METHODOLOGY OF DETERMINING THE METABOLOMIC PROFILE OF TUMOR-ASSOCIATED MACROPHAGES AND MONOCYTES IN ONCOLOGICAL DISEASES

Frankevich VE[1,2,3] ✉, Novoselova AV[1], Starodubtseva NL[1], Patysheva MR[2,4], Larionova IV[2,3,4], Rakina MA[2], Bragina OD[4], Kzhyshkowska JG[2,3,5,6]

[1] Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology, Moscow, Russia
[2] Laboratory of translational cellular and molecular biomedicine, National Research Tomsk State University, Tomsk, Russia
[3] Laboratory of Genetic Technologies, Siberian State Medical University, Tomsk, Russia
[4] Cancer Research Institute, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russia
[5] Institute of Transfusion Medicine and Immunology, MI3, Mannheim Faculty of Medicine, University of Heidelberg, Germany
[6] German Red Cross Blood Service Baden-Württemberg–Hessen, Mannheim, Germany

Breast cancer is the leading cause of cancer-related death among women worldwide. Tumor-associated macrophages (TAMs) constitute the primary component of innate immunity in breast cancer tissue. During the development of new approaches for breast cancer treatment aimed at editing the epigenome of TAM, precise methods for the analysis of macrophage metabolome are required to examine the effect on new approaches on macrophage metabolism. Our study aimed to develop an HPLC-MS/MS-based analytical approach to characterize the metabolome of human innate immune cells (TAMs and their precursors, monocytes). Analysis of lipid extracts was conducted on a Dionex UltiMate 3000 liquid chromatograph connected to a Maxis Impact qTOF mass analyzer with an ESI ion source. Quantitative analysis of 38 amino acids in the cells was conducted using the Jasem Amino Acids LC-MS/MS Analysis Kit and an HPLC-MS/MS chromatographic system consisting out of an Agilent 6460 triple quadrupole mass spectrometric detector (Agilent), and an Agilent 1260 II liquid chromatograph (Agilent) with Amino acids-HPLC Column (Jasem). The modified Folch method with double extraction was found to be the optimal approached for the sample preparation, since it enables to simultaneously isolate the lipid extract and water-soluble substances, in particular, amino acids. The method of reversed-phase chromatography yielded more useful data on the cell lipid composition than the method of hydrophilic interaction liquid chromatography (HILIC). The minimum number of cells required to determine the metabolome of immune system cells (TAM and monocytes) was identified as 2×10^6. Thus, we have developed the approach to determine the lipid and amino acid composition of modelled human TAMs and primary monocytes isolated out of breast cancer patients using minimal amount of clinical material.

Keywords: mass spectrometry, metabolomics, lipidomics, tumor-associated macrophages

Funding: the study was financially supported by the Russian Federation represented by the Ministry of Science and Higher Education of the Russian Federation (agreement dated 29 September 2021 № 075-15-2021-1073 on the topic "Genetic and epigenetic editing of tumor cells and the microenvironment in order to block metastasis ").

Author contribution: Frankevich VE, Kzhyshkowska JG — study planning and coordination, manuscript writing; Bragina OD — cancer patient selection and clinical characteristic preparation; Novoselova AV — sample preparation, HPLC-MS/MS; Larionova IV, Patysheva MR — monocyte and macrophage sample preparation and characterization, model TAM system set-up; Rakina MA — obtaining conditioned media of breast cancer cell lines; Starodubtseva NL — HPLC-MS/MS data analysis, manuscript writing; Frankevich VE, Larionova IV, Patysheva MR — discussion of results; Frankevich VE, Kzhyshkowska JG, Starodubtseva NL — manuscript editing.

Compliance with ethical standards: the study was approved by ethical review board of the Federal State Budgetary Educational Institution of Higher Education of the Siberian State Medical University of the Ministry of Health of Russia (protocol № 7 of 14 January 2017), federal laws of the Russian Federation (№ 152, 323, and others), and the 1964 Declaration of Helsinki.

