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REVIEW ARTICLE

Human Blood Plasma Lipidome: Opportunities and Prospects of Its Analysis in Medical Chemistry

T. I. Torkhovskaya[a, b, 1], T. S. Zakharova[a], E. I. Korotkevich[a], O. M. Ipatova[a], and S. S. Markin[a]

[a] *Institute of Biomedical Chemistry, Moscow, 119121 Russia*

[b] *Scientific Research Institute of Physical–Chemical Medicine, Moscow, 119435 Russia*

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Abstract—The emerging development of new analytical mass spectrometry technologies resulted in the possibility of a detailed fractionation of biologically important compounds. As applied to lipids, it promoted the development of opportunities for identification of the whole set of lipid molecular species, or the lipidome of a biological object. The review summarizes approaches to and results of human blood plasma lipidome analysis in medical studies. The main principal approaches of MS lipid analysis are considered, including targeted lipidomics after preliminary HPLC or direct MS of the whole lipid extract, termed recently "shotgun lipidomics." New methods reveal the diversity of individual species (more than 1000) of blood plasma lipids due to many variants of combinations of polar and fatty acid fragments of lipid molecules in each lipid class. Some of them change abundance in different ways in some diseases. Analytical and physiological factors influencing lipidome analysis are also shortly considered, including sample preparation, normalization, and effect of genes associated with lipid metabolism. The prospects of lipidome analysis in clinical studies are noted, as well as the need for standardization of used processes and conditions. Due to the involvement of lipids in many cellular and metabolic processes, their detailed analysis will contribute to unveiling of new biomarkers, as well as to increased understanding of pathogenetic mechanisms of a number of diseases.

Keywords: clinical lipidomics, molecular lipid species, biomarkers, plasma lipid classes, mass spectrometry

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1. LIPIDOMICS AND ITS PLACE IN MODERN OMICS STUDIES

Investigation of informative specific indicators (recently termed biomarkers), which would allow accurate detection of diseases or evaluation of the organism's response to therapy, has been a major focus of medical science from its early days. Approaches to solving this problem, at various levels, have been determined by the degree of development of currently available analytical techniques. In the course of the many years of its existence, clinical biochemistry has created a range of biochemical indicators, mainly blood plasma and serum parameters, that correlate with certain diseases and are currently used in clinical practice. Together with expansion of knowledge and completion of new studies, including epidemiological ones, on large groups of participants (called cohorts in the literature), new additional parameters have been introduced and implemented in practice.

For example, to reveal patients at risk of cardiovascular diseases and monitor therapeutic changes in such patients, a parameter that had not been used previously, i.e., high-density lipoprotein cholesterol (Chol) level, has been used in clinical practice for the past twenty years, in addition to levels of total Chol and triglycerides (TG) [1, 2]. Discovery of the fact that the disease risk is associated with the plasma level of a specific lipoprotein, termed lipoprotein (a), which was determined using monoclonal antibodies, also led to its application as an informative criterion in many clinical studies [3, 4]. Nevertheless, for both cardiovascular and a number of other diseases the accepted criteria are not sufficient since they show normal values in a number of diseased patients [5, 6]. Moreover, analysis of total lipid classes does not take into account that various molecular species (within the same class) can have different biological effects. Due to involvement of lipids, especially oxidized ones, in a number of pathologies, important information can be lost upon analysis of total lipid classes in a certain patient [5].

In the search for new sensitive criteria of diseases, a huge leap has been made owing to the rise and development of mass spectrometry (MS) methods of inves-

Abbreviations: FA, fatty acids; SL, sphyngolipids; SM, sphyngomyelin; TG, triglycerides; PL, phospholipids; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; lyso-PA, lysophosphatidic acid; PE, phosphatydilethanolamine; Chol, cholesterol; Chol-E, cholesterol esters; n:m, fatty acid containing n carbon atoms and m double bonds.

[1] Corresponding author: phone: +7(499)248–40–08; e-mail: torti@mail.ru.

