LipidOne 2.0: a web tool for discovering biological meanings hidden in lipidomic data

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ABSTRACT:

LipidOne 2.0 (https://lipidone.eu) is a new web bioinformatic tool for the analysis of lipidomic data. It facilitates the exploration of the three structural levels of lipids: classes, molecular species and lipid building blocks (acyl, alkyl or alkenes chains). The tool's flexibility empowers users to seamlessly include or exclude experimental groups and lipid classes at any stage of the analysis. LipidOne 2.0 offers a range of mono- and multivariate statistical analyses, specifically tailored to each structural level. This includes a novel lipid biomarker identification function, integrating four diverse statistical parameters.
LipidOne 2.0 incorporates Lipid Pathway analysis across all three structural levels of lipids. Users can identify lipid-involved reactions through case-control comparisons, generating lists of genes/enzymes and their activation states based on Z scores. Accessible without the need for registration, LipidOne 2.0 provides a user-friendly and efficient platform for exploring and analyzing lipidomic data.

Basic Protocol 1: Dataset preparation for LipidOne 2.0
Basic Protocol 2: Uploading a dataset into LipidOne 2.0
Basic Protocol 3: Data mining of lipidomic dataset by LipidOne 2.0
Support Protocol 1: lipid nomenclature suitable for LipidOne 2.0

Keywords: Lipidomics, System Biology, Data Mining, Lipid Pathway, R

INTRODUCTION

Lipids constitute one of the largest groups of small, naturally occurring molecules with amphiphilic properties. They are characterized by vast structural diversity resulting from the combination of a certain number of building blocks: polar heads and hydrophobic chains. According to the Lipid Maps database, more than 48,400 unique lipid structures are recorded, classified into seven main categories, further subdivided into classes and subclasses based on the type of polar head (Conroy et al., 2024).

Lipids play an essential role in numerous cellular functions and are key elements in major biological systems. They serve as primary constituents of cell membranes, actively participate in intracellular signalling pathways, and act as energy reserves. The comprehensive study of lipids in a biological context is known as lipidomics.

Lipidomics has gained significant attention in recent years as a robust investigative tool for elucidating the roles of lipids in humans. It helps in understanding the functions of lipids and their involvement in various physiological processes and diseases, as well as identifying new therapeutic targets. This heightened interest is reflected in a substantial increase in research articles dedicated to lipidomics within the scientific community (Géhin et al., 2023).

Although lipidomics is included in the broader context of metabolomics, it can be considered a distinctive discipline for a number of technical-analytical reasons. Lipid extraction and analysis methodologies differ significantly from those used for polar metabolites such as amino acids, amines, and peptides, mainly due to differences in their chemical-physical characteristics. Furthermore, lipids are distinguished by their uniqueness and functional specificity compared to other metabolites. Therefore, lipidomics requires specific analytical approaches to capture the complexity and diversity of lipids in biological systems (Cajka et al., 2023). Within lipidomics, several techniques are employed to study lipids, including liquid chromatography coupled with mass spectrometry (LC/MS) and NMR spectroscopy, which are among the most commonly used for this purpose. Through the use of LC/MS, biological samples can be analysed to profile thousands of lipids. This field is not limited to the mere characterization and quantification of lipid species present but plays a key role in investigating the molecular mechanisms of lipid metabolism in a certain biological system.

Several dedicated web-based platforms are available for the analysis of metabolomic data, such as the well-established MetaboAnalyst (Pang et al., 2021). This excellent bioinformatics tool incorporates robust statistical modules and supports, among other features, functional and metabolic pathway analyses. However, in the context of lipidomic data analysis within this platform, lipids are treated similarly to other metabolites, where lipids are exclusively considered at the level of molecular species. However, important information can be obtained by studying lipids grouped at the level of lipid classes or by studying the building blocks of which individual molecular species are composed: acyl, alkyl, and alkenyl chains. This implies that conducting statistical or metabolic analyses for each of these three levels requires the prior application of time- and labour-intensive calculations to generate submatrices corresponding to lipid classes (LCL), lipid molecular species (LMS), and lipid building blocks (LBB).

Due to the growing interest in lipidomics and its potential to unravel the complexities of human health and disease, the demand for user-friendly bioinformatics tools capable of efficiently mining elaborating lipidomic data has increased. The desktop version of LipidOne (Pellegrino et al., 2022), marked our initial attempt to address the gap in lipid analysis tools, providing the scientific community with a tool for comprehensive analysis of lipid Building Blocks composition.

To meet the growing demand for lipid-focused study tools, we are now introducing LipidOne 2.0, a comprehensive, customized web-based platform for lipid-oriented data analysis. This new platform allows users to interpret their lipidomic data in the context of systems biology for each of the three levels of lipids. The platform's user-friendly interface and its focus on contextualizing lipidomics data within the framework of systems biology make it a valuable addition to the repertoire of bioinformatics tools available for lipid research and analysis.

LipidOne 2.0 is easily accessible at https://lipidone.eu without the need for registration, providing an intuitive and efficient platform for the exploration and analysis of lipidomic data generated in a typical LC/MS workflow.

STRATEGIC PLANNING

The LipidOne 2.0 project seamlessly integrates into the downstream phase of a typical LC-MS based lipidomic workflow. It commences with the input of a lipidomics data matrix that reports the concentrations of annotated lipid molecules extracted from two or more experimental replicate groups. Upon data loading, LipidOne 2.0 automatically performs two fundamental functions:

1. Reads and presents to the user both the experimental groups and the lipid classes within the input matrix, enabling the selection/deselection of these elements in any subsequent analytical context.
2. After processing the input data, it automatically generates three sub-matrices with the contributions relevant to the LCL, LMS and LBB. These three submatrices are the basis for the three levels of analysis of lipidomic data in LipidOne 2.0. This is possible provided that the lipid nomenclature adheres to the molecular species level guidelines of the Lipidomics Standards Initiative (LSI) (Liebisch et al., 2020).

   The lipid class (**LCL**) sub-matrices are derived by aggregating the contributions of all molecular species within the same class, on a sample-by-sample basis.

   The Lipid Molecular Species (**LMS)** sub-matrices resemble the input data, but numeric values that are absent or equal to '0' are automatically substituted with a value equal to one-tenth of the lowest value within the respective row.

   The Lipid Building Block (**LBB)** sub-matrices are generated by breaking down each row corresponding to a molecular species into multiple rows corresponding to the constituent chains of the lipid (e.g., one for lyso-phosphocholines, two for phosphocholines, three for triglycerides, and four for cardiolipins). The numerical value of each cell is subsequently divided by one, two, three, or four as appropriate. For the row headers, we have devised pseudo-lipids that display the class name and the name of the individual chain composing the original molecular species. For instance, the lipid PC 16:0_18:1 will result in two rows with the pseudo-lipid headers 'PC_16:0' and 'PC_18:1'. Any identical pseudo-lipids are summed together on a sample-by-sample basis.

These two functions empower users to explore and analyze lipidomic data with high selectivity at each of the three levels. Additionally, users can freely choose both the experimental groups and lipid classes to be included in various analyses, facilitating the swift identification of the biological significance hidden in lipidomic data. Figure 1 provides an overview of the LipidOne 2.0 project.

