Feature-based molecular networking in the GNPS analysis environment

Molecular networking has become a key method to visualize and annotate the chemical space in non-targeted mass spectrometry data. We present feature-based molecular networking (FBMN) as an analysis method in the Global Natural Products Social Molecular Networking (GNPS) infrastructure that builds on chromatographic feature detection and alignment tools. FBMN enables quantitative analysis and resolution of isomers, including from ion mobility spectrometry.

Since its introduction in 2012 (ref. 1), molecular networking has become an essential bioinformatics tool to visualize and annotate non-targeted mass spectrometry (MS) data. Molecular networking, uniquely, goes beyond spectral matching against reference spectra, by aligning experimental spectra against one another and connecting related molecules by their spectral similarity. In a molecular network, related molecules are referred to as a ‘molecular family’, differing by simple transformations such as glycosylation, alkylation and oxidation/reduction. Molecular networking became publicly accessible in 2013 through the initial release of GNPS, a web-enabled MS knowledge capture and analysis platform (https://gnps.ucsd.edu/), and has been widely applied in MS-based metabolomics to aid in the annotation of molecular families from their fragmentation spectra (MS²).

Powered by more than 3,000 CPU cores at the University of California San Diego Center for Computational Mass Spectrometry and the MassIVE data repository, GNPS has provided researchers from more than 150 countries with the ability to perform molecular networking. To build upon the success of the first molecular networking method referred to as ‘classical’ molecular networking (classical MN), which is based on the MS-Cluster algorithm, we introduce a complementary tool named FBMN. FBMN leverages the capability of well-established MS processing software and improves upon classical MN by incorporating not only MS¹ information, such as isotope patterns and retention time, but also ion mobility separation when performed. By relying on processed spectral information, molecular networks obtained with FBMN can (1) distinguish isomers producing similar MS² spectra that are resolved by chromatographic or ion mobility separation, which may have remained hidden in classical MN, (2) facilitate spectral annotation, and (3) incorporate relative quantitative information that enables robust downstream metabolomics statistical analysis. Whereas users of the classical MN would have had to perform molecular networking and MS¹ analysis separately before performing a cumbersome linking of the outputs, the FBMN method accepts the output of feature detection and alignment tools, making them directly compatible with annotation tools and the entirety of the analysis pipeline.

To fully utilize the MS¹ and MS² data collected during a non-targeted metabolomics experiment in liquid chromatography coupled to tandem MS (LC–MS²), we have created an online and streamlined workflow (Fig. 1a) infrastructure that supports the outputs of feature detection and alignment tools for FBMN analysis (https://ccms-ucsd.github.io/GNPSdocumentation/featurebased-molecularnetworking/), including the standard output format for analysis of small molecules (mzTab-M). The diversity of supported software, each offering different functionalities and modules, serves experimentalists, bioinformaticians, and software developers. FBMN is the second most commonly used analysis tool within the GNPS environment (Fig. 1b), with more than 6,767 jobs performed.
in 2019, and has already been used in more than 80 publications since its introduction in November 2017.

The molecular networks generated with FBMN enable the efficient visualization and annotation of isomers in LC–MS² datasets, as demonstrated below with LC–MS² data from a drug discovery project from *Euphorbia* plant extract⁷ (Fig. 2a,b) and the detection of human microbiome-derived lipids belonging to the commendamide family⁸, detected in fecal samples from the American Gut Project (AGP⁹; a crowd-sourced citizen-science microbiome project; Fig. 2c,d). In both cases, FBMN resolved positional isomers/stereoisomers in the molecular networks that have similar MS² spectra but distinct retention times, that would not have been resolved with classical MN. The uses of FBMN facilitated the discovery of antiviral compounds⁷ (Fig. 2c), and the annotation of commendamide isomers⁹ and of a putative new derivative, the compound (that is, precursor isotope patterns, adduct annotation). FBMN enables robust statistical analysis by providing accurate relative ion intensities across a dataset. This capacity is demonstrated with a serial dilution series dataset of the NIST 1950 serum reference standard, containing 150 spiked standards. Here the LC–MS² data were processed with MZmine¹¹ or OpenMS¹² for FBMN (Fig. 2g,h). A linear regression analysis was used to evaluate the relative quantification between classical MN and FBMN. Figure 2h shows that for FBMN, relative quantification had a coefficient of determination ($R^2$) value distribution mostly above 0.7, whereas this was not found when the precursor ion abundance was obtained from classical MN via spectral counts (Fig. 2g). The improved distribution of correlation coefficients toward 1 indicates a more linear response between molecular concentration and ion abundance, which improves the accuracy and precision of the quantification of results. In addition, FBMN facilitates
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Feature-based molecular networking

