REVIEW

Metabolomics and Lipidomics for Studying Metabolic Syndrome: Insights into Cardiovascular Diseases, Type 1 & 2 Diabetes, and Metabolic Dysfunction-Associated Steatotic Liver Disease

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Summary

Metabolomics and lipidomics have emerged as tools in understanding the connections of metabolic syndrome (MetS) with cardiovascular diseases (CVD), type 1 and type 2 diabetes (T1D, T2D), and metabolic dysfunction-associated steatotic liver disease (MASLD). This review highlights the applications of these omics approaches in large-scale cohort studies, emphasizing their role in biomarker discovery and disease prediction. Integrating metabolomics and lipidomics has significantly advanced our understanding of MetS pathology by identifying unique metabolic signatures associated with disease progression. However, challenges such as standardizing analytical workflows, data interpretation, and biomarker validation remain critical for translating research findings into clinical practice. Future research should focus on optimizing these methodologies to enhance their clinical utility and address the global burden of MetS-related diseases.

Key words

Metabolomics • Lipidomics • Mass spectrometry • Metabolic syndrome • Cardiovascular diseases • Type 1 diabetes • Type 2 diabetes • Metabolic dysfunction-associated steatotic liver disease

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Introduction

Metabolic syndrome (MetS), also known as insulin resistance syndrome, is defined as a cluster of risk factors for cardiovascular disease and diabetes. The main risk factors include raised blood pressure, visceral obesity, hyperglycemia, and dyslipidemia (reduced high-density lipoprotein cholesterol or raised triacylglycerols) [1-3]. These features are often related to insulin resistance, which can lead to prediabetes or type 2 diabetes [4]. Recent studies have shown that even non-obese patients may suffer from insulin resistance, with visceral adiposity being considered the primary contributor to MetS pathology. Visceral adiposity is strongly associated with hepatic fatty infiltration, indicating that the amount of fatty acids in the liver is indirectly linked with MetS, both as a cause and a consequence of the syndrome [5]. Furthermore, in recent decades, MetS has become a significant health concern with a high prevalence worldwide [4,6-8]. To properly understand MetS metabolism and the relationships between the aforementioned risk factors [9,10], metabolomics and lipidomics can be applied.

Metabolite profiling is conducted using either untargeted or targeted approaches, applied to biological samples through various analytical methods and platforms [11]. Large-scale metabolomics and lipidomics studies, which involve extensive populations or numerous samples (over 1000), have demonstrated their effectiveness in various scientific fields. These studies have defined individual phenotypes and shown the effects of genetic, environmental, intervention, or aging factors. They have also discovered biomarkers and validated metabolite patterns associated with specific biological

states [11]. Integrating newly identified metabolite biomarkers with clinical characteristics can potentially enhance the prediction of disease development [12].

In this review, we examine metabolomics and lipidomics human cohort studies and their application in MetS research. We introduce the analytical workflow and provide examples of recent MetS studies on cardiovascular diseases, type 1 and type 2 diabetes, and metabolic dysfunction-associated steatotic liver disease.

Metabolomics and lipidomics in large cohort studies

Large-scale metabolomics and lipidomics studies analyze hundreds to thousands of human samples containing thousands of metabolites. These samples are often processed in multiple batches over several weeks or months. No single analytical platform can cover all metabolites in a biological sample due to the complexity, diversity, and size of the human metabolome and lipidome. Therefore, multiple analytical platforms are employed to increase metabolite coverage [13]. Figure 1 shows metabolomics and lipidomics workflow, consisting of sample handling, instrumental analysis, data processing, and bioinformatics.

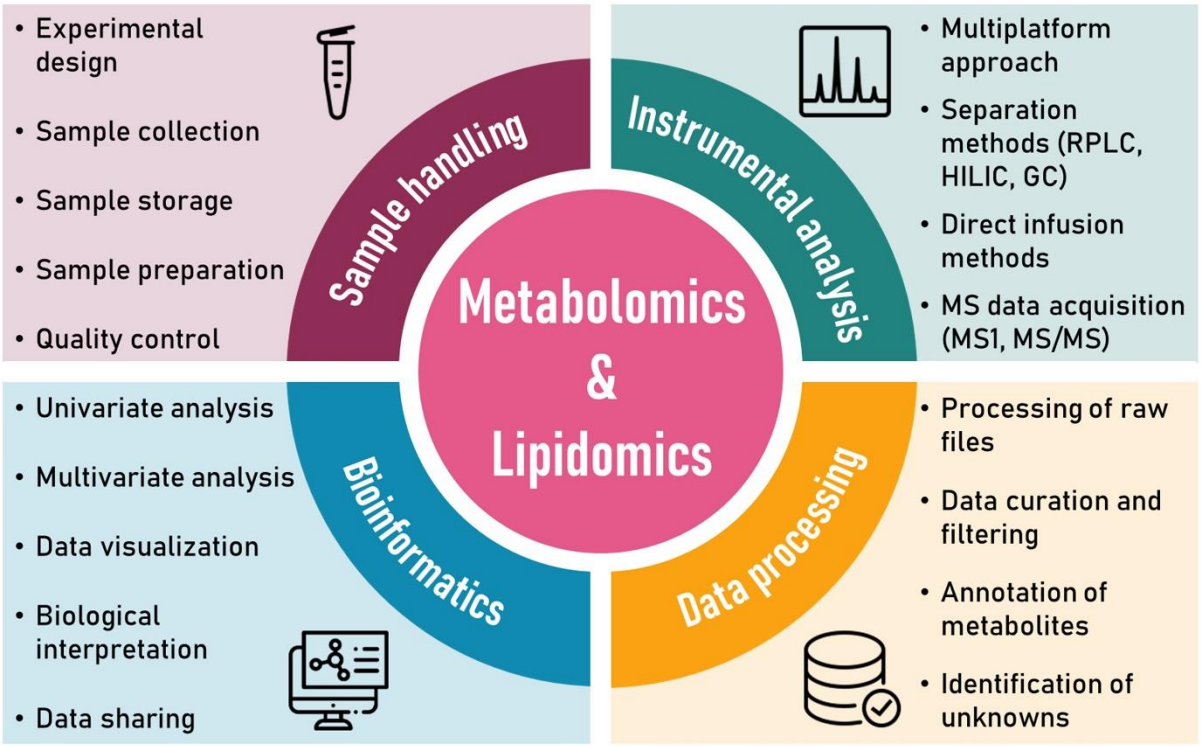

Fig. 1. Metabolomics and lipidomics workflow.

Sample handling

The first step in metabolomics and lipidomics studies is creating a proper experimental design, including sample size, sample collection and storage, sample preparation, quality control, and analytical techniques [14].

Determining the appropriate sample size, both overall and for each group, is essential. Insufficient sample size can lead to errors and lack of precision. Conversely, even small, insignificant differences might appear statistically significant with a larger sample size, while clinically important effects might seem statistically non-significant with a small sample size [15]. A high sample size may also waste resources for minimal information gain [16]. The minimal sample size is calculated using power analysis, taking into account the significance level (e.g., α=0.05), statistical power (e.g., 0.8), and effect size (d=0.8, 0.5, 0.2 for large, medium, small effect size,

respectively) [17]. To this end, freely available software such as G*Power can be used [18]. However, for untargeted metabolomics and lipidomics studies with *a priori* unknown number of measured metabolites [19], alternative strategies have become available, such as the Data-driven Sample size Determination (DSD) algorithm for MATLAB and GNU Octave [20], MetSizeR [21], or the online tool SSizer (idrblab.org/ssizer) [22].

Generally, at least 20-30 samples per group are advised for human studies, although the number of samples can range from hundreds to even thousands to achieve reasonable statistical power. On the other hand, for cell and animal studies with tightly controlled conditions, 3-6 and 5-10 samples per group, respectively, are recommended [23-25].

Another crucial aspect to consider is sample collection and storage. These steps must be decided during preanalytical processing to ensure reliable results [26]. Collection procedures differ based on the type of samples and planned analysis. For human cohort studies, samples typically consist of plasma or serum. The selection of a specific anticoagulant for plasma (e.g., EDTA, citrate, heparin) should be decided in advance and maintained consistently throughout the study. Inaccurate sample collection or improper storage may cause metabolite degradation, increased variability, or interference with instrumentation [27].

It is important to quench the metabolism of samples as soon as possible prior to their storage. Quenching should stop all enzymatic and chemical activities and maintain the current metabolite levels during harvesting [28]. The recommended method for quenching is to rapidly freeze the samples using liquid nitrogen, dry ice, or freeze clamping. After that, samples should be stored at -80 °C [29].

The next step is sample extraction to capture as many metabolites as possible in the sample. Various sample preparation techniques are available [30]. Minimal sample preparation methods, such as dilution, are sufficient for some matrices like urine. Water is a suitable diluent for reversed-phase liquid chromatography platforms, which start with a high percentage of water in the mobile phase. On the other hand, acetonitrile as a diluent is preferred for hydrophilic interaction chromatography, which begins with a high percentage of organic solvent (acetonitrile). Additionally, normalization to creatinine or osmolality values is a common strategy for urine due to its high variability in concentration, which correlates with metabolite composition [31]. On the other hand, plasma and serum, often used in large human cohort studies, contain many interfering proteins and require an extraction step to remove these before instrumental analysis. Common preparation methods like buffering, dilution, evaporation, and centrifugation may lead to metabolite losses and issues such as high salt concentration and instrument disruption, which can be reduced by adding an extraction step [32].

Extraction techniques in metabolomics and lipidomics commonly include organic solvent-based protein precipitation, liquid–liquid extraction (LLE), or solid-phase extraction (SPE). Isolation can also be performed in single or multiple fractions [33]. Single-phase extraction uses methanol, acetonitrile, isopropanol, a mixture of isopropanol/acetonitrile/water, acetonitrile/methanol, butanol/methanol [27,34,35]. This method enables simultaneous extraction of lipids and polar metabolites, but such extracts are very complex and can be challenging during instrumental analysis.

