Supplementary Note

This tutorial exemplifies application and interoperability of tools described within the **Lipidomics Tools Guide** for targeted and untargeted lipidomics workflows. These examples are meant to illustrate interconnectivity between different tools within the workflows and thus we do not provide here full details on data processing. For the detailed description of each tool, its GUI and related documentation please refer to the corresponding sections within the **Lipidomics Tools Guide**.

Tutorial 1: Targeted Method Design and Data Analysis in Lipidomics

Are you interested in performing targeted analysis and quantification for specific set of lipids?

For instance, you might ask a question like this:

"Are there any differences in the level of cholesteryl esters (CE) in subcutaneous (SAT) and visceral (VAT) white adipose tissue of lean and obese individuals?" [1]**.**

Using this case study example, the tutorial below will guide you through the main steps within the Lipidomics Tools Guide for targeted lipidomics method design and data analysis (Figure 1).

Detailed description and links to the tools used in this example can be found via the **Lipidomics Tools Guide** (the number of the corresponding section is shown at the right side of the page for each step described in this tutorial).

[1] This tutorial relies on the parallel reaction monitoring (PRM) data acquired for cholesteryl esters (CE) from the AdipoAtlas study by Lange et al[1]. The authors used targeted lipidomics to quantify CE in human white adipose tissue (WAT). The sample set included subcutaneous (SAT) and visceral (VAT) WAT of lean and obese individuals. The dataset was uploaded to Metabolomics Workbench (ST001738) and is freely accessible.

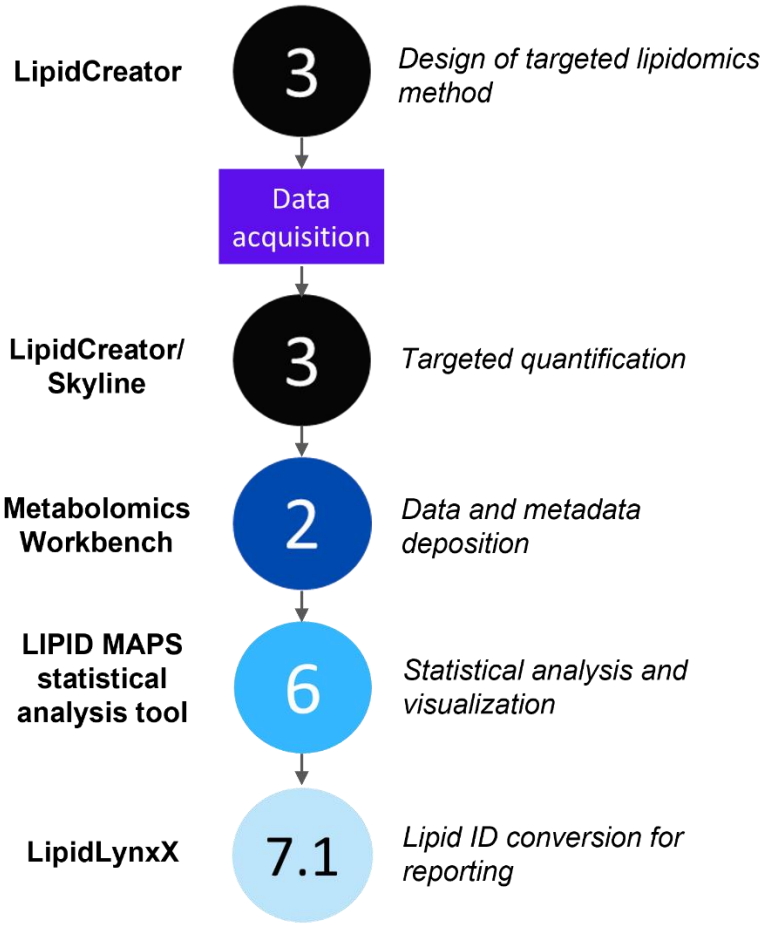


Figure 1. Overview of tools used for the targeted lipidomics method design and data analysis workflow exemplified in Tutorial 1. Number of the corresponding sections within the **Lipidomics Tools Guide** is illustrated in the middle together with the name of the exemplified tools (left) and performed tasks (right side).

Step1: Generate transitions for the selected list of lipids

List of transitions (pairs of selected precursor and corresponding product ions) can be generated using **LipidCreator** (Version 1.2.1 within **Skyline**[2]). 

Figure 2 illustrates such a transition list generated for cholesteryl esters (CE) of interest (here, CE 18:1, CE 18:2, CE 20:3, CE 20:4 CE 22:4, CE 22:5, CE 22:6). To this end, the class of 'sterol lipids' with the precursor 'cholesterol' and type 'ester' is selected. The number of fatty acyl chain carbons and the number of double bonds are selected accordingly to the molecular species of interest and the sterol lipid is added to the transition list. In the same way the deuterated internal standard (CE 18:1 [D7]) deuterated at the fatty acyl moiety) is added. The list can be stored and further used for the generation of a targeted acquisition method as well as quantification workflow in Skyline.

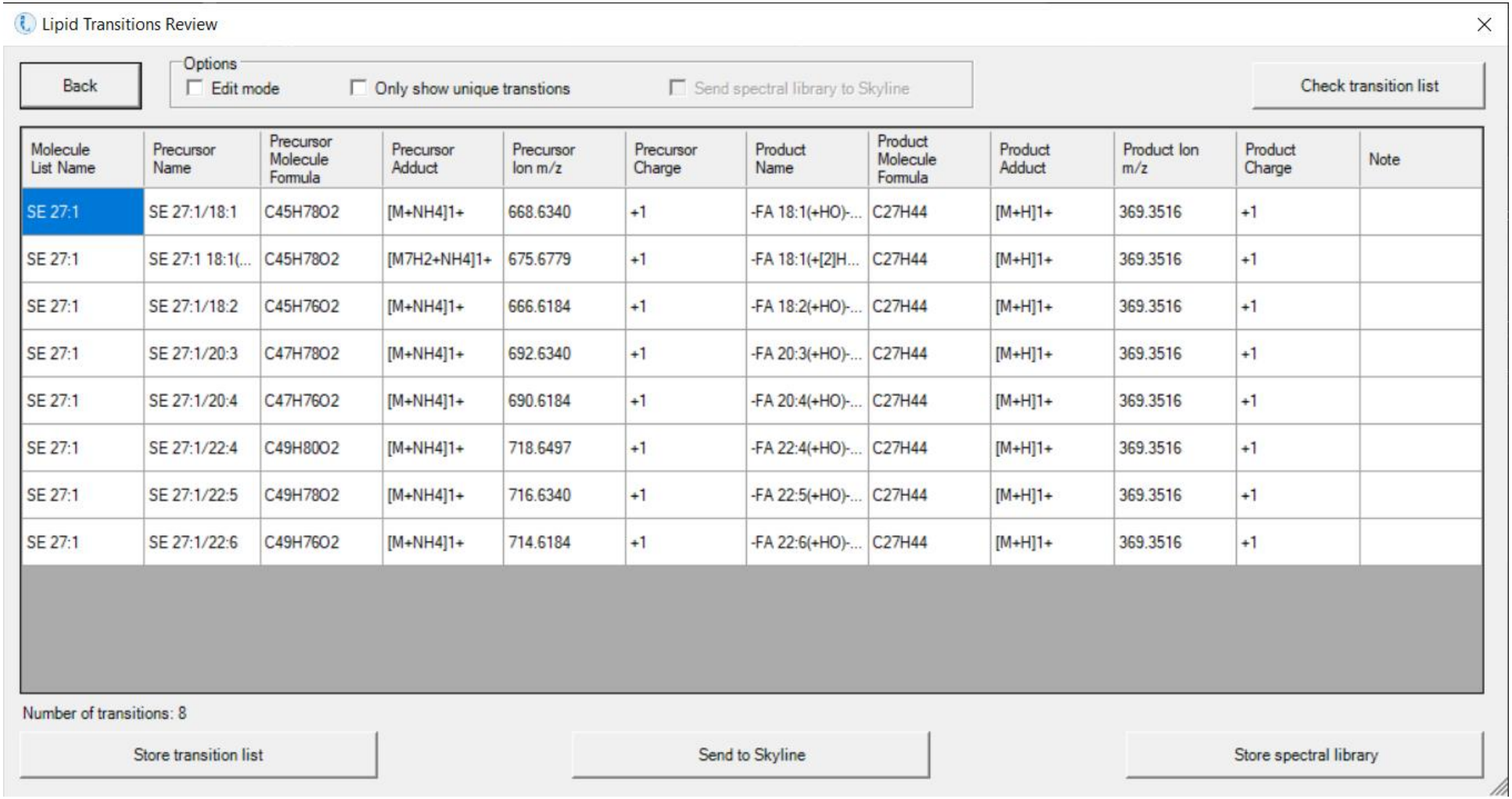


Figure 2: Transition list for selected CEs used for the setup of a targeted acquisition method generated with **LipidCreator** within **Skyline**.

