) using a HESI source was operated in positive ion mode using the following parameters: spray voltage – +3.5 kV, ion transfer tube temperature – 250°C, sheath gas – 30 arbitrary units, aux gas – 10, sweep gas – 1, arbitrary units, vaporizer temperature – 300°C. Full MS were performed by the Orbitrap mass analyzer, operated at a resolution setting of 120,000 for m/z 200, scan range of m/z 500–1200, AGC target 4e5 counts, maximum IT 100 ms, RF level: 25%, data type profile, EASY-IC internal calibration. For MS/MS resolution setting of 15,000 for m/z 200, HCD (collision energy: 20, 35, 50%), isolation window of 1.2 m/z, AGC target 2e4, Maximum IT 40 ms, data type centroid were used. The filters used were MIPS small molecule, precursor selection range (m/z 500-1200), charge state 1, dynamic exclusion for 6 s, exclude isotopes, mass tolerance ± 10 ppm, and Xcalibur Acquire X generated exclusion and inclusion lists (mass tolerance 25 ppm). The MS3 and relative MS4 spectra were acquired only for ions which fulfilled the two following filters: acquisition neutral loss ion trigger and loss trigger (list of FA, tolerance ± 20 ppm). MS3 spectra were acquired in the orbitrap mass spectrometer at resolution settings of 15000 for m/z 200 (HCD, Collision Energy: 35%; Isolation Window: 1; automatic gain control target: 2e4 counts; max. injection time: 25 ms, centroid). MS4 analysis were performed in the ion trap at normal scan rate (CID, Collision Energy: 30%; Activation time: 10 ms; Activation Q: 0.25, Isolation Window: 1; automatic gain control target: 2e4 counts; max. injection time: 35 ms, centroid).

Targeted identification of cholesteryl esters

The total lipid extract (unpolar fraction) was analyzed via C30 RPC coupled on-line to a Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a HESI probe. Mass spectra were acquired in positive mode with the following ESI parameters: sheath gas – 40 au, auxiliary gas – 10 au, sweep gas – 1 au, spray voltage – 3.5 kV, ion transfer temperature – 300°C, S-lens RF level – 35% and aux gas heater temperature – 370°C. For cholesteryl ester identification data were acquired in parallel reaction monitoring (PRM) mode. An inclusion list of the ammoniated adducts of 36 cholesteryl esters covering the range of fatty acids from 2 up to 22 carbons and 0 to 6 double bonds was employed. PRM data was acquired with a resolution setting of 17 500 at 200 m/z, AGC target 2e5 counts, Maximum IT 200 ms, isolation window 1.2 m/z and a normalized collision energy of 20% in profile mode.

Co-elution of short chain TGs, MGs, and acylcarnitine’s with corresponding internal standards

The retention behavior of short chain TGs, MGs and acylcarnitines was additionally studied in C18 RPC for verification of their identification. The short chain TG mixture TAG-MIX-10 (Larodan, Solna, Sweden) was dissolved in CHCl3/MeOH (2:1, v/v) and an amount corresponding to 2.5 µg total TG was transferred to an HPLC vial and dried in vacuo. Dried TAGs were redissolved in pure i-PrOH.
Lipid identification

DDA and Acquire X LC-MS/MS datasets were used for lipid molecular species identification. Identification strategy relied on three independent software tools followed by manual annotation.

LipidSearch

Lipids were identified using Thermo Scientific LipidSearch software version 4.1 SP1 using the following key processing parameters: target database – general, precursor tolerance ±5 ppm, product tolerance ±20 ppm, product ion threshold 1, Quan m/z tolerance ±5 ppm, Quan RT (retention time) range ±0.5 min. According to the lipid class, the ion mode and the structure of the lipids the results were filtered according to the “Grades.” (Lyso)phospholipids were identified with Grade A (diacyl forms) and Grade B (ether forms). Sphingomyelins were accepted at Grade C. Ceramides were used after filtering with Grade A and Grade C for corresponding water loss ions, and TG/DG lipids were identified with Grade A only.

LipidHunter

LipidHunter 2 RC source code version was used (https://github.com/SysMedOs/lipidhunter) to identify phospholipids in negative mode ([M+HCOO]− for PC and [M+H]+ all others) and glycerolipids ([M+NH₄]+) in positive mode. Raw files were converted into mzML format using MSConvert from Proteowizard project (version 3.0.9134). Lipids were identified using the following parameters: mass accuracy at MS level -5 ppm, MS intensity threshold -3e3 counts, mass accuracy at MS/MS level -20 ppm, MS/MS intensity threshold -100 counts. Identifications were filtered for isotopic score ≥75, and rank score ≥30. The white list of considered fatty acyl chains included 123 fatty acids corresponding to the LipidSearch default configuration file “FattyAcidDefinition.xml” for inter-software compatibility. Weight Factors assigned to each FA neutral loss fragment ions were 40, 48 and 30% for PLs, DGs and TGs, respectively. The identification results were reviewed using interactive HTML report. The table output files from LipidHunter were filtered and merged. Additional filters applied for PLs identification included: isotope score ≥80, rank score ≥40, both FA residues identified (for O/P containing PLs, only one FA residue should be present, rank score ≥30). For DGs and TGs, all FA residues have to be identified, the isotope score filter was set to ≥85.