The most utilized method for reducing extract complexity is two-phase liquid extraction, where the separate phases are created by combining immiscible solvents: methyl *tert*-butyl ether (MTBE)/methanol/water [36], chloroform/methanol/water [37], and dichloromethane/methanol/water [38]. After centrifugation, the organic phase primarily contains nonpolar metabolites, such as lipids, while the polar (water) phase mainly consists of polar metabolites (Fig. 2A). In 2019, Vale *et al.* [39] introduced three-phase extraction using hexane, methyl acetate, acetonitrile, and water. After centrifugation, the upper organic phase is enriched with neutral lipids such as triacylglycerols and cholesteryl esters; the middle organic phase contains the glycerophospholipids, and the bottom aqueous phase contains polar metabolites.

While organic solvent-based protein precipitation and LLE methods are typically used for untargeted methods, SPE is the first choice for targeted methods, usually covering trace concentrations of metabolites [40].

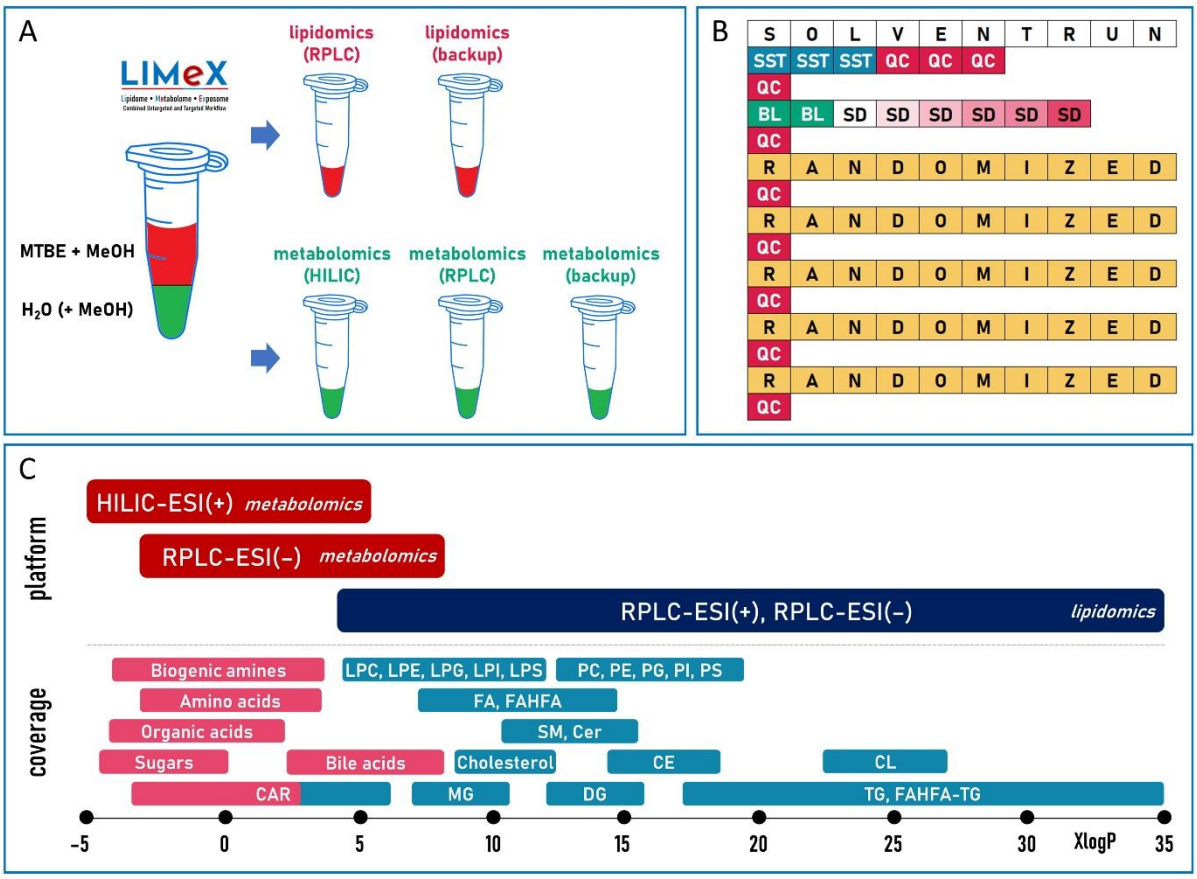

Fig. 2. (**A**) Example of sample extraction using MTBE, methanol, and water [41], leading to two phases for subsequent metabolomics and lipidomics platforms. (**B**) Example of a typical LC-MS sequence during metabolomics and lipidomic analysis, consisting of solvent injection for general platform equilibration, followed by a system suitability test (SST), platform equilibration using pooled QC samples, analysis of method blanks (BL), a diluted series of QC samples (SD), randomized study samples with regular QC sample injections after every 10 study samples. (**C**) Example of different LC-MS platforms [41,42] for metabolomic and lipidomic analysis in relation to the XlogP (predicted octanol/water partition coefficient) range of subgroups of polar metabolites and complex lipids.

The success of any research study also depends on an effective quality control (QC) process. Using internal standards in the extraction and resuspension solvents helps control the method's performance. These standards verify that aliquots are collected correctly from all extracts, the autosampler injects the correct volume, chromatographic and mass accuracy drifts are monitored, signal intensity fluctuations are tracked, and the quality of generated data is assessed during data processing [29]. They can also be used for quantification using a single-point calibration approach if added during the extraction step. Internal standards are essential because they represent true positives in the sample.

QC samples are crucial for obtaining high-quality data in high-throughput analytical chemistry laboratories [43]. They help assess the precision and stability of the analysis. QC samples are used to equilibrate the analytical platform, monitor signals for precision (within and between days), correct signals (normalization), and standardize methods. QC data can also help indicate random errors or fluctuations during the analytical run [44].

QC samples can be created by pooling aliquots of each study sample, reflecting the composition of all samples during analysis. Another option is to employ external QC using a matrix that matches the study samples, which can be useful in large-scale studies where pooling is challenging. In such studies, pooling QC samples can be simplified by using pooled aliquots from only a portion of the samples. Additionally, commercially accessible QC samples (e.g., human plasma NIST SRM 1950 standard reference material [45]) can be applied, though there is a risk of missing some metabolites compared to pooled QC samples [46]. These approaches can be combined; however, they should be planned in advance and not modified during the study.

As Figure 2B shows, a typical metabolomics and lipidomics sequence consists of pre-injection steps (injection of solvents, QC sample) to equilibrate a particular platform, followed by a system suitability test (e.g., a mixture of selected metabolites or biological samples with known composition), analysis of method blanks, a diluted series of QC samples, randomized samples, and regular injection of QC samples [42]. All these steps are essential to generate reliable metabolomics and lipidomics data.

Instrumental analysis

A multiplatform approach using various analytical techniques and platforms is necessary due to the diversity and complexity of the metabolome and lipidome. This approach can improve the overall coverage and reliability of detected metabolites [47]. Liquid chromatography-mass spectrometry (LC-MS) dominates metabolomics and lipidomics. Other commonly applied platforms are gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), and nuclear magnetic resonance (NMR). However, NMR does not offer as broad metabolite coverage as MS-based approaches [29].

LC-MS separates metabolites with a wide range of polarities due to its versatility in stationary phases, column dimensions, mobile phase modifiers, and solvents [48]. Commonly used LC-MS separation platforms are reversed-phase LC (RPLC) and hydrophilic interaction chromatography (HILIC). RPLC separates polar to semi-polar metabolites using C18, C8, or C30 columns, whereas HILIC separates highly polar metabolites using silica, alkyl amide, aminopropylsilane, or sulfobetaine groups as the stationary phase [48]. Efficient chromatographic separation enhances the sensitivity of MS detection, while background noise reduction improves the quality of MS data [49]. For the analysis of polar metabolites (Fig. 2C), RPLC and HILIC are preferred, with mobile phases containing water, acetonitrile, and methanol. On the other hand, for RPLC-based lipidomics, stronger mobile phases are needed, typically containing a high percentage of isopropanol [41,42]. The column formats vary around 50-150 mm in length, with an internal diameter of 2.1 mm, packed with sub-2 μm particles. The separation process takes between 10 and 30 min [50]. However, fast, high-throughput LC-MS methods (<5 min), combined with 96-well plate sample preparation, are preferred for large cohort studies since they allow for hundreds of injections to be performed daily [29,42].

Once separated, analytes are ionized in an ion source to create charged particles. In LC-MS, electrospray ionization (ESI) is typically used, allowing ion formation for small molecules (<2,000 Da) and large molecules, such as peptides and proteins. Due to the chemical diversity of the metabolome and lipidome, ESI is usually applied in both positive and negative modes for more efficient coverage. ESI is a soft ionization technique, minimizing the fragmentation of molecular ions compared to electron ionization (EI) in GC-MS. However, ESI is sensitive to non-volatile salts, leading to limited use of only volatile mobile phase modifiers (e.g., formic acid, acetic acid, ammonium formate, ammonium acetate) in the chromatography part of the method. Due to the possible occurrence of ion suppression, metabolites with lower affinity for electrons or protons can be masked or undetected when competing for ionization [51].

MS techniques used for analyte detection can be either in a simple MS system with a single mass analyzer or in a tandem MS/MS system with multiple analyzers. These systems fall into low-resolution (LRMS) and high-resolution (HRMS) techniques. The main difference between LRMS and HRMS is their mass accuracy, i.e., the precision in determining the mass. HRMS can reach the accurate mass and increase confidence during metabolite annotation, whereas LRMS can only differentiate compounds based on nominal mass, which can cause false positives for compounds that share mass but are structurally unrelated [52]. Therefore, untargeted metabolomics and lipidomics rely on HRMS and HR-MS/MS using time-of-flight or orbital ion trap analyzers and operating in data-dependent acquisition (DDA) or data-independent acquisition (DIA) modes. In DDA mode, precursor ions above a pre-set threshold are selected using a narrow isolation window, making connecting product and precursor ions easier. However, low-abundance ions can be missed, and the settings are more complex than in DIA, which may lead to errors [53,54]. Conversely, in DIA mode, all precursor ions within the wide isolation window are fragmented, covering more low-abundance ions; however, this results in more complex spectra that are harder to interpret [55]. Tools like MS-DIAL [56], DecoMetDIA [57], and DecoID [58] help to deconvolute these complex MS/MS spectra. For the targeted LC-MS method, LRMS triple-quadrupole (QqQ) and quadrupole/linear ion trap (QLIT) are used, usually operating in a multiple reaction monitoring (MRM) mode to

improve sensitivity and selectivity of monitored ions [50].