Ion mobility spectrometry (IMS) experiments coupled within classical MN resulted in one node for the ion at m/z 589.313, while FB MN was able to detect seven isolomers. AU, arbitrary units; EIC, extracted ion chromatogram. Classical MN with data from the AGP (201 samples; n=1 LC–MS2 experiment per sample) showed two different N-acetyl amides, while the use of FB MN allowed the annotation of three different isomers per N-acetyl amides. Classical MN (e) and FB MN (f) were used to analyze the network of EDTA in plasma (373 samples; n=1 LC–MS2 experiment per sample). By merging MS2 spectra of EDTA eluting over 2.5 min into one representative MS2 spectrum, FB MN recovered the molecular similarity of in-source fragments observed for EDTA. Evaluation of quantitative performance using multiple dilutions of a reference serum sample (5 dilutions; n=3 LC–MS2 experiments per sample). The plots show the distribution of the coefficient of determination (R²) from the ordinary least squares (OLS) linear regression analysis between the observed and expected ion abundances for molecular network nodes in classical MN (g) or FB MN (h). The upper charts present the distribution of the R² for the network nodes with classical MN (n=3,367) and FB MN (n=877), and the lower charts show the R² distribution for the annotated reference compounds with classical MN (n=49) and FB MN (n=54).

The FB MN workflow not only offers automated spectral library search and spectral library entry curation, but is also integrated with other annotation tools available on the GNPS environment, such as MASST, while promoting data analysis reproducibility by saving the FB MN jobs on the user’s private online workspace. The GNPS environment conveniently enables the user to evaluate different parameters and share the results via a URL for publication.

the direct application of existing statistical, visualization and annotation tools, such as QI IM2 (ref. 12), MetaboAnalyst3,ili4, SIRIUS5, DEREPLICATOR16, MS2LDA17 and Qemistree18.

FB MN further enables the creation of molecular networks from ion mobility spectrometry (IMS) experiments coupled within LC–MS2 analysis. As an orthogonal separation method, the use of ion mobility offers additional resolving power to differentiate isomeric ions in the molecular network based on their collisional cross-section. The integration of ion mobility with FB MN on GNPS can currently be performed with MetaboScape, MS-DIAL19, and Progenesis QI. An example of such isomer separation using trapped IMS (TIMS) coupled to LC–MS2 is shown in Supplementary Fig. 1.

Available on the GNPS web platform at https://gnps.ucsd.edu/ FB MN is ideally suited for advanced molecular networking analysis, enabling the characterization of isomers, incorporation of relative quantification and integration of ion mobility data. FB MN analysis is recommended for a single LC–MS2 metabolomics study, but its applicability is limited when applied across multiple studies due to different experimental conditions and possible batch effects. Moreover, the use of FB MN for the analysis of very large datasets (containing several thousand samples) is limited by the scalability of most feature detection and alignment software tools. Thus, while FB MN offers an improvement upon many aspects of molecular networking analysis, classical MN remains essential for meta-analysis of large-scale datasets and is convenient for rapid analysis of LC–MS2 data with less user-defined parameters; one important aspect of molecular networks obtained with FB MN is the use of adequate processing steps and parameters, which otherwise could negatively affect the resulting molecular networks. To facilitate dissemination and education of the FB MN method and the supported processing software, we have created detailed tutorials and step-by-step instructions, available at https://ccms-ucsd.github.io/GNPSDocumentat on/featurebasedmolecularnetworking/.

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Methods

Development of FBMN. The FBMN method consists of two main steps: (1) LC–MS feature detection and alignment (and (2) a dedicated molecular networking workflow on GNPS. Our first prototype for FBMN was developed with the Optimus workflow\(^1\), using OpenMS tools.\(^2\) Following the first step, two files are exported: a feature quantification table (.TXT format) and a MS\(^2\) spectral summary (.MGF format). The feature quantification table contains information about LC–MS features across all considered samples including a unique identifier (feature ID) for each feature, m/z value, retention time and intensity. The MS\(^2\) spectral summary contains a list of MS\(^2\) spectra, with one representative MS\(^2\) spectrum per feature. The mapping of information between the feature quantification table and the MS\(^2\) spectral summary is stored in these files using the feature ID and scan number, respectively. This simple mapping enabled us to relate LC–MS feature information or statistically derived results to the molecular network nodes. This approach was also used for the integration of other tools with FBMN and does not require third-party software, as proposed previously.\(^3\) Finally, the FBMN workflow also supports the mzTab-M format\(^4\), a standardized output format designed for the report of metabolomics MS data-processing results. In this case, the mzTab-M file is used instead of the feature quantification table and requires the input of the mzML files instead of the MS\(^2\) spectral summary file. Support for the mzTab-M format enables one to perform FBMN with any existing and future processing tools that support this standardized format.