Step 2: Data acquisition

Generated list of transitions can next be used to design a targeted acquisition method. Depending on the vendor of the MS instrument, required fields may slightly vary. In this example, PRM data was acquired on a Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer[1]. In PRM, the precursor ion defined by the transition list is fragmented and an MS/MS spectrum is recorded. Other acquisition methods such as single or multiple reaction monitoring (SRM/MRM) will record defined transitions (precursor to product ions) from the list. For a PRM method, it is necessary to provide precursor formula, precursor adduct (for CEs $[M+NH_4]^+$) and corresponding retention times (if performed in a scheduled manner).

Step 3: Quantification

The transition list generated in Step 1 as well as acquired raw data are imported to **Skyline**[2]. In case of CEs, the fragment ion at *m/z* 369.3516, corresponding to cholestene ion, is the dominating ion in the MS/MS spectrum, and is therefore used for quantification. The peak area integration of this signal in each file and for each analyte can be easily verified (Figure 3), integrated and exported in the form of a .csv file.

[2] For a detailed tutorial on the use of Skyline the reader is referred to the website of Skyline (https://skyline.ms/wiki/home/software/Skyline/page.view?name=tutorials).

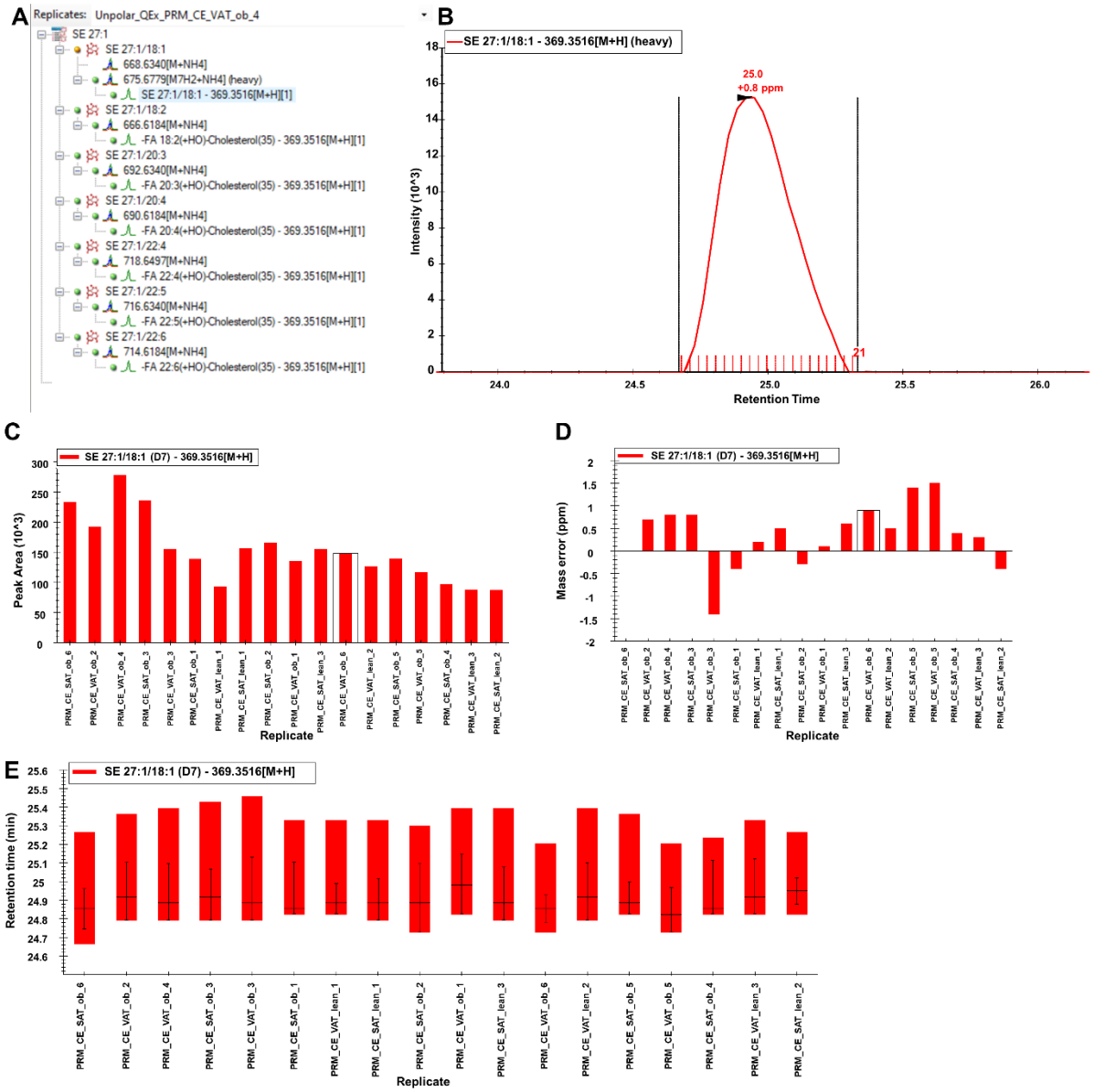


Figure 3: Illustrative representation of quantification from PRM data in **Skyline**. List of targeted lipid (A) contains transitions of interest for each analyte. The extracted ion chromatogram (B) shows peak area integrated for the selected product ion. Additional plots such as retention time comparison (C), mass errors (D) and peak areas (E) of all samples can be observed.

Step 4: Data deposition

The dataset can now be uploaded into the data repositories. This way data will be available for re-use by the community. Additionally, depending on the journal, it is often required to upload both raw and processed data supporting the results presented in manuscripts submitted for the publication. For example, this dataset was uploaded to **Metabolomics Workbench** (Figure 4A, ST001738) and is freely accessible together with its associated metadata (Figure 4B and C).