Lipostar

Lipostar (version 1.0.6, Molecular Discovery, Hertfordshire, UK) equipped with LIPID MAPS structure database (version December 2017) was used. The raw files were imported directly, and aligned using default settings. Automatic peak picking was performed with SDA smoothing level set to low and minimum S/N ratio 3. Automatic isotope clustering settings were set to 7 ppm with RT tolerance 0.2 min. An “MS2 only” filter was applied to keep only features with MS/MS spectra for identification. Following parameters were used for lipid identification: 5 ppm precursor ion mass tolerance and 10 ppm product ion mass tolerance. The automatic approval was performed to keep structures with quality of 3-4 stars. Identification results of each lipid class were exported separately into 3 files using the Lipostar export function: feature table, best 3 matches of each feature (with check chain fragments enabled), and all approved matches. Exported tables were used for additional filtering and generation of merged identification list. For all classes, a fragmentation score filter of 60 and at least 2 fragment matches were applied (for TG at least 3 fragment matches).

Merge of identification results

Results of software assisted lipid identification were merged, and only lipids fulfilling the following parameter were kept in the final list: lipid must be identified by at least two software, within ∆RT < 0.3 min. A chain length filter according to the FA included 58 fatty acids (from C4 to C26; maximum of six double bonds) were applied to all software results. A set of customized python scripts was used to filter and merge the output files. The corresponding source code is available on GitHub (https://github.com/SysMedOs/AdipoAtlasScripts). Ether PLs identified by the software were manually corrected based of the retention time mapping.

Manual lipid annotation

Manual annotation and retention time mapping were performed as described at https://github.com/SysMedOs/AdipoAtlasScripts/blob/main/LipidIdentification/AdipoAtlas_Lipid_Identification_Manual.pdf.
Lipid quantification

**LC-MS quantification of acylcarnitines, polar and unpolar lipids**

For quantification purposes, the respective lipid classes were separated on a RPC C30, C18 or HILIC as described above. MS data were acquired in Full MS mode on a Q Exactive Plus Hybrid Quadrupol Orbitrap mass spectrometer in the positive and negative ion mode at the resolution of 140,000 at m/z 200, AGC target of 1e6 and a Maximum IT of 100 ms in the mass range from m/z 100 – 1500. Data were acquired in profile mode.

**Generation of calibration curves of employed ISTD**

In order to ensure linear response of the employed standards, internal calibration curves were generated for the respective ISTD. Varying concentrations of ISTD were spiked into ≈50 mg pooled WAT prior to lipid extraction to generate a 5-point internal calibration curve (Table 2). Lipids were extracted and ISTD derived signals were quantified. Additionally, isobaric or isomeric overlap with endogenous compounds during LC-MS analysis was excluded by close inspection of LC-MS derived data. Only calibration points resulted in a calibration curve with R > 0.98 were approved. Final ISTD concentration to spike for subsequent quantification had to display a relative standard deviation of < 20% and represent the linear response range. Final spiked concentrations are marked in bold in Table 2.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

For quantification raw datasets of Full MS measurements were processed using Thermo Scientific TraceFinder 4.1 (Thermo Fisher Scientific, Bremen, Germany). Quantification was based on determination of area under curve (AUC) using following settings: mass tolerance – 5 ppm, area noise factor – 5, peak noise factor – 10, baseline window – 150, S/N ≥ 3 using ICIS detection algorithm. Signals to be quantified (adducts and in-source fragments) were determined based on measurement of representative lipid standards for each class as shown below (Table S3).

For quantification of cholesteryl esters, raw datasets were acquired in PRM mode as described above. Measurements were processed using TraceFinder™ 4.1 (Thermo Fisher Scientific, Bremen, Germany). Quantification was based on determination of area under curve (AUC) using following settings: mass tolerance – 20 ppm, area noise factor – 5, peak noise factor – 10, baseline window – 150, S/N ≥ 3 using ICIS detection algorithm.