The FBMN workflow has been integrated into the GNPS ecosystem and thus benefits from the connection with other GNPS features, for example, the possibility to perform automatic MS\(^2\) spectral library searching, the direct addition and customization of libraries, the search of a spectrum against previously defined sets with MAST\(^5\) and the visualization of molecular networks directly in the web browser\(^6\) or with Cytoscape\(^7\). The FBMN workflow is available on the GNPS platform (https://gnps.ucsd.edu) via a web interface (Supplementary Fig. 2). Jobs are computed and stored on the computational infrastructure of the University of California San Diego Center for Computational Mass Spectrometry. Each finished job is saved in the private user space for future examination and has an permanent static link that enables data sharing and collaborative analyses. We strongly recommend the sharing of this static link along with data publications using GNPS workflows to facilitate accessibility and reproducibility of results. Instructions to perform FBMN with the supported tools and input file format requirements are provided in the GNPS documentation (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking/Supplementary Fig. 3).

Processing mass spectrometry data for FBMN. FBMN supports the input from several feature detection and alignment processing software programs. Depending on the type and size of MS data and the intended user (for example, bioinformatician, mass spectrometrists and biologists), different software might be more appropriate. In general, tools with a graphical user interface (GUI) for example, MZmine\(^8\), MS-DIAL\(^9\), MetaBoscope and Progenesis QC, are convenient for these large datasets, tools that were designed to operate on a cluster/cloud environment are preferred (XCMS\(^10\), OpenMS) and, to some extent, MZmine. Regardless of the software or application, the processing steps and parameters used should be determined according to the recommendations from tool developers and experienced users through community feedback. Finally, automated optimization modules can be used to finely tune parameters, which is particularly valuable when using command-line interface tools.\(^11\) When we acknowledge that many tools and configurations are available to analyze MS data, we provide a summary of processing steps on the supported tools in the FBMN documentation (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking). These steps constitute an aggregation of institutional knowledge from tool developers and experienced tool users that do not encompass all possible applications, but rather provide a starting point for new users.

Generation of a representative MS\(^2\) spectrum from a LC–MS\(^*\) file. The selection of the representative MS\(^2\) spectrum for detected features in the MS\(^*\) spectral summary file is performed using several methods. Available in all tools supported, the default method (most intense) uses the MS\(^2\) spectrum with the highest precursor ion intensity or total ion current, in the specified mass and retention time range, as the representative MS\(^2\) spectrum for a LC–MS feature. Experimental spectral ‘clustering’ methods for the creation of the representative MS\(^2\) spectrum in FBMN are implemented in MZmine, OpenMS and XCMS. The spectral clustering method implemented in MZmine (‘merge’ option in the GNPS/SIRIUS export modules) and OpenMS (‘merge option’ in OpenMS export tool) works as follows: for each LC–MS\(^*\) feature, the purity of each fragmentation spectra is calculated with a function inspired by mSpurity.\(^12\) Briefly, adjacent MS\(^2\) scans are examined to determine if other isobaric ions were co-fragmented. In these MS\(^2\) scans, the ratio between the precursor ion intensity and the other isobaric ions in the precursor ion isolation range is calculated. Then, the purest MS\(^2\) spectrum (highest purity score) is selected as the final processing option. (Cosine score) is computed between the purest spectrum and all the other MS\(^2\) spectra for the feature. All MS\(^2\) spectra reaching a cosine score threshold with the reference MS\(^2\) spectra are then merged into one representative MS\(^2\) spectrum. The mass accuracy, isolation width for the ion filtering and cosine score are defined by the user.

FBMN after MZmine processing. MZmine\(^13\) is an open-source cross-platform software for MS data processing with an advanced GUI that enables the users to visually optimize parameters and examine the results of each processing step. Moreover, MZmine allows for the export of a batch file containing all the steps and parameters used in the processing, thus enabling reproducibility. To support FBMN in the post-processing feature detection (peak deconvolution), MZmine is modified to provide the ability to pair a feature with its MS\(^2\) scans using an m/z and retention time range defined by the user (Supplementary Fig. 4). Due to a new data structure and to support older projects (before version 2.38), an additional specific filtering module (group MS\(^2\) scans with features) was developed to assign all MS\(^2\) scans to the feature for the existing peak list (for instructions see https://www.youtube.com/watch?v=EL5pmYyPtTE). Moreover, a GNPS export and direct submission module was created (Supplementary Fig. 5), which offers two modes: (1) export of the feature quantification table and the MS\(^2\) spectral summary file and (2) direct FBMN analysis on the GNPS web platform (version 2.37+). The direct GNPS job submission generates all the files and uploads them together with an optional metadata table and default parameters (Supplementary Fig. 6) to the FBMN workflow on GNPS. By providing the user’s GNPS login credentials (optional), a new job can be created in the personal user space (https://www.youtube.com/watch?v=rFVGcGq7_44E&list=P L A1_2Xw_x88iTaD99hs5XiP94vP1xj3alSalD&index=4&tl=0). Otherwise, the user can be notified by email or redirected to the job web page after the submission. With the optimized module release, the MS-DIAL export tool uses the most intense MS\(^2\) spectrum as a representative spectrum for each LC–MS feature. When using the ‘merge MS\(^2\) spectra option (version 2.40+), a representative high quality MS\(^2\) spectrum is instead generated from all spectra and exported as a representative MS\(^2\) spectrum. For detailed documentation, see https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-mzmine2/.