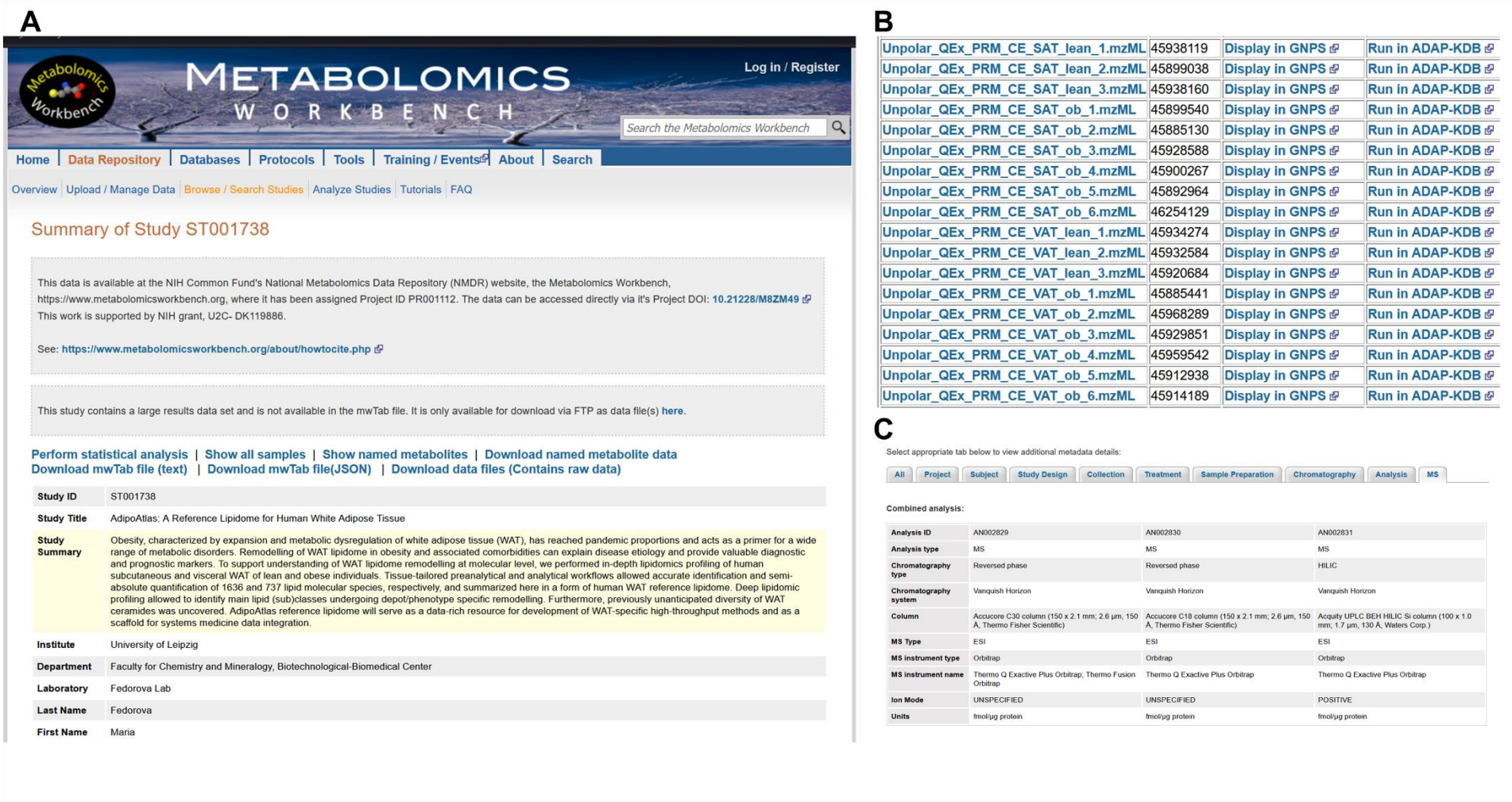


Figure 4: Study summary example uploaded to **Metabolomics Workbench** (A), PRM raw files selected for this tutorial (B) and metadata uploaded together with the study (C).

Step 5: Statistical Analysis

To explore obtained results for trends and significance, statistical and visualization tools can be next applied. Here the **LIPID MAPS statistical analysis tool** was applied to evaluate the difference in CE levels between the studied groups of samples.

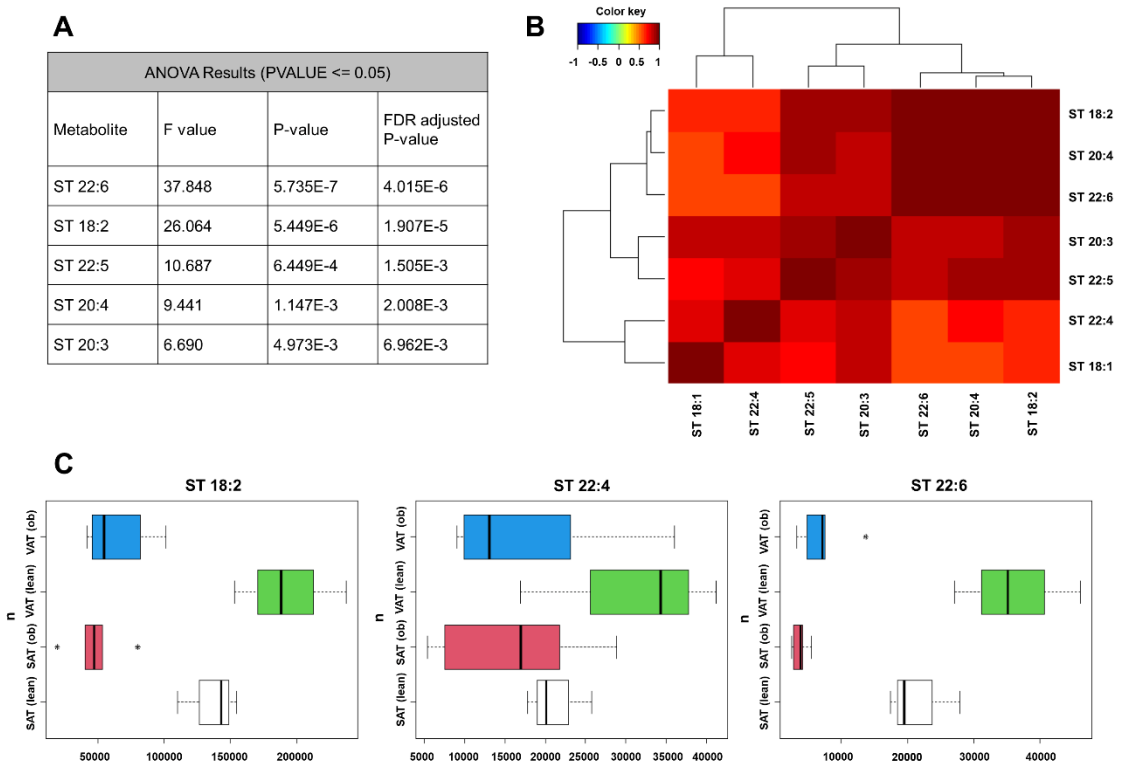


Figure 5: Statistical operations performed using the **LIPID MAPS statistical tool** including ANOVA (A), clustering correlation (B), and box-plots for three different cholesteryl esters (C).

Measured lipid concentrations are first uploaded in a form of tab-delimited text. The LIPID MAPS statistical tool allows different statistical operations (univariate analysis, multivariate analysis, clustering, and correlation) to be performed (Figure 5). The data can be exported (as PDFs) and used further for data interpretation.

Step 6: Lipid IDs and notation conversion.

Often different tools and software provide different ways of lipid notations, based on developers' preferred styles. To unify shorthand notation styles and to make results comparable with and searchable by others, lipid annotations can be converted into standard shorthand notation style or the style of your choice.

TextInput	TextInput_converted
CE 18:1	ST 18:1
CE 18:2	ST 18:2
CE 20:3	ST 20:3
CE 20:4	ST 20:4
CE 22:4	ST 22:4
CE 22:5	ST 22:5

Showing 1 to 6 of 6 entries

7.1

Figure 6: Output from **LipidLynxx** after conversion of lipid annotations to shorthand notation.

Here, a list of lipids is provided as .csv file and used by the Lipid ID Converter within **LipidLynxx** to convert lipid IDs to the shorthand notations recommended by Liebisch et al [3] (Figure 6). The updated lipid notations together with associated data (semi-absolute quantities, statistically significant changes etc) can then be used for the reporting.

The examples provided above illustrate the application of **LipidCreator**, **Skyline**, **Metabolomics Workbench**, **LIPID MAPS statistical analysis tools**, and **LipidLynxX** for the design, analysis, and reporting of a targeted lipidomics experiment.

Tutorial 2: Data Processing for Untargeted Lipidomics

Are you interested in performing untargeted analysis to find out what kind of lipids are present in your sample of interest?

For instance, you might ask a question like this: "What kind of ceramides are present in subcutaneous (SAT) and visceral (VAT) white adipose tissue of lean and obese individuals?"[3].

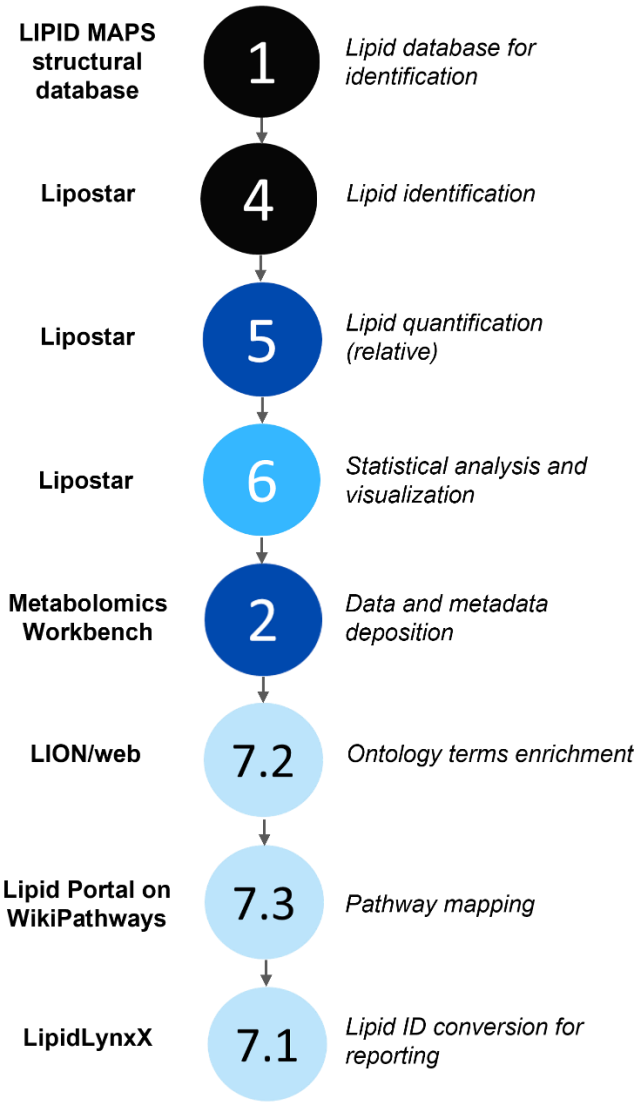


Figure 7. An overview of tools used for untargeted lipidomics data analysis workflow exemplified in Tutorial 2. The umber of the corresponding sections within the **Lipidomics Tools Guide** is illustrated in the middle together with the name of the exemplified tools (left) and performed tasks (right side).