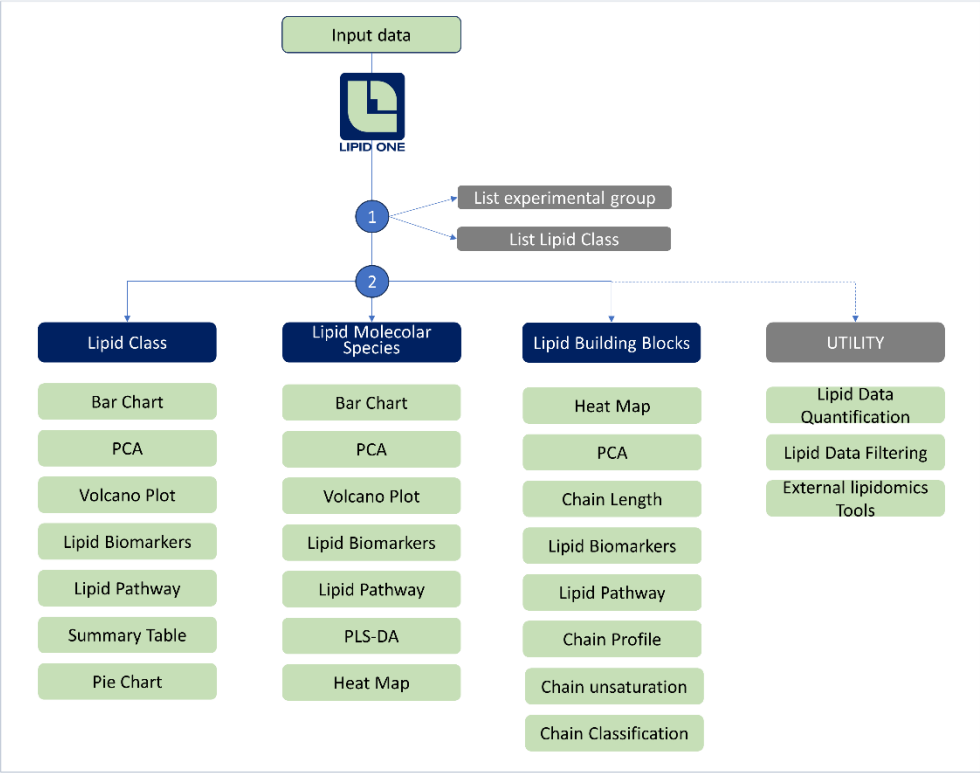

Figure 1: Overarching structure of the LipidOne 2.0 project. Upon data loading, LipidOne 2.0 executes two crucial actions: 1) Reading Experimental Groups and Lipid Classes: This step involves the extraction and presentation of information pertaining to

the experimental groups and lipid classes from the input matrix; 2) Creating Three Sub-Matrices (depicted as blue rectangles): These sub-matrices consist of Lipid Classes, Lipid Molecular Species, and Lipid Building Blocks. Each sub-matrix plays a pivotal role in facilitating in-depth lipidomic analysis and exploration. The green rectangles in the figure represent the functional analysis associated with each sub-matrix. Additionally, LipidOne 2.0 features a Utility section designed to perform essential functions on lipidomic data and provides links to other lipidomics web tools. All operations executed by LipidOne 2.0 yield graphical outputs and tables that are downloadable as high-resolution PNG images (resolution > 300dpi) and CSV files respectively.

BASIC PROTOCOL 1: Dataset preparation for LipidOne 2.0

In this section, we explain how data must be prepared for uploading into the bioinformatics web tool LipidOne 2.0.

Necessary Resources:

* *Hardware: A PC with any operating system*
* *software* – any spreadsheet such as Microsoft Excel or LibreOffice Calc.
* *files* – a table showing a list of lipids with peak areas or lipid concentrations. It can be generated by any LC/MS data processing and lipid annotation programme such as MS-DIAL (RIKEN), LipidAnnotator/MassProfinder (Agilent), LipidSearch (Thermo), LipidHunter, SimLipid (PERMIER Biosoft), Lipid Data Analyzer (Graz University), LipidFinder etc.

LipidOne 2.0 has been designed to accept data tables in either txt or csv format. The table should list samples in columns and lipid names in rows. Class labels (e.g., control, experiment) should be placed in the row immediately following the sample names. Quantitative data can be reported either as concentration, peak area, or ion intensity.

We recommend preparing the data table using a spreadsheet program such as Excel or Calc.

Carefully observe Figure 2 which shows an example of a table in the appropriate format.

	A	B	C	D	E	F	G	H	I	J
1	Metabolite	SN.01.WT	SN.06.WT	SN.10.WT	SN.11.WT	SN.16.WT	SN.03.HOM	SN.07.HOM	SN.12.HOM	SN.13.HOM
2	Label	WT	WT	WT	WT	WT	HOM	HOM	HOM	HOM
3	CE 18:1	0.0174256	0.01476097	0.01543425	0.00912359	0.00916384	0.13787724	0.15749813	0.05649067	0.10143616
4	CE 18:2	0.06763296	0.11784167	0.11284725	0.01528067	0.01658074	0.77272721	0.68761121	0.19489135	0.33834694
5	CE 20:1	0.00708205	0.00352643	0.0055915	0.00396984	0.00261916	0.02338907	0.03354751	0.01352688	0.02128104
6	CE 20:2	0.00489203	0.00158106	0.00414173	0.00220132	0.00224378	0.05351047	0.08262451	0.02489983	0.03974087
7	CE 20:3	0.00270499	0.00187404	0.0035676	0.00233152	0.00227301	0.00654512	0.00663433	0.00448426	0.00561917
8	CE 20:4	0.00378937	0.00340459	0.00815683	0.00078437	0.00296762	0.8154958	0.86751402	0.05076486	0.24644674
9	Cer[NS] 18:1;O2_22:0	0.01006187	0.00775429	0.00552381	0.01688459	0.01850825	0.00480389	0.00515579	0.02416877	0.02482555
10	Cer[NS] 18:1;O2_23:0	0.00164885	0.00171273	0.00109718	0.00446686	0.00396998	0.00084784	0.00101287	0.00331515	0.00385042
11	Cer[NS] 18:1;O2_23:1	0.00041703	0.00036281	0.00023642	0.00107058	0.00116651	0.00027705	0.00028996	0.0014999	0.00186466
12	CL 18:0_18:4_18:1_18:1	0.00047165	0.00048781	0.00061896	0.00032125	0.00049646	0.00072242	0.00068664	0.00039115	0.00057366
13	CL 18:1_18:1_18:1_18:1	0.00095046	0.00132343	0.0010144	0.0006969	0.00088792	0.00330479	0.00312999	0.00181654	0.00272954
14	CL 18:1_18:1_18:1_18:2	0.00309745	0.00342302	0.00308062	0.00178223	0.00180205	0.00557791	0.0058053	0.00269738	0.00362239
15	CL 18:1_18:2_18:2_18:2	0.00322379	0.00633393	0.00705403	0.00174313	0.0011686	0.00336135	0.00314461	0.00246545	0.00257728
16	CL 18:2_18:2_18:2_18:2	0.00178396	0.00538074	0.00519974	0.00090158	0.00043987	0.00190809	0.00165789	0.00169341	0.00167855
17	DG 16:0_16:1	0.02471122	0.03172728	0.01638558	0.02361204	0.02632509	0.00548461	0.00651622	0.00733964	0.00659471
18	DG 16:0_18:1	0.01504505	0.0163336	0.01240597	0.01939405	0.02950869	0.01019671	0.00989629	0.0116004	0.00947244
19	DG 16:0_18:2	0.04454175	0.05781916	0.02778505	0.0355858	0.0226389	0.01024288	0.00834684	0.02756485	0.0122921
20	DG 18:0_18:1	0.04752203	0.02856281	0.02910078	0.03081223	0.04974735	0.01340258	0.01383877	0.00882621	0.00954193
21	DG 18:0_18:2	0.06704756	0.05016986	0.03655798	0.0364034	0.03961449	0.02159319	0.02050744	0.02207531	0.01559211
22	DG 18:0_20:3	0.05309676	0.02468742	0.03027596	0.02611372	0.03268271	0.03555572	0.03261245	0.02108259	0.02370976
23	Hex-Cer 16:0;O2_26:0	0.01154203	0.00806145	0.00807741	0.00635271	0.00826323	0.00071841	0.00086529	0.00104852	0.00104115
24	Hex-Cer 18:0;O2_24:1	0.00560069	0.0037762	0.00325175	0.00270426	0.003535	0.00057813	0.00075049	0.00079454	0.00078573
25	Hex-Cer 18:1;O2_22:0	0.00893218	0.00673666	0.0078237	0.0057433	0.0069516	0.00220686	0.00281141	0.00293221	0.00303012
26	LPC 16:0	0.22979667	0.19689836	0.14605176	0.10066158	0.16844741	0.20780751	0.19974242	0.1662844	0.24880633
27	LPC 18:0	0.07290413	0.0676504	0.04670711	0.03553638	0.0582542	0.09436958	0.09124748	0.06952485	0.11656711
28	LPC 18:1	0.41823113	0.32164818	0.22073727	0.08758721	0.16573826	0.08070864	0.08845958	0.06355501	0.11677891
29	PA 16:0_18:1	0.5086378	0.29226935	0.15396731	0.05756427	0.10958709	0.03792336	0.05325893	0.01046853	0.01794782
30	PA 16:0_20:4	0.03202692	0.02295292	0.01142811	0.00356926	0.00417192	0.01096372	0.01143474	0.00211329	0.00261825
31	PA 18:0_18:1	4.70891454	2.77801186	1.66588599	0.81897415	1.7514279	0.16183938	0.33529389	0.07558709	0.14004516
32	PA 18:0_20:4	0.61452742	0.3934483	0.17650446	0.07563346	0.12639167	0.03786442	0.05885535	0.00986829	0.02045342
33	PA 18:0_22:6	0.17556897	0.1436987	0.06284557	0.02699531	0.05779999	0.02160206	0.03810956	0.00952973	0.01011507
34	PA 18:1_18:1	1.89801934	1.07275722	0.55607726	0.2633222	0.5187726	0.0475593	0.0833724	0.02583104	0.0376368
35	PA 18:1_20:1	0.11813179	0.06888185	0.04034095	0.02558338	0.04477821	0.00720613	0.01050811	0.00626455	0.00675525