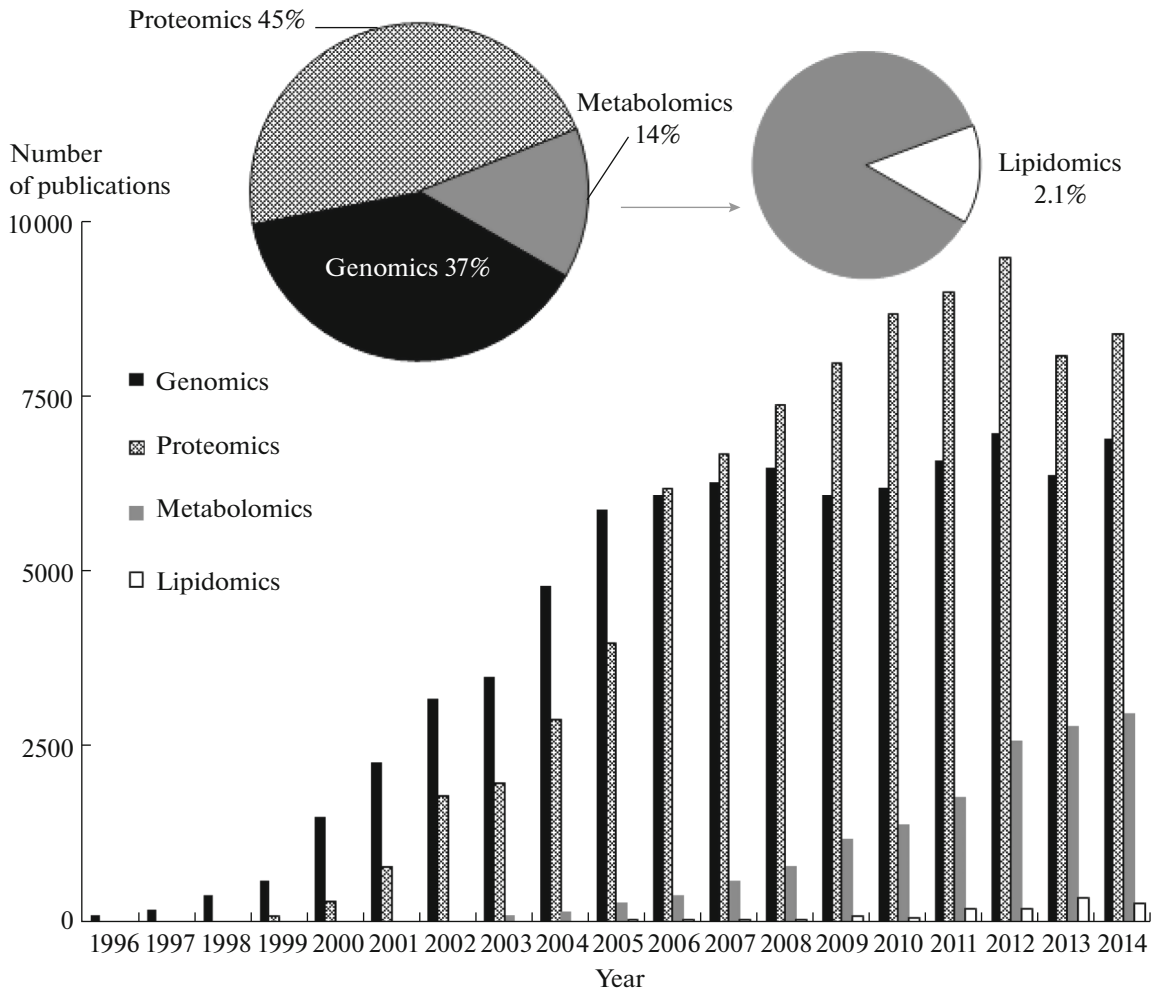

Fig. 1. Growth of the number of publications in the area of metabolomics, including lipidomics, against the works on genomics and proteomics. Percentage of the total number of studies in 2013 is shown in the upper part (adapted from [5]).

tigation exhibiting extremely high sensitivity and resolving power and enabling the separation of the compounds according to molecular mass-to-charge ratio (m/z). This stimulated development of modern postgenomic technologies, in particular, metabolomics, an MS analysis of a wide range of metabolites in search for the most informative ones, which has allowed identification of a number of new biomarkers [7–9]. The object of major metabolite studies was a wide range of rather easily analyzed low-molecular-weight compounds and only a small fraction of works was aimed at studies of plasma lipidome, which is illustrated in Fig. 1 showing the analysis of a number of publications in the area of the so-called OMICS studies before the year 2014 reported in the work of Hinterwirth and coauthors [5].

As follows from Fig. 1, the main works in metabolomics began in 2003–2004; during the first years these studies rarely touched on the lipid compounds. In the course of the following 10 years, the number of metabolomic studies grew progressively remaining however much lower in number than studies in genomics and proteomics. By 2013, the percentage of works in metabolomics increased overall from 0 to 14%, yet only 2.1% to the total number of studies concerned lipidomics, despite the well-known role of various lipids in cell and organism functioning. This is explained by the extreme diversity of individual molecular species of lipids, the possibility of multiple combinations of various parts of their molecules—nonpolar parts (various fatty acid chains) and several types of more polar fragments typical of individual lipid classes. This is the reason why in a single biological sample under study, for example, in blood plasma, there may be hundreds of individual molecular species of lipids.

Approaches to such a complicated fractionation became physically feasible only in recent years owing to the development of individual variants of MS technology, sometimes in combination with HPLC or, less often, GLC [10, 11]. This allowed identification and quantitative analysis in a sample of not only classes of lipids but also individual molecular species within

each class. Such studies were performed on various biological subjects, including cells and tissues of experimental animals or in model experiments, obtaining new information on the involvement of a number of lipids in regulation of multiple biological processes, including cell signaling or proliferation [12–15]. The involvement of individual types of lipids in pathological processes, e.g., excessive cell proliferation [16, 17] or the formation of atherosclerotic plaques [18], has been revealed. This indicated the possibility of their clinical importance.

Works on lipidome analysis of samples—mainly of blood plasma collected from patients with various diseases—in comparison with various clinical characteristics have been performed; this led to the formation of a new individual field of study, clinical lipidomics [19], in parallel with translation to clinics of other OMICS approaches (genomics, proteomics, and metabolomics) altogether called multiomics [20, 21]. The term includes a wide area of integrated studies performed at the levels of cells, tissues, organs, and the whole body. Translation into clinics with relevant incorporation and analysis of the results in combination with clinical data, that is, clinical multiomics, is sometimes called (in contrast to a more general discipline of biological multiomics) clinical trans-omics [20, 22]. Due to the indispensable role of lipids in most metabolic processes, clinical lipidomics is its important part.

In recent years, a number of works and reviews in the area have been published [19, 23–28], including those involving epidemiological studies [25]. Roles of such factors as age, gender, ethnicity [29], and specific features of lipid molecular species composition in a number of diseases, in particular cardiovascular system and metabolic disorders, Alzheimer's disease, oncology-related conditions, etc. [14, 15, 19, 23], including pre-eclampsia [30] and respiratory conditions [31], have been revealed. Considerable attention is being paid to the development of the procedure of lipidomic analysis, which is particularly important for lipids hydrolysable by lipolytic enzymes present in sample, as well as containing easily oxidized unsaturated fatty acids [10, 11, 32, 33].