[3] This tutorial relies on data acquired using positive data dependent acquisition mode from the AdipoAtlas study by Langer et al[1]. The authors used untargeted lipidomics to identify and (semi-absolutely) quantify lipids in human white adipose tissue (WAT). The sample set included subcutaneous (SAT) and visceral (VAT) WAT of lean and obese individuals. The dataset was uploaded to metabolomics workbench (ST001738) and is freely accessible.

Using this case study example, the tutorial below will guide you through the main steps within our **Lipidomics Tools Guide** for untargeted lipidomics data analysis (Figure 7). Detailed descriptions and links to the tools used in this example can be found via the **Lipidomics Tools Guide** (the number of the corresponding section is shown at the right side of the page for each step shown in this tutorial). For simplicity, we focus here only on one particular lipid subclass (ceramides) but using similar workflows, one can explore the whole lipid diversity in a sample.

Step 1: Lipid oriented databases

Lipid oriented databased are often required for software assisted lipid identification. User can download (Figure 8) the latest version of the database and upload it into the identification software of choice. For this tutorial the **LIPID MAPS structural database** (LMSD) is used within Lipostar 2.0 [4] identification software.

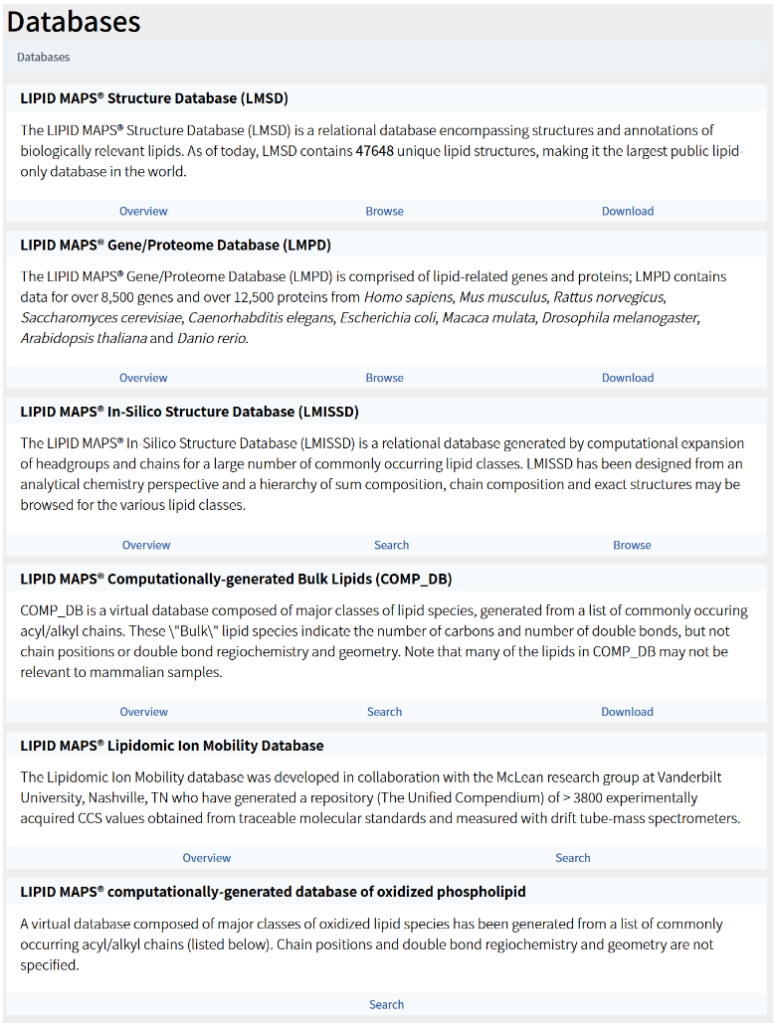


Figure 8: LIPID MAPS databases available for download under Resources: Databases section of LIPID MAPS website (https://www.lipidmaps.org/databases).

Step 2: Lipid identification

Ceramides are identified using data acquired in positive ionization mode with data dependent acquisition (DDA; top 15) workflow on a Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer[1]. For lipid identification **Lipostar 2.0** is first used (Figure 9).

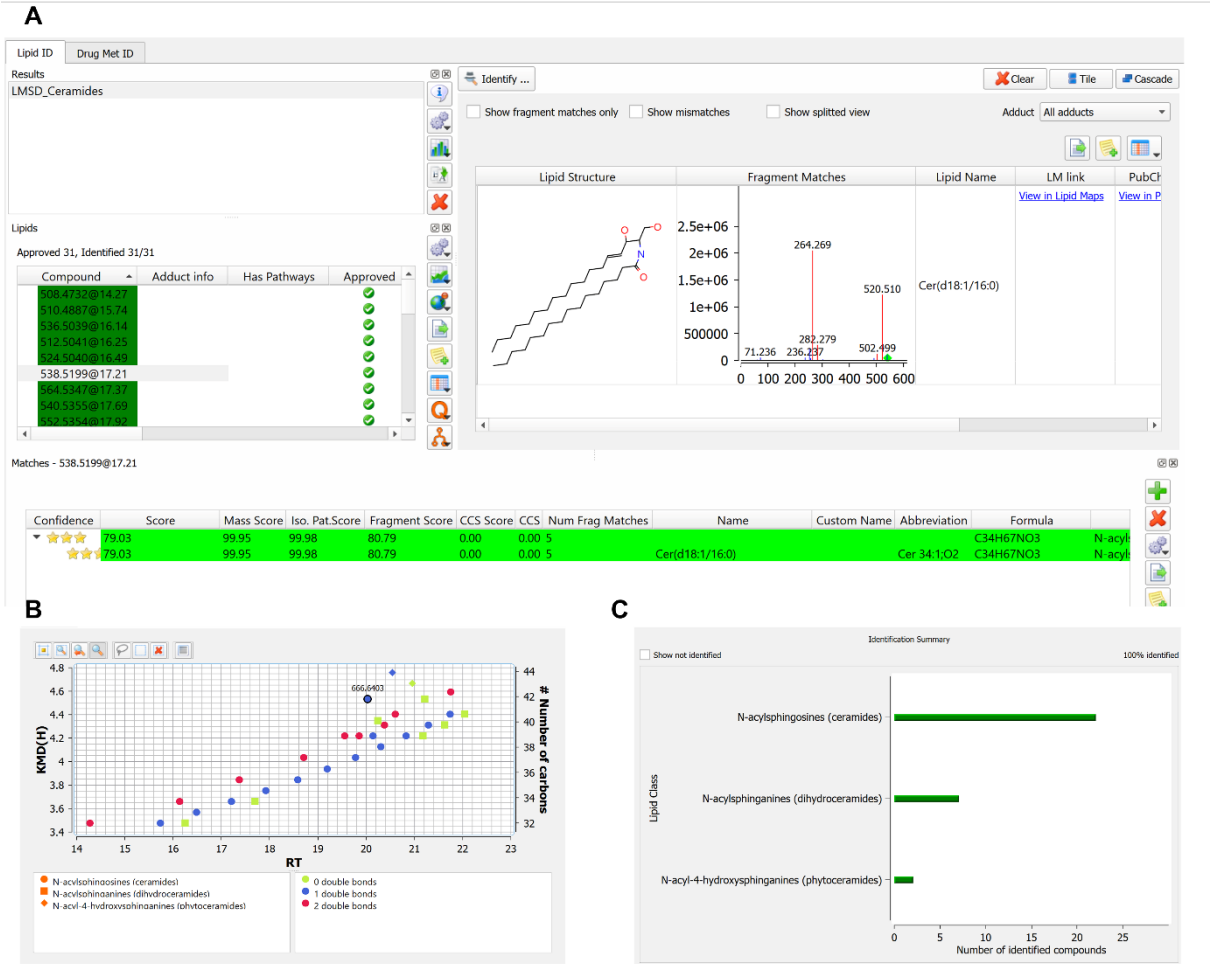


Figure 9: Lipostar 2.0 identification results for ceramides searched against **LMSD database** (A), the corresponding Kendrick mass defect plot (B; e.g. feature with *m/z* 666.6403 does not follow the expected elution trend and thus can be excluded from the list of identified lipids), and an identification summary providing the number of identified lipids per subclass of ceramides (C).