✉ **Correspondence should be addressed:** Vladimir E. Frankevich
Akademika Oparina, 4, Moscow, 117997, Russia; vfrankevich@gmail.com

Received: 22.08.2022 **Accepted:** 13.09.2022 **Published online:** 13.10.2022

DOI: 10.24075/brsmu.2022.049

МЕТОДИКА ОПРЕДЕЛЕНИЯ МЕТАБОЛОМНОГО ПРОФИЛЯ ОПУХОЛЕАССОЦИИРОВАННЫХ МАКРОФАГОВ И МОНОЦИТОВ ПРИ ОНКОЛОГИЧЕСКИХ ЗАБОЛЕВАНИЯХ

В. Е. Франкевич[1,2,3] ✉, А. В. Новоселова[1], Н. Л. Стародубцева[1], М. Р. Патышева[2,4] , И. В. Ларионова[2,3,4], М. А. Ракина[2], О. Д. Брагина[4], Ю. Г. Кжышковска[2,3,5,6]

[1] Национальный медицинский исследовательский центр акушерства, гинекологии и перинатологии имени В. И. Кулакова, Москва, Россия
[2] Лаборатория трансляционной клеточной и молекулярной биомедицины, Национальный исследовательский томский государственный университет, Томск, Россия
[3] Лаборатория генетических технологий, Сибирский государственный медицинский университет, Томск, Россия
[4] Научно-исследовательский институт онкологии, Томский национальный исследовательский медицинский центр Российской академии наук, Томск, Россия
[5] Институт Трансфузионной медицины и иммунологии, медицинский факультет Маннгейм, университет Гейдельберга, Германия
[6] Германский Красный Крест Служба крови Баден-Вюртемберг-Гессен, Маннгейм, Германия

Рак молочной железы — ведущая причина смерти от рака среди женщин во всем мире. Опухолеассоциированные макрофаги (ОАМ) представляют собой основной компонент иммунных клеток в ткани при раке молочной железы. Новые подходы к лечению направлены на редактирование эпигенома ОАМ. Для изучения изменений в клетках иммунной системы при репрограммировании необходимы точные методы анализа метаболома. Целью исследования было создать на базе ВЭЖХ-МС/МС аналитический подход для определения особенностей метаболома иммунных клеток человека (ОАМ и моноцитов). Липидные экстракты анализировали на жидкостном хроматографе Dionex UltiMate 3000, соединенном с масс-анализатором Maxis Impact qTOF с ЭРИ источником ионов. Для количественного анализа 38 аминокислот в клетках использовали набор Jasem Amino Acids LC-MS/MS Analysis Kit и хроматографическую систему ВЭЖХ-МС/МС, состоящую из тройного квадрупольного масс-спектрометрического детектора Agilent 6460 (Agilent) и жидкостного хроматографа Agilent 1260 II (Agilent) с колонкой Amino acids-HPLC Column (Jasem). Модифицированный метод Фолча с двойной экстракцией позволяет одновременно выделить экстракт липидов и водорастворимых веществ, в частности аминокислот. Наиболее информативные данные о липидном составе клеток были получены методом обращенно-фазовой хроматографии по сравнению с гидрофильной хроматографией (HILIC). Для определения метаболома клеток иммунной системы (ОАМ и моноциты) минимальное число клеток оказалось равным 2 млн. Таким образом, разработан подход для определения особенностей липидного и аминокислотного состава ОАМ и моноцитов пациентов с раком молочной железы.

Ключевые слова: масс-спектрометрия, метаболомика, липидомика, опухолеассоциированные макрофаги

Финансирование: исследование выполнено при финансовой поддержке Российской Федерации в лице Министерства науки и высшего образования Российской Федерации (соглашение от 29 сентября 2021 г. № 075-15-2021-1073 на тему: «Генетическое и эпигенетическое редактирование клеток опухоли и микроокружения с целью блокировки метастазирования»).

Вклад авторов: В. Е. Франкевич — планирование исследования, обсуждение результатов, редактирование рукописи; А. В. Новоселова — пробоподготовка, проведение ВЭЖХ-МС/МС; Н. Л. Стародубцева — анализ ВЭЖХ-МС/МС данных, написание и редактирование рукописи; М. Р. Патышева, И. В. Ларионова — подготовка и характеристика образцов моноцитов и макрофагов, постановка модельной системы ОАМ, обсуждение результатов; М. А. Ракина — получение кондиционных сред клеточных линий РМЖ; О. Д. Брагина — подбор и клиническая характеристика онкологических пациентов, Ю. Г. Кжышковска — планирование исследования, редактирование рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБОУ ВО СИБГМУ Минздрава России (протокол № 7 от 14 января 2017 г.), федеральных законов Российской Федерации (№ 152, 323 и др.) и Хельсинкской декларации 1964 г.