Obtained AUC values for all adducts and in-source fragments of each lipid species were summed up in order to display all-ion abundance of studied lipid species. AUC values were corrected for $^{13}$C abundance (Type I correction following the guidelines of Lipidomics Standards Initiative\(^7\) as described before:\(^68\)

\[
AUC_{n(k)}^{\text{total}} = AUC_{n(k)}^{\text{total}} = \frac{AUC_{n(k)}^{\text{total}}}{C_{18}} + 0.0109^n(n-1) + 0.0109^{2}n(n-1)\]

AUC_{n(k)}^{\text{total}} = total ion area under curve, AUC_{n(k)} = quantified area under curve of monoisotopic mass, n = No. of C-Atoms, k = No. of double bonds

Due to incomplete labeling of ISTDs, AUC of deuterated ISTD for phospholipids, sphingomyelins and acyl carnitines were determined by summing up the AUC of [M-2]\(^+\) (d\(_k\)-2), [M-1]\(^+\) (d\(_k\)-1), [M]\(^+\) (d\(_k\)), [M+1]\(^+\) (d\(_k\); $^{13}$C\(_1\)) and [M+2]\(^+\) (d\(_k\); $^{13}$C\(_2\)).

AUC of non-labeled ISTD for different ceramide classes were corrected for $^{13}$C abundance (Type I correction following the guidelines of Lipidomics Standards Initiative\(^7\) as described before:\(^68\)

\[
AUC_{n(k)}^{\text{total}} = AUC_{n(k)}^{\text{total}} = \frac{AUC_{n(k)}^{\text{total}}}{C_{18}} + 0.0109^n(n-1) + 0.0109^{2}n(n-1)\]

Quantitative values for lipid species were determined by relating AUC of the used ISTD to the lipid specie AUC:

\[
C_{\text{lipid}} = \frac{AUC_{\text{lipid}}}{AUC_{\text{STD}}} + C_{\text{STD}}
\]

C_{\text{STD}} = concentration of ISTD, AUC_{\text{lipid}} = corrected area under curve for lipid specie, AUC_{\text{STD}} = area under curve for ISTD.

**Response factors calculations for triacylglycerols**

TGs were quantified using a single $^{13}$C\(_3\) labeled TG molecular species (TG 18:1/18:1/18:1 ([13]C3)). Type II isotopic correction was applied for determination of AUC of $^{13}$C\(_3\) labeled TG, due to coelution with the native TG and isobaric overlap of [M]\(^+\) of $^{13}$C\(_3\) labeled TAG and [M+3]\(^+\) of native TG.

TG molecular species experience differential ionization and ion transmission yielding varying intensities depending on the molecular structure. Response factors of different native and isotopically labeled standards (Table S4) relative to the used $^{13}$C\(_3\) labeled ISTD TG 18:1/18:1/18:1 ([13]C3) were determined in order to increase accuracy of TG quantification. Dilution series of 34 different labeled and unlabeled standards spanning over three orders of magnitude were recorded (Table S4). Calibration curves were generated for each species to yield linear regression models with a minimum R\(^2\) > 0.99 and RSD < 10% for each point of the dilution curve. Obtained
## Table 2. Spiked concentrations to generate an internal calibration curve

| Lipid Spiked in | Spiked in ≈ 50 mg WAT [nmol] |
|-----------------|-------------------------------|
| **SPLASH LIPIDOMIX** |                                |
| **PC 15:0,18:1 (d7)** | 0 0.107 0.213 1.067 2.134<sup>a</sup> 4.268 |
| **PE 15:0,18:1 (d7)** | 0 0.004 0.008 0.040 0.080<sup>a</sup> 0.160 |
| **PS 15:0,18:1 (d7)** | 0 0.003 0.005 0.027 0.054<sup>a</sup> 0.108 |
| **PG 15:0,18:1 (d7)** | 0 0.019 0.038 0.190 0.381<sup>a</sup> 0.762 |
| **PI 15:0,18:1 (d7)** | 0 0.005 0.011 0.054 0.107<sup>a</sup> 0.215 |
| **PA 15:0,18:1 (d7)** | 0 0.005 0.011 0.054 0.107<sup>a</sup> 0.215 |
| **LPC 18:1 (d7)** | 0 0.024 0.048 0.241 0.482<sup>a</sup> 0.965 |
| **LPE 18:1 (d7)** | 0 0.005 0.011 0.054 0.109<sup>a</sup> 0.218 |
| **CE 18:1 (d7)** | 0 0.271 0.541 2.705 5.411<sup>a</sup> 10.821 |
| **MG 18:1 (d7)** | 0 0.003 0.006 0.028 0.055<sup>a</sup> 0.110 |
| **DG 15:0,18:1 (d7)** | 0 0.008 0.016 0.080 0.160<sup>a</sup> 0.320 |
| **TG 15:0,18:1,15:0 (d7)** | 0 0.035 0.071 0.353 0.705<sup>a</sup> 1.411 |
| **SM d18:1,18:1 (d9)** | 0 0.021 0.042 0.209 0.419<sup>a</sup> 0.837 |
| **Chol (d7)** | 0 0.125 0.250 1.250 2.499<sup>a</sup> 4.999 |

