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General Review

Metabolomics – an overview. From basic principles to potential biomarkers (part 1)

Métabolomique – un aperçu. Des principes de base aux biomarqueurs potentiels. 1re partie

A. Agin [a,*,b,c], D. Heintz [d], E. Ruhland [a,b], J.M. Chao de la Barca [e], J. Zumsteg [d], V. Moal [c,e], A.S. Gauchez [c,f], I.J. Namer [a,b]

[a] *Service de biophysique et de médecine nucléaire, hôpital de Hautepierre, hôpitaux universitaires de Strasbourg, avenue Molière, 67200 Strasbourg, France*
[b] *ICube, CNRS, UMR 7357, fédération de médecine translationnelle de Strasbourg, faculté de médecine, université de Strasbourg, 67412 Strasbourg, France*
[c] *Groupe de biologie spécialisée, SFMN, 75270 Paris, France*
[d] *CNRS, UPR 2357, institut de biologie moléculaire des plantes, 67000 Strasbourg, France*
[e] *Département de biochimie et génétique, CHU d'Angers, 49933 Angers, France*
[f] *UMR-S Inserm 1039, pôle de biologie, CHU de Grenoble, 38043 Grenoble, France*

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Abstract

The metabolome, final downstream product of the genome and exogenous sources, is characterized by a large number of small molecules exhibiting a huge diversity of chemical structures and abundances, requiring complementary analytical platforms to reach its extensive coverage. The metabolome is dynamic, reflecting the continuous fluxes of metabolic and signalling pathways. Metabolomics is the detection and quantitation of metabolites in biological samples. Furthermore, recently emerged technologies have enabled us to determine spatiotemporal distribution of biological molecules present in tissues. In a two-part article, we present a description of usual metabolomics technologies, workflows and strategies leading to the implementation of new clinical biomarkers. In this first part, after introducing metabolomics concepts, we review analytical techniques used in this setting.

Keywords: Metabolomics; Nuclear magnetic resonance; Mass spectrometry; Mass spectrometry imaging; Biomarker

Résumé

Le métabolome, produit terminal issu du génome et de sources exogènes, est caractérisé par un nombre important de petites molécules. Ces molécules présentent une très grande diversité de structures chimiques et d'abondances, nécessitant des plateformes analytiques complémentaires pour une description globale. Le métabolome est dynamique, reflétant les flux continus de voies métaboliques et de voies de signalisation. La métabolomique consiste à détecter et quantifier les métabolites présents dans des échantillons biologiques. Des technologies récentes nous permettent de déterminer la distribution spatiotemporelle des molécules présentes dans les tissus. Dans deux articles, nous présentons une description des technologies utilisées en métabolomique, des *workflows* et des stratégies menant à l'introduction de nouveaux biomarqueurs en clinique. Dans cette première partie, après avoir introduit les concepts liés à la métabolomique, nous passerons en revue les techniques analytiques utilisées dans ce domaine.

Mots clés : Métabolomique ; Résonance magnétique nucléaire ; Spectrométrie de masse ; Imagerie par spectrométrie de masse ; Biomarqueur

* Corresponding author.
E-mail address: agin@unistra.fr (A. Agin).

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1. Basic concepts

1.1. Metabolomics

Systems biology aims at model complete biological systems and pathological processes by overcoming the current limitations of organism and disease complexity with tools enabling high-throughput and large-scale analysis of complex molecular composition [1–3]. This discipline includes so-called omics sciences: genomics (DNAs) and transcriptomics (mRNAs) for the ''blueprint'' assessment; proteomics (proteins) for measuring the means by which these ''orders'' are carried out; and metabolomics (metabolites) for assessing the ''true real-time'' processes [2,4]. As genes and proteins are subject to epigenetic and post-translational modulations, respectively, metabolites reflect the functional level of a biological system (e.g. cell, tissue, body fluid, organism) taking into account environmental stresses [5,6]. Metabolites as downstream products provide a direct, integrated and most ''up-to-date'' insight into the state of a system namely the phenotype [2,7].