✉ **Для корреспонденции:** Владимир Евгеньевич Франкевич
ул. Академика Опарина, д. 4, г. Москва, 117997; vfrankevich@gmail.com

Статья получена: 22.08.2022 **Статья принята к печати:** 13.09.2022 **Опубликована онлайн:** 13.10.2022

DOI: 10.24075/vrgmu.2022.049

Breast cancer is the most commonly diagnosed malignant disease and the leading cause of cancer-related death among women worldwide [1]. Tumor microenvironment plays a crucial role in breast cancer progression, regulating tumor growth, vascularization, metastasis, and response to therapy [2-4]. Tumor-associated macrophages (TAM) are a major component of the innate immune system in breast cancer tissue; their role in breast cancer progression has been shown in animal models and in patients Larionova et al 2020 [5]. Both the overall number of macrophages and, in particular M2-like macrophages were found to indicate poor prognosis in most studies of breast cancer, which however do not account for intratumoral heterogeneity [2, 5, 6]. Our previous studies showed that macrophages can retain antitumor properties in certain intratumoral compartments, as supported by the negative correlation of breast tissue TAMs with local metastases [6]. Macrophages control tumor growth and metastasis by secreting cytokines, extracellular matrix components, and growth factors; the profile of secreted components is defined by programming on the transcriptional level as well as on the epigenetic and metabolic levels [2, 9–11].

The primary tumor is generally surgically removed without any additional exposure, since such exposure will not have any effect on the micrometastases. TAM metabolize chemotherapeutic agents and significantly increase the growth factors and proangiogenic factors secretion to stimulate tumor growth and further metastasis in response to chemotherapy [12]. The macrophage epigenetic program is the link between the genetic code and interaction with stressful factros, endogenous pathological factors and infectious agents. Macrophage pathological programming can create the conditions, in which even single-cell micro-metastases can develop into deadly secondary tumors resistant to chemotherapy and immunotherapy. New approaches for breast cancer treatment aim to edit the TAM epigenome, using advanced CRISPR/dCas technology for targeted delivery of epigenome editors to target gene promoters. The authors of this article developed a model system of human primary macrophages *ex vivo* and demonstrated long-term programming of human macrophages using a new biomaterial-based cytokine release system [13].

Precise methods of metabolome analysis — in particular of lipids, the main energy component and building material of membranes, and of amino acids, the monomeric units of proteins — are required to study changes in immune system cells during reprogramming. A technique for metabolome analysis in model TAM and in monocytes of patients with tumors of varied localization will allow to study the molecular composition of monocytes in patients with tumors of varied localization and will contribute to the development of technology for clinical application of any discovered correllations. Data that can be obtained with such a technique is of interest for solving both fundamental and applied problems in biology and medicine; it may be particularly useful for studies of mechanism of pathogenesis in malignant neoplasm. Liquid chromatography combined with mass spectrometry (HPLC-MS/MS), the method this study employs, is currently the most effective approach to determining the molecular composition of biological samples [14]. The key problem of existing approaches is the lack of consensus on the minimum number of cells for analysis, which is especially relevant for monocytes and TAM of patients with cancer, since the complex procedure of obtaining monocytes and TAM requires a large amount of whole blood, long-term post-treatment and differentiation in in culture conditions ex vivo in the absence of cell proliferation.

Our study aimed to identify the minimum number of human immune cells (TAM and monocytes) necessary for the metabolomic profile analysis of these cells in oncological diseases using HPLC-MS/MS.

METHODS

Samples from healthy volunteers were used to determine the optimal number of cells to obtain results in metabolomic spectrum study. The inclusion criteria for healthy volunteers: no history of cancer; absence of chronic diseases in the acute phase. In the study of a group of patients, samples were obtained from four patients with breast cancer (BC) T1–4N0–3M0 (stages IIA–IIIB) luminal B-subtype undergoing treatment at the Oncology Research Institute clinic of the Tomsk National Research Medical Center in 2021. Selection criteria: age 45–55 years; no history of cancer of another localization; no hereditary predisposition to breast cancer (based on family history and genetic testing for the 5382insC, 4153delA, 185delAG mutations in the BRCA1 gene); perimenopause or menopause; morphological verification of the nonspecific invasive carcinoma diagnosis (according to the WHO 2019 classification); estrogen receptors (ER) expression ≥ 5 points; Ki-67 ≥ 40%; no hematogenous metastases. Exclusion criteria: age < 45 years or > 55 years; history of cancer; hereditary predisposition; premenopause; all histological subtypes except non-specific invasive carcinoma; ER expression < 5 points; Ki-67 < 40%; hematogenous metastases. An immunohistochemical study was performed before treatment to determine the tumor molecular subtype in accordance with the ASCO/CAP recommendations. BC stage was determined according to the AJCC TNM classification (8th edition, 2017).