Figure 2: An example of a table with the appropriate format to upload in LipidOne 2.0.

Here are some rules to follow:

1) The first column (A) should display the names of the lipids. The lipid nomenclature must adhere to the "Molecular species level" or “sum composition” of the Lipidomics Standards Initiative (LSI) Guidelines. If the lipid nomenclature is “sum composition”, detailed analysis at the level of lipid building blocks is prevented. Please see SUPPORT BASIC PROTOTOL 1.

2) Samples should be organized in columns, starting from the second column (B). There is no limit to the number of samples or the number of lipids. We tested datasets containing information of 2500 samples/1000 lipids without any problems. However, with such large datasets, the operations performed by LipidOne are somewhat slowed down.

3) The first row should contain the names of the samples. Sample names should be simple, formatted as characters (not numeric), without spaces between characters.

4) The second row should contain the labels of the groups to which the samples belong. Normally, each group consists of experimental replicas. In LipidOne, the minimum number of replicates is 2. Note that the second row starts with "Label". Group names should be simple, formatted as characters (not numeric), without spaces or special characters such as "@", "#", "$", "%", "^", "&", "*", and "=". There is no limit to the number of groups that can be entered, however we recommend not exceeding 10 to avoid graphics problems.

5) Once the table is prepared, save it in CSV (comma-separated) or TXT (tab-separated) format.

Users should be aware of the following:

- Two or more lines with the same lipid name are not allowed. If duplicates are present, when user upload the dataset in LipidOne 2.0 an error message will appear indicating which names are duplicates.
- Numeric values that are absent or have a value of "0" are automatically replaced with a random number between one-tenth and one-twentieth of the minimum value in the same row.
- Rows containing only '0' cause an error. They must be deleted from the dataset.

SUPPORT PROTOCOL 1

Lipid nomenclature from spectrometric experiments

Lipids constitute a broad group of molecules that are divided into categories, classes, and subclasses. Briefly, they are organized into homologous series composed of a combination of building blocks: *polar heads* and *hydrophobic tails*. The types of polar heads define the category, classes, and subclasses to which the class belongs. Hydrophobic tails consist of linear or branched acyl or alkyl, alkenyl chains of varying lengths (expressed by the number of carbon atoms) and degrees of unsaturation (expressed by the number of double bonds).

In a typical workflow based on spectrometric techniques, after the data acquisition phase, the bioinformatics part is implemented, comprising pre-processing, lipid annotation and finally data analysis and interpretation.

Regarding lipid annotation, two levels of information can emerge, depending on the degree of analysis, acquisition and annotation performed:

- The **species level** (or sum composition level) is where the *polar head*, and thus the lipid's class or subclass, is represented by an abbreviation, while the *hydrophobic tails* are summarized using numbers separated by ":". For instance, PC 34:1 denotes a single lipid molecule belonging to the phosphatidylcholine (PC) class, specifying the total carbon atoms (34) and double bonds (1) within its two hydrophobic tails.
- The **molecular species level** adds information on the building blocks of the lipid. The type of chains is indicated as in the following example: 16:0 indicates a 16 carbon-atom and 0 unsaturation of acyl chain, linked to glycerol with an ester bond; O-16:0 indicates an alkyl chain (Plasmanyl) and P-16:0 indicates an alkenyl chain (Plasmenyl), both linked to glycerol with an ether bond. For example, the phosphatidylcholine PC 16:0_18:1 indicates the same lipid as the example before but with more details: the two chains are both Acyl chains; the first chain has 16 atoms of carbon and 0 unsaturation (saturated chain), and the second one has 18 atoms of carbon and one double bond within the chain (monounsaturated chain). For the phospholipid classes in which both chains are acyl type, the spectrometric information allows us to determine the position of the substituents at the sn-1 and sn-2 positions of the glycerol backbones. In these cases, the two chains are separated by the "/" symbol: the first is at the sn-1 position, the second at the sn-2 position: PC 16:0/18:1.

It should be noted that with highly specialized analytical techniques, it would also be possible to obtain information on the position of unsaturation within the chains and other stereochemistry information; however, this level of analysis has not yet become routine practice in lipidomic laboratories. Typically, lipid analysis using spectrometric techniques provides information up to the lipid species or lipid molecular species level or the sn-position level. See for example the excellent work by Zullig and colleagues in which this analytical aspect of lipids is very well explained (Züllig et al., 2020).

Lipid nomenclature suitable for LipidOne 2.0

Unfortunately, there is no common line for the nomenclature of lipid molecular species. To address this problem, Liebisch and colleagues have recently introduced a standardized shorthand annotation method for lipid species structures obtained from mass spectrometry techniques (Liebisch et al., 2020). They applied a hierarchical approach to nomenclature, depending on the available information.

Following this method, they established various levels, including species level (or sum composition level), molecular species level, sn-position level, DB-position level, Structure defined level, Full structure level, and Complete structure level.

Therefore, during the development of LipidOne, we adopted this nomenclature model. Specifically, we prioritize the molecular species level, allowing for the full utilization of all analysis functions within LipidOne. While the species level remains feasible, it may limit the functionality of LipidOne.

Table 1: Some examples of suitable lipid nomenclature for LipidOne 2.0:

Systematic Name	Species level or Sum Composition	Molecular species level	sn-position level
1-hexadecanoyl-2-(10E,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine	PC 34:2	PC 16:0_18:2	PC 16:0/18:2
1,2-di-(9Z-octadecenoyl)-3-(8Z,11Z,14Z-eicosatrienoyl)-sn-glycerol	TG 56:5	TG 18:1_18:1_20:3	TG 18:1/18:1/20:3
10-Octadecenoic acid	FA 18:1	FA 18:1	FA 18:1
N-(hexadecanoyl)-4E,14Z-sphingadienine	Cer 34:2;2O	Cer 18:2;O2/16:0	Cer 18:2;O2/16:0

1-(1Z-hexadecenyl)-2-(9Z-octadecenoyl)-glycero-3-phosphoethanolamine	PE O-34:2	PE P-16:0_18:1	PE P-16:0/18:1

Some software such as LDA (Hartler et al., 2011) or MS-DIAL (Tsugawa et al., 2020) already adhere to this type of lipid nomenclature. However, other programs provide lipid names with similar but not identical annotations. In these cases, LipidOne users can either manually "translate" the lipid names (using the "find and replace" function in Excel or Calc) or utilize one of the available online services for this purpose (see, for example, LipidLynxX or Goslin Web Application).For the sake of clarity, we show in table 2 how lipid names must be reported in order to be loaded into the LipidOne 2.0 tool.