In general, untargeted methods provide semi-quantitative data, meaning that the results are reported as peak areas or heights in arbitrary units within the linear dynamic range of the detector. In contrast, targeted methods report quantitative data in molar concentrations [29]. Although quantification is often requested, it is not necessary for many studies since both semi-quantitative and quantitative data can be used for statistical analysis. However, the advantage of quantitative data is that it allows for the immediate distinction between major and minor metabolites and enables direct comparisons of results between laboratories and studies.

Data processing

Properly handling complex datasets produced by metabolomics and lipidomics experiments is crucial, as this process significantly impacts metabolite annotation and quantification, consequently affecting the biological interpretation of results [59]. A standard untargeted metabolomics and lipidomics study can generate hundreds of annotated metabolites and numerous unknown features characterized by retention time and mass-to-charge ratio (*m*/*z*). Data handling can be divided into data processing and data analysis. Data processing uses signal processing methods to refine the raw data and combine them between measurements, converting data into a format that is easier for further analysis. This includes feature detection, chromatogram building, deisotoping, peak alignment, and gap-filling. Data analysis involves examining and interpreting processed data from previous steps, using methods like clustering metabolic profiles or finding key differences between sample groups [59]. Over the last decade, numerous processing tools have been introduced, such as MarkerLynx, MarkerView, MassHunter Profiling, Compound Discoverer, MS-DIAL (Fig. 3A), MZmine, XCMS, MetAlign, GeneDATA, Matlab and R scripts [29].

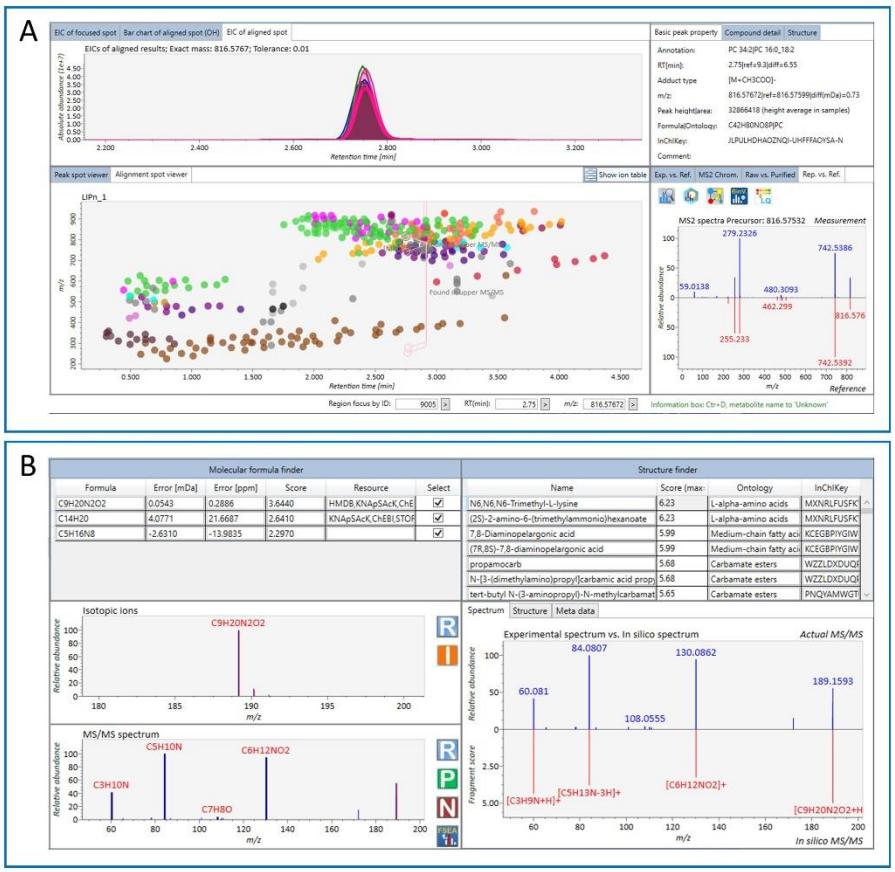

Fig. 3. (**A**) Example of MS-DIAL software [56] used for processing lipidomics data acquired using the RPLC-ESI(–)-MS [41], with annotated PC 16:0_18:2 in human serum. Using ammonium acetate and acetic acid as mobile phase modifiers led to the detection PC 34:2 as an acetate adduct ($[M+CH_3COO]^-$) (*m*/*z* 816.576). The MS/MS spectrum of PC 34:2 provided a fragment ion $[M–CH_3]^-$ (*m*/*z* 742.539) and a series of fragments for elucidating fatty acyl chains (e.g., *m*/*z* 255.233 for 16:0 and *m*/*z* 279.233 for 18:2). The use of the underscore "_" indicates certainty in the composition of the fatty acyl constituents but not their specific placement on the glycerol backbone. (**B**) Example of MS-FINDER software [60] used for the structure elucidation of an unknown compound (*m*/*z* 189.1597, retention time 4.57 min) in human serum acquired using the HILIC-ESI(+)-MS platform [41], with tentative annotation as N^6,N^6,N^6-trimethyl-L-lysine.

Due to the structural variability and diversity of metabolites, detecting and annotating metabolites can be challenging. On average, successful annotation occurs for only approximately 10 % of the molecules, underscoring the importance of accurately identifying most molecular structures [61]. It should also be noted that in LC-MS, each metabolite can be detected in multiple ion forms, which can be annotated if present in spectral libraries or based on accurate mass differences. For instance, phosphatidylcholines (PC) can be detected during lipidomics profiling in positive ESI as $[M+H]^+$ (major peak) and $[M+Na]^+$ (minor peak), while negative ESI provides $[M+CH_3COO]^-$ (major peak in the presence of ammonium acetate in the mobile phase [41]) and $[M+Cl]^-$ (minor peak). Depending on the data processing workflow, various options are possible for reporting these ion forms, such as providing all annotated species separately, species from one ionization mode only (e.g., the one with a lower relative standard deviation in QC samples), or combining adducts (summing peak intensities) for each ionization mode.

The Metabolomics Standardization Initiative (MSI) describes community-based guidelines for reporting and performing metabolomics workflows, proposing four confidence levels [62]: Level 1 – matching based on retention time, MS1, and MS/MS spectrum; Level 2 – matching based on MS1 and MS/MS spectrum; Level 3 – annotation based on matching MS1 accurate mass only; Level 4 – unknown compound characterized by retention time and *m*/*z*. However, multiple researchers have suggested revisions and modifications [63-65]. The Lipidomics Standards Initiative (LSI) has recently been introduced to create standardized lipid species annotations and unify community efforts [66].

The most reliable approach for metabolite annotation represents the use of spectral libraries containing retention time, *m*/*z* (MS1 accurate mass), and MS/MS fragmentation spectra (MSI – Level 1). However, it is virtually impossible to obtain all three pieces of information for every possible metabolite. Thus, commercial or open-access MS/MS libraries (with MS1 precursor ions and MS/MS spectra) are crucial in confident compound annotation in metabolomics and lipidomics (MSI – Level 2). In recent years, spectral libraries and databases have grown in both coverage and diversity [67]. METLIN Gen2 is the most extensive spectral library (metlin.scripps.edu), containing over 900,000 molecular standards and MS/MS data, comprising over 4 million tandem spectra [68]. Other extensive MS/MS libraries include the National Institute of Standards and Technology (NIST) MS/MS library (chemdata.nist.gov) and MassBank of North America (MoNA, massbank.us). Additional resources include MassBank (massbank.jp), ReSpect (spectra.psc.riken.jp), RIKEN PlaSMA (plasma.riken.jp), mzCloud (mzcloud.org), GNPS (gnps.ucsd.edu), MSforID (msforid.com), and HMBD (hmdb.ca).

Furthermore, numerous software and tools have been developed to help annotate unknown compounds, such as MS-FINDER (Fig. 3B), CFM-ID, MetFrag, ChemDistiller, and CSI:FingerID. These tools convert mass data into molecular fragments using combinatorial structure generation techniques and search against existing structures in various databases. Potential candidates can be filtered using additional orthogonal filters based on retention time prediction [69] or hydrogen/deuterium exchange mass spectrometry (HDX-MS) [70,71]. Nevertheless, confirmation should always follow by analyzing an analytical standard under identical instrumental conditions [72].

Bioinformatics

Statistical analysis is essential to properly extract relevant information from the obtained data. Statistical analyses can be categorized as univariate and multivariate methods. Univariate statistical methods include *t*-test, ANOVA, and fold-change analysis to compare different sets of samples. These methods are used for sets of tens to hundreds of metabolites, which increases the chances of false positives [73]. Therefore, correction methods such as Bonferroni correction [74] or the Benjamini-Hochberg [75] false discovery rate should be applied. These corrections have been addressed in multiple studies [74-76]. Commonly used multivariate methods include principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) [77]. A routinely employed web-based platform for comprehensive metabolomics and lipidomics data analysis and interpretation is MetaboAnalyst (metaboanalyst.ca) [78].

Next, the biological relevance of the measured metabolites is interpreted using pathway and enrichment analysis. Enrichment analysis identifies functionally relevant metabolites and links their changes to biological contexts, suggesting key pathways or disease conditions for further study. Pathway analysis, on the other hand, finds pathways that significantly affect specific biological

processes [79]. Both analyses are performed using various software tools such as MetaMapR, MetabNet, GNPS, MS2LDA, MetaboAnalyst, or MetFlow to map the metabolic pathways. New tools, such as an ontology database and enrichment analysis (LION, lipidontology.com) and lipid over-representation analysis (LORA, lora.metabolomics.fgu.cas.cz), are also available to interpret complex lipids [80].