The review briefly discusses major results of studies in the area of clinical lipidomics with emphasis made on blood plasma lipidome as the biological material used most frequently in clinical studies. Questions associated with technical aspects of MS analysis procedure, equipment applied, identification and quantitative determination of lipid molecular species have been described in detail in other works and reviews [10–12, 15, 28] and thus are touched upon here only briefly. The review is focused on the biochemical aspects of lipidome fractionation, including the main principles of methodology, diversity of individual lipid molecular species present in blood plasma, and factors affecting the results. Understanding these issues is necessary for utilization of plasma lipidome analysis in medical chemistry and the identification of new informative lipid biomarkers [5, 14, 17].

2. MAJOR APPROACHES TO MASS SPECTROMETRY ANALYSIS OF BLOOD PLASMA LIPIDOME

In the first works on lipidomics in the early 2000s, groups of lipids isolated from biological material, including plasma, were subjected to MS analysis and identification after preliminary separation using HPLC. Later, along with improved MS techniques, another approach has gained attention, that is, analysis of lipid extract performed upon its direct and single injection into the system. This became possible owing to introduction of various methods of mild ionization, such as electrospray [36] and matrix-assisted laser desorption ionization (MALDI) [28, 34], which was first applied for large labile molecules of proteins and peptides and then, to lipids [14]. In a number of works, atmospheric pressure chemical ionization (APCI) or photoionization (APPI) have been performed [14]. Subsequent MS analysis is performed upon simultaneous registration of positive and negative ions, which enables the separate detection of various lipid classes.

In clinical lipidome studies, MS of lipids in a sample (mainly, blood plasma) is performed both after preliminary HPLC fractionation (HPLC-MS) and without it. These approaches are characterized as either targeted lipidome analysis, aimed at detailed characterization of a certain group of lipids, or global analysis, which provides the fullest information possible about the set of all lipids present in the biological sample [14].

In recent years, the number of works utilizing the approach previously known as direct infusion mass spectrometry (DIMS) [28, 35] utilizing injection of the lipid extract into the ionization source of a mass spectrometer has been growing; today, another name of the approach, shotgun lipidomics, has become widespread [15, 36]. The term was introduced in 2004 [37]. This is the type of analysis utilizing electrospray ionization that is used the most in clinical lipidome studies aimed mainly at detailed analysis of blood plasma lipids. Technically, in different works it has been implemented in different experimental setups utilizing various types of mass spectrometers and MS systems, including tandem mass spectrometry (MS/MS) detecting not only ionized lipid molecules, but also, in a number of cases, their fragments formed upon collision with high-energy gas molecules (usually argon) in what is called higher-energy collision dissociation (HCD). Such methods, and the HPLC-MS mentioned above, allow separation and identification of isobaric lipids, which have the same formulas, but different structures. These processes and the applied equipment have been described in detail in reviews of Lokhov and coauthors [7, 8] and others [5, 14, 36].

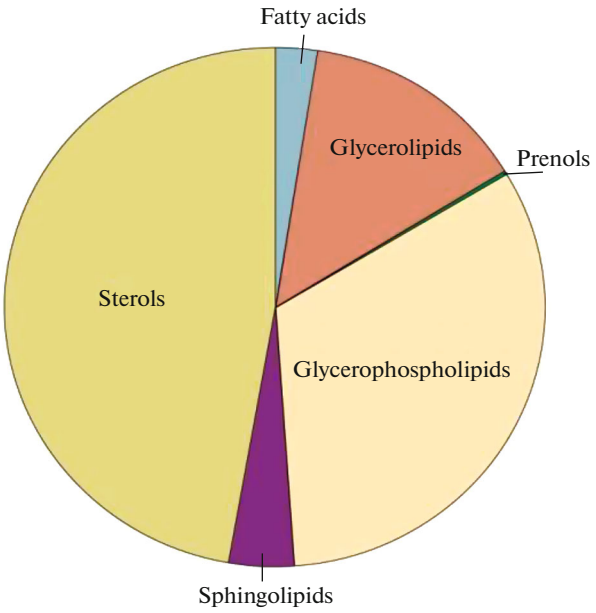

Fig. 2. Average molar distribution of the levels of various lipid categories according to the Lipid MAPS classification [38–40] found in human blood plasma lipidome analysis. Adapted from [41].

3. BLOOD PLASMA LIPIDS AS THE MAIN SUBJECT OF CLINICAL LIPIDOMICS: DIVERSITY OF INDIVIDUAL MOLECULAR SPECIES OF LIPIDS

To systematize the results of studies of lipidomics commencing in 2005–2007, a scientific consortium named Lipid MAPS (Lipid Metabolites and Pathways Strategy) [38] was organized in the University of San Diego, United States, which, together with International Lipids Classification and Nomenclature Committee (ILCNC), developed a somewhat modified comprehensive system of lipid classification compared to the one previously in use [39, 40]. It grouped all lipids from various natural sources into eight main categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, glycolipids, and polyketides (polycarbonyl secondary metabolites formed in cells of bacteria, fungi, animals, and plants) [39, 40]. In blood plasma, the first six of the eight classes are present in various ratios (Fig. 2, adapted from [41]).

As follows from the figure, specific features of the new classification are mainly applied to phospholipids (PL). A sphingosine-containing PL sphingomyelin (SM) (with the second highest concentration in plasma after phosphatidylcholine) was moved from the general class of phospholipids to the group of sphingolipids (SLs) together with other lipids containing sphingosine. The rest of the PLs based on glycerol thus form a class now called glycerophospholipids. Therefore, while the previous classification was based on the presence of a phosphate in the lipid molecule (determined by the presence of inorganic phosphate after PL oxidation), the modern one is based on the type of hydroxyl-containing fragment: in glycerophospholipids it is represented by glycerol and in sphingolipids, a long-chain alcohol sphingosine.