We studied the TAM model system [13], blood monocytes from breast cancer patients before treatment and blood monocytes from healthy volunteers. Monocytes were isolated from the whole blood (patients) or from the buffy coat (donors). The $CD14^+$ cell fraction was obtained by positive magnetic separation using the Miltenyi Biotec protocol. Model differentiated M2 macrophages were obtained by culturing monocytes from healthy volunteers at a concentration of 10^6 cells per 1 ml of X-VIVO macrophage medium (Lonza; Germany) supplemented with 1 ng/ml M-CSF (Peprotech; Germany) and 10^{-8} M dexamethasone (Sigma-Aldrich; Germany). Differentiation of macrophages into protumor M2 macrophages was induced by 10 ng/ml IL4 (Peprotech; Germany). Model TAM was obtained by adding conditioned media obtained from MCF7 breast carcinoma cells to the obtained macrophages in a volume of 10% of the total medium amount. Macrophages differentiated for 6 days at 7.5% CO_2 and were afterwards removed from the culture plates with a rubber scraper in the cold.

The resulting monocytes and macrophages were centrifuged in a solution of cold 0.3% ammonium acetate and 0.3% ammonium formate for 5 min at 311g thrice. After separation of the supernate, the precipitate was lyophilized at –197 °C using liquid nitrogen. Samples were stored at –80 °C until further analysis.

Sample preparation for HPLC-MS/MS analysis

Metalobome (lipid and amino acid composition) of TAM and monocytes from patients with breast cancer was determined by HPLC-MS/MS analysis of cell extracts. Lipid extraction was carried out by the modified Folch method [15–17] with double extraction: cells were mixed with 480 µl of chloroform-methanol mixture (2 : 1, v/v) with 20 µl of IS (internal standard) at 4 °C and subjected to ultrasonic treatment for 10 min; then 150 µl of water was added and the sample was centrifuged