An important part of every experiment is data sharing. Data should be shared following the Findable, Accessible, Interoperable, and Reusable (FAIR) Guiding Principles for scientific data management and stewardship [81]. Public repositories such as Metabolomics Workbench (metabolomicsworkbench.org), MetaboLights (ebi.ac.uk/metabolights), and MassIVE (massive.ucsd.edu/ProteoSAFe/static/massive.jsp) enable data sharing. A newly introduced dynamic checklist (lipidomicstandards.org/reporting_checklist) summarizing key details of lipidomic analyses can be stored or shared in the supporting materials of papers or at a general-purpose open repository Zenodo (zenodo.org).

Recently introduced metabolomics and lipidomics atlases should also serve as open-access resources [29]. These atlases monitor the quantities and relationships of metabolites in different biological matrices, highlighting the importance of reusing and sharing data [82].

Metabolomics and lipidomics for studying metabolic syndrome

In recent years, MetS has become a major health risk with its increasing prevalence, reaching pandemic proportions [83]. The disease affects around 25 % of the global population, making prevention and management essential [84]. Understanding its pathophysiology is crucial in this effort. Metabolomics and lipidomics have been employed to investigate various diseases by identifying diagnostic biomarkers. Recently, research efforts have focused on cardiovascular diseases (CVD), type 2 diabetes (T2D), and metabolic dysfunction-associated steatotic liver disease (MASLD), all of which are associated with MetS. Wishart's comprehensive review in 2019 further underscored the significance of metabolomics studies in understanding physiological and pathophysiological processes [19]. Supplementary Tables S1-S3 overview metabolomics and lipidomics large-cohort studies focusing on CVD, T1D/T2D, and MASLD. Next, we briefly highlight some of these studies to elucidate key findings and advancements, emphasizing how metabolomic and lipidomic profiles have provided deeper insights into disease mechanisms and potential therapeutic targets.

Cardiovascular diseases

CVDs are the leading cause of death globally. In 2022, CVDs caused approximately 19.8 million deaths, accounting for about one-third of all global mortality that year. Major contributors to this toll were ischemic heart disease (9.2 million deaths) and ischemic stroke (3.5 million deaths) [85]. More than three-quarters of CVD deaths occur in low- and middle-income countries, compared to high-income countries, where the CVD death rate has declined [86,87].

CVDs are disorders of the heart and blood vessels, including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism. Heart attacks and strokes are usually considered acute events, primarily resulting from a blockage that obstructs blood flow to the heart or brain [86]. The risk factors for cardiovascular diseases often include an unhealthy diet, physical inactivity, tobacco use, and harmful use of alcohol. These factors can be controlled, reducing the risk of CVD occurrence [86].

One area in CVD research involves exploring the role of different metabolites in disease promotion and progression. For instance, amino acids (alanine, glutamine, glycine, histidine, isoleucine, leucine, lysine, valine, phenylalanine, and tyrosine) have been identified as predictors of incident CVD risks [88-92]. Other discovered biomarkers of CVD are choline, trimethylamine *N*-oxide (TMAO), and betaine [93-96]. Similarly, compounds such as trimethyllysine [97], phenylacetyl glutamine [98], and niacin metabolites (N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide) [99] have been linked to CVD risks. Moreover, in recent studies, the endogenous sugar alcohols erythritol and xylitol were both clinically and mechanistically linked to CVD [100,101].

The association of diet-linked metabolites with CVD has also been explored. Fu *et al.* [102] investigated metabolites connected with a healthy lifestyle and their effect on CVD incidence. They identified and validated 111 metabolites associated with overall lifestyle, 65 of which were related to CVD risk. Healthy lifestyle-linked metabolites were also studied by Lu *et al.* [90]. Diabetes patients free of CVD were divided into groups based on

the healthy level of five lifestyle factors and observed. Adherence to healthy lifestyle factors was associated with 44 plasma metabolites (e.g., 3-hydroxybutyrate, alanine, glutamine, glycine, branched-chain amino acids), and approximately half of them mediated between at least one lifestyle factor and CVD risk. Both studies suggest that a healthy diet positively affects the incidence of CVD.

Additionally, the effects of legume [103] and walnut [104] consumption on CVD risk were researched. Walnut consumption was found to lower the risk of incident CVD and T2D, while legume consumption was associated with a lower risk of T2D but not CVD. Furthermore, gut microbiome-derived metabolites such as *p*-cresol sulfate and indoxyl sulfate have garnered attention [105]. This study shows that these abundant microbiome-derived metabolites have a greater impact on CVD than previously thought. It also suggests targeting the gut microbial pathways that produce *p*-cresol and indole as a potential strategy for treating CVD.

Lipidomics profiling also reveals characteristic lipid signatures associated with increased CVD risk. Harm *et al.* [106] focused on the platelet lipidome of coronary artery disease patients and found alterations in the lipid composition of patients with adverse cardiovascular events. The results showed that the platelet lipidome of CVD patients with increased cardiovascular risk is changed, and specific platelet lipids may indicate adverse events. These findings may help discriminate the individual risk of patients with coronary artery disease. Eichelmann *et al.* [88] investigated associations of plasma lipid alterations with incident cardiometabolic diseases and studied the effect of dietary fat modulation on discovered risk-associated lipids. The results suggest that dietary fat intervention can alter lipids, which may serve as a potential tool for primary disease prevention. Furthermore, Seah *et al.* [107] suggested that certain classes of sphingolipids may also affect CVD risk.

Type 1 & 2 diabetes

As of 2021, the global prevalence of diabetes was estimated at 10.5 % (537 million people), projected to rise to 12.2 % (783 million people) by 2045. Diabetes was responsible for approximately 6.7 million deaths worldwide in 2021, with global healthcare expenditures amounting to approximately USD 966 billion [108]. However, the majority of these cases are attributed to T2D, while T1D affected approximately 8.4 million individuals globally in 2021 [109]. In the future, access to and affordability of insulin may become challenging, particularly in underdeveloped and developing countries, due to the increasing prevalence and incidence of T1D [110].

Diabetes is a complex chronic metabolic disease characterized by high prevalence and mortality, encompassing T1D, T2D, and gestational diabetes occurring during pregnancy. T1D results from insufficient insulin production by the pancreas, necessitating daily insulin administration. T2D arises from inadequate insulin secretion and the body's ineffective use of insulin, leading to elevated blood sugar levels. T2D impacts the metabolism of glucose, lipids, and amino acids [111,112].

Metabolomics and lipidomics studies of T1D aim to identify biomarkers for predicting T1D risk and aiding in early disease detection. Orešič *et al.* [113] analyzed the lipidome profile of cord serum samples to investigate associations between lipid profile changes and β-cell autoimmunity development or clinical T1D. Their study found that progression to T1D correlated with decreased concentrations of major choline-containing phospholipids (sphingomyelins and phosphatidylcholines) in cord blood. The study also indicated that phospholipid reduction is associated explicitly with T1D progression rather than general β-cell autoimmunity.

La Torre [89] and Tapia [90] also studied cord blood samples. La Torre *et al.* [114] discovered that decreased levels of phospholipids at birth, especially phosphatidylcholines and phosphatidylethanolamines, may contribute to early induction of islet autoimmunity and increased T1D risk. Conversely, Tapia *et al.* [115] focused more on changes in the metabolome profile than lipidome alterations. However, the research showed no strong associations of selected polar metabolites with T1D. Nevertheless, Webb-Robertson *et al.* [116] identified multiple metabolites associated with T1D progression by age 6, primarily comprising sugar metabolism compounds such as fructose, levoglucosan, glycerol-α-phosphate, and xylulose.

Recent studies have explored metabolomics' potential in predicting T2D risk based on dietary patterns and corresponding biomarkers. One study involving nearly 6,000 participants identified 29 plasma metabolites associated with inflammatory and insulinemic dietary patterns [117]. The top five biomarkers included PE 36:4, CAR 5:0, PC 34:4, 1-methylguanosine, and N^4-acetylcytidine. Additionally, investigations into the lipid profile of lean and obese individuals with

T2D revealed significant lipidome changes (lyso-, diacyl- and ether-phospholipids, and 1-deoxyceramides), aiding in T2D diagnosis [118].

Lipid profiles containing 69 odd-chain saturated fatty acids (OCFA) among 15 lipid subclasses were also examined for their potential as T2D biomarkers [119], revealing variations dependent on lipid class and sex, correlating with food consumption. Sun *et al.* [120] investigated plasma acylcarnitines' role in early T2D prediction, identifying long-chain acylcarnitines as significantly linked to future T2D risk.

Moreover, interventions targeting weight loss have shown promise in altering metabolite signatures associated with T2D. Studies have noted positive associations between changes in branched-chain amino acids (valine, leucine, isoleucine) and branched-chain ketoacids (α-ketoisovalerate, α-ketoisocaproate, α-keto-β-methylvalerate) with glycated hemoglobin (HbA1c) levels following weight loss [121]. Branched-chain amino acids are frequently studied due to their association with increased T2D risk [122-127]. 3-Hydroxybutyrate is another frequently studied metabolite, often alongside branched-chain amino acids [122-126,128]. Similar to its association with CVD, TMAO has also been investigated in relation to T2D [129]. Lemaitre *et al.* [129] explored the connections of TMAO, carnitine, crotonobetaine, and γ-butyrobetaine with insulin resistance, and betaine and choline with enhanced insulin sensitivity. However, they did not establish a definitive association.