Isolation of SLs into a separate, and also heterogenic, group is caused by the considerable attention paid to these lipids lately due to their involvement in pathogenesis of a number of diseases, especially neurodegenerative ones, particularly Alzheimer's disease, through participation in many intra- and intercellular processes and interactions, such as differentiation and signal transduction. The diversity of this class of lipids comprising sphingomyelin, ceramides, glycosylceramides, sphingosine, and sphyngosine-1-phosphate, led to the rise of sphingolipidomics as an independent branch of lipidomic studies [42].

Modern sensitive MS methods also allow for analysis of prenols, a minor class of plasma lipids containing the isoprene (3-methyl-2-buten-1-ol) group. Few papers have considered prenols due to their extremely low content (0.06–0.08% of total plasma lipids). However, according to a number of authors [11, 32], in the search for biomarkers it is the minor lipids not having been evaluated previously due to lack of required methods that can turn out to be informative. The presence of eicosanoids, highly active mediators of a lipidic nature (prostaglandins, leukotrienes, thromboxanes) among plasma lipids, at yet even lower concentrations of ~0.001% of total plasma lipids, has also been noted [32].

Table 1 presents the molecular species of lipid categories present in blood plasma of healthy volunteers evaluated both in total and individually for their subclasses, as well as their molar and weight concentrations. Among represented lipid classes, only total Chol and TG have been determined for medical purposes previously; in research works, free and esterified Chol (Chol-E), glycerophospholipids (sometimes their individual subclasses), and rarely free (not esterified) fatty acids (FA) have been assayed. These lipid subclasses for decades have been being determined in total, without separation into individual components differing by hydrocarbon chains; in individual studies fatty acid composition has been analyzed using GLC. The whole biological sample (plasma) or individual lipid classes upon preliminary isolation by column chromatography or preparative TLC were analyzed.

This experimental setup resulted in the fact that for a long time researchers have been obtaining limited data on only nine FA acyl groups dominant in plasma lipids: C18–C22 with up to four double bonds; arachidonic acid (20:4) was the longest to be analyzed. Later, thanks to the increased interest in polyunsaturated FA, the range of FA expanded to include C24–C26, not excluding pentaenoic and hexaenoic FA 20:5, 22:5, and 22:6 (the level of which in plasma is only fractions of a percent, 40–50 times lower than that of major FA, C14–C18). As a result, at best the total per-

Table 1. Number, total concentrations, and main individual molecular species of lipids of various classes in human blood plasma (adapted from [32] and [50])

Lipid class	Plasma concentration[a]		Total number of molecular species[a]	Main individual molecular species[b]
	nmol/mL	mg/dL		
Total lipids	8023	471	588	
Among them				
Sterols[c] (total)	**3780**	**146**	**36**	
free	826	31.8	14	
esterified	2954	114	22	18:2, 18:1, 20:4
Glycerophospholipids (total):	**2596**	**201**	**160**	
phosphatidylcholine (PC)	1974	157	31	34:2, 36:2, 38:4
phosphatidylethanolamine (PE)	435	32.7	38	38:5, 38:4, 38:4-plasmalogen, 38:5-plasmalogen
lyso-PC	103	5.25	12	16:0, 18:0, 18:1
lyso-PE	36.6	1.78	7	18:2, 18:0, 20:4
phosphatidylinositol (PI)	31.5	2.74	19	38:4, 36:2, 34:2,
phosphatidylglycerol (PG)	6.12	0.480	16	36:1, 38:6, 38:5
phosphatidic acid (PA)	2.50	0.173	15	34:0, 36:2, 36:0
phosphatidylserine (PS)	7.0	0.559	20	36:0, 36:1, 40:6
N-acyl-PS	0.013	0.001	2	52:1, 54:2
Glycerolipids	**1100**	**93.7**	**73**	
triglycerides (TG)	1058	90.6	18	(16:1/18:1/18:1), (16:1/18:0/18:2), (16:1/18:1/18:2)
1,2-diglycerides (DG)	39	2.36	28	36:3, 36:4, 36:2
1,3-diglycerides	13	0.806	27	36:3, 36:4, 34:1
Sphingolipids (total)	**318**	**23.7**	**204**	
sphingomyelins (SM)	303.47	22.817	101	(18:1/16:0), (18:1/24:1), (18:2/24:1)
monohexosylceramides	2.31	0.180	56	(18:1/22:0), (18:1/24:0), (18:1/16:0)
ceramides	11.59	0.732	41	(18:1/24:0), (18:0/24:0), (18:1/22:0)
sphingoid bases	0.57	0.02	6	Sphingosine 18:1, sphingosine-1-phosphate (S1P)18:0, S1P 18:1
Free fatty acids	214	5.82	31	18:1, 16:0, and 18:0 (78% in total)
Eicosanoids (prostaglandins, leukotrienes, thromboxanes)	0.071	0.002	76	15-deoxy-prostaglandin D_2 and others [32]
Prenols (isoprenoids)	4.62	0.397	8	Ubiquinones, dolichols [32]

[a] Data from the work [32] for the standard reference material obtained from plasma samples of 100 individuals 40–50 years old of a representative group of the US population.
[b] Data from the work [50]; total number of C atoms and double bonds in FA chains is indicated; it thus includes several possible isobaric variants.
[c] Mainly cholesterol, with 2–3% intermediate products of its biosynthesis or metabolism (latosterol, desmosterol, 7-dehydrocholesterol, lanosterol, sitosterol) [32].

cent ratio of FA residues in a lipid class could be determined, without information on their combination in certain lipid molecules (most of which contain two fatty acid chains present in various combinations). Only MS methods provided the possibility—owing to hydrocarbon chain-derived differences in molecular masses of a certain lipid molecular species—to isolate and identify an individual peak and separate it from the others, with the same polar group and other FA residues. According to the Lipid MAPS classification [38–40], free FA (typically transported with albumin) and eicosanoids present in much lower (more than 1000-fold) amounts are related to fatty acids.