Table 2: Some examples of lipid nomenclature and translation in the format compatible with LipidOne 2.0:

This type of lipid nomenclature	**Must be reported as:**	
	Molecular species level	**or sum composition**
PC(16:0/18:1) or PC(16:0/18:1)[H+]+ or (16:0/18:1) PC or PC(16:0/18:1(9Z))	PC 16:0_18:1	PC 34:1
DG(16:0/18:2/0:0)	DG 16:0_18:2	DG 34:2
LPE(18:1)	LPE 18:1	LPE 18:1
SM(d16:1/18:2)	SM 16:0;O2/18:2	SM 34:1;O2
PC(36:1) or PC_(36:1)	-	PC 36:1
Mixed forms such as PC 36:1|PC 18:0_18:1 (from MS-DIAL)	PC 18:0_18:1	PC 36:1
CL(36:0/36:1)	-	CL 72:1
PE(P-16:0/18:1) or PE(P-34:1)	PE P-16:0_18:1	PE O-34:2

BASIC PROTOCOL 2

Uploading a dataset into LipidOne 2.0

In this protocol, we describe the dataset upload functions in LipidOne 2.0

Necessary Resources:

* *Hardware:* A PC with any operating system, internet connection.
* *software* – Any internet browser such as Chrome, Firefox, Opera, Edge, Safari etc. with JavaScript enabled.
* *files* – a lipidomic dataset file prepared as described in BASIC PROTOCOL 1

1. Using your favourite browser, go to LipidOne.eu webpage.
2. Select "START NOW" on the top of LipidOne homepage;
3. Choose "Load Data Matrix". The following page appears:

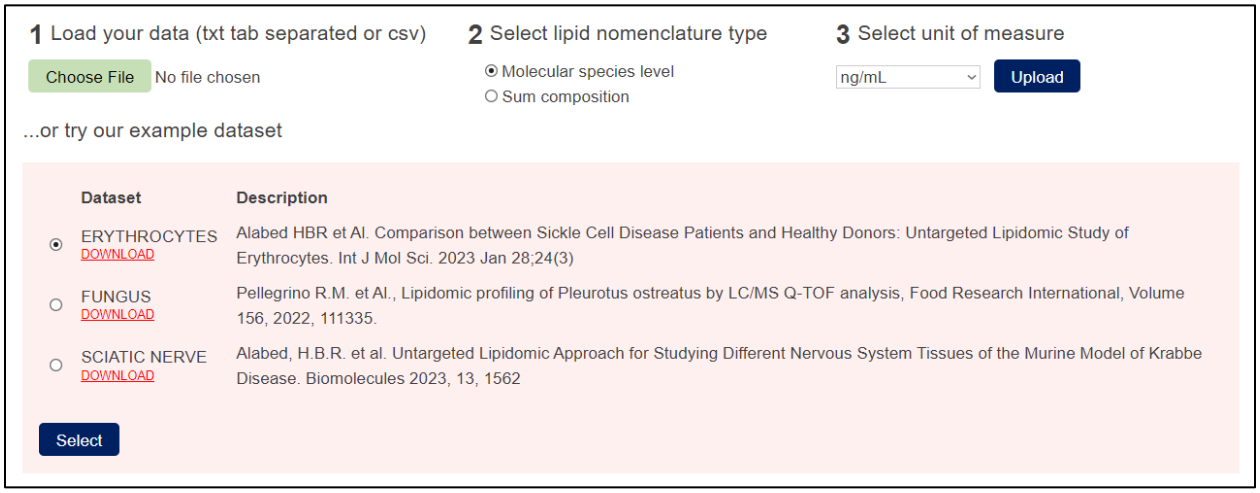

Figure 3: LipidOne loading data page.

4. Two options a) and b) are possible here:
    a) **Load user dataset**: follow the three steps in order:
        1) Load your data: Click on “Choose file”. This opens a window to navigate through the folders on your PC. Select the file to upload.
        2) Select lipid nomenclature type: select the option that corresponds to the nomenclature type of your dataset. The default value is “Molecular species level”. In this case, all LipidOne functions are active. If “Sum composition” is selected, the functions at the Lipid Building Block level are inhibited.
        3) Select unit of measure: a drop-down menu will show different types of units. Select the one that corresponds to your dataset. If your dataset is not quantified, you can select either Peak Area or Peak Hight. The selected unit of measure will appear in all bar chart types available in LipidOne 2.0
        4) Now click on the 'Upload' button

    b) **Load one of three example datasets**: three example datasets from recent publications are available. This makes it possible to explore the various analysis functions available in LipidOne 2.0. Click on the radio button of the dataset you wish to explore and press the “select” button. Note that it is possible to download each of the three datasets. This is useful to better understand the structure of the data as illustrated in BASIC PROTOCOL 1

5. After pressing “Upload” or “Select”, LipidOne 2.0 will execute the upload script. The duration of the "processing" phase depends on the size of the dataset. For example, 200KB file take 4 seconds, 5000KB files take 25 seconds, 10000KB files take 50 seconds. Note that if the dataset contains rows with duplicate lipid names, an error message and the list of duplicate names will appear. In this case you have to edit your dataset by removing duplicates and then reload it into LipidOne.

6. At the end of “processing” phase, a screen very similar to the following is displayed. It represents the starting point for the lipidomics analysis as described in BASIC PROTOCOLS 3.

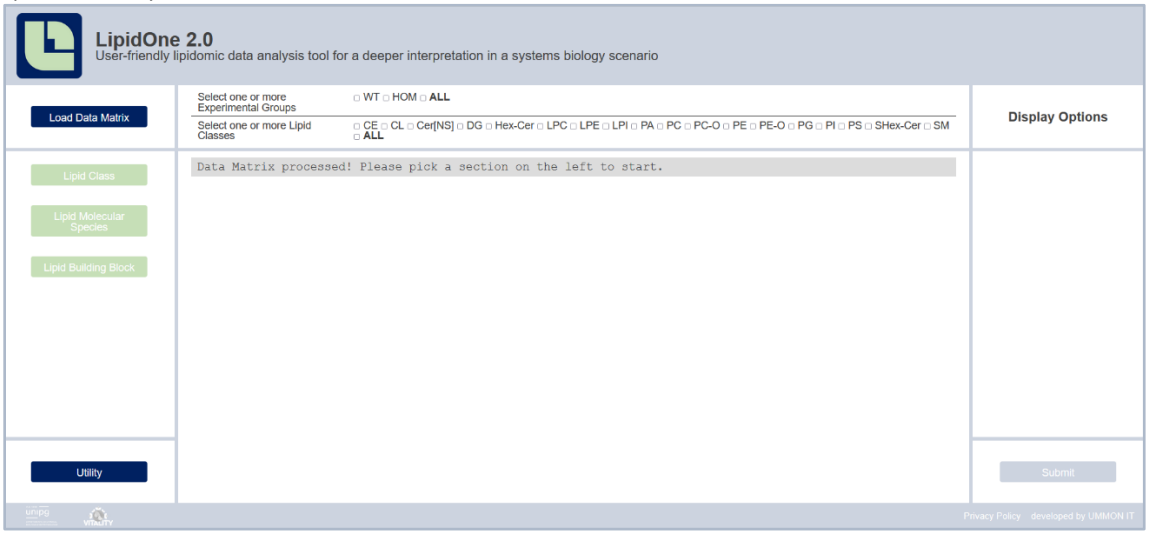

Figure 4: LipidOne page appearance after the data loading and processing.