Metabolic dysfunction-associated steatotic liver disease

MASLD is the latest term used to describe steatotic liver disease associated with MetS, encompassing various metabolic risk factors and often coexisting with other chronic liver conditions [130]. Historically, the term nonalcoholic fatty liver disease (NAFLD) was used. In 2020, Eslam *et al.* [131] proposed the term metabolic dysfunction-associated fatty liver disease (MAFLD), which was further modified to MASLD in 2023 [132]. Both MAFLD and MASLD identify patients with hepatic steatosis and metabolic dysfunction [133]. There are slight differences in the definitions of MASLD and MAFLD, which have been discussed in several articles [130,132-134]. Notably, MAFLD encompasses patients with fatty liver regardless of alcohol consumption pattern or amount [132], whereas MASLD introduces the term MetALD for patients who meet alcohol-related fatty liver disease criteria [134]. MASLD diagnosis requires meeting one of five cardiometabolic risk factors [132], while MAFLD requires meeting two out of seven metabolic dysfunction parameters [131]. De *et al.* [135] suggest that MASLD and SLD (steatotic liver disease) criteria may better suit lean patients with NAFLD than MAFLD criteria. Consequently, both MASLD and MAFLD terms are used in literature to classify liver diseases associated with metabolic dysfunction, although NAFLD remains prevalent in many studies since the new nomenclature's introduction.

The global prevalence of NAFLD was estimated to be approximately 30 % between 1990 and 2019, with a continuing upward trend [136]. This increasing prevalence of NAFLD is likely associated with rising rates of diabetes and obesity. However, the global mortality rate declined from 2.39 per 100,000 population in 1990 to 2.09 per 100,000 population in 2019 [137].

MASLD includes a range of steatotic liver conditions, from isolated hepatic steatosis to metabolic dysfunction-associated steatohepatitis (MASH), with varying levels of liver fibrosis that can potentially lead to cirrhosis. MASLD is associated with a higher risk of liver complications (e.g., cirrhosis), end-stage liver disease, and hepatocellular carcinoma, as well as an increased risk of developing extrahepatic issues such as cardiovascular disease (CVD), chronic kidney disease, and certain extrahepatic cancers [138].

Recent large-scale cohort studies aim to identify risk factors and biomarkers for MASLD, aiding in its challenging diagnosis. Commonly identified biomarkers include amino acids, particularly aromatic amino acids (tyrosine, tryptophan) and branched-chain amino acids (isoleucine, leucine, valine) [139-143]. Studies by Hirata [142] and Martínez-Arranz [144] examined the association of NAFLD with cardiovascular risk, identifying metabolomic signatures aligning with known CVD risk factors. Hirata *et al.* [142] found that NAFLD was positively associated with the cardio-ankle vascular index (CAVI), an indicator of subclinical atherosclerosis, and identified ten metabolites involved in both NAFLD and CAVI: branched-chain amino acids (valine, leucine, and isoleucine), aromatic amino acids (tyrosine and tryptophan), alanine, proline, glutamic acid, glycerophosphorylcholine, and 4-methyl-2-oxopentanoate. Martínez-Arranz *et al.* [144] investigated lipidomic profile changes, particularly in triacylglycerols, phosphatidylcholines, and sphingomyelins, providing evidence of distinct metabolic mechanisms associated with NAFLD progression that vary between subtypes.

McGlinchey *et al.* [145] observed lipidomic and metabolomic profile changes across different stages of NAFLD progression, highlighting unique metabolites and 27 common metabolites across all stages, including significant alterations in cholesteryl esters, ceramides, lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamine, sphingomyelins, and triacylglycerols. Hu *et al.* [146] discovered correlations between NAFLD and uric acid, as well as oleic acid-hydroxy oleic acid (OAHOA), identifying OAHOA as a novel biomarker for NAFLD prevalence in a cohort of 1,479 patients (aged 18-80 years). Other studies have explored potential biomarkers, such as anandamide [147] or taurochloric acid [148].

Conclusions

Metabolomics and lipidomics represent effective tools for studying MetS and related disorders. The comprehensive multiplatform-based profiling of polar metabolites and complex lipids in large cohorts has enabled the identification of novel biomarkers and enhanced our understanding of disease mechanisms. Key advancements include the discovery of metabolic signatures associated with CVD, T1D, T2D, and MASLD.

Regarding polar metabolites, branched-chain amino acids (valine, leucine, isoleucine), TMAO, betaine, choline, and 3-hydroxybutyrate have been identified in multiple studies as promising biomarkers. For complex lipids, a panel or combination of affected lipids is expected to be useful as biomarkers, including acylcarnitines, phospholipids, sphingomyelins, and triacylglycerols as key lipid subclasses.

Further research is needed to validate these reported biomarkers in diverse populations and clinical settings, ensuring their robustness and clinical utility. Standardization of experimental protocols and data analysis methods will be critical to facilitate data comparability and reproducibility across studies. Based on a review of multiple studies, we also advocate for the inclusion of authoritative identifiers such as InChI keys or identifiers from bioinformatics resources such as the Human Metabolome Database (hmdb.ca) and LIPID MAPS (lipidmaps.org). This will expedite the comparison of potential biomarkers within studies, making the process faster and more effective.

In addition, further advances in analytical technologies and computational tools will continue to drive innovation in metabolomics and lipidomics, offering new opportunities for early disease detection and personalized therapeutic interventions.

Supplementary Materials

The supporting information (Suppl Table S1-S3) can be downloaded at <link>.

Conflict of Interest

There is no conflict of interest.

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Metabolomics and Lipidomics for Studying Metabolic Syndrome: Insights into Cardiovascular Diseases, Type 1 & 2 Diabetes, and Metabolic Dysfunction-Associated Steatotic Liver Disease

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Supplementary Materials

Table S1. Metabolomics and lipidomics cohort studies focused on cardiovascular disease

Table S2. Metabolomics and lipidomics cohort studies focused on type 1 and type 2 diabetes

Table S3. Metabolomics and lipidomics cohort studies focused on metabolic dysfunction-associated steatotic liver disease

Table S1. Metabolomics and lipidomics cohort studies focused on cardiovascular disease