FBMN after OpenMS processing. OpenMS is an open-source cross-platform software specifically designed for the flexible and reproducible analysis of high-throughput MS data analysis, including more than 200 tools for common mass spectrometric data-processing tasks.\(^14\) Building on our experience with the Optimus development, the integration of OpenMS and FBMN was achieved by creating a GNPSExport tool (TOPP tool) as a part of the OpenMS tool collection (https://github.com/OpenMS/OpenMS). A detailed description of the GNPSExport module and instructions for use with FBMN is available at https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-openms/. Briefly, after running an OpenMS non-targeted metabolomics pipeline, the GNPSExport TOPP tool can be applied to the consensusXML file resulting from FeatureLinkerUnlabeledKD or FeatureLinkerUnlabeledQT tools (alignment step) and the corresponding mzML files. For each consensusElement (LC–MS\(^*\) feature) in the consensusXML file, the GNPSExport tool generates one representative MS\(^2\) spectrum that will be exported in the MS\(^2\) spectral summary file (using either the option ‘most intense’ or ‘merged spectra’). The TextExport tool is applied to the same consensusXML file to generate the feature quantification table. Note that GNPSExport requires the use of the IDMapper tool on the featureXML files (from the feature detection step) before feature linking; to associate MS\(^2\) scans (peptide annotation in OpenMS terminology) with each feature. These MS\(^2\) scans are then merged into the GNPSExport tool for the generation of the representative MS\(^2\) spectrum. Additionally, the FileFilter has to be run on the consensusXML file before the GNPSExport, to remove consensusElements without associated MS\(^2\) scans. The two exported files (feature quantitation table and MS\(^2\) spectral summary) can be directly used for FBMN analysis on GNPS. The OpenMS-GNPS workflow for metabolomics data processing was implemented as a Python wrapper around OpenMS TOPP tools (https://github.com/Bioinformatic-squad-DorresteinLab/openms-gnps-tools/) and released as a workflow (https://github.com/Bioinformatic-squad-DorresteinLab/openms-gnps-workflow/) on the GNPS/MassIVE web platform and could be run in OpenMS TOPPAS workflow.\(^15\) The OpenMS and GNPS workflow can be accessed and run at https://proteomics2.ucsd.edu/ProteoSAFe/

FBMN after XCMS processing. XCMS (for the most recent version, see https://github.com/sneumann/xccms/) is one of the most widely used software packages for processing of MS-based metabolomics data.\(^16\) The integration of XCMS and FBMN is currently possible using a custom utility function that allows a format of spectraForGNPS to create the MS\(^*\) spectral summary. This function is available on GitHub (https://github.com/sorainer/xccms-gnps-tools/) and is compatible with the CAMERA algorithm for isotopes and adduct annotation.\(^17\) The GNPSExport R scripts in Markdown and Jupiter notebook formats and the GNPSExport R tool (https://github.com/GNPS/gnps-export-tool) were developed to support FBMN after XCMS processing. Both the exported files (feature quantitation table and MS\(^2\) spectral summary) can be directly used for FBMN analysis on GNPS. The detailed documentation is available at https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-xccms/.

FBMN after MS-DIAL processing. MS-DIAL is an open-source MS data-processing software\(^18\) (available for Windows only; https://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/). The integration of MS-DIAL and FBMN
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FBMN after MetaboScape processing. MetaboScape is a commercial MS metabolomics data-processing software commercialized by Bruker and available on Windows. MetaboScape can perform feature detection, alignment, and annotation of non-targeted LC–MS data acquired on Bruker mass spectrometers. Support for the full data-processing pipeline, including LC–MS (LC-TIMS-MS) was added in MetaboScape 4.0, which results in LC-TIMS-MS features. FBMN can be performed on LC–MS or LC-TIMS-MS data by exporting the feature quantitation table and MS/MS spectral summary from the 'bucket table' using the 'export to GNPS format' function. These files can be uploaded to GNPS for FBMN analysis. Information from MetaboScape, such as the collision cross-section values or other spectral annotations, can be mapped into the molecular networks using Cytoscape. The detailed documentation is available at https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-metabscape/

FBMN after Progenesis QI processing. Progenesis QI is a commercial feature detection and alignment software developed by Nonlinear Dynamics (Waters) that is compatible with various proprietary and open MS data formats. Progenesis QI can perform feature detection, alignment and annotation of non-targeted LC–MS data acquired either in data-dependent acquisition or data-independent analysis and can also utilize the IMS dimension. FBMN can be performed on any of these data types processed with Progenesis QI (v4.0), by exporting the feature quantitation table (CSV format) and MS/MS spectral summary (.MSP format). These two files can be exported from the 'identify compounds' submenu by using the functions 'export compound measurement' and 'export fragment database', respectively. These files can be uploaded to GNPS for FBMN analysis. Information from Progenesis QI, such as the collision cross-section values or other spectral annotations, can be mapped into the molecular networks using Cytoscape. The detailed documentation is available at https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-progenesisQI/