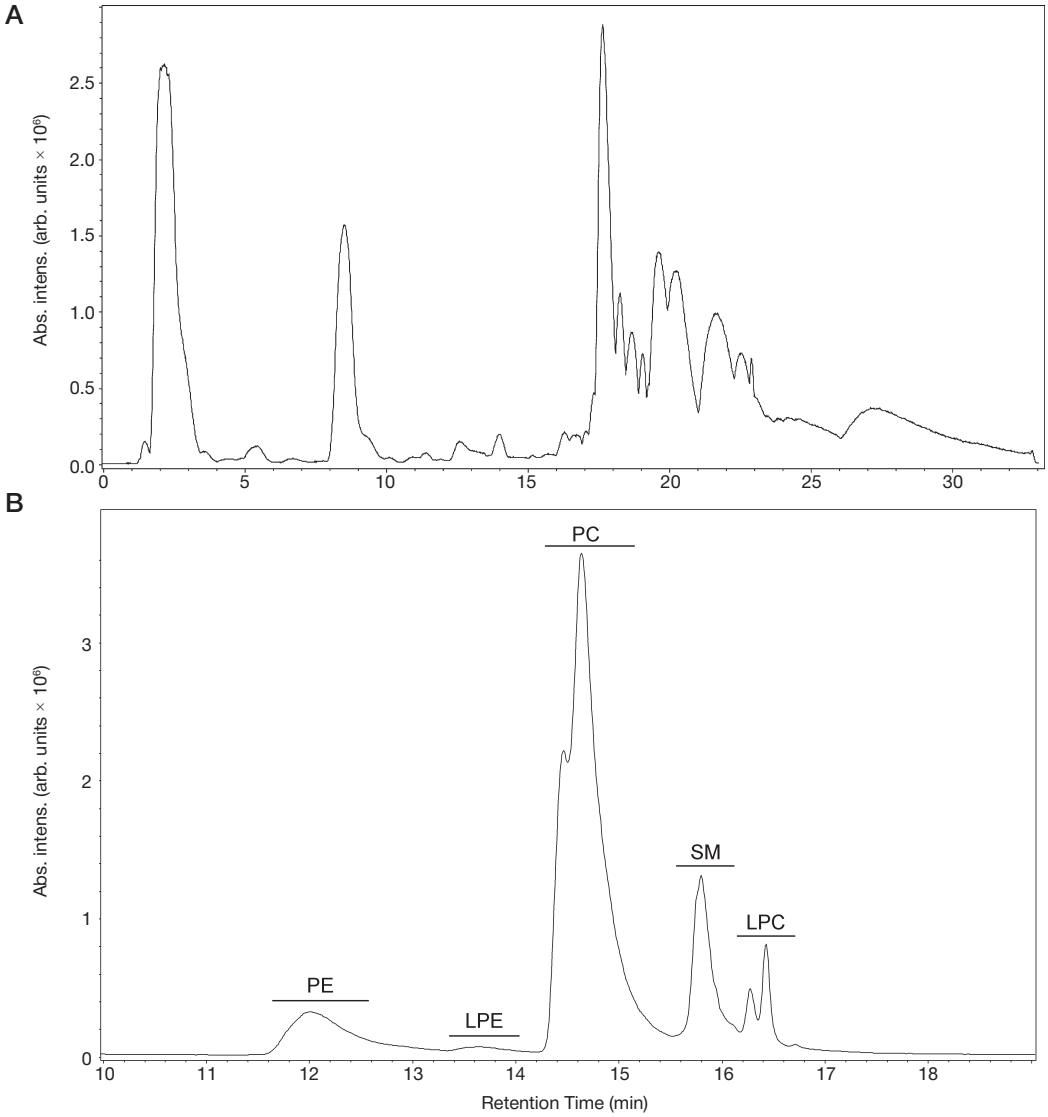

Fig. 1. Characteristic total ion current chromatograms of lipid extracts from monocyte cells recorded in the positive ion mode. (**A**) Reversed-phase chromatography; (**B**) HILIC chromatography. PC — phosphatidylcholines; PE — phosphatidylethanolamines; SM — sphingomyelins; LPC — lysophosphatidylcholines; PE — phosphatidylethanolamines

at 13,000 g for 5 min at room temperature; the lower organic layer containing lipids was taken and the extraction procedure was repeated (adding 250 µl of chloroform-methanol mixture (2 : 1, v/v, 10 min on a vortex; centrifugation at 13,000 g for 5 min); the water-methanol layer was taken into separate tubes; the organic layer was combined with the sample obtained in the first stage and lyophilized in a nitrogen stream. The dried lipid extracts (organic layer) were re-dissolved in 100 µl of isopropanol solution with acetonitrile (1 : 1, v/v). The extracts of water-soluble compounds (water-methanol phase) were used to analyze the amino acid profile: they were redissolved in 100 µl of water with acetonitrile (1 : 1, v/v) and 1% formic acid.

HPLC-MS/MS analysis

Lipid extracts were analyzed on a Dionex UltiMate 3000 liquid chromatograph (Thermo Scientific; Germany) connected to a Maxis Impact qTOF mass analyzer with an ESI ion source (Bruker Daltonics; Germany). Lipid separation by HILIC was performed according to a modified procedure described previously [18, 19]: 3 µL of the sample was injected into the system. Separation was performed on a Spherisorb Si column (150 — 2.1 mm, 5 µm; Waters, USA) at a flow rate of 50 µl/min using acetonitrile as solvent A and aqueous ammonium acetate solution (5 mmol/l) as solvent B (linear gradient from 6 to 23% (v/v) solvent B) for 25 min (column temperature 40 °C). Separation of lipids by reversed-phase chromatography was carried out on a Zorbax C18 column (150 — 2.1 mm, 5 µm; Agilent, USA) with a linear gradient from 30 to 90% of eluent B (solution of acetonitrile/isopropanol/water, 90/8/2 vol., with the addition of 0.1% formic acid and 10 mmol / l ammonium formate) for 20 minutes. A solution of acetonitrile/water (60/40 vol.) with the addition of 0.1% formic acid and 10 mmol/l ammonium formate was used as eluent A. The elution flow rate was 40 µL/min, the injected sample volume was 3 µL. Mass spectra were obtained in the positive and negative ion mode for 100–1700 m/z (capillary voltage — 4.1 kV; spray gas pressure — 0.7 bar; drying gas flow rate — 6 l/min; drying gas temperature — 200 °C). To identify lipids, tandem mass spectrometry was performed in dependent scan mode with a window width of 5 Da.