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Discovery cohort: n = 4,824 (27.8% female) Replication cohort: n = 1,716 (56.3% female)	Participants with 15.8 years follow-up	Serum	NMR	41	Isoleucine Leucine Phenylalanine Glycerol Cholesterol Total lipid concentrations, Glycerides and other Phospholipids, Fatty acids, Fatty acids ratios — see the original paper.	• Association with adherence to dietary recommendations provided by the Alternative Healthy Eating Index	[1]
European cohort: n = 352 USA cohort: n = 1,777	European participants (100% Caucasian) with either abdominal aortic aneurysm or sub-aneurysmal aortic dilations, and healthy non-aneurysm subjects US participants (96% Caucasian) with abdominal aortic diameter of 3.0 cm or greater, and subjects with history of dilated aorta with measurements of abdominal aortic diameter less than 3 cm or no prior aortic aneurysm, and no MI, stroke or death over the following 3 years	Plasma	LC-MS/MS	3	Choline Trimethylamine *N*-oxide Trimethylamine	• Association of elevated TMAO with increased abdominal aortic aneurysm incidence	[2]
Low-risk cohort: n = 620 Borderline-risk cohort: n = 110 Intermediate-risk cohort: n = 225 Highrisk cohort: n = 147 (53.3% female)	Participants with LDL levels less than 190 mg/dl and no pre-existing coronary artery disease or myocardial infarction	Plasma	LC-MS/MS	50	Alanine Arginine Aspartic acid CAR 4:0-DC CAR 8:1 CAR 16:0-OH Citrulline Glutamic acid Glutamine Glycine Histidine Phenylalanine Threonine Tryptophan	• Association with the 10-year ASCVD risk score • Identification of metabolic pathways associated with the development of 10-year ASCVD events	[3]
EPIC-Potsdam Study cohort: Common reference subcohort: n = 1262 T2D subcohort: n = 1886 (775 incident cases) CVD subcohort: n = 1671 (551 incident cases) **DIVAS study cohort:** CVD risk subcohort: n = 113 (on 3 different isoenergic diets)	General population Patients with estimated moderate CVD risk	Plasma	DMS-MS/MS	282	CE 20:3 DG 16:0 DG 18:0 FA 15:0 FA 20:4 LPC 18:2 MG 15:0 MG 20:4 PC 20:3 PE 20:3 TG 16:0 TG 18:0 TG 18:2 TG 18:3 TG 22:1	• Association with cardiometabolic disease risk and T2D risk • Dietary fat intervention as a potential tool for primary disease prevention	[4]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Discovery cohort: n = 1,162 (36.3% female) Validation cohorts: n = 2,331 (US, 33.2% female), n = 832 (European, 29.9% female)	Stable participants undergoing elective diagnostic cardiac evaluation	Plasma	LC-MS/MS	5	N^1-Methyl-2-pyridone-5-carboxamide N^1-Methyl-4-pyridone-3-carboxamide Phenylacetylglutamine Trimethylamine *N*-oxide Trimethyllysine	• Association of terminal breakdown products of excess niacin with residual CVD risk	[5]
Phase I: Discovery cohort: n = 3,613 Validation cohorts: n = 121,733 **Phase II:** n = 118,120	UK Biobank participants have undergone a wide range of physical measures, provided information on their lifestyle and medical history (follow-up)	Plasma	NMR	111	Multiple markers – see the original paper.	• Association with a healthy lifestyle • Association of healthy lifestyle-associated metabolites with coronary artery disease (CAD)	[6]
Discovery cohort: n = 1,028 Validation cohort: n = 1,670	Discovery cohort: Participants free of coronary heart disease (10 years follow-up)	Plasma	LC-MS/MS	32	LPC 18:1 LPC 18:2 MG 18:2 SM d28:1	• Association of MG 18:2 with coronary heart disease • Association of LPCs with body mass index, C-reactive protein and with less evidence of subclinical CVD	[7]
Discovery cohort: n = 1,833 (57% female) Validation cohorts: n = 1,522 Low walnut intake subcohort: n = 691 High walnut intake subcohort: n = 467	Participants at high cardiovascular risk	Plasma	LC-MS	385	4-Hydroxy-3-methylacetophenone Cyclohexylamine Guanine Isocitric acid *N*-Acetylaspartic acid Piperine Serine Sorbitol Succinic acid Bilirubin Biliverdin CAR 10:2 LPC 14:0 LPC 16:1 MG 22:1 PC 36:4 PE 36:5 PS 40:6 TG 54:6	• Association of walnut consumption with a lower risk of incident T2D and CVD in a Mediterranean population at high cardiovascular risk	[8]
Study cohort: n = 1,057	Participants with symptomatic coronary artery disease	Blood-platelets	LC-MS/MS	767	CAR 10:0 CAR 14:0 CAR 14:1 CAR 16:0 CAR 16:1 FA 18:1 FA 18:2 FA 18:2;2O LPE 18:1|LPE 0:0/18:1 LPE 18:1|LPE 18:1/0:0 LPE 18:2|LPE 0:0/18:2 LPE 18:2|LPE 18:2/0:0 LPE 20:1|LPE 20:1/0:0 LPE 20:3|LPE 0:0/20:3 LPE 20:3|LPE 20:3/0:0 LPE 20:4 LPE 20:5 LPE 22:4|LPE 0:0/22:4 LPE 22:4|LPE 22:4/0:0 LPE 22:5	• Association of adverse cardiovascular events with alterations in the platelet lipidome	[9]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
					LPE 22:6 LPS 18:1|LPS 0:0/18:1 PC 34:2;O PE 34:3|PE 16:1_18:2;O PI 36:4|PI 16:0_20:4 PI 38:5|PI 18:1_20:4 TG 48:1|TG 14:0_16:0_18:1 TG 48:2|TG 16:0_14:1_18:1		
Study cohort: n = 1,021 (48.3% female)	Participants with T2D and were followed up for CVD over the subsequent 10 years	Serum	NMR	228	3-Hydroxybutyric acid Acetic acid Creatinine Glycine Lactic acid Leucine Phenylalanine	• Association with 10-year cardiovascular risk in people with type 2 diabetes • Metabolite-based risk score created	[10]
Malmo Diet and Cancer-Cardiovascular cohort: n = 4,067	General population followed up to 23 years and stratified into risk groups	Plasma	DI-MS/MS	184	*Sum of lipid subclasses:* Ceramide Cholesteryl ester Cholesterol Diacylglycerol Ether-phosphatidylcholine Ether-phosphatidylethanolamine Lysophosphatidylcholine Lysophosphatidylethanolamine Phosphatidylcholine Phosphatidylethanolamine Phosphatidylinositol	• Possible identification of lipidomic risk before disease incidence (CVD and T2D)	[11]
Discovery cohort 1: n = 99 Discovery cohort 2: n = 1,162 Validation cohort: n = 2,140	Sequential stable subjects without evidence of acute coronary syndrome undergoing elective diagnostic coronary angiography for evaluation of CAD with longitudinal (3–5 years) follow-up	Plasma	HILIC-MS/MS LC-MS/MS		Trimethyllysine Trimethylamine *N*-oxide	• Association with CVD risks	[12]
Study cohort: n = 2,278 (50% female)	Participants were followed up for CVD incident (almost 10 years)	Plasma	LC-MS/MS	790 (37)	Dimethylglycine *N*-Acetylmethionine (top findings)	• Association with CVD risks	[13]
Study cohort: n = 5,072	Participants with diabetes	Plasma	NMR	44	3-Hydroxybutyric acid Acetic acid Acetoacetatic acid Acetone Alanine Citric acid Creatinine Glucose Glutamine Glycine Histidine Isoleucine Lactic acid Leucine Phenylalanine Pyruvic acid Tyrosine Valine	• Association of multiple healthy lifestyle factors with improved circulating metabolites from different pathways	[14]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Discovery cohort: n = 1,833 (57.6% female) Validation subcohort: n = 1,522	Participants at high risk of CVD (1-year follow-up)	Plasma	LC-MS/MS	382	1-Methylguanine γ-Aminobutyric acid (GABA) Aminoisobutyric acid Asparagine Cortisol Creatine Cytosine Glycodeoxycholic acid Hippuric acid Homoarginine Hypoxanthine Lactic acid Lysine N^1-Acetylspermidine *N*-Acetylaspartic acid *N*-Acetylornithine *N*-Carbamoyl-β-alanine Piperine Pyroglutamic acid Sorbitol Sucrose Trimethylbenzene CAR 7:0 CAR 18:2 CAR 18:0 DG 34:3 DG 36:0 LPC 16:1 MG 22:1 PC 34:3 PC 36:4 PC 38:4 PE 32:0 PE 38:6 PE 40:7 SM d34:2|SM d18:1/16:1 TG 50:3 TG 50:4 TG 55:2 TG 56:2	• Association of legume consumption with T2D incidence, but not with CVD incidence risk	[15]
Study cohort: n_1 = 5,991; n_2 = 3,779 (38.9% female)	Participants with an 8-year follow-up	Plasma	LC-MS/MS	342	CE 24:0 LPI 18:2 PC 38:5 PC O-34:2 PC O-36:1 PC P-40:6 PE 38:6 PI 38:3 SM d42:1	• Association with future cardiovascular events and cardiovascular death	[16]
Discovery cohort: n = 1,162 Validation cohort: n = 4,000	Sequential stable subjects undergoing elective diagnostic cardiac evaluation with longitudinal (3 years) follow-up	Plasma	HILIC-MS LC-MS/MS	5 (top-ranked)	Phenylacetylglutamine	• Association with cardiovascular disease and death in humans	[17]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Discovery cohort: n = 1,149 Validation cohort: n = 3,954	Participants with preserved kidney function undergoing elective diagnostic cardiac evaluation with longitudinal follow-up (5 years)	Plasma	GC-MS LC-MS/MS	N/A	*p*-Cresol sulfate Indoxyl sulfate	• Association with CVD risk and overall mortality	[18]
Study cohort: n = 2,627	Participants were invited to attend a health examination for additional tests and collection of 8–12 h fasting blood samples (mean 12.9 years follow-up)	Plasma	HILIC-MS LC-MS/MS	79	Hex2Cer d34:2|Hex2Cer d18:2/16:0 HexCer d36:1|HexCer d18:1/18:0 HexCer d34:1|HexCer d18:1/16:0 HexCer d42:2|HexCer d18:2/24:0 SM d34:1|SM d18:1/16:0 SM d36:1|SM d18:1/18:0 SM d36:2|SM d18:2/18:0 SM d42:1|SM d18:1/24:0	• Association with higher CVD risk	[19]
Study cohort: n_1=50; n_2=4,007	Healthy participants before and after the suppression of intestinal microbiota with oral broad-spectrum antibiotics underwent phosphatidylcholine challenge (ingestion of two hard-boiled eggs and deuterium [d_9]-labeled phosphatidylcholine) Participants undergoing elective diagnostic cardiac catheterization with no history of acute coronary syndrome	Plasma	LC-MS/MS	3	Betaine Choline Trimethylamine *N*-oxide	• Association among intestinal microbiota-dependent metabolism of dietary phosphatidylcholine, TMAO levels, and adverse CVD events	[20]
Discovery cohort: n = 3,867 Validation cohort: n = 3,569	Participants were free of known CVD at baseline	Serum	NMR	N/A	1,5-Anhydrosorbitol 1-Methylhistidine 3-Hydroxybutyric acid 5-Oxoproline Acetaminophen + glucuronide Alanine Aspartic acid Citratic acid Glucose Glutamatic acid Glutamine Glycerol Glycine Histidine Lactic acid Lysine Mannose Methionine myo-Inositol Dimethylglycine Phenylalanine Glyceryl groups of lipids Lipids (CH2-CO) Lipids (CH2-CH2-C=, CH2-CH2-CO) Lipids (CH2-CH2-CH=CH) Lipids (CH3-CH2-R, (CH2)n) Lipids (CH3-CH2-R, CH3-CH2-C=)	• Association with atherosclerosis and incident CVD	[21]
Discovery cohort: n = 50 Validation cohort: n = 25	Stable patients undergoing elective cardiac evaluation who subsequently experienced a heart attack, stroke or death over the ensuing three-year period vs. age- and gender-matched subjects who did not	Plasma	LC-MS/MS	18	Betaine Choline Trimethylamine *N*-oxide	• Identification of markers as predictors of CVD risk • Discovery of a relationship between gut-flora-dependent metabolism of dietary	[22]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
						phosphatidylcholine and CVD pathogenesis	
Discovery cohort: n = 1,157 Validation cohorts: n_1 = 2,149; n_2 = 833	Stable subjects undergoing cardiac risk assessment Healthy volunteers (n = 8)	Plasma	GC-MS LC-MS/MS	N/A	Creatinine Erythritol Xylitol	• Association with major adverse cardiovascular event	[23]
Discovery cohort: n = 1,157 Validation cohort: n = 2,149	Stable subjects undergoing elective diagnostic cardiac evaluations Healthy volunteers (n = 10)	Plasma	GC-MS LC-MS/MS	N/A	Creatinine Erythritol Xylitol	• Association with major adverse cardiovascular event	[24]
Discovery cohort: n = 7,256 Validation cohorts: n_1 = 2,622; n_2 = 3,563	Participants were followed up for CVD incident (15 years)	Serum	NMR	68	3-Hydroxybutyric acid Acetic acid Acetoacetatic acid Alanine Citratic acid Glucose Glutamine Glycerol Glycine Histidine Isoleucine Lactic acid Leucine Phenylalanine Pyruvic acid Tyrosine Valine Docosahexaenoic acid (FA 22:6) Linoleic acid (FA 18:2) Monounsaturated FA Omega-3 FA Omega-6 FA Polyunsaturated FA Saturated FA	• Association with incident CVD	[25]
Study cohort: n = 4,007	Participants undergoing elective diagnostic cardiac catheterization with no history of an acute coronary syndrome	Plasma	LC-MS/MS	18	Choline Trimethylamine Trimethylamine *N*-oxide	• Discovery of increased levels of TMAO as a predictor of incident risk for thrombotic events • Association between specific dietary nutrients, gut microbes, platelet function, and thrombosis risk	[26]

Table S2. Metabolomics and lipidomics cohort studies focused on type 1 and type 2 diabetes