Running time and scalability of the FBMN method. While the molecular networking part of the FBMN method is performed online on the GNPS web server (runtime of 5 to several hours depending on the number of features and job parameters), the data-processing part has to be performed with the computational resources available to the researcher (laptop or desktop computer, workstation and cloud infrastructure). The computational cost of the data-processing part depends on (1) the software employed, (2) the number of samples in the dataset and (3) the parameters set for this reason. The computational cost of the method in all scenarios cannot be comprehensively established. Nevertheless, our experience and feedback from the FBMN community with open-source tools such as MZmine or MS-DIAL showed that small data sets (<500 files) can be processed within 5 to 10 minutes using a desktop equipped with 8–16 GB RAM. Medium-sized datasets (>100 samples) require the use of a workstation equipped with 16–32 GB RAM, and large datasets (>500 samples) require 32–64 GB RAM. For very large datasets (>1,000 samples), it is currently recommended to use OpenMS or XCMS on a cluster/cloud infrastructure.

Integration with other computational mass spectrometry annotation tools. The .MGF file format is accepted by numerous computational MS annotation tools. The use of these annotation tools with the MS/MS spectral summary file enables (1) a reduction in the computation time compared to when using the unprocessed MS files (a) and (2) the export of MS/MS molecular networks produced by the FBMN method. Some of these tools are directly available in the GNPS environment, including SIRIUS, DEREPLICATOR, Network Annotation Propagation (NAP), MS2LDA, MolNetEnhancer and Qemistree (see below for details), as well as other software such as MetWork, CFM-ID and MetFrag.

SIRIUS. SIRIUS is an advanced software for the computational annotation of small molecules from LC–MS data. It is capable of identifying compounds at the molecular formula, and annotating substructural, class and structural levels from the compound MS/MS spectra. The MS/MS spectral summary file (.MGF format) generated for the FBMN compatible with OpenMS or with the dedicated GNPS workflow (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-gnps/) results from SIRIUS can be mapped on the molecular networks, which is essential since spectral library matching usually results frequently in a 1–5% annotation rate. In addition, a dedicated SIRIUS export function compatible with FBMN was created in MZmine and MetaboScape that exports a modified MS/MS spectral summary file with representative MS/MS spectra for each feature. The MS/MS spectra contains information about the detected isotopic pattern and can be used for automated detection of adduct/rare elements in SIRIUS, which restricts the two-features formula space to speed up computation and improve molecular formula identification rates.

DEREPLICATOR. DEREPLICATOR, along with DEREPLICATOR VarQuest, is a collection of computational MS tools specialized in the annotation of peptide small molecules often produced by microorganisms endowed with various biological activities. DEREPLICATOR tools can be run directly through the FBMN workflow results on GNPS. Alternatively, and for advanced parameterizing, the DEREPLICATOR workflow on GNPS accepts the MS/MS spectral summary file (.MGF format) as input and can directly map into the FBMNs (https://ccms-ucsd.github.io/GNPSDocumentation/derePLICATOR/).

Network annotation propagation. NAP uses MetFrag/MetFusion for the prediction of putative structures and the network topology to rerank structure predictions by propagating the expected structural similarity. NAP is available on GNPS as a dedicated workflow and offers direct support to FBMN (https://ccms-ucsd.github.io/GNPSDocumentation/nap/).

Unsupervised substructure annotation with MS2LDA. MS2LDA uses the latent Dirichlet allocation algorithm to mine for motifs (Mass2Motifs) of co-occurring fragmentations and neutral losses in MS2 spectra. MS2LDA accepts the outputs of MS1 or MS2, allowing the direct annotation of MS1 and MS2 in the molecular networks. MS2LDA can be run on the GNPS web platform (https://ccms-ucsd.github.io/GNPSDocumentation/ms2lda/) and/or in the MS2LDA web application.

MolNetEnhancer. MolNetEnhancer combines the outputs from molecular networking, substructure annotation with MS2LDA and other structural annotation tools, including SIRIUS, NAP and DEREPLICATOR, together with automated chemical classification through ClassyFire into a single molecular network. MolNetEnhancer accepts input files from classical MN and FBMN. The MolNetEnhancer accepts the MS/MS spectral summary from FBMN and is available through the GNPS web platform (https://ccms-ucsd.github.io/GNPSDocumentation/molnetenhancer/).

Qemistree. Qemistree is an MS data exploration strategy based on hierarchical organization of structural fingerprints predicted from fragmentation spectra. The fingerprints are predicted with SIRIUS/CSI:FingerID and the tree-based structure comparison allows the application of ecological tools to study the chemical composition. Qemistree is available as a GNPS workflow and directly accepts the outputs of FBMN (https://ccms-ucsd.github.io/GNPSDocumentation/qemistree/).