Eicosanoids are formed from arachidonic acid and other C20 polyunsaturated FA under the effect of enzymes, cyclooxygenase, lipoxygenase, and cytochrome P450–epoxygenase. They possess extremely high biological activity, and their level in plasma often correlates with various, mainly inflammatory, conditions; therefore, despite very low concentrations, eicosanoids are considered as an important—in terms of significance, not amount—part of the plasma lipidome [32].

Among glycerophospholipids, a minor (below 0.04%) but functionally important lysophospholipid, lyso-phosphatidic acid (lyso-PA) should be noted [43], which is involved in many biological processes through the specific G protein coupled receptors (GPCR) leading to activation of a range of intracellular effectors not yet completely elucidated [44]. In a number of works, an increased level of lyso-PAs has been demonstrated in several oncology-related and cardiovascular diseases [13, 43, 45], particularly including molecular species lyso-PA/22:6, lyso-PA/18:2, and lyso-PA/20:4, that is docosahexaenoic, linoleic, and arachidonic acids [45].

The total number of various individual molecular species of lipids in human blood plasma based on the analysis of a standard reference material was 588 [32]. Weir and coauthors [46] studied plasma microsamples (10 μL) of 1430 volunteers (part of a population-based San Antonio Family Heart Study, SAFHS, investigating genetic and other risk factors of cardiovascular diseases) and isolated 312 individual lipids. According to other authors, the number of lipid molecular species in plasma is higher [47] and may be over 1000 [14, 48], although the figure is indicated as approximate and theoretically predicted. A recent paper by Kim and coauthors [49] reporting a study of plasma lipidome in cases of dementia mentions the presence of 3225 individual molecular species, including dietary lipids. For each individual class of lipids the number of individual species, according to [32], varies from 15 to 100 on average (see Table 1). Each class of lipids is dominated by 3–5 main species [32, 50], which, as noted in [50], can be the most informative lipid metabolites with respect to biomarker identification in clinical lipidome studies (Table 1).

As follows from Table 1, mostly traditional well-known FAs, with chain length from C16 to C20 and number of double bonds up to four, are present in plasma lipids, while the species diversity is created by the variants of the FA and double bond positions. For glycerolipids and glycerophospholipids, total number of carbon atoms in the FA also creates several variants. For example, PC C36:2 can contain two C18:1 chains (oleic acid) or a combination of C18:0 (stearic) and C18:2 (linoleic acids), or two chains of different lengths, but with the same total number of carbon atoms and double bonds, that is, six different molecular species with coinciding molecular ion peaks are possible for such a PC in blood plasma.

Separation of such isobaric molecular species of lipids of the same class can be achieved, as mentioned above (section 2) through application of tandem MS, yielding different MS/MS spectra reflecting various ion fragments that are formed upon induced dissociation of initial ionized lipids (in the bottom-up lipidomics, which is described in detail in the review [5]).

Classes of lipids indicated in Table 1 are analyzed by MS separately after their preliminary separation by HPLC or totally upon direct injection of total lipid extract of plasma (section 2). Minor lipid classes, such as monohexosylceramides, sphingoid bases, prenols, and, particularly, eicosanoids, are studied in detail in a few research papers by techniques employing various targeted analytical approaches [32].

4. ANALYTICAL FACTORS INFLUENCING THE RESULTS OF LIPIDOME ANALYSIS AND REQUIRING STANDARDIZATION TO TRANSLATE LIPIDOMICS INTO CLINICAL PRACTICE

In view of high lability of lipid compounds, in many works and reviews on lipidome analysis of plasma, considerable attention is paid to not only the MS procedure, which is not being considered here [10–12, 15], but also the methodology of sample preparation; for the methodology determines completeness of extraction of different types of molecular species or the possible risk of double bond oxidation in FA and therefore affects the final result [51–54]. Extraction of lipids from plasma by insufficiently hydrophobic organic solvents leads to loss of such important lipids as TG and Chol-E; on the contrary, insufficient polarity of solvent will not allow complete extraction of lyso-PCs and some SLs. Completeness of extraction can be influenced by conditions of plasma or serum sample collection and storage, due to the possibility of changes in lipid–protein interactions in lipoproteins. Prolonged storage of samples can lead to destruction or oxidation of double bonds, which changes the mass-to-charge ratio (m/z) essential for further MS fractionation. Moreover, in view of the nonequality of the ratio between MS spectrum peak size and the amount of lipid present in the sample for

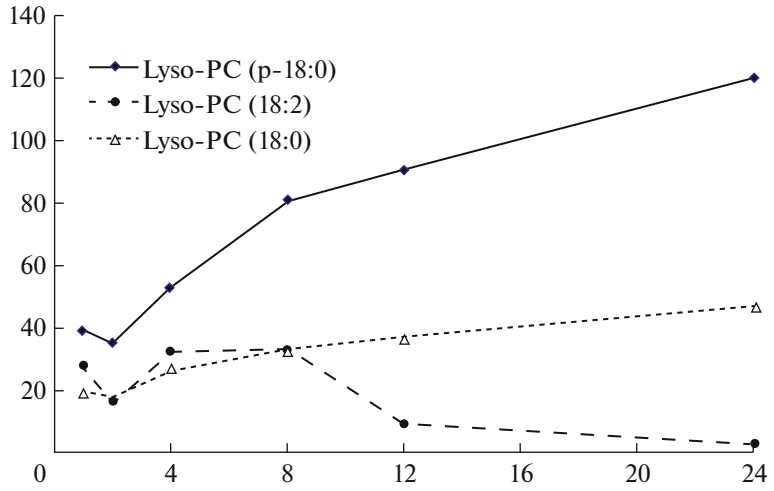

Fig. 3. Changes in the level of various lysophosphatidylcholine species in a plasma sample over 24 h at 4°C. Time, h, is plotted along the *x* axis and percent increase compared to the initial level, along the *y* axis (upon direct analysis of the plasma collected). Analysis was performed by HPLC-MS after precipitation of plasma proteins with acetonitrile. Lyso-PC(p-18:0) is lyso-PC-plasmalogen. Adapted from [50].