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Study cohort: n = 170 (48% female)	Children with high genetic risk for T1D	Plasma	GC-MS LC-MS/MS	91	γ-Aminobutyric acid (GABA) Glycine Tagatose Arabitol myo-Inositol Adipic acid Cer d38:1 Cer d39:1 LPC 18:3 LPC 20:3 LPC 20:5 SM d41:2	• Utilization of multi-omics data for the modeling of complex, multifactorial diseases, like T1D	[27]
Study cohort: n = 152 (47.4% female)	Children with T1D (n=76) and healthy control children (n=76)	Cord blood serum	LC-MS/MS	106	PC 32:1 PC 36:4 PC 38:4 PC 38:5 PC 38:5 PC 38:6 PC 40:4 PC 40:5 PC 40:5 PC 40:7 PC 40:8 PC sum PE 38:4 PE 38:4 PE 40:4	• Cord-blood metabolic patterns may be a valuable measure of type 1 diabetes risk	[28]
Study cohort: n = 101 (37.6% female)	Children who progressed to T1D (PT1D; n = 30), children who developed at least one islet autoantibody but did not progress to T1D during the follow-up (P1Ab; n = 33), and their age-matched controls (CTR; n = 38)	Cord blood plasma	LC-MS/MS	232 lipid species	CE 18:2 TG 46:2 TG 46:2 TG 48:1 TG 51:3	• Identification of lipids that can be predictive of the risk of progression to T1D • Comparison of lipidomic profiles of all subcohorts	[29]
Study cohort: n = 120	Children progressed to T1D; children developed at least a single islet autoantibody but did not progress to T1D during the follow-up; matched controls	Plasma	LC-MS/MS	45	CE 20:5 PC 33:0 TG 54:4|TG 18:2_18:1_18:1 TG 56:5	• Children who progress to T1D in the follow-up tend to have a distinct and persistently dysregulated lipid profile as compared to those who later progress to islet autoimmunity but not to T1D	[30]
Study cohort: n = 120	Progressors to T1D (n = 40); children tested positive for at least one antibody in a minimum of two consecutive samples but did not progress to clinical T1D during the follow-up (n = 40); control children remained islet autoantibody-negative during the follow-up (n = 40)	Plasma	GC-MS	94	2-Ketoisocaproic acid 3,4-Dihydroxybutanoic acid Aspartic acid Bisphenol A Glutamic acid Glycerol-2-phosphate Levoglucosan Malic acid Methionine Pyruvic acid	• Association of unique metabolomic profile with T1D	[31]
Study cohort: n = 2,124	Children with high genetic risk for T1D	Plasma	GC-MS LC-MS/MS	357	5-Methoxytryptamine Alanine Glutamic acid Isoleucine Leucine	• Studying autoantibodies and metabolomic markers, which are associated with the risk of progression to T1D	[32]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
					Methionine Proline Valine Vitamin E α-Ketoglutaric acid		
Study cohort: n = 166	T1D patients (n = 85) and healthy controls (n = 81). All patients had a stable dose of insulin usage for more than 3 months (dose change <10%)	Serum Urine	LC-MS/MS	54 (serum) 45 (urine)	4-(2-Aminophenyl)-2,4-dioxobutanoic acid 4-Pyridoxic acid 5-Hydroxytryptophan 5-Methoxyindole-3-acetic acid Hypoxanthine Thromboxane B3	• Identification of altered metabolic profiles in T1D individuals with different time in range (TIR)	[33]
Study cohort: n = 286	Infants later developed T1D (n=33); infants developed different numbers of islet autoantibodies during the follow-up (n=110); controls matched for sex, HLA-DQB1 genotype, city of birth, and period of birth (n=143)	Cord blood serum	LC-MS/MS	137	PC 32:0|PC 16:0_16:0 PC 32:1|PC 16:0_16:1 PC 34:1|PC 16:0_18:1 PC 34:3|PC 16:0_18:3 PC 36:1|PC 18:0_18:1 PC 38:3|PC 18:0_20:3 SM d34:1|SM d18:1/16:0 SM d36:1|SM d18:1/18:0 SM d38:0|SM d18:0/20:0 SM d38:1|SM d18:1/20:0 SM d42:1|SM d18:0/24:1 SM d42:2|SM d18:1/24:1 SM d42:2|SM d18:0/24:2 SM d42:3|SM d18:2/24:1	• Association with high risk for progression to T1D	[34]
Study cohort: n = 343	Children, who later developed type 1 diabetes (n=166), and random control children in the Norwegian Mother, Father, and Child cohort (n=177)	Cord blood plasma	LC-MS/MS	27	Aminoadipic acid Indoxyl sulfate Tryptophan	• Association with T1D	[35]
Study cohort: n = 655	Children with high genetic risk for T1D	Plasma	GC-MS	139	Ascorbic acid Piperidone	• Association with progression to T1D	[36]
Study cohort: n = 141	Children with T1D (n=76) and gender- and age-matched healthy controls (n=65)	Serum	GC-MS	70	1,5-Anhydroglucitol Adenine Fructose Glycerol-α-phosphate Inosine Levoglucosan Pyruvic acid Uridine Xylulose	• Association with T1D and with the duration of the disease	[37]
Study cohort: n = 11,896	Participants from four prospective population-based cohorts in Finland (follow-up for 7.8–15 years)	Serum	NMR	229	3-Hydroxybutyric acid Acetatic acid Acetoacetatic acid Citratic acid Creatinine Glutamine Glycerol Glycine Histidine Isoleucine Lactic acid Leucine Phenylalanine Pyruvic acid	• Association with risk of developing diabetes • Association with deterioration in post-load glucose and insulin resistance than with future fasting hyperglycemia	[38]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
					Tyrosine Valine		
Study cohort: n = 1,016	General population	Plasma	NMR	49	3-Hydroxybutyric acid Acetatic acid Alanine Citratic acid Creatine Creatine phosphate Creatinine Cysteine Glutamine CH2CH2CO- CH2N- Isobutyratic acid Isopropanol Leucine *N*-Acetylglutamine *O*-Phosphoethanolamine Phenylpropionic acid Proline Pyruvic acid	• Strong inverse association of healthy lifestyle with incident T2D	[39]
Study cohort: n = 1,138	Participants from four prospective population-based cohorts	Plasma	LC-MS/MS	70	2-Methylbutyroylcarnitine Cortisol Deoxycholic acid Tyrosine γ-Glutamyl-leucine Barogenin CerPE 38:2 LPC 20:2 MG 18:2 PC 42:7 SM d33:1 SM d34:2 SM d36:3|SM d18:2/18:1	• Association with incident T2D	[40]
Study cohorts: n_1 = 1,261; n_2 = 2,580	Clinically healthy participants (follow-up for 3 years)	Plasma	LC-MS/MS	N/A	2-Hydroxybutyric acid LPC 18:2	• Association with insulin resistance and glucose intolerance	[41]
Study cohort: n = 2,282 Incident T2D cohort: n = 800	General population	Serum	FI-MS	163	Glycine Hexose Phenylalanine LPC 18:2 PC O-34:3 PC O-40:6 PC O-42:5 PC O-44:4 PC O-44:5 PC O-32:1 PC 36:1 PC 38:3 PC 40:5 SM d34:2|SM d18:1/16:1	• Association with increased or decreased risk of T2D	[42]
Study cohort: n_1 = 1,813; n_2 = 451	1,813 participants without any signs of T2D 451 participants with newly diagnosed T2D	Serum	FI-MS LC-MS/MS	134	Alanine/glycine	• Association of analine/glycine ration with T2D	[43]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Study cohort: n = 5844 (90% female)	Female and male nurses	Plasma	LC-MS/MS	186	1-Methylnicotinamide 1-Methylguanosine Aminoisobutyric acid Caffeine CAR 2:0 CAR 5:0 CAR 5:0-DC Cortisone Dimethylglycine Guanidoacetic acid N^2,N^2-Dimethylguanosine N^4-Acetylcytidine *N*-Acetylspermidine *N*-Acetyltryptophan *N*-Carbamoyl-β-alanine Piperine Ribothymidine Tryptophan Biliverdin Cer d34:1|Cer d18:1/16:0 LPE 18:2 PC 34:2 PC P-34:4 PC P-38:4 PE 36:4 PE P-36:2 SM d38:1|SM d18:1/20:0	• Association between inflammatory and insulinemic dietary patterns, plasma inflammatory/insulin biomarkers, plasma metabolomics and risk of type 2 diabetes.	[44]
Study cohort: n = 2240	T2D participants, prediabetes participants, and normal glucose tolerance participants	Serum	FI-MS LC-MS/MS	123	Glycine CAR 16:0 LPC 18:2 PC O-36:0	• Association with incident T2D	[45]
Study cohort: n = 4,442 (61% female)	Participants without diabetes at baseline	Plasma	LC-MS/MS	6	Betaine Carnitine Choline Crotonobetaine γ-Butyrobetaine Trimethylamine *N*-oxide	• Association with incident T2D	[46]
Study cohort: n = 1571	Healthy participants (follow-up for 14 years)	Plasma	NMR LC-MS	24	1,5-Anhydroglucitol 2-Hydroxybutyric acid 2-Oxoglutaric acid Glycerol Glycine betaine Isoleucine Lactic acid Methionine Pyruvic acid Tyrosine PC 34:2;O TG 48:0 TG 48:1 TG 50:5	• Increase of the long-term prediction performance in combination with classical measurements	[47]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Discovery cohort: n = 3,821 Validation cohort: n = 14,651	Participants with normal glucose regulation	Serum	LC-MS/MS	667 (discovery cohort) 250 (validation cohort)	CE 14:0 LPI 16:1 PC 34:3 PE 38:4|PE 18:0_20:4 TG 48:1 (16:0) TG 48:1 (16:1) TG 48:2 (16:0) TG 48:2 (16:1) TG 48:2 (18:1) TG 48:3 (16:1) TG 50:0 (18:0) TG 50:1 (16:0) TG 50:1 (16:1) TG 50:1 (18:0) TG 50:2 (16:0) TG 50:2 (16:1) TG 50:2 (16:2) TG 50:2 (18:1) TG 50:3 (16:0) TG 50:3 (16:1) TG 50:3 (16:2) TG 51:0 (17:0) TG 51:2 (17:0) TG 51:3 (17:1) TG 53:2 (19:0) TG 53:3 (16:0) TG 54:3 (16:0) TG 54:3 (16:0) TG 54:4 (16:0) TG 54:4 (16:1) TG 54:5 (16:0) TG 54:5 (16:1) TG 54:6 (20:4) TG 54:7 (20:4) TG 54:7 (22:6) TG 55:6 (19:3) TG 56:5(18:1) TG 56:5(22:4) TG 56:6(22:5)	• Association of biomarkers and lipid pathway dysregulation with T2D onset	[48]
Study cohort: n = 2,204 (100% female)	Participants with T2D or impaired fasting glucose + normoglycemic control participants	Plasma Urine	LC-MS/MS GC-MS	447	2-Hydroxybutyric acid 1,5-Anhydroglucitol Arabinose Citrulline Dimethylarginine Erythritol Fructose Glucose Isoleucine Lactic acid Leucine Malic acid Mannose *N*-Acetylglycine Octanoylcarnitine Proline	• Association with incident T2D and IFG	[49]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
					Uric acid Valine 10-Heptadecenoic acid (FA 17:1n7) 15-Methylpalmitic acid (FA iso-17:0) 3-Methyl-2-oxobutanoic acid 3-Methyl-2-oxovaleric acid 4-Methyl-2-oxopentanoic acid 5-Dodecenoic acid (FA 12:1n7) Adrenic acid (FA 22:4n6) Arachidonic acid (FA 20:4n6) Cholesterol Heptanoic acid (FA 7:0) Myristic acid (FA 14:0) Myristoleic acid (FA 14:1n5) Palmitoleic acid (FA 16:1n7) SM d34:1|SM d18:1/16:0 Pelargonic acid (FA 9:0) Pentadecanoic acid (FA 15:0)		
Study cohort: n = 1,150	Participants with normal fasting glucose (follow-up for 20 years)	Plasma	LC-MS/MS	N/A	5-Hydroxyindoleacetic acid Glucose Glycine Isocitric acid Phenylalanine Taurine 2-Aminodipic acid 3-Methyladipic acid CE 20:3 DG 36:1 LPC 18:1 LPC 18:2 PC 36:4 SM d42:1|SM d18:1/24:0 TG 48:0 TG 48:1 TG 52:1 TG 54:8 TG 58:11	• Association with improved prediction of T2D beyond conventional risk factors	[50]
Discovery cohort: n = 543 Validation cohort: n = 1,044	Non-diabetic participants (follow-up)	Serum	LC-MS/MS GC-MS	568	2-Hydroxybutyric acid Bilirubin Glucose Glutamic acid Glutamine Histidine Isoleucine Mannose Trehalose Valine α-Tocopherol	• Association with positive or negative impact on progression to T2D	[51]
Study cohort: n = 1,248	Participants with 6.5 years follow-up	Plasma	DMS-MS/MS GC-MS	N/A	*Lipid classes containing species with FA 15:0 and FA 17:0:* CE 15:0 CE 17:0 DG 15:0 FA 15:0	• Association with incident T2D	[52]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
					FA 17:0 LPC 15:0 LPC 17:0 LPE 17:0 MG 15:0 MG 17:0 PC 15:0 PC 17:0 PE 17:0 PL-OCFA (phospholipid species containing odd-chain fatty acids) TG 15:0 TG 17:0		
Study cohorts: n_1 = 1,039; n_2 = 520	Participants with mean follow-ups: 4.61 and 7.57 years	Plasma	LC-MS/MS	166	CE 16:1 LPC 15:0 LPC 18:2 PC 33:3 PC 35:3 PC 40:7 PC 43:6 PC 44:1 SM d34:2 SM d41:2 TG 46:1 (12:0) TG 48:1 (16:0) TG 48:2 (14:0) TG 49:7 (16:0) TG 50:1 (16:0) TG 50:2 (16:0) TG 50:3 (18:1) TG 51:7 (16:0) TG 52:5 (18:2) TG 52:6 (18:2) TG 54:3 (18:0) TG 54:4 (18:2) TG 54:5 (18:2) TG 54:6 (18:2) TG 54:7 (18:3) TG 56:5 (20:4)	• Association with incident T2D	[53]
Study cohort: n = 2,939	Participants without diabetes prevalence	Serum	LC-MS/MS	245	3-(4-Hydroxyphenyl)lactic acid Asparagine Erythritol Isoleucine Leucine Trehalose Valine	• Association with incident T2D (protective biomarker of diabetes risk)	[54]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Study cohort: n = 2,103	Participants with a 6-year follow-up	Plasma	LC-MS/MS	34	Carnitine 3-Dehydroxycarnitine 3-Dehydrocarnitine CAR 2:0 CAR 3:0 CAR 3:0-DC CAR 4:0 CAR 5:0 CAR 5:0-OH CAR 5:1 CAR 6:0 CAR 6:0-OH CAR 6:0-DC CAR 7:0-DC CAR 8:0 CAR 8:1 CAR 10:0 CAR 10:0-DC CAR 12:0 CAR 12:0-OH CAR 12:1 CAR 12:0-DC CAR 14:0 CAR 14:0-OH CAR 14:1-OH CAR 16:0 CAR 16:1 CAR 16:2 CAR 18:0 CAR 18:0-OH CAR 18:1 CAR 18:2 CAR 20:0 CAR 20:4	• Association with improved predictive ability for type 2 diabetes beyond conventional risk factors	[55]
Study cohort: n = 3,234	Participants were assigned to 1) intensive lifestyle, 2) metformin, or 3) placebo (all followed up for 3.2 years)	Plasma	HILIC-MS/MS	84	Betaine Methionine sulfoxide Serine	• Association with incident T2D	[56]