Amino Acids LC-MS/MS Analysis Kit (Jasem; Turkey) (1-methyl-L-histidine, 3-aminoisobutanoic acid, 3-methyl-L-histidine, β-alanine, DL-5-hydroxylysine, DL-homocystin, ethanolamine, γ-aminobutanoic acid, L-2-aminoadipic acid, L-2-aminobutanoic acid, L-alanine, L-anserine, L-arginine,

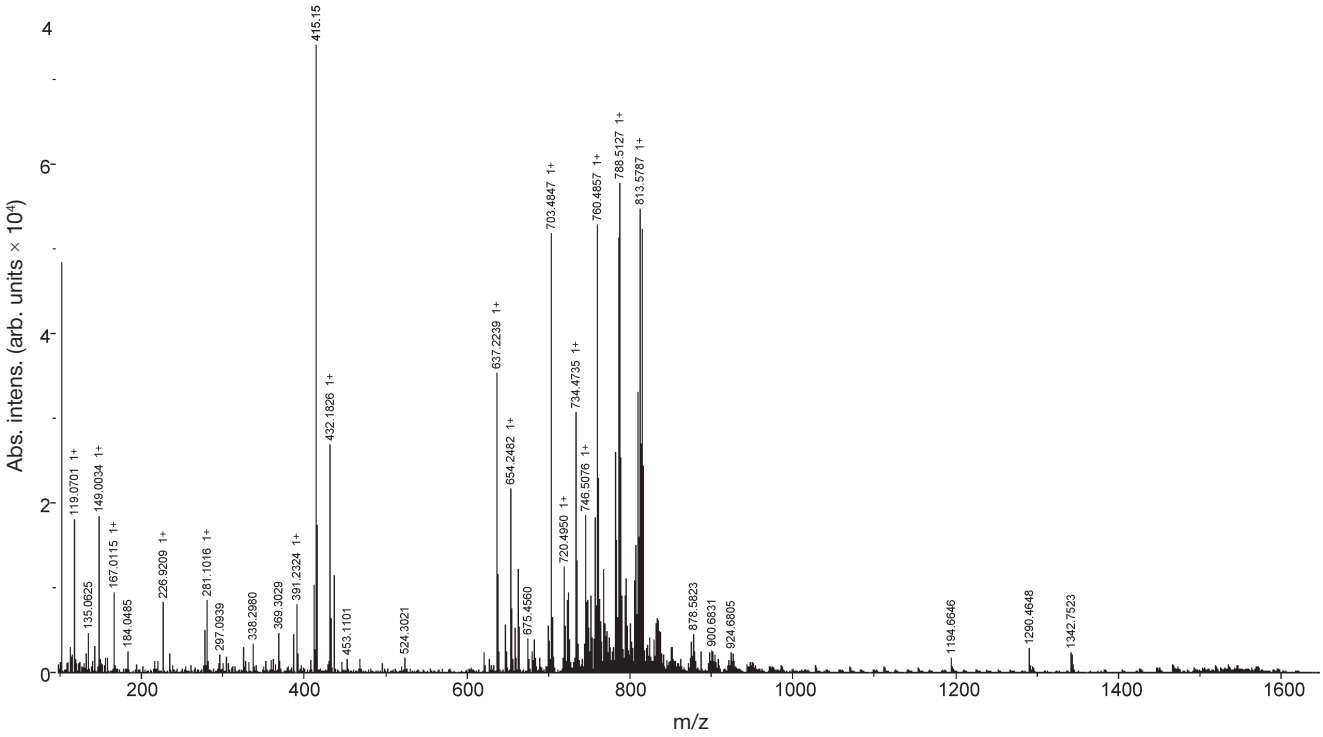

Fig. 2. Time-averaged HPLC-MS mass spectrum of positive ions of a studied sample of macrophage lipid extract, obtained by reversed-phase chromatography

L-asparagine, L-aspartic acid, L-carnosine, L-citrulline, L-cystathionine, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-homocitrulline, L-lysine, L-norvaline, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, sarcosine, taurine, trans-4-hydroxy-L-proline) was used for quantitative analysis of 38 amino acids in the cells. 40 µl of aqueous cell extract was mixed with 50 µl of internal standard solution and 300 µl of Reagent № 1 (Jasem; Turkey); the resulting mixture was thoroughly shaken for 2 min, then centrifuged at a rotation speed of 13,000 rpm for 10 min; 200 µl of the supernatant was taken into a vial with an insert, 15 µl was injected into an HPLC-MS/MS chromatographic system consisting of an Agilent 6460 triple quadrupole mass spectrometric detector (Agilent; USA) and an Agilent 1260 II liquid chromatograph (Agilent; USA) with a column Amino acids-HPLC Column (Jasem; Turkey).