different lipids, a poor choice of the internal standard can also produce false results [14]. These issues are important for clinical lipidomics and are briefly discussed below.

4.1. Type of Biosample, Sample Preparation, and Storage

Various protocols of sample, mainly blood plasma or serum, collection and primary treatment differing by blood clotting conditions (temperature and time) and further centrifugation have been used. Since in studies on large clinical cohorts it is not always possible to maintain the same conditions at all stages, it is important to determine the possible effect of each of the stages of the procedure, including blood collection, preliminary treatment, and sample storage, on the levels of lipid molecular species.

It turned out that lipid profiles for serum and plasma obtained under the effect of different anticoagulants differed. In most works, total concentration of lipids, particularly PCs and TGs, was higher in serum than in plasma, although it is still not clear which treatment is preferable for lipidomic analysis [29, 51, 52].

Some species of PC, PE, SM, and TG have been determined in larger amounts in citrated plasma than in plasma under EDTA. Reproducibility of lipidomics data, according to some authors, is higher in serum, while others claim, in plasma. It is hypothesized to be connected to blood maintenance at room temperature; therefore, blood samples are proposed to be kept on ice; EDTA treatment is considered preferable especially for SL, particularly hexosylceramide, analysis [53].

The effect of storage conditions has also been demonstrated. For example, maintenance of plasma at 4°C for 24 h resulted in changes in the ratio between certain types of lyso-PC [50], a functionally important phospholipid; changes in plasma concentrations of some types of lyso-PCs have been recorded in various diseases [25, 46, 50] (Fig. 3, adapted from [50]). This is accompanied by formation of lyso-PC in the course of hydrolysis of phosphatidylcholines by phospholipase A2 in parallel with oxidation of the double bond in its unsaturated molecular species, lyso-PC(C18:2). An increase in the level of lyso-PC in plasma after 48 h has been demonstrated in other works as a result of enzymatic hydrolysis of PE catalyzed by phospholipase A2 [50, 52, 54]. A similar result was obtained upon transporting noncentrifuged blood [51]. All other glycerophospholipids showed higher stability when maintained in cold and even at room temperature for at least 24 h. The number of freezing–thawing cycles is also an important factor. For most molecular species of lipids, a single freezing procedure does not affect the result of analysis, however, repeated freezing decreases the level of a number of lipids determined in plasma and serum [29]. In practice, plasma samples are frozen and thus stored prior to analysis [14].

4.2. Extraction of Lipids from Samples

Extraction of the sum of lipids from plasma or serum by organic solvents is a necessary stage prior to injection of the analyzed mixture into the mass spectrometer for lipidome analysis. Storage of extracts (at –20°C) is considered safer than storage of the original plasma [55]. To extract lipids from plasma various methods are used. In each method, prior to extraction, available internal standards are usually added to samples to have the possibility of quantitative evaluation of final mass spectra. Several methods of lipid extraction from a biological sample accompanied (for blood

plasma) by precipitation of protein have been reported. The quantity of plasma used for analysis varied from 5 to 100 μL [14].

In most of the works, classical methods of extraction by a chloroform–methanol mixture were used. Most often, a 20-fold excess (with respect to plasma) of chloroform–methanol 2 : 1 mixture was used, followed by washing of the extract with 0.9% NaCl (0.2 volumes) and further separation of phases (according to Folch). The upper (methanol–water) layer is removed; the lower layer evaporated, dissolved in convenient solvent, and analyzed. For example, such a method was used in [24] after the addition of butyloxytoluene as an antioxidant to plasma.

The final state of the sample suitable for injection into the analyzer (choice of the solvent and its quantity) is determined by the implied MS analysis method: either direct injection of total lipid extract into the mass spectrometer, without HPLC (shotgun lipidomics), or the LC-MS (HPLC-MS) approach [32, 56].

A variation of the chloroform–methanol extraction is the use of these solvents in equal volumes, 1 : 1 (according to Bligh and Dyer) in 2 : 1 or 4 : 1 ratio as reported in different works. After mixing and centrifugation, the lower layer is collected; the upper (aqueous) layer is washed with chloroform for more complete extraction. Lower layers are combined, evaporated, and again dissolved for the MS analysis.

According to some authors, the disadvantage of these methods is the use of chloroform requiring (due to its high density) collection of the lower layer, which is not always convenient. Other methods used are described in works [14, 15]. One of the methods uses methyl-*tert*-butyl ester (MTBE). The plasma sample is extracted with 4.5 volumes of MTBE–methanol, 5 : 1.5 mixture.

Another common method of extraction in lipidome analysis is the BUME method, or extraction with a butanol–methanol mixture in 3 : 1 to 10 : 1 ratio with plasma. After treatment, another mixture of solvents is added, that is heptane–ethyl acetate (3 : 1) with further collection (after phase separation) of the upper phase. The approach is considered more convenient than using chloroform mixtures, however this treatment has its limitations, particularly, the possibility of some water-soluble components to be captured, as well as laborious evaporation of the organic phase due to the presence of butanol [14].