7. The user can exit from this page in two ways: by clicking on the LipidOne 2.0 icon at the top right (you will be taken to the start page); by clicking on the Load Data Matrix button (you will be taken to the upload page).

BASIC PROTOCOL 3

In the following protocol, we describe step-by-step how to use LipidOne to perform an in-depth analysis of lipidomic data. The user can autonomously explore their own data and use all the functions we have implemented in this bioinformatics tool. Next, we will decrypt the functions implemented in the Utility section.

Necessary Resources:

* *Hardware:* A PC with any operating system, internet connection.
* *software* – Any internet browser such as Chrome, Firefox, Opera, Edge, Safari etc. with JavaScript enabled.
* *files* – a lipidomic dataset file loaded into LipidOne 2.0 as described in BASIC PROTOCOL 2
* *files* – Additional two files for "Lipid data Quantification" function (Utility section) to be prepared as described in the corresponding paragraph.

Step-by-step data mining of lipidomics dataset by LipidOne 2.0

First of all, let us look at the various sections of the LipidOne work screen (Figure 5). In section 1, the user can select one of three levels to be analysed: lipid classes, lipid molecular species or lipid building blocks. In section 2, the user can select the type of analysis to be performed (they depend on the chosen analysis level). In section 3, the user can select experimental groups and lipid classes to be included in the analysis. In section 4 “Display Option”, the user can set certain parameters (these depend on the type of analysis chosen). Button 5 starts the analysis. In section 6, the result will appear. Graphs and tables generated can be downloaded using button 7.

Performing a step-by-step analysis:

1. Select the level of analysis by clicking one of the three buttons Lipid class, Lipid Molecular Species or Lipid Building Block; once selected, a series of buttons appear in section 3. These function keys depend on the analysis level chosen.
2. Select function type by clicking on one of the buttons. Once selected, the button turns blue.
3. Select the experimental groups and lipid classes of interest. Selecting ALL in either case selects or deselects all possibilities. The selection depends on the type of function chosen. If the function requires only two experimental groups (comparison between experimental and control group), a drop-down menu will appear. In other contexts, it will only be possible to select one lipid class at a time, in others no selection at all.
4. Go to “Display Option” area and select, if necessary, the options related to the selected function.
5. Click “Submit” button. This action will activate the R script related to the chosen function. The execution time of the script depends on the size of the dataset. It usually takes a few seconds.
6. At this point, the required graph appears in the central part of the display.
7. In the same area, the DOWNLOAD button appears at the top right. Any graph produced and related tables can be downloaded. The graphs are in PNG format at a resolution of 300 dpi, the data in csv format.

The entire process can be repeated indefinitely. Each time, the generated image overwrites the previous one.

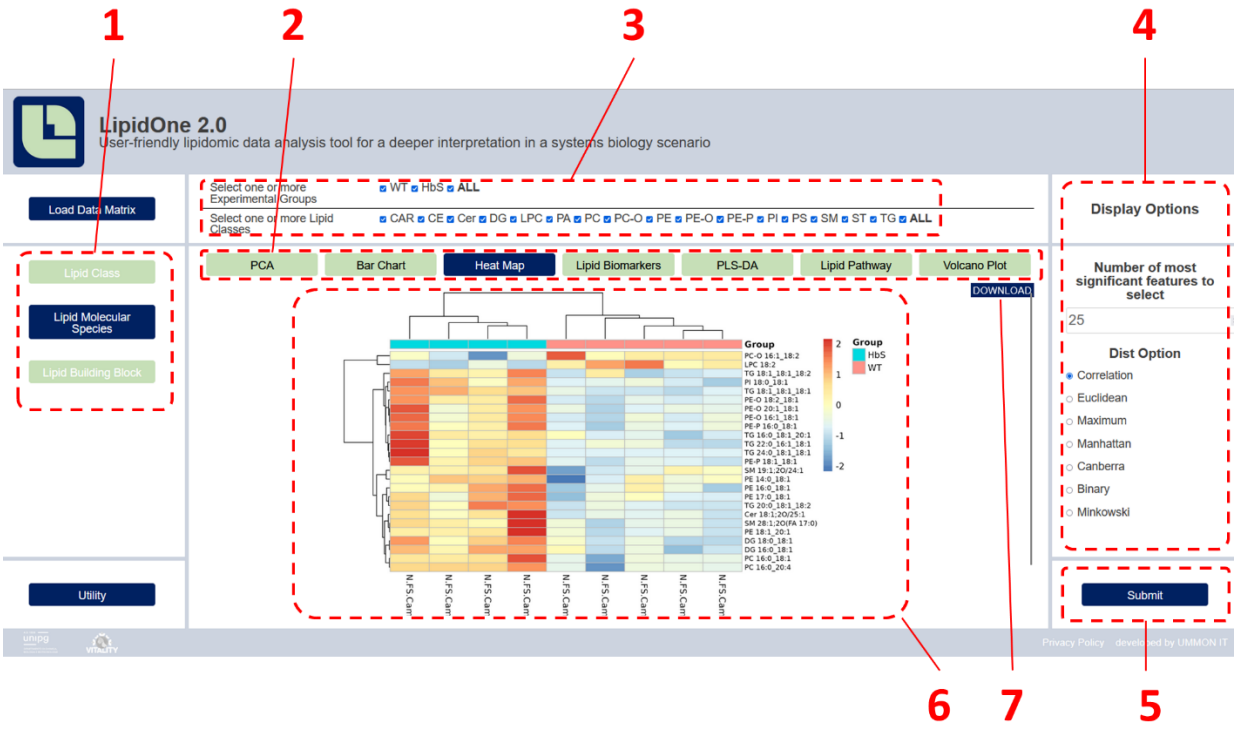

Figure 5: Sections of the LipidOne work screen. See text above for a complete description.

Functions implemented in LipidOne 2.0

All the functions implemented in LipidOne 2.0 are shown in table 1. Since users have the option to select experimental groups and lipid classes, the number of results that can be obtained will be much greater than those listed in Table 1.

Table 1: The following table shows the general outline of all functions implemented in LipidOne 2.0 by analysis level.

Level of analysis	Functions	Description
Lipid Class	Bar chart	The quantitative representation of lipid classes.
	PCA	Principal Component Analysis.
	Volcano Plot	Demonstration of over- or under-expressed lipid classes.
	Lipid Biomarkers	Searching for biomarkers among lipid classes.
	Lipid Pathway	Network of interactions and transformations among lipid classes.
	Pie Chart	Percentage representation of lipid categories and classes (pie chart and bar graph)
	Summary Table	Lipid class summary table.
Lipid Molecular Species	Bar chart	The quantitative representation of the molecular species of a lipid class.
	PCA	Principal Component Analysis.
	Heat Map	Expression representation of molecular species.
	Lipid Biomarkers	Searching for biomarkers among lipid molecular species.
	PLS-DA	Partial Least Square - Discriminant Analysis
	Lipid Pathway	Network of interactions and transformations among lipid molecular species.
	Volcano Plot	Demonstration of over- or under-expressed lipid molecular species.
Lipid Building Blocks	Heat Map	Expression representation of lipid building blocks.
	Lipid Biomarkers	Search for biomarkers among lipid building blocks.
	PCA	Principal Component Analysis.
	Chain length	The quantitative representation of chain lengths in one or more classes based on the number of carbon atoms.
	Chain unsaturation	The quantitative representation of the number of unsaturation of chains in one or more classes.
	Lipid Pathway	Network of interactions and transformations between building blocks.
	Chain Profile	The quantitative representation of the type of chains in a lipid class.
	Chain Classification	The quantitative representation of chain length and unsaturation of Acyl, Alkyl and Alkenyl chains.

In the following paragraphs, we describe in detail each function implemented in LipidOne 2.0. The interpretation of the results is delegated to the user, who is familiar with the biological context from which their lipidomic dataset originates.