FBMN applications. FBMN makes it possible to resolve isomers in a drug lead discovery effort. The examination of LC–MS data (MSV00008050) from the E. dendroides plant extract showed the presence of numerous chromatographic peaks for ions in the range of m/z 500–900, corresponding to diterpene ester derivatives. These specialized metabolites consist of a polyhydroxylated diterpene core acylated with various acidic moieties, which are typically found as positional isomers based on their acylation pattern. The EIC for the ion m/z 589.31 in the E. dendroides extract data (Supplementary Fig. 7) shows the presence of at least seven distinct LC–MS peaks between 24.5 min and 27.3 min, including five peaks with associated MS3 spectra. The analysis of the extract and the fractions where these molecules were originally isolated (fractions 13 and 14) with classical MN resulted in a molecular network with two nodes for the m/z 589.31 ions (Fig. 2a and Supplementary Fig. 8). These MS3 spectra (cluster index of 5332 and 5333) resulted from the merging of 96 fragmentation spectra spanning 23.6 min to 26.5 min by MS–Cluster (Fig. 2b and Supplementary Fig. 9). Close examination of the cluster spectra revealed that, while all MS3 spectra for the precursor m/z 589.31 present fragment ions m/z 501.26, 423.21, 335.16 and 295.17, three distinct spectral types could be detected based on the fragmentation intensities of (1) the C-12 acyl chain (Supplementary Fig. 9), (2) the C-13 aromatic ring (Fig. 10), FBMN of the dataset with MZmine processing (see the GNPS job) enabled the differentiation of the MS3 spectra of seven isomers (Fig. 2b and Supplementary Fig. 11; see the molecular network view). A detailed discussion of the differences observed between the two methods can be found in the Supplementary Note 1 and Supplementary Table 1. Interestingly, in the original study, OpenMS was used for FBMN and resulted in the observation of three different positional isomers instead of seven, which shows that different processing methods and/or parameters can lead to different results with FBMN. These three isomers were subsequently isolated and differed by the position of one double bond on the C-12 acyl chain or from carbon C-4 configuration. Because FBMN connects the accurate relative abundance of ion across the fractions and the molecular networks, it allowed us to create bioactivity-based molecular networks, which were used to predict and target potentially antiviral compounds. For a detailed description of the extraction, MS analysis and structural elucidation, see the original paper. The MZmine project and parameters used can be accessed on the MassIVE submission (MSV000080502).
FBMN resolves isomers in large-scale metabolomics studies. FBMN was applied on a cohort of the AGP, a citizen-science research project that enabled the observation of commendamide in humans, along with other new N-acetyl amide derivatives using molecular networking. Commandamide is a recently discovered bacterial N-acetyl amide that has been shown to modulate host metabolism via G-protein–coupled receptors in the murine intestinal tract. The use of FBMN for the AGP data (Fig. 2d) allowed the observation of two additional commendamide isomers (m/z 330.26) and of an analog, (N-hydropyruvamide) (m/z 344.28). This was performed in the observation of one single consensus spectrum for all the isomers (Fig. 2c). In addition, FBMN allowed the observation of a putative commendamide derivative N-(dehydroxeadecanoyl)glycine (CCMISLB00005436498; Supplementary Fig. 12) in the commendamide molecular network. The sample collection and MS acquisition methods are described in the original manuscript. The data were downloaded from https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=8a43db5891a4a84d7a4596ab4896f666. All MS data were processed with MZmine (v2.37). The MZmine project data, along with parameters and export files, were deposited to the MassIVE repository (MSV000084095). The chromatograms for m/z 330.26 and m/z 344.28 displayed in Fig. 2c,d are from samples 43076, P3_R90_01_314.mzML and 38131_P5_RA_01_358.mzML, respectively. Chromatograms were exported within MZmine. The results were exported with the ‘Export for/submit to GNPS’ module for FBMN analysis on GNPS. The corresponding job can be accessed at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=8a43db5891a4a84d7a4596ab4896f666 (only logged-in users can access all the input files). The mzML files used for the classical MN job are available at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=3c27e43d9048c40bace605c39c4d25. FBMN reduces spectral redundancy and de-obfuscates spectral similarity relationships: the case of EDTA. The benefit of using FBMN can be illustrated with the metal chelating agent EDTA, widely used in beauty products, food and scientific protocols. A search for its occurrences in public spectral datasets with the MS search tool (MASST) showed that it is frequently observed in plasma samples where it is used during the sample preparation. Using classical MN, we analyzed a public dataset of human plasma where EDTA was observed (MSV00008263; see Supplementary Note 2 for protocol and MS parameters); the results showed that the EDTA ions are found in two molecular networks: one network consisting of M+H spectral data and the other of [M+Na]+ spectral data. Interestingly, each of these networks have one node with a large number of clustered spectra (node 91,205 for 4,665 spectra and node 116,470 for 571 spectra), yet EDTA ions are represented by multiple nodes, although these nodes have the same precursor ion mass and retention time. Detailed analyses showed that while the median pairwise cosine values between EDTA spectra were high (median values of 0.93 and 0.94), the spectra were not clustering into a single node. Examination of the multiple fragmentation spectra for EDTA ions showed that (1) some are chimeric spectra that are ‘contaminated’ by fragment ions produced by coeluting isobaric ions and (2) other spectra were dominated by low-intensity fragment ions resulting from MS+ spectra acquired at low intensity.