Data is imported using “Thermo DDA pos” default settings. The data matrix is filtered by “super sample filter” to retain only lipids with MS/MS and approved isotopic pattern. For identification, automatic approval only for lipid annotations graded with 3 and 4 stars (reflecting the quality of identification) is applied. Next, lipid identifications are manually evaluated based on the tandem mass spectrum and the observed fragment ions. For instance, the tandem mass spectrum of ceramide ***Cer 18:1;O2/16:0*** (Figure

9A) contains besides the precursor ion (*m/z* 538.5199), also two characteristic consecutive water losses (*m/z* 520.510 and *m/z* 502.499) and the loss of water and formaldehyde (*m/z* 490.499). Furthermore, fragment ions from the sphingosine base (18:1;O2, *m/z* 300.289) with the loss of up to two water molecules (*m/z* 282.279 and *m/z* 264.269) are observed. Further evaluation of annotation accuracy for approved lipids can be done for example by Kendrick mass defect plot, which takes into account retention time for each identified lipid versus its Kendrick mass defect by hydrogen, allowing the user to exclude possible false positive identifications (Figure 9B). Finally, an identification summary for approved lipids can be generated (Figure 9C).

Step 3: Relative quantification

Relative quantification can be further performed within the **Lipostar 2.0** environment. First, peak integrations for approved lipid identifications can be manually validated and adjusted if necessary (Figure 10). Data can be normalized using variety of options. Obtained results can be exported (in a form of .csv tables) or further processed within Lipostar.

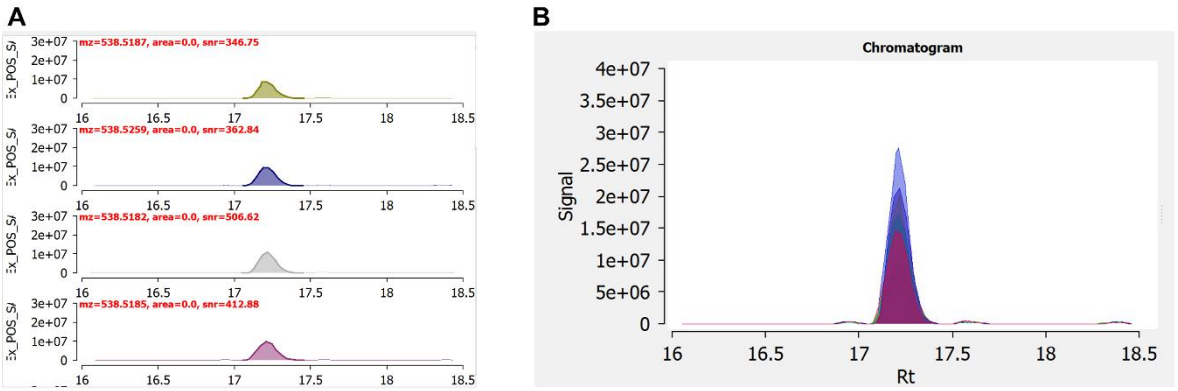


Figure 10: Lipostar 2.0 generated extracted ion chromatogram for Cer 18:1;O2/16:0 in the individual samples (A) and overlayed from all DDA files that were used for the identification (B). If necessary, peak integration can be adjusted.

Step 4: Statistical analysis

Within **Lipostar 2.0**, a set of statistical operations is available. For example, univariant analysis using fold change can be applied (Figure 11A), and visualized using volcano plot (Figure 11B). Multivariant analysis can be performed using for instance principal component analysis (PCA). In this case study, the PCA score plot (Figure 11C) reveals a distinct separation between SAT (blue dots)

and VAT (orange dots) samples driven by specific species of ceramides such as Cer(d18:1/24:1) (*m/z* 648.6299), Cer(d18:2/22:0) (*m/z* 620.5982) and Cer (d18:1/16:0) (*m/z* 538.5199) as revealed by the corresponding loading plot (Figure 11D).

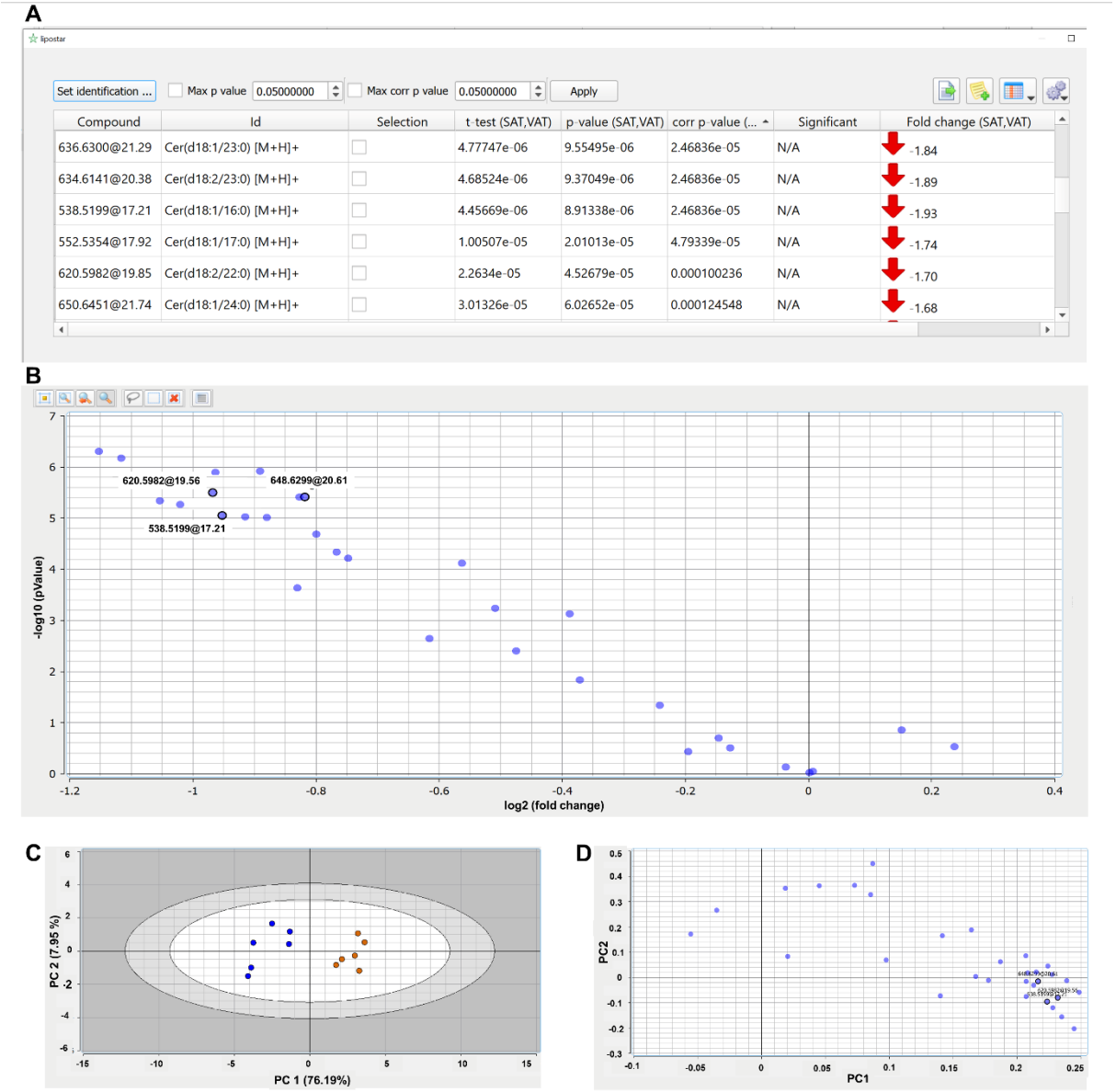


Figure 11: Illustrative representation within **Lipostar 2.0** of different statistical tools available including ANOVA/Fold change analysis (A), volcano plot (B), and principal component analysis (PCA) with score plot (C) and loading plot (D). Highlighted features correspond to Cer(d18:1/24:1) (*m/z* 648.6299), Cer(d18:2/22:0) (*m/z* 620.5982) and Cer (d18:1/16:0) (*m/z* 538.5199).