1. **Bar Chart**: LipidOne 2.0 can generate diverse types of bar graphs across the three levels of analysis, shown in Table 2. In many instances, users have the flexibility to choose experimental groups and/or lipid classes for inclusion in the plot. Each bar graph incorporates an experimental error bar. Furthermore, when comparing two or more experimental groups, t-tests or ANOVAs are executed, resulting in the calculation of a p-value. One, two, or three asterisks denoting statistical significance are assigned to bar based on the p-value thresholds (<0.05, <0.01, <0.001), two-tailed and with equal variance calculated (Figure 6 A). The download button enables the retrieval of tables in Comma Separated Values (CSV) format containing t-statistic and FDR values in the case of t-tests. For ANOVA comparisons, the tables include Fisher values, FDR, and results from the Tukey post hoc analysis.

Table 2: the table shows the type of bar graph that can be produced with LipidOne with the description at each level.

Level	Key	Description
Lipid Class	Bar Chart	A bar graph representing lipid classes. Users can select any experimental group and any lipid class to plot
Lipid Molecular Species	Bar Chart	A bar graph of lipid molecular species. User can select any experimental group and one lipid class to plot
Lipid Building blocks	Chain Length	A bar graph displaying the number of carbon atoms in lipid chains. Users can select any experimental group and any lipid class to plot.
	Chain Unsaturation	A bar graph displaying the number of unsaturation of lipid chain. User can select any experimental group and any lipid class to plot
	Chain Profile	A bar graph of all chains type. User can select any experimental group and any lipid class to plot

	Chain Classification	Two bar graphs showing the classification of chains (length and unsaturation). The chains are divided into acyl, alkyl and alkenyl. Users can select any experimental group and any lipid class to plot.

2. **PCA** is based on the prcomp R command which is part of the basic R package. In LipidOne 2.0, PCA is possible for each of the three levels of analysis. Lipidomic data are automatically normalized by the median. Depending on the context, the user can select any experimental group and any lipid class to be subjected to PCA. In addition, the user can choose whether to apply a scaling method to the samples (Autoscaling, Pareto Scaling or None) and can select which pairs, from the first five components, to plot in the score and loading plots (Figure 6 J and M). The score plot depicts samples with distinctively coloured points corresponding to their respective experimental groups. When the input data matrix includes at least four replicates for each experimental group, the score plot displays groupings using ellipsoids at the 95 percent confidence level. The PCA loading diagram at lipid class level shows each class with a label. The PCA loading plot of LMS and LBB shows each feature with a colour corresponding to the lipid class to which it belongs. Finally, the scree plot for the first five components can be obtained (Figure 6 K).

3. **Partial Least Square Discriminant Analysis (PLS-DA)** is based on the R package KODAMA (Cacciatore et al., 2014). This analysis is possible for the LMS level only. As with the PCA analysis, the user can select the experimental groups and lipid classes to be analysed, as well as the scaling method and component pair to be plotted. The user will obtain both the score plot and loading plot with similar characteristics to those described for PCA (Figure 6 L and M). In addition, a bar graph is provided with Accuracy, Coefficient of Determination for Y (R2Y) and Predictive Ability for Y (Q2Y), representing the result of cross validation for the first five components (Figure 6 N). Cross validation is performed using 100 as the number of cross-validations with permutated samples and 10 as the number of cross-validations loops (Szymańska et al., 2012).

4. The **Heatmap** graphs are produced by LipidOne 2.0 on the basis of the pheatmap R libraries (Kolde, 2019). The HeatMap of LMS or LBB is generated by LipidOne 2.0 after the user selects a specific group and class of interest. Additionally, users have the flexibility to determine the number of features displayed in the graph (ranging from 2 to 200) based on the most significant features identified through a t-test or ANOVA. Furthermore, users can choose from seven grouping metrics (Correlation, Euclidean, Maximum, Manhattan, Canberra, Binary, Minkowski) to group experimental groups and features according to their preferences (Figure 6 F).

5. **Lipid Pathway**: LipidOne 2.0 facilitates the examination of lipid transformations across the three levels of analysis. This allows users to explore metabolic pathways involving lipids by comparing an experimental group to a control group and obtaining a list of genes/enzymes associated with these transformations (Table 3). In other words, LipidOne 2.0 enables the hypothesis of a dynamic association between lipid phenotype, genes and enzymes involved in transformations, within a Systems Biology perspective. The analysis is grounded in algorithms outlined in the study by A. Nguyen et al. (Nguyen et al., 2017). Lipid pathways are visualized as a network graph, wherein nodes represent, based on the context, Lipid Classes, Lipid Molecular Species, or Lipid Building Blocks (Figure 6 B, C and D). In the case of the latter, users have the flexibility to choose any lipid class, even a single one, allowing for the analysis of metabolic pathways within each lipid class. The construction of the graph is implemented using the R igraph library (Csárdi et al., 2023). The edges connecting nodes are color-coded in blue or red, corresponding to positive or negative activation status, respectively. The edge thickness is categorized as thin if the p-value is less than 0.05, medium if less than 0.01, and thick if less than 0.001. Users have the ability to fine-tune the graph's complexity by specifying their preferred level of statistical significance to filter the results of the lipid pathway analysis.

Table 3: Association between each lipid transformation, its corresponding genes and/or enzymes involved in transformations, and the bibliographic references.

Lipid Class transformation				Chains transformation			
From	**To**	**GENE/ENZYME**	**Reference**	**From**	**To**	**GENE/ENZYME**	**Reference**
Cer	SM	SMsyn	(Cui and Houweling, 2002)	16:0	18:0	ELOVL1/3	(Bond et al., 2016)
CL	PG	MIGA complexes & PLD6	(Milacic et al., 2024)	18:0	20:0	ELOVL1/3	(Bond et al., 2016)
CL	PA	MIGA complexes & PLD6	(Milacic et al., 2024)	20:0	22:0	ELOVL1/3	(Bond et al., 2016)
DG	PA	DGK/AGK	(Milacic et al., 2024)	22:0	24:0	ELOVL1/3	(Bond et al., 2016)
DG	PE	EPT	(Vance, 2015)	16:0	16:1	SDC1-4	(Bond et al., 2016)
DG	PC	CPT	(Vance, 2015)	16:1	18:1	ELOVL1/3/6	(Bond et al., 2016)
DG	TG	DGAT	(Maan et al., 2018)	18:1	20:1	ELOVL1/3	(Bond et al., 2016)
DG	MG	DAGLA/DAGLB	(Milacic et al., 2024)	18:0	18:1	SDC1,2,4,5	(Bond et al., 2016)
TG	DG	PNPLA2/3	(Milacic et al., 2024)	20:1	22:1	ELOVL1/3	(Bond et al., 2016)
LPA	PA	AGPAT5	(Milacic et al., 2024)	22:1	24:1	ELOVL1/3	(Bond et al., 2016)
LPC	PC	LPCAT	(Milacic et al., 2024)	18:1	18:2	D6D/D12D	(Kim et al., 2018; Bond et al., 2016)
LPE	PE	LPEAT	(Milacic et al., 2024)	18:2	22:2	ELOVL5	(Bond et al., 2016)
LPS	PS	LPSAT	(Milacic et al., 2024)	22:2	22:3	D5D	(Bond et al., 2016)
LPG	PG	LPGAT	(Milacic et al., 2024)	18:2	18:3	D6D	(Bond et al., 2016)
PA	DG	LPIN/PAP-1	(Vance, 2015)	18:3	20:3	ELOVL5	(Bond et al., 2016)
PA	LPA	PLA1/PLA2	(Fisher and Jain, 2009)	20:3	20:4	D5D	(Bond et al., 2016)
PA	PI	CDS1/CDIPT	(Milacic et al., 2024)	20:4	22:4	ELOVL2	(Bond et al., 2016)
PA	PS	CDS/PSS	(Milacic et al., 2024)	22:4	22:5	D6D	(Bond et al., 2016)