The method of FBMN was applied on that same dataset using the OpenMS-GNPS workflow (see the job), and the results showed that it efficiently reduced the appearance of these redundant node patterns from the same molecule (see the FBMN job; Fig. 2f), both for the molecular networks containing the [M+H]+ and [M+Na]+ spectra. FBMN recovered the molecular similarity of in-source fragments observed for EDTA, which were not displayed with classical MN, as they now fall within a single node in the FBMN network. The sample preparation and MS methods are described in Supplementary Note 3. The files and parameters used, can be accessed on the MassIVE submission (MSV000080186; Creative Commons CC0 1.0 Universal license). The classical MN and FBMN jobs can be accessed via the GNPS website at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=198e86f1a41f5578b0a900f1c4f44a4 and https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=672da5a5732384fc84f2c74972048d789. The LC–MS+ data for the AGP were downloaded from MassIVE (MSV000080186; see Supplementary Fig. 13 for the CoelutionScope and compared to classical MN (v2.37). The MZmine project along with parameters and export files were deposited (MSV000084095; Creative Commons CC0 1.0 Universal license). The classical MN and FBMN jobs can be accessed via the GNPS website at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5c27e43d9048c40bace605c39c4d25. The MS+ data for the AGP were deposited from MassIVE (MSV000080186; see Supplementary Note 4, Supplementary Table 2 and Supplementary Fig. 19). Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The LC–MS+ data for the E. dendroides dataset, along with the MZmine project and protocol used, can be accessed on the MassIVE submission (MSV000080186; Creative Commons CC0 1.0 Universal license). The classical MN and FBMN jobs can be accessed via the GNPS website at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=198e86f1a41f5578b0a900f1c4f44a4 and https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=672da5a5732384fc84f2c74972048d789. The LC–MS+ data for the AGP were downloaded from MassIVE (MSV000080186; see Supplementary Fig. 13 for the CoelutionScope and compared to classical MN (v2.37). The MZmine project along with parameters and export files were deposited (MSV000084095; Creative Commons CC0 1.0 Universal license). The classical MN and FBMN jobs can be accessed via the GNPS website at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5c27e43d9048c40bace605c39c4d25. The MS+ data for the AGP were deposited from MassIVE (MSV000080186; see Supplementary Note 4, Supplementary Table 2 and Supplementary Fig. 19).

Code availability

The FBMN workflow is available as a web interface on the GNPS web platform (https://gnps.quickstart.ucsd.edu/featurebasednetworking/). The workflow code is open source and available on GitHub (https://github.com/CCMS-UCSD/GNPS_Workflows/tree/master/feature-based-molecular-networking/). It is released under the license of The Regents of the University of California San Diego and free for non-profit research (https://github.com/CCMS-UCSD/GNPS_Workflows/blob/master/LICENSE). The workflow was written in Python (v3.7) and deployed with the ProteoSAFe workflow manager used by GNPS (https://proteosafe.ucsd.edu/Software/ProteoSAFe). We also provide documentation, support files and additional information on the GNPS documentation website (https://ccms.github.io/GNPSDocumentation/featurebasedmolecularnetworking/). The source code of the GNPSExport module in MZmine is available at https://github.com/mzmine/mzmine3/- under the GNU General Public License. The source code of the GNPSExport tool in OpenMS is available at https://github.com/CCMS-UCSD/GNPS_Workflows/tree/master/feature-based-molecular-networking/). The source code for the GNPSExport custom function for XCMS is available at https://github.com/jorainer/xcms-gnps-tools/- under the GNU General Public License.