### Cer/Sph mix I (Avanti)

| Lipid Spiked in | Spiked in ≈ 50 mg WAT [nmol] |
|-----------------|-------------------------------|
| **SPB 17:1;O2** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **SPB 17:0;O2** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **SPBP 17:1;O2** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **SPBP 17:0;O2** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Lac-Cer 18:1;O2/12:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Gluc-Cer d18:1;O2/12:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **SM 18:1;O2/12:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:1;O2/12:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:1;O2/25:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |

### Acylcarnitine NSK-1-B mix

| Lipid Spiked in | Spiked in ≈ 50 mg WAT [nmol] |
|-----------------|-------------------------------|
| **Free carnitine (d9)** | 0 0.008 0.016 0.081 0.162<sup>a</sup> 0.324 |
| **CAR 2:0 (d3)** | 0 0.002 0.004 0.018 0.037<sup>a</sup> 0.074 |
| **CAR 3:0 (d3)** | 0 0.000 0.001 0.004 0.007<sup>a</sup> 0.015 |
| **CAR 4:0 (d3)** | 0 0.000 0.001 0.004 0.007<sup>a</sup> 0.015 |
| **CAR 5:0 (d9)** | 0 0.000 0.001 0.004 0.007<sup>a</sup> 0.015 |
| **CAR 8:0 (d3)** | 0 0.000 0.001 0.004 0.007<sup>a</sup> 0.014 |
| **CAR 14:0 (d9)** | 0 0.000 0.001 0.004 0.007<sup>a</sup> 0.015 |
| **CAR 16:0 (d3)** | 0 0.001 0.002 0.008 0.015<sup>a</sup> 0.030 |

### Additionally added

| Lipid Spiked in | Spiked in ≈ 50 mg WAT [nmol] |
|-----------------|-------------------------------|
| **FA 18:0 ([13]C1)** | 0 0.500 1.000 5.000 10.000<sup>a</sup> 20.000 |
| **TG 18:1/18:1/18:1 (13)[C3]** | 0 50.000 100.000 500.000 1000.000<sup>a</sup> 2000.000 |
| **TG 16:0/16:0/16:0 (13)[C3]** | 0 2.500 5.000 25.000 50.000<sup>a</sup> 100.000 |
| **DG 18:1/18:1/0:0 (13)[C3]** | 0 0.500 1.000 5.000 10.000<sup>a</sup> 20.000 |
| **Cer 18:0;O2/12:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:0;O2/8:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:1;O/6:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:0;O3/16:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:0;O3/8:0** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |
| **Cer 18:1;O2/17:0,O([2R-OH])** | 0 0.019 0.038 0.188 0.375<sup>a</sup> 0.750 |

<sup>a</sup>Final used ISTD concentration
slopes of concentration-response curves were related to the concentration-response curve of the isotopically labeled ISTD to establish response factors (RF). RF were normalized to the number of carbons and double bonds (DB) by dividing RF with the equivalent-carbon-number (ECN) yielding the normalized response factor (RF\text{norm}). Deviations in response mainly arise from increasing unsaturation and therefore for each DB number RF\text{norm} was separately related to m/z of [M+NH₄]⁺. Resulting slopes and y-intercepts for each double bond number were used to establish a linear regression model for determination of slopes and y-intercepts for which no standard were available. Subsequently, RF\text{norm} is calculated for each identified TG species and used for the quantification. A detailed description of the workflow provided in Figure S4. Finally, accurate quantification of TG was performed by relating the intensity of the ISTD and the RF-corrected intensity of the TG molecular species as expressed by the following equation:

\[ AUC_{TG\text{,corrected}} = \frac{AUC_{TG}}{RF_{TG}} \]

\[ C_{TG} = \frac{AUC_{TG\text{,corrected}}}{AUC_{ISTD}} \times C_{ISTD} \]

**Software**

Tracefinder 4.1 (Thermo Fisher Scientific, Bremen, Germany) was used for targeted lipid quantification. Quantitative data analysis including isotopic correction, ISTD normalization and response factor normalization was performed with Microsoft Excel 2016. Graphical representations were generated with Graphpad Prism® 5.02 and OriginPro® 2017. Metaboanalyst (https://www.metaboanalyst.ca/) was used to generate heatmaps and perform statistical analysis. A lipid was found to be statistically significantly regulated by Students t test with a threshold of \( p \leq 0.05 \) (FDR adjusted) assuming equal variances and a fold change \( \geq 2 \), or with an ANOVA \( p \leq 0.05 \).