Conditions for chromatographic separation: temperature — 30 °C, eluent A — 0.1 vol.% formic acid in water; eluent B — acetonitrile 100%, gradient elution from 78% B to 20% B in 4.5 min; return to 78% B and isocratic mode. Amino Acid LC-MS/MS Analysis Kit (Jasem; Turkey) was used for amino acid analysis. Ion source parameters ESI: positive mode; drying gas temperature — 150 °C; capillary voltage — 2 kV. Mass spectrometry was performed in the multiple reaction monitoring (MRM) mode.

Lipid identification and analysis of GC-MS data

For preprocessing of chromato-mass spectrometric data, the msConvert programs from the Proteowizard 3.0.9987 package were used to convert files into the MzXml format, which contains information about the mass spectrum at any moment in time, and the ms2 format, which contains information about tandem mass spectra. In addition, the MzMine program was used to isolate the peaks and create a table containing information on the registered ions: the mass of the ion, the area of its chromatographic peak, and the release time. To identify lipids, the LipidMatch program [20] was used, which took into account the exact mass (0.01 Da) of the corresponding ion, the mass of fragment ions from the tandem mass spectra of the identified ion falling within the specified mass window (3 Da), and the specified vicinity of the retention time (0.5 min). Semi-quantitative analysis of lipids was performed using previously developed R language scripts [11–23].

RESULTS

To develop an optimal methodology for studying human primary macrophage lipidome by LC/MS analysis of lipid extracts, several extraction/chromatography separation methods were compared, including reversed-phase chromatography and hydrophilic interaction liquid chromatography (HILIC), and the minimum required cell number was determined. The efficiencies for three extraction methods were compared in advance. One protocol, adapted from an earlier study [19], used a modified Folch method [2] with double extraction (see Methods). The second protocol was similar to another previously described approach [15], also Folch method-based [16], with single extraction. Shortly, the cells were mixed with 200 µL of ammonium acetate aqueous solution (155 mmol/L) and 990 µL of cold chroloform/methanol (10 : 1, v/v) and sonicated for 10 min; the sample was diluted with 150 µL of water and centrifuged at 13,000 g for 5 min at room temperature; the lipid-containing lower organic phase was dried in a nitrogen flow and re-dissolved in 200 µL of isopropanol/acetonitrile (1 : 1, v/v).

The third protocol employed the Bligh and Dyer extraction principle [24]. Shortly, the cells were mixed with 300 µL of chroloform/methanol (1 : 2); then 100 µL of chloroform were added and the sample was homogenized for 1 min; then 100 µL of water were added, the sample was homogenized for another 1 min and centrifuged at 1,000 g for 10 min at 4 °C. The organic phase was collected and the polar phase was re-extracted with 2 mL of cold chloroform; the organic phase was collected, mixed with the extract obtained at the previous step, dried in a nitrogen flow, re-dissolved in 4 mL of

Table 1. Number of identified lipids in analyzed samples

Cell type	Cells (in millions)	Number of lipids identified by exact mass and characteristic tandem mass spectrum	Number of lipids identified by exact mass
Monocytes	1	80	638
	2	92	701
	5	111	726
	10	183	2727
TAM	1	137	825
	2	176	916
	5	194	900
	10	205	933

cold chloroform/methanol (1 : 1) with added 1.8 mL of NaCl aqueous solution (20 mmol/L) and double-filtered using 0.2 μm PFTE syringe filter.

The second method with a single Folch extraction showed low efficiency for weakly polar lipids (triglycerides, diglycerides and ceramides). The advantages of the third method include the possibility of successful extraction of cardiolipins on a par with other classes of lipids. However, the approach is laborious and involves more stages than the first two protocols, which hampers the reproducibility and extraction yields. Overall, the first protocol turned out to be the most universally applicable and optimal in terms of reproducibility, as well as labor time costs, and accordingly was used as a method of choice in subsequent experiments. Importantly, the water-methanol phase (discarded in the course of lipid extraction) can be preserved for analysis of polar metabolites, notably amino acids, in the same sample.