Step 5: Data deposition

The dataset can now be uploaded into data repositories. This way data will be available for re-use by the community. Additionally, it is often required to upload both raw and processed data supporting the results presented in the manuscript submitted for publication, depending on the journal. For example, this dataset was uploaded to **Metabolomics Workbench** (ST001738) and is freely accessible together with associated metadata (Figure 12).

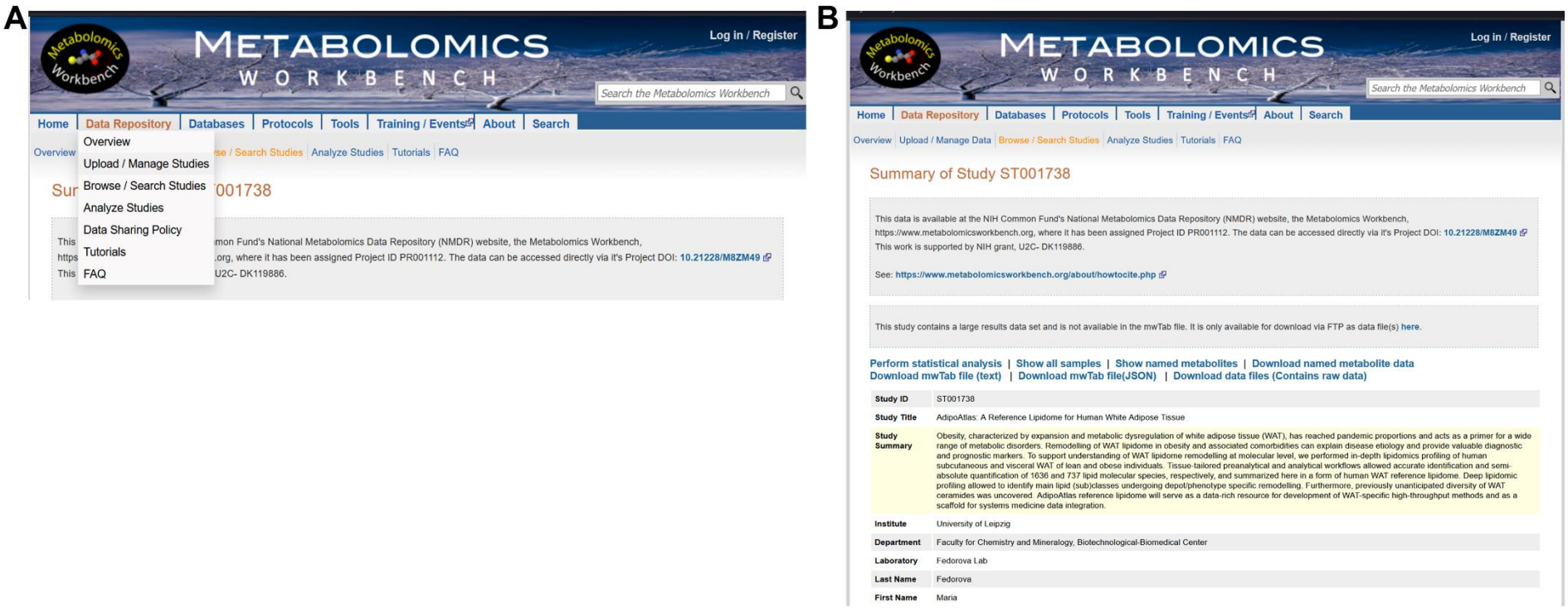


Figure 12: Metabolomics Workbench data repository where data can be uploaded (A) and shared with the community (B).

Step 6: Lipid ontology using LION/web rank mode

As **LION/web**[5] uses the LIPIDMAPS lipid annotations, ontology analysis can be directly performed using normalized data exported from Lipostar.

7.2

A .csv file containing the experimental sample names (in replicates) and the normalized values per lipid are loaded into the web tool. Figure 13 illustrates how to perform data analysis and visualization using the classical LION-enrichment (Figure 13 A and B). The same results can be displayed as volcano-type plot (Figure 13 A and B) or as a LION-heatmap, displaying all four conditions in one plot, which might be useful if space is limited or one want to compare all conditions at once (Figure 14).

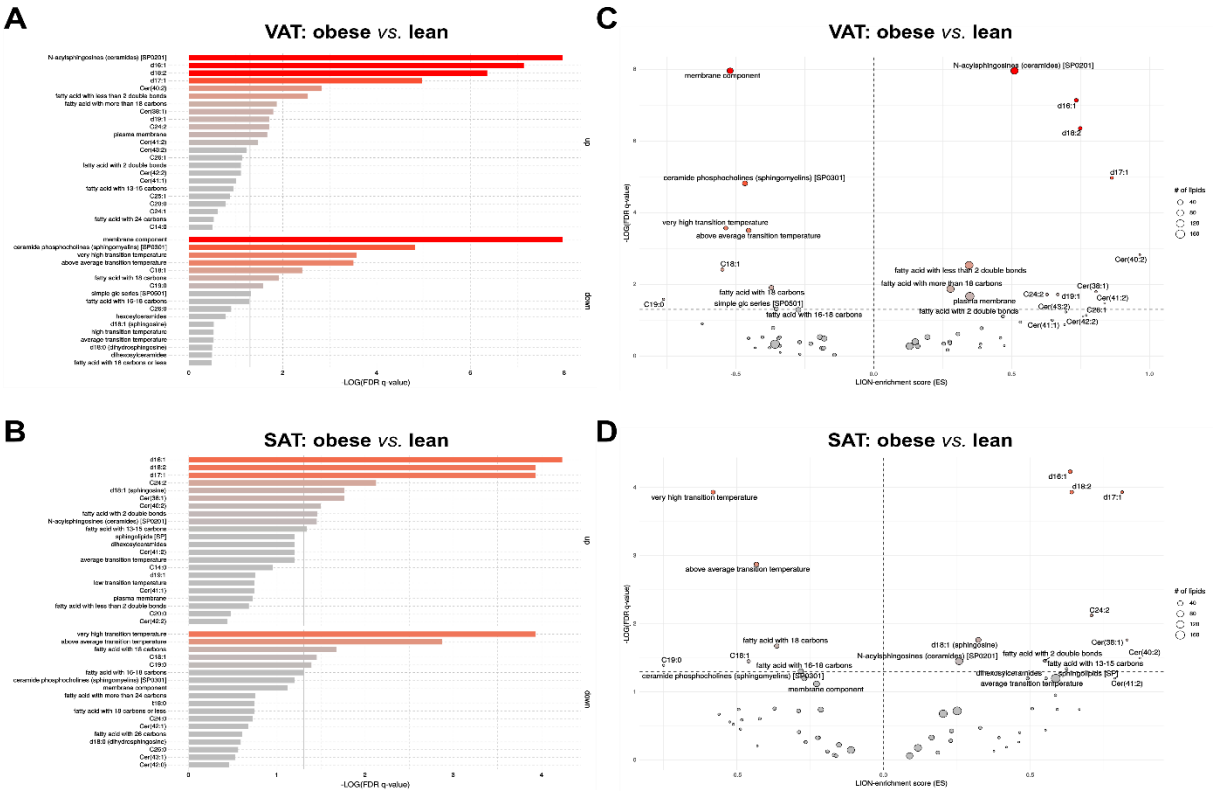


Figure 13: Illustrative representation of possibilities for data analysis and ontology mapping within **LION/web** including the classical LION-enrichment (VAT (A) and SAT (B)), and volcano-type plot (VAT (C) and SAT (D)).