PA	PG	CDS2/PGS1/PTPMT1	(Milacic et al., 2024)
PC	LPC	PLA1/PLA2	(Fisher and Jain, 2009)
PC	PA	PLD	(Fisher and Jain, 2009)
PC	SM	SMS1/SMsyn	(Vance, 2015; Cui and Houweling, 2002)
PC	PS	PSS1	(Vance, 2015; Cui and Houweling, 2002)
PC	PG	PLD1-4/6	(Milacic et al., 2024)
PC	DG	PLC	(Fisher and Jain, 2009)
PE	DG	PLC	(Fisher and Jain, 2009)
PE	LPE	PLA1/PLA2	(Fisher and Jain, 2009)
PE	PS	PSS2	(Vance, 2015; Cui and Houweling, 2002)
PE	PC	PEMT	(Vance, 2015; Cui and Houweling, 2002)
PE-O	PE-P	PEDS1	(Schooneveldt et al., 2022)
PE-P	PC-P	PEMT	(Schooneveldt et al., 2022)
PG	CL	CRLS1	(Milacic et al., 2024)
MG	DG	PNPLA2/3 / AWAT2	(Milacic et al., 2024)
MG	LPA	AGK	(Milacic et al., 2024)
PI	LPI	PLA1/PLA2	(Fisher and Jain, 2009)
PI	DG	PI4 K/PLC	(Fisher and Jain, 2009)
PI	PC	PITPNB	(Milacic et al., 2024)
PS	PE	PSD	(Vance, 2015; Cui and Houweling, 2002)
PS	PA	PLD	(Fisher and Jain, 2009)
PS	LPS	PLA1/PLA2	(Fisher and Jain, 2009)
PS	DG	PLC	(Fisher and Jain, 2009)
SM	Cer	Smase	(Cui and Houweling, 2002)
Chol	CE	CAT	(Maan et al., 2018)
CDP-DG	PI	PIS	(Vance, 2015)
CDP-DG	PG	PGPS/PGP-Pase	(Vance, 2015)
PG	BMP	Still unclear[a]	(Milacic et al., 2024)

22:4	24:4	ELOVL2,4	(Bond et al., 2016)
24:4	24:5	D6D	(Bond et al., 2016)
24:5	22:5	Beta-Ox	(Bond et al., 2016; Robichaud et al., 2018)
18:3	18:4	D6D	(Kim et al., 2018; Robichaud et al., 2018)
18:4	20:4	ELOVL5	(Kim et al., 2018; Robichaud et al., 2018)
20:4	20:5	D5D	(Kim et al., 2018; Robichaud et al., 2018)
20:5	22:5	ELOVL2,5	(Kim et al., 2018)
22:5	24:5	ELOVL2	(Kim et al., 2018; Robichaud et al., 2018)
24:5	24:6	D6D	(Kim et al., 2018; Robichaud et al., 2018)
24:6	22:6	Beta-Ox	(Bond et al., 2016; Robichaud et al., 2018)

In addition to the network graph, LipidOne 2.0 also provides a bar graph indicating the activation status of genes based on the t-student values (Figure 6 E) and a table containing all individual transformations, the associated gene, the Z-score and the activation status (+ or – respectively) (Figure 6 H).

6. **Summary table**: LipidOne 2.0 generates a user-friendly summary table presenting the count of lipid molecular species for each class, the average concentration for each class along with experimental error, the p-value (t-test or ANOVA), and asterisks denoting statistical significance. The last row of the table provides cumulative totals. Users have the flexibility to choose specific experimental groups and lipid classes to be included in the Summary Table.

7. **Lipid Biomarker**: LipidOne 2.0 helps discover possible lipid biomarkers. Top 20 Biomarkers of an experimental group compared to a control group is calculated for LCL, LMS and LBB. A table shows each of the top 20 features with the p-value, Area Under Curve (AUC) of an ROC analysis, Cohen's d-factor, and test power calculated for a p-value less than 0.001.

8. **Pie Chart**: LipidOne 2.0 can create, at the Lipid Class level, a pie chart with two concentric layers. The inner layer represents the lipid categories, the outer layer the lipid classes. Each sector shows the percentage (Figure 6 I). The Pie chart is based on the R MoonBook (Moon, 2015). In addition, we have included two stacked bar graphs representing the lipid categories respectively in absolute and normalised concentration.

9. **Volcano Plot**: In LipidOne 2.0, a volcano plot graph is created at the Lipid Class level and at the Lipid Molecular Species level. The user must select an experimental group and a control group. In addition, the user must set a significance limit level below which features are represented in grey. Significant features are represented in blue or red depending on whether they are overexpressed or underexpressed compared to the control. With the "download" button it is possible to obtain the 300dpi png chart and a CSV table showing the Fold Change and p-value values for all features (Figure 6 G).

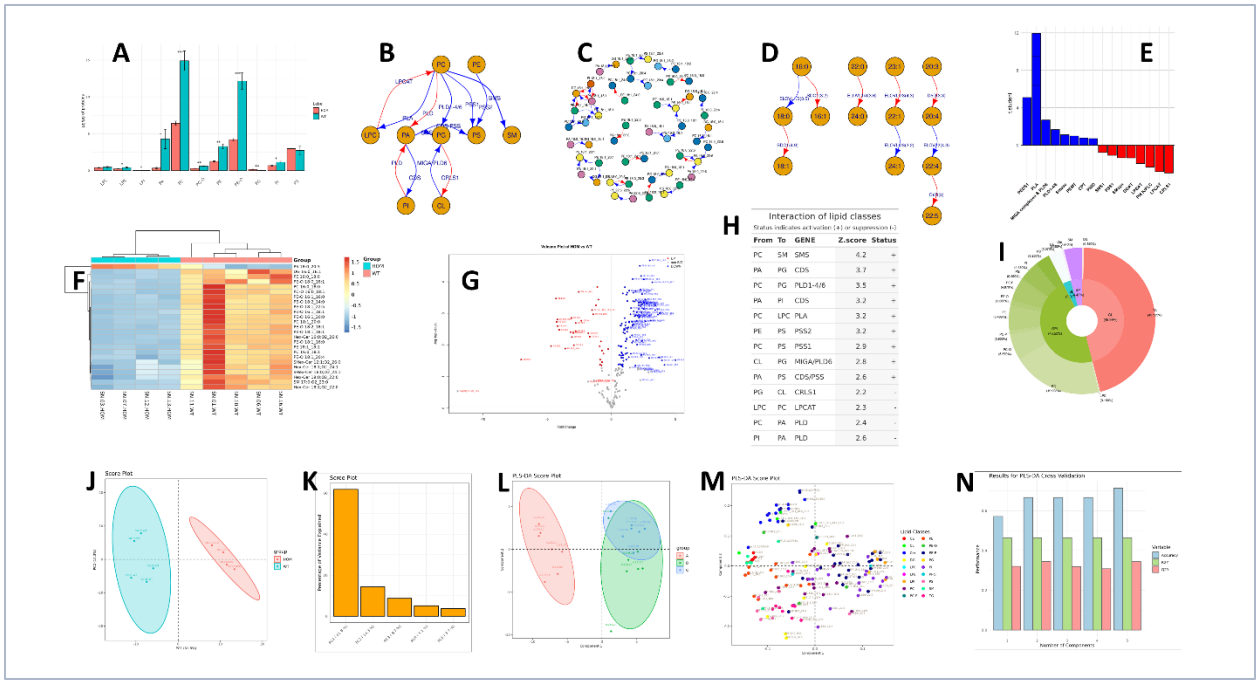

Figure 6: Some different examples of graphic output. Bar Graph (A); Lipid Pathway at the level of Classes (B), Molecular Species (C) and Lipid Building Blocks (D); Bar graph of genes involved in order of average Z score (E); Heat Map at Lipid molecular species level (F); Volcano plot (G); Results table of Lipid Class Pathway analysis (H); Pie Chart of Lipid categories and Class (I); Score plot of a PCA (J); Scree Plot of a PCA (K); Score plot of PLS-DA (L); Loading Plot of a PLS-DA (M); Cross Validation Bar graph of a PLS-DA (N).