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Author contributions
L.-F.N., D.P., M.W. and P.C.D. conceived the method and supervised its implementation and wrote the manuscript. I.P., L.-F.N., M.E. and T.A. created the FBMM prototype in Optimus. M.W., L.-F.N., D.P. and Z.Z. created the FBMM workflow on GNPS. R.S., L.-F.N., M.W., D.P., A.K., M.E. and S.R. created the FBMM workflow on MS-DIAL. H.T., M.W., M.L., Z.Z. and P.C.D. created the FBMM workflow on OpenMS. T.A., M.W. and L.-F.N. enabled the FBMM workflow with MS-DIAL. S.R. and V.V.P. created the FBMM workflow with Progenesis QI. L.-F.N., D.P., M.W. and P.C.D. conceived the method and supervised its implementation and wrote the manuscript. I.P., L.-F.N., M.E. and T.A. created the FBMM workflow on GNPS. R.S., L.-F.N., M.W., D.P., A.K., M.F., Z.Z., A.S. and T.P. developed the GNPSexport tool in MZmine. K.D., A.K., M.L. and S.B. developed the spectral clustering algorithm and SIRIUS export in MZmine. A.S. and L.-F.N. created the GNPSexport tool in OpenMS, with guidance from E.A., O.A. and O.K. J.R. and M.W. created the XCMS export tool. H.T., M.W., M.L. and L.-F.N. enabled the FBMM workflow in MetaboScape. T.A. and V.V.P. enabled the FBMM workflow in MetaboScape as a plugin. L.-F.N. D.P. and R.d.S. performed the FBMM workflows on the XCMS processing of the forensic dataset. L.-F.N. and M.W. created the FBMM documentation. The serum sample analysis in PASEF mode and the data processing with MetaboScape were performed by E.Z., and the subsequent FBMM analysis was performed by L.-F.N., D.P., L.-F.N. and R.d.S. created the MZmine documentation. K.B.K. and H.Y. created the MS-DIAL documentation. F.V., J.M.G., K.W. and A.K.J. prepared the MS-DIAL video tutorial. M.W., R.S. and D.P. prepared the MZmine video tutorials. M.J.T., M.W., M.L., O.M. and S.B. created the XCMS documentation. L.-F.N. and A.S. created the OpenMS documentation. L.-F.N., N.H.N. and T.D. created the MetaboScape documentation. A.M.C.-R. and L.-F.N. documented the FBMM interface workflow. M.N.-E., I.K. and C.M. created the Cytoscape documentation. H.M., A.G., M.W. and L.-F.N. made the integration with DEREPPLICATOR. M.W., J.J.v.d.H., M.E. and S.R. made the integration with MS2LDA. R.d.S made the integration with NAP M.M., N.B., J.C., X.C., V.V.P., J.P., N.G., R.A.Q., A.A.A., Z.K. and S.N. tested and provided suggestions on how to improve the methods. J.J.v.d.H., A.K.J., T.P., V.V.P., A.L.G., L.-F.N., P.M.A., S.B. and N.S. improved the manuscript. All authors contributed to the final manuscript.

Competing interests
P.C.D. is a scientific advisor for Sirenas, Galileo and Cybele and scientific advisor and founder of Ometa labs and Enveda. M.W. is a founder of Ometa Labs. T.P. is a consultant for Ginkgo Bioworks. A.A.A. is a consultant for Ometa Labs. T.A. is on the Scientific Advisory Board of SGL5, a Brucker company. K.D., M.L., M.E. and S.B. are founders of Bright Giant. A.B., S.W.M., H.N. and E.Z. are employees of Bruker Daltonics. G.I., J.M. and R.S. are employees of Waters.

Additional information
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The mass spectrometry data, acquisition method files, and results files used in this study were deposited to GNPS/MassIVE repository. The accession numbers are: MSV000080502, MSV000080186, MSV00008263, MSV000084092, MSV000084402, MSV000080030.

Data analysis

The results for the molecular networking jobs are accessible via their web link provided in the manuscript. The parameters used for the feature detection/tools are described in the manuscript and deposited to GNPS/MassIVE and at all softwares used is this study are open source and free to use, except for MetaboScape (Bruker Daltonics GmbH) and Progenesis QI (Nonlinear dynamics, Waters Corporation). The FBMIN workflow is available as a web-interface on the GNPS web platform (https://gnps-quickstart.ucsd.edu/featurebasednetworking). The workflow code is open source and available on GitHub (https://github.com/CCMS-UCSD/GNPS_Workflows/tree/master/feature-based-molecular-networking). It is released under the licence of The Regents of the University of California and free for non-profit research (https://github.com/CCMS-UCSD/GNPS_Workflows/blob/master/LICENSE). The workflow was written in Python (ver. 3.7) and deployed with the ProteoSAFe workflow manager employed by GNPS (http://proteomics.ucsd.edu/Software/ProteoSAFe/). We also provide documentation, support, example files, and additional information on the GNPS documentation website (https://github.com/CCMS-UCSD/Documentation/blob/master/featurebasedmolecularnetworking/). The source code of the GNPSExport module in MZmine is available at [https://github.com/mzmine/mzmine2] under the GNU General Public License. The source code of the GNPSExport tool in OpenMS is available at [https://github.com/Bioinformatic-squad-Dorresteinlab/OpenMS] under the BSD licence. The source code for the GNPSExport custom function for XCMS is available at [https://github.com/jbarreiro/xcms-gnps-tools] under the GNU General Public License.

The custom code used for the evaluation of of relative quantification between classical molecular networking and feature-based molecular networking (python version 2.7.13) is available as jupyter notebook at [https://github.com/luothias/FeatureBasedMolecularNetworking_RelativeQuantEval] for manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
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All the data were deposited on the GNPS/MassIVE public repository (https://gnps.ucsd.edu) under the following accession numbers: MSV000080502, MSV000080186, MSV00008263, MSV000084092, MSV000084402, MSV000084030.

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