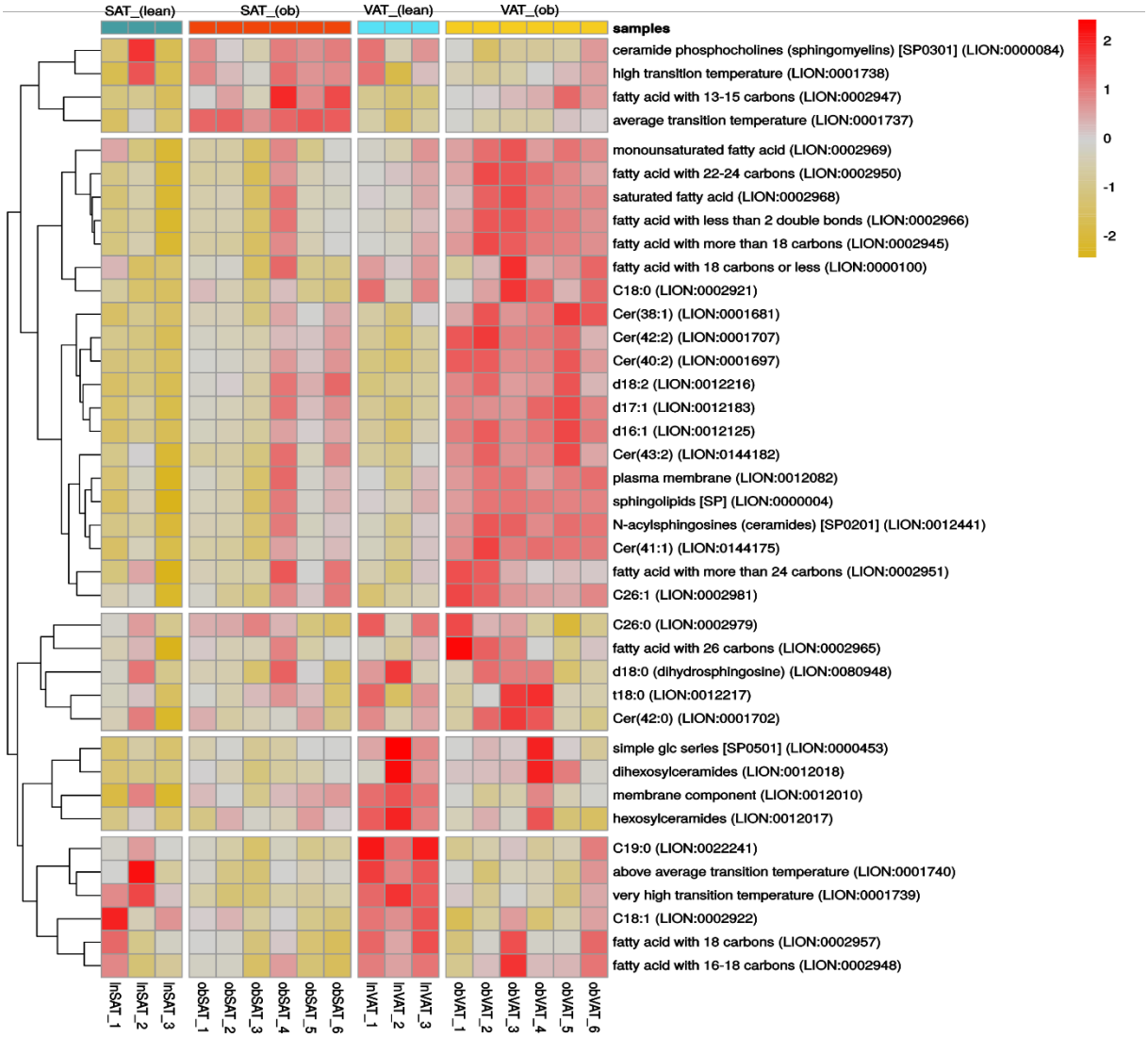


Figure 14: Illustrative representation of a LION-heatmap.

Step 7: Pathway analysis using WikiPathways (Lipid portal)

7.3

The data analysis up to this point revealed significant differences in ceramides between SAT and VAT with ceramides containing the sphingosine base (18:1;O2) upregulated in VAT. To understand the biological meaning behind this observation, the **Lipid Portal on WikiPathways** can be used (Figure 15A).

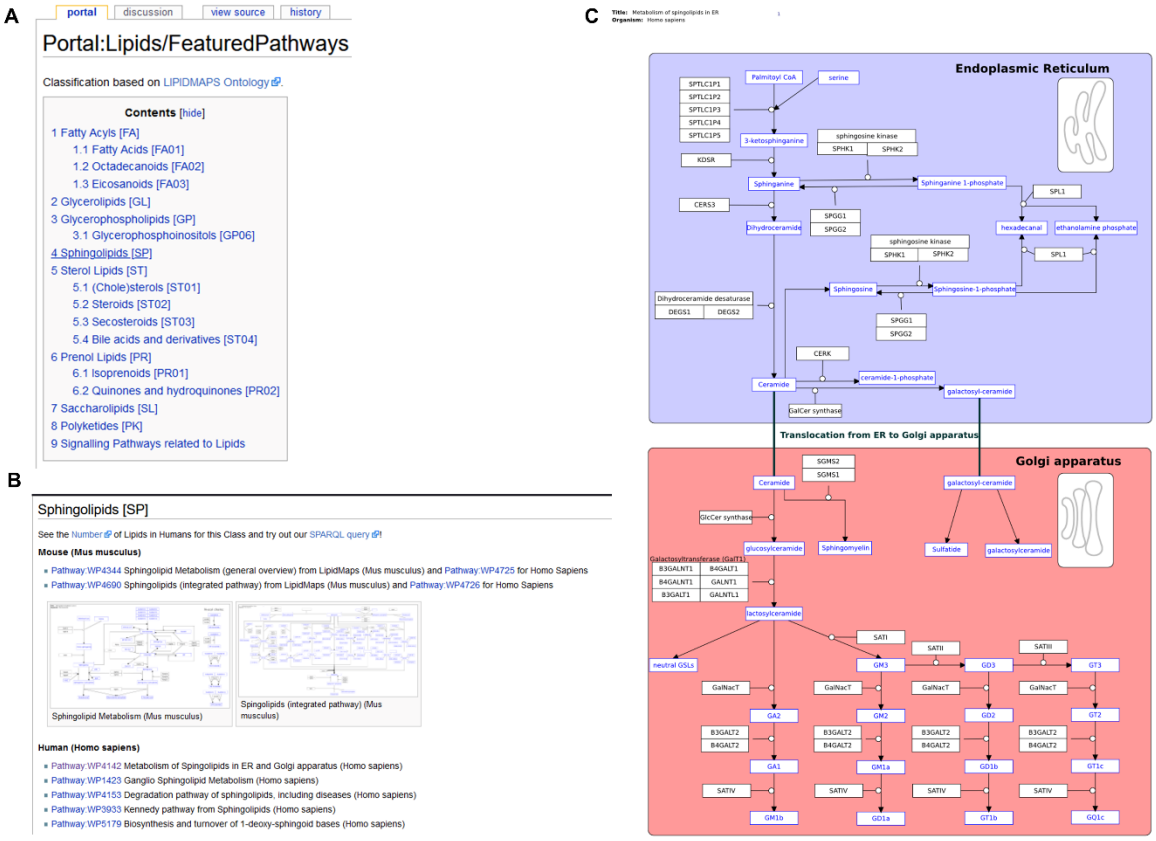


Figure 15: Overview of pathways available at **Lipid Portal on WIkiPathways** classified according to LIPIDMAPS ontology (A), sphingolipids related pathways (B), and a detailed view on the selected WP4142 pathway: Metabolism of sphingolipids in the ER and Golgi apparatus (Homo sapience) (C).

The Lipid Portal on WikiPathways contains manually curated collection of lipid related pathways for mouse and human. One can browse available pathways (Figure 15A) based on the LIPID MAPS ontology, and select the one of interest for the corresponding species (e.g., here WK4142: Metabolism of sphingolipids in ER and Golgi apparatus (*Homo sapience*) is selected; Figure 15B). Detailed view of the pathway (Figure 15C) illustrates biochemical reactions involved in lipid synthesis and catabolism with corresponding enzymes.