Therefore, in different works, authors introduced their modifications using combinations of organic solvents of different polarities, such as hexane, isopropanol, ethyl acetate, methanol, and acetonitrile. The dependence of the solubility of lipids on FA chain length and their composition determining the hydrophobicity of the molecule, as well as the presence of polar functional groups of phosphate or sugars imparting the molecules with somewhat hydrophilic properties, should be kept in mind. For example, for TG and Chol-E being neutral lipids dissolve well in nonpolar or weakly polar solvents (e.g., hexane, diethyl ether, chloroform), but have low solubility in methanol. Most polar lipids, such as glycerophospholipids and sphingolipids, dissolve better in polar solvents. Therefore, the most efficient extraction is achieved in a combination of solvents of different polarities. Good results were demonstrated for extraction with isopropanol, the advantage of which is the possibility of direct injection in the analyzer. Partial evaluation of extraction efficiency of lipids of this or that kind by the selected solvent is possible upon addition of known concentrations of internal standards, sometimes radioactively labeled [14].

In a number of works the above-indicated methods have been used in combination with solid-phase extraction [14], a rather new method representing in essence not the extraction of lipids from the biosample, but separation of total extracts into groups of analytes (here, lipids), which is a modern variant of column chromatography. Preconditioned columns (cartridges) filled with various stationary phases based on silica gel and synthetic polymers are used. For lipid extracts these are typically phases containing amino groups adsorbing lipids of varying polarity, with varying content of OH groups in a molecule. Prior to MS analysis, lipid extract is either separated into two parts, so that the sample is analyzed with and without the SPE treatment, or adsorbed lipids are extracted by a more polar eluent and analyzed separately. Such treatment decreases the number of peaks in the mass spectra, facilitating their identification, which is particularly important in the shotgun lipidomics of the total lipid extract. Moreover, a number of authors used partial fractionation of lipids reached by SPE for the following targeted MS analysis of individual groups of steroids and eicosanoids [57].

4.3. Normalization of Individual Lipid Levels

Mass spectra of lipid extracts injected in the spectrometer with a large number of peaks of ions or fragments thereof [14, 15] are analyzed to identify peaks of individual lipids according to their m/z values and quantify the amount of lipids using relevant databases and several special software packages for calibration (XCMS, MZmine, etc.) described in the review [50]. The results of quantitative analysis in MS are affected by differing efficiencies of ionization (and thus peak magnitude) for different lipid molecules; therefore, peaks are normalized by peak values for internal standards, that is, lipids added to the sample in known amounts. Since it is impossible to have internal standard for each of the individual species (for there may be over 1000 of them in a sample as noted above [46–49]), nontarget general lipid profiling mainly used in clinical lipidomics is considered semiquantitative.

There are sets of internal standards (Quality Control samples) [30, 50] containing control samples spe-

cific for some lipid classes. Lipids with an uneven number of carbon atoms in fatty acid chains—C13, C15, C17—not typical of endogenous lipids of plasma, are used as internal standards so that they produce separate peaks that are further quantitatively processed using the calibration software [30]. Utilization of a limited number of internal standards does not allow for ideally precise and absolutely correct quantitative lipidome analysis. The rate of ionization depends on the lipid structure, in particular, FA chain length and the number of double bonds. However, the composition of polar head groups of lipids has the most influence. Therefore, addition of a control set comprising internal standards for each of the lipid classes is considered sufficient for the lipidome analysis [58].

5. PHYSIOLOGICAL FACTORS AFFECTING BLOOD PLASMA LIPIDOME

When planning clinical lipidome analysis, one should take into account, along with the patient's main disease, a number of physiological factors affecting the plasma lipidome, which may interfere with correct interpretation of the results; these include diet type, medication, gender, and age. Some specific features of lipidome, as those of the metabolome in general, can be correlated with genetic features [59, 60] often associated with the diseases [60].

5.1. Genetic Factors

In a number of works on clinical lipidomics—in a modest number compared to other metabolomics studies—a link between changes in a number of plasma parameters with genetic conditions has been revealed. As noted in the work [61], lipidome changes being detected can often be due to impairment in the expression of genes of proteins associated with lipid metabolism. In a genome-wide association study (GWAS) of 284 men performed by Gieger and coauthors, 12% of variations observed in the metabolome (including the lipidome) were associated with cases of single nucleotide polymorphisms (SNPs) in a number of genes. For example, the content of FAs of a certain chain length and number of double bonds in lipid classes is linked to polymorphism in several genes (denoted as *FADS1*, *LIPC*, *SCAD*, and *MCAD*), encoding desaturases and dehydrogenases, through their influence on the processes of synthesis and beta-oxidation of a number of fatty acids [62]. SNP in one of these genes (rs174548) significantly influences the level of glycerophospholipids [59].

Another GWAS on 4400 individuals of the Caucasoid population revealed several genes (*SPTLC3*, *LASS4*, *SGPP1*, *ATP10D*, and *FADS1-3*), expression of which was associated with the level of SM, dihydro-SM, ceramides, and glycosylceramides, through participation in regulation of metabolism of SL [63]. Specific features in the content of several plasmalogens of smoking individuals revealed in [64] were shown to be associated with expression of the gene of alkyl dihydroxyacetone phosphate (alkyl-DHAP) synthase in lung cells.