Utility section

LipidOne 2.0 features a Utility section designed to enhance the operability of lipidomic data. Currently, three functions are available: Lipid Data Quantification; Lipid Data Filtering; External Lipidomic Tools.

1. **Lipid Data Quantification**: In this section, users are guided through the process of quantifying lipidomic data. Put simply, if lipidomics data is presented as peak area or peak height, and the dataset includes information from standards added to the samples (i.e. SPLASH Lipidomix or Equisplash by Avanti Polar Lipids), it becomes feasible to convert peak area or peak height values into concentration values. To facilitate this conversion, the data designated for quantification must be organized in a format closely resembling that suitable for use in LipidOne 2.0. There are only two distinctions to note: 1) Lipid molecular species must incorporate one or more lipid standards; 2) The numerical data in the matrix must represent areas or peak heights. An example of data to be quantified can be found clicking DOWNLOAD button in section 1. Users are required to prepare the "standard.txt" table, comprising three columns with the exact names "Standard," "Class," and "Conc." These columns respectively represent the name of the standard lipid, the associated lipid class for quantification, and the concentration of the standard. An example of standard.txt table can be found clicking DOWNLOAD button in section 2.

   Once these data are provided, clicking “Choose File” and “Upload” buttons in sections 1 and 2, LipidOne 2.0 will execute the quantification process by clicking the START button. Upon completion, rows containing standards are automatically excluded from the data matrix. Furthermore, in the case of duplicate entries for molecular species, these entries are consolidated into a single row through summation. Users can obtain the quantified data matrix, ready for upload to LipidOne 2.0, by clicking the DOWNLOAD button in section 4.

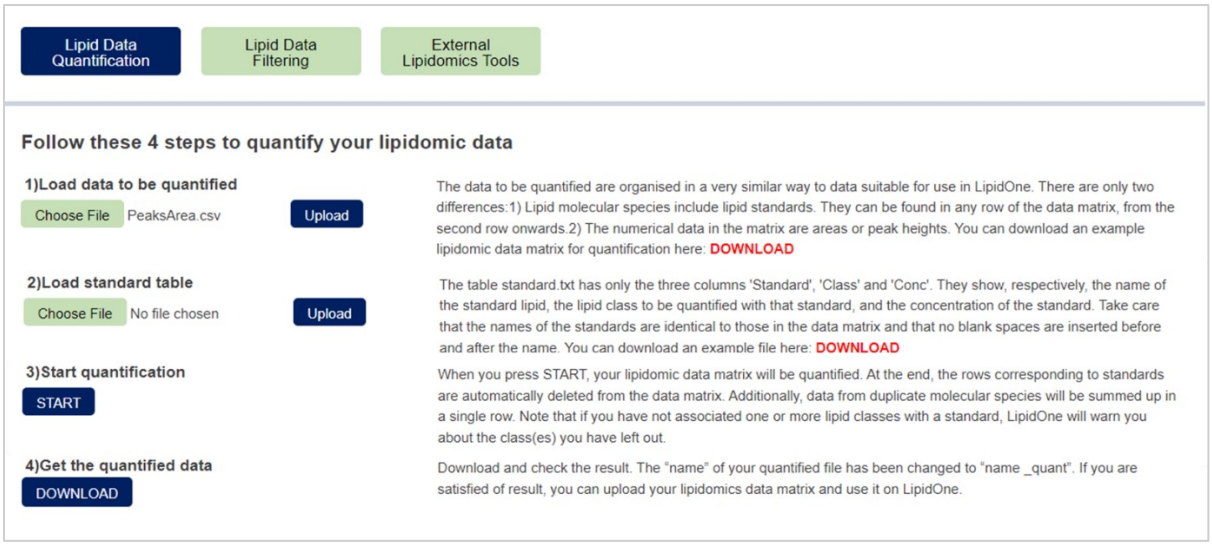

Figure 7: The figure shows the lipid data quantification tool from the utilities section.

2. **Lipid Data Filtering**: Sometimes LipidOne 2.0 users, when running PCA or HeatMap, may find that one or more samples in the lipidomic dataset should be treated as outliers. In the Lipid Data Filtering section, users can remove outliers from the input data matrix. Once the filter is applied, users can return to working on the main functions because LipidOne 2.0 automatically loads the filtered data matrix. In any case, users can obtain the filtered data matrix by clicking on the download button.

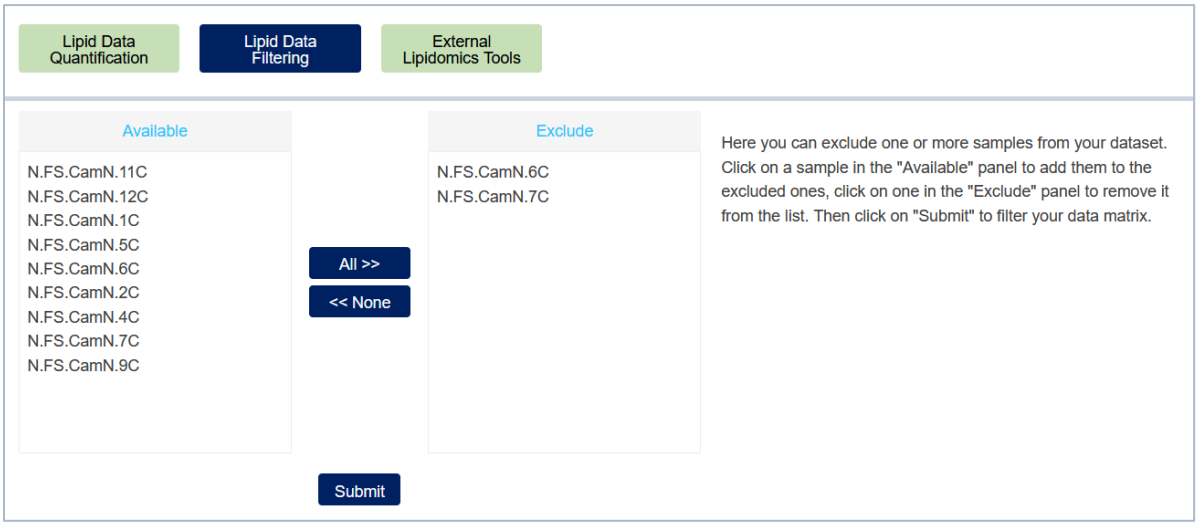

Figure 8: The figure shows the lipid data filtering tool from the utilities section.

3. **External Lipidomic Tools**: In this section of Utilities, LipidOne 2.0 users can find links to useful web services, such as lipid nomenclature translators (to obtain a list of lipid names in standard form), computational tools for creating libraries of lipid MS2 spectra, bioinformatics tools for lipid ontological analysis, links to LipidMaps and LSI consortia, and various others.

CONCLUSION

The introduction of LipidOne 2.0 represents a significant advancement in the field of lipidomics, offering a web-based solution that addresses the evolving needs of researchers and scientists engaged in lipid analysis. The integration of diverse functions, encompassing statistical analysis, exploration of lipidomic pathways, and biomarker identification, within LipidOne 2.0 web platform aims to streamline the interpretation of lipidomic data within the broader context of systems biology. We hope that LipidOne 2.0 will provide valuable support to researchers worldwide engaged in lipidomics studies. Our primary objective was to create an intuitive and user-friendly bioinformatics tool suitable for both experts and non-experts. We welcome feedback and suggestions to continuously enhance our platform.

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AUTHOR CONTRIBUTIONS

Roberto Maria Pellegrino and Husam B.R. Alabed designed and implemented the R scripts of the LipidOne 2.0 tool, wrote the manuscript, and prepared the figures and tables. Dorotea Frongia Mancini performed the software validation tests. Carla Emiliani supervised the project and Sabata Martino financed it. All other authors revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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