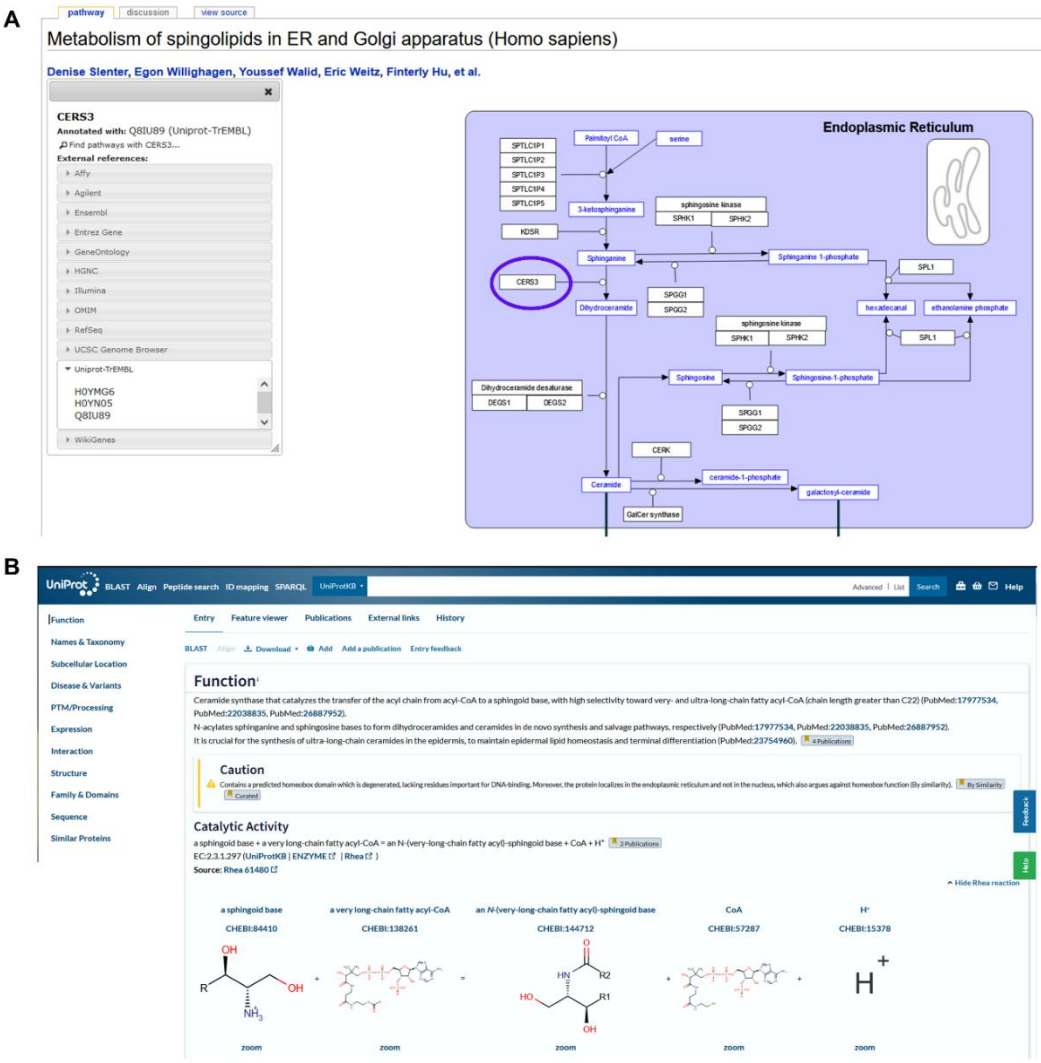


Figure 16: A zoomed view of the pathway WP4142 showing the information available for the selected enzyme CERS3 (highlighted in blue) (A). By clicking at the selected UniProt identifier (e.g. Q8IU89) user is redirected to the corresponding UniProt database entry and can review the information available for quired proteins (B).

A particular enzyme within the pathway can be selected (Figure 16A; e.g. ceramide synthase 3, CERS3), and information about various identifiers for this protein is provided. By clicking on selected identifier user will be re-directed to the corresponding database. Here, UniProt ID for CERS3 was selected allowing to retrieve corresponding UniProt database page (https://www.uniprot.org/) where one, among other information, can get informed about enzyme function as well as catalyzed reaction with the link to Rhea database (Figure 16B).

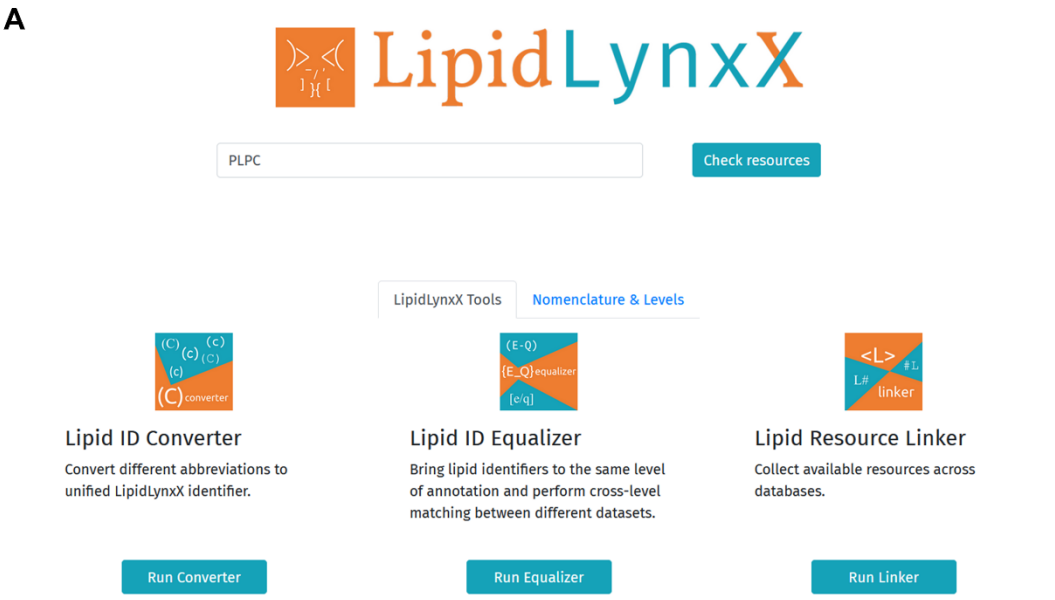
Step 8: Conversion of lipid annotations using LipidLynxx.

7.1

Often different tools and software provide different ways of annotating lipids. To unify shorthand notation styles and to make your results comparable with and searchable by others, you can convert your annotations into standard shorthand notation style or the style of your choice.

Here lipid annotations are converted with **LipidLynxx** using the Lipid ID Converter to the shorthand notation recommended by Liebisch et al [3] (Figure 17). The updated notations together with associated data can be used for further analysis and reporting.

A



B

TextInput	TextInput_converted
Cer(t18:0_22:0)	Cer 18:0;3_22:0
Cer(d18:2/26:0)	Cer 18:2;2_26:0
Cer(d18:2/23:0)	Cer 18:2;2_23:0
Cer(d18:2/22:0)	Cer 18:2;2_22:0
Cer(d18:2/22:0)	Cer 18:2;2_22:0
Cer(d18:2/20:0)	Cer 18:2;2_20:0
Cer(d18:2/18:0)	Cer 18:2;2_18:0
Cer(d18:2/16:0)	Cer 18:2;2_16:0
Cer(d18:2/14:0)	Cer 18:2;2_14:0
Cer(d18:1/24:1)	Cer 18:1;2_24:1

Showing 1 to 10 of 27 entries Previous 1 2 3 Next

Figure 17: LipidLynxx web interface containing a Lipid ID converter, an ID Equalizer, and a Resource linker (A) and the output of the Lipid ID converted for Ceramides (B).

Thus, example provided above illustrates application of **LIPID MAPS structural database** (LMSD), **Lipostar 2.0**, **Metabolomics Workbench**, **LION/web, Lipid Portal on WikiPathways**, and **LipidLynxX** for analysis, and reporting of untargeted lipidomics experiment.

References

1. Lange, M. *et al.* AdipoAtlas: A reference lipidome for human white adipose tissue. *Cell Reports Medicine* **2**, 100407 (2021).
2. Adams, K. J. *et al.* Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics. *Journal of Proteome Research* **19**, 1447–1458 (2020).
3. Liebisch, G. *et al.* Update on LIPID MAPS classification, nomenclature, and shorthand notation for MS-derived lipid structures. *Journal of lipid research* **61**, 1539–1555 (2020).
4. Goracci, L. *et al.* Lipostar, a Comprehensive Platform-Neutral Cheminformatics Tool for Lipidomics. *Analytical Chemistry* **89**, 6257–6264 (2017).
5. Molenaar, M. R. *et al.* LION/web: a web-based ontology enrichment tool for lipidomic data analysis. *GigaScience* **8**, 1-10 (2019).