In a recent work [60], blood plasma lipidome of lung cancer patients was studied and compared with the previously obtained genomic data for such patients [65]. A number of differences in the lipidome compared to that of healthy volunteers, in particular, a pronounced increase in the level of phosphatidylserines, PS/40:6 (containing apparently arachidonic or a longer FA), PS/34:5 (with rather short FA), and lyso-PS/22:6 (with docosahexaenoic acid), as well as a decreased level of lyso-PE/20:4 and several others, were ascribed by the authors to changes in a family of genes associated with enzymes of lipid metabolism, ACOT (acyl-CoA-thioesterase), ACSL (long-chain acyl-CoA-synthetase), and PKD (protein kinase D) [60], which had been previously demonstrated in patients with some types of cancer [65]. In future, this information can become significant for interpretation of lipidome data analysis in clinics.

5.2. Influence of Other Factors

Naturally, similar to traditional blood lipid analysis, fewer variations (and more correct results) are produced under fasting conditions. In the latter case, dietary lipids, which also contribute to plasma lipidome, have less influence. Under the same analysis conditions, levels of major plasma lipids remain constant; the amount of glycerophospholipids and sphingolipids has been shown constant upon repeated analysis within 14 days (for plasma samples of three persons) while retaining a number of interindividual differences [51].

A number of age-related changes have been reported: along with a known general increase of TG in plasma with age, increased values for several species thereof have been shown in women. In plasma (not in serum) age-related changes of some lyso-PCs have been observed. In a large cohort study, higher levels of Chol-E, TG, lyso-PC, and ceramides, along with a lower content of SM, trihexosylceramides, and PS, has been demonstrated in men compared to women. Seventeen species of lipids turned out to be associated with body weight index and some species of PC and PS differed in plasma of smoking individuals compared to nonsmokers [46]. Differences due to the effects of smoking have been shown for some species of PC-plasmalogen [64]. Some racial differences have been reported; for example, in the work [66], in representatives of the Negroid race a two-times higher level of plasmalogens and two-times lower level of PC (16:0/18:1) have been shown.

Such features should be taken into account in the course of interpretation of the results of clinical lipidomics studies.

6. PROSPECTS OF CLINICAL LIPIDOMICS IN THE STUDY OF DISEASES

To translate lipidome analysis into clinics, that is, to implement and utilize it in clinics in order to reveal new diagnostic lipid markers, elaboration of a number of standard methodological approaches and protocols is needed. Today, due to the extreme variation in the equipment, methods, protocols, sample preparation procedures, and storage conditions used, data of different laboratories on the lipidome profile of biosamples obtained from patients with the same pathology can differ. To date, there are few studies of the plasma lipidome on large patient cohorts and the absence of relevant databases does not allow us to define the "normal lipidome" [5, 19] and reveal possible ranges of values in health and pathology. However, the growing interest in clinical lipidomics and the large number of studies in the area in recent years suggest that it will be actively developing and gradually solving these problems, thus opening prospects for clinical use of the lipidome profile [5, 19].

The importance of lipidome studies in general, including the medical aspects, is evidenced by a recent monograph devoted to lipidomics [67] written by Dr. Han, the author of many publications in the area, including the abovementioned [12, 14, 15, 36, 37, 47, 56]. In the work, along with an extensive consideration of lipidome studies of various biological subjects, a large section deals with issues of clinical lipidomics. Based on the data available on the role of lipids in cell signaling as secondary messengers, Dr. Han emphasizes the importance of lipidome studies in such diseases as diabetes, obesity complications, steatosis, nonalcoholic steatohepatitis, Alzheimer's disease, cardiovascular disorders, and oncology-related diseases. Due to the sensitivity of lipids to many physiological and analytical factors, the author stresses the need for standardization of the procedure of clinical lipidomic studies as a prerequisite to implementation of plasma lipidome analysis data in clinical practice.

Standardization of all stages of lipidome analysis, creation of a common database, and large numbers of studies can widen the concept of dyslipidemia, along with the elucidation of various aspects of lipid dysregulation. Being a case of integration of clinical medicine and modern lipidology, the development of which in recent decades has widened our knowledge of lipid involvement in many biological processes, including those associated with disease progression [19, 68], implementation of lipidomics in clinical practice is strongly hoped for. Supposedly, lipidomics will not only help to reveal new informative markers for diagnostics and treatment control, but also provide for better understanding of the disruptions in many processes involving lipids and the possibility to detect new therapeutic targets. Sometimes, such targets are being indicated by lipidomic changes caused, as noted above (section 5.1), by impaired gene expression of proteins associated with lipid metabolism [59–64, 69].

Also, in future, the possibility of joint integrated analysis of lipidomics data together with the results of genomic and proteomic studies is supposed, although in a number of cases inconsistences therein are allowed [20]. In general, clinical lipidomics is becoming an indispensable part of the scientific area called clinical multiomics, or trans-omics, which has originated recently [20, 22]; it represents translation into clinics of modern biochemical approaches based on MS analysis. In future, development of automated systems to obtain and analyze the multiplicity of data taking into account simultaneous analysis of all available OMICS studies is the aspiration [19]. This should promote not only the rise of new—including lipidic—biomarkers, but also a deeper interpretation of the molecular nature of pathogenesis and the progression of diseases, response to therapy, and thus its improvement and development. According to Dr. Wang [20], progress expected in the area will lead to development of new therapeutic strategies, improved understanding of disease symptoms, and treatment alternatives. For lipidomics, still less advanced compared to other areas, intense development is predicted in the near future [70].

Detailed analysis of data available today on the plasma lipidome profile in certain diseases, due to the increased number of publications for some of them (e.g., cardiovascular diseases, diabetes, pre-eclampsia), as well as some differences in data reported therein, requires careful consideration.

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COMPLIANCE WITH ETHICAL STANDARDS

The work contains no studies involving humans or animals as study subjects.

Conflict of Interests

Authors declare no conflict of interest.

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