Table S3. Metabolomics and lipidomics cohort studies focused on metabolic dysfunction-associated steatotic liver disease

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Study cohort: n = 121,032	Participants with a mean 12.6-year follow-up	Plasma	NMR	170	3-Hydroxybutyric acid Acetic acid Acetoacetatic acid Acetone Alanine Citric acid Creatinine Glucose Glutamine Glycine Histidine Isoleucine Lactic acid Leucine Phenylalanine Pyruvic acid Tyrosine Valine Docosahexaenoic acid (FA 22:6) Linoleic acid (FA 18:2) Omega-3 FA Omega-6 FA	• Positive and negative association with MASLD	[57]
Study cohort: n = 10,809	Participants with and without MASLD	Plasma	NMR	123	Tyrosine	• Association with MASLD	[58]
Study cohort: n = 3,048	Participants have been followed up since birth, including questionnaires and clinical assessments starting from age 7 years	Plasma	NMR	154	3-Hydroxybutyric acid Acetic acid Acetoacetatic acid Alanine Creatinine Glutamine Histidine Isoleucine Leucine Phenylalanine Tyrosine Valine	• Association with incident MASLD	[59]
Study cohort: n = 928 (67% female)	Participants with and without MASLD	Plasma	CE-MS	94	4-Methyl-2-oxopentanoic acid Alanine Glutamic acid Isoleucine Leucine Proline Tryptophan Tyrosine Valine Glycerophosphorylcholine	• Association with both MASLD and cardio-ankle vascular index (CAVI)	[60]
Study cohort: n = 1,479 Study subcohort: n = 447 (known age)	Participants were not treated for cancer or infectious disease or had undergone surgery in the previous year, and they had no history of cancer or an infectious disease.	Serum	LC-MS/MS	N/A	Oleic acid-hydroxy oleic acid (OAHOA) Sphingosine Uric acid	• Association with MASLD	[61]
Study cohort: n = 997 (53% female)	Participants free of prevalent myocardial infarction or congestive heart failure at the first examination cycle	Plasma	HILIC-MS/MS	179	Anandamide	• Association with MASLD severity, the presence of nonalcoholic steatohepatitis, and fibrosis	[62]

Subjects (n)	Cohort	Matrix	Platforms	Reported metabolites (n)	Markers	Outcomes	Ref.
Study cohort: n = 559	Participants with and without MASLD	Plasma	LC-MS/MS	11	Dihydrothymine Serine Tryptophan LPC 18:1 LPE 20:0	• Screening tool for MASLD	[63]
Study cohort: n = 1,154 (50% female) Control cohort: n = 350	Participants with biopsy-proven MASLD and participants from the general population with similar gender and age to the cohort of patients with MASLD	Serum	LC-MS NMR	105	PC 32:0|PC 16:0_16:0 PC 32:2|PC 14:0_18:2 PC 34:2|PC 16:0_18:2 PC 36:1|PC 18:0_18:1 PC 36:3 PC 36:6|PC 18:3_18:3 PC 37:5 PC 38:2|PC 20:0_18:2 PC 38:3|PC 18:0_20:3 SM d32:1 SM d39:1 TG 48:3	• Identification of three MASLD subgroups, independent of histological disease severity	[64]
Study cohort: n = 627	Histologically characterized participants. Participants include the full spectrum of disease, from histologically normal liver tissue through NAFL to NASH-F4 (cirrhosis)	Serum	LC-MS/MS GC-MS/MS	211	*Markers of fibrosis 0–1 vs. 2–4:* 2-Hydroxybutyric acid 3-Hydroxybutyric acid LPC O-16:0 LPC P-16:0 LPC 18:2 LPC 20:4 Oleic acid PC 32:0|PC 16:0/16:0 PC 32:1 PC 37:4 PC O-34:2 PC O-34:3 PE 16:0/18:1 PE 34:2 PE 38:6 SM d42:1|SM d18:1/24:0 SM d36:0 SM d41:1 TG 56:4 TG 58:6	• Identification of a key metabolic 'watershed' in the progression of liver damage, separating severe disease from mild	[65]
Discovery cohort: n = 1,546 Internal validation cohort: n = 377 Prospective validation cohort: n = 749	Participants with and without MASLD (4 years follow-up	Feces	LC-MS/MS	198	Taurocholic acid	• Positive association with both a higher microbiome risk score and MASLD risk	[66]

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