In this study, we tested two principal techniques of chromatographic separation as applied to lipidome: reversed-phase chromatography and HILIC. Characteristic chromatograms and mass spectra of the studied lipid extracts are given on Figures 1 and 2. In the positive ion mode, about 200 lipids were identified using characteristic tandem mass spectra and about 1000 lipids were identified by exact mass alone. The identified lipids were classified as mono, di- and triglycerides, ceramides and various modifications of phosphatidylcholines and phosphatidylethanolamines including oxidation products. HILIC advantages include default separation of lipids into classes; however, the MS peak intensity obtained by this technique is on average 30% lower compared to reversed-phase chromatography. In addition, HILIC method is of questionable suitability for non-polar lipids (notably triglycerides), poorly bound by the column and often lost (being eluted at a timepoint close to the 'dead time' of the column). Accordingly, the number of lipid identifications by HILIC was 25% lower. Subsequent tests were carried out using reversed-phase chromatography.

Table 1 summarizes the lipidomic data for the analyzed samples. Extracts prepared from 2×10^6 monocytes presented with 15% more lipids than 1×10^6 cell extracts, whereas for 5×10^6 vs 2×10^6 and 10×10^6 vs 5×10^6 the differences constituted 21% and 65%, respectively. For TAM, 2×10^6, 5×10^6 and 10×10^6 cells provided 28%, 10% and 6% more identifications than 1×10^6, 2×10^6 and 5×10^6 cells, respectively. Thus, with limited access to biological material (primary human macrophages), it is necessary to analyze at least 2×10^6 cells, as the 1×10^6 to 2×10^6 transition provides the maximal increment in the number of identifications. For monocytes, no corresponding increment dynamics were encountered.

The results of amino acid profiling of the monocyte and TAM samples are provided in Table 2. The chromatographic peak areas correlate with cell number, with the exception of the smallest monocyte samples containing 1×10^6 cells, which is apparently due to LC/MS sensitivity limits. In this regard, samples suitable for the analysis should contain at least 2×10^6 cells.

CONCLUSIONS

We developed a HPLC-MS/MS-based method for monocyte and cultured TAMs metabolome analysis. The modified Folch

Table 2. Areas of chromatographic peaks of amino acids obtained from the analysis of samples of monocytes and TAM

Sample	Monocytes				TAM			
Cells (in millions)	1	2	5	10	1	2	5	10
3-Methyl-Histidine	7	2	64	187	4	45	76	169
L-Arginine	167.432	85.895	169.003	261.068	192.217	425.313	775.226	798.153
L-Cystine	496	144	158	423	85	386	184	400
L-asparagine	1199	758	1674	3884	70	1443	1265	3290
L-Glutamine	97.176	39.931	105.365	288.808	53.604	145.366	465.023	472.766
L-Histidine	18.103	8496	17.941	34.787	12.253	33.254	114.909	65.534
L-Lysine	97.176	39.931	105.365	288.808	53.604	145.366	465.023	472.766
L-Ornithyne	1354	562	1988	3836	725	2043	2720	1437
L-Phenylalanine	6023	6793	8427	30.896	3393	3052	0	5769
L-Serine	2675	2503	5180	14.425	2658	3134	2617	10.421
L-Threonine	2438	1531	2835	8252	1614	2111	2089	7324
L-Tyrosine	646	1038	884	3756	278	326	0	9464
Sarcosine	6044	5857	12.512	34.552	5858	8329	10.555	28.340
Taurine	92	333	462	1311	204	247	201	944

method with double extraction was chosen as the optimal sample preparation method because it allows to simultaneously isolate the lipid extract (i.e. the organic phase) and the extract of water-soluble substances, in particular amino acids (i.e. the water-methanol phase). The most intense peaks in the mass spectra correspond to lipids and other metabolites, proving the effectiveness of this method of metabolomic profile obtainment. Within the framework of this study, use of both HILIC and reversed-phase chromatography was analyzed: the two methods yield different chromatographic separations for HPLC-MS/MS of lipid extracts of cells, with data yielded by reversed-phase chromatography proving more useful. A comparative analysis of lipid and amino acid composition of monocyte and macrophage samples containing 1×10^6, 2×10^6, 5×10^6, and 10×10^6 cells was carried out; lipids and amino acids were found in all studied samples, but the most effective identification can be performed for samples containing at least 2×10^6 cells. The developed HPLC-MS/MS-based approach for cellular metabolome analysis will be used to determine the metabolomic composition of macrophages and monocytes, and to search for characteristic differences in the metabolomic profile of TAM and monocytes in patients with cancer.

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