Metabolomics is an interdisciplinary ''omics'' approach described as the global, unbiased, qualitative and quantitative analysis of the metabolome in a biological system [1,8–10]. Metabolome is the total metabolite content of a biological sample. As the body is composed of approximately 500 different cell types, we can consider that there are at least as many different cellular metabolomes, as well as those of the extracellular fluid compartments and the various secretory and excretory biological fluids [10]. However, metabolic profiles are not limited to endogenous metabolites but also contain metabolites originating from exogenous sources such as diet, drug and gut microbiota. Mammalian gut microflora metabolic cross-talks represent an increasingly studied major determinant in health or pathologic conditions [4,11,12]. Therefore, metabolomics covers a broad range of small molecules (metabolic intermediates, hormones, signalling molecules and secondary metabolites) such as amino acids, peptides, lipids, nucleic acids, carbohydrates, organic acids, ketones, aldehydes, amines, steroids, vitamins, etc. [4,6,9,11]. It is still difficult today to estimate the number of metabolites in human metabolome [11]. The most comprehensive database on human metabolome (Human Metabolome Database, HMDB) currently includes more than 40,000 metabolite entries. While other ''omics'' study sets of relatively chemically similar biopolymers (4 different nucleotides and 22 amino acids), metabolomics covers structurally heterogeneous and physico-chemical diverse molecules. Furthermore, the dynamic range of metabolite concentrations exists across nine orders of magnitude [13–15]. The chemical variability of the metabolome requires the use of multiple analytical platforms allowing simultaneous measurement of such a broad spectrum of bioactive compounds [1]. Moreover, metabolomics offers a holistic approach to clinical medicine and relies on multiple science areas like analytical biochemistry, biology, bioinformatics, epidemiology and clinical research [10,16].

1.2. Strategies to assess the human metabolome

Metabolomic studies can be divided into two different approaches for the detection of metabolites: untargeted and targeted metabolomics [16–18].

The untargeted or global approach has the aim of simultaneously measuring as many metabolites as possible (including analyte identification of unknown signals) without having prior knowledge of the nature and the identity of assessed metabolites. Datasets are particularly complex and a number of metabolites remain uncharacterized. Minimal pretreatment has to be applied to prevent the loss of metabolites [6,17,18]. Numerous analytical platforms have been used, such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) or high-resolution mass spectrometry (HRMS). Although untargeted metabolomics is limited to qualitative and semi-quantitative information, this approach enables novel areas of metabolism to be identified and is therefore often hypothesis generating [16,17].

Targeted approach refers to the exact quantification of known and expected metabolites by employing analytical standards. In this strategy, predefined metabolite-specific signals are used to quantify, precisely and accurately, concentrations of a limited number of metabolites [16,18]. Sample preparation and optimal instrument adjustment are key elements for accurate quantification of selected metabolites. In contrast to untargeted metabolomics, the targeted approach is driven by specific hypothesis that motivates the investigation of particular biochemical pathways [6].

2. Insight into technology

2.1. Introduction

Identification and quantification of metabolites can be achieved by sophisticated methods, which are supplementary and complementary to one another [8,19]. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies offer multifaceted and powerful approaches to describe parts of the metabolome [19,20]. However, none of these techniques alone can cover the entire metabolome of an organism. The choice is guided by the strengths and limitations of the different techniques [14]. Depending on the goals of the study, these analytical tools may be used either alone, in parallel or in combination [8,20].

2.2. NMR-based metabolomics

NMR spectroscopy is an analytical tool for the structure elucidation of a molecule. This technique is based on the energy absorption and re-emission of the atom nuclei due to variations in an external magnetic field [21]. In the metabolomics study, the principal analysis is the proton (^{1}H NMR) due to its naturally abundance in biological sample. However, other experiments can be used like HSQC (^{1}H-^{13}C correlation) or TOCSY (^{1}H-^{1}H correlation) for 2D spectra or ^{13}C NMR and ^{31}P NMR for 1D spectra. These analyses provide supplementary

information about metabolites [22]. NMR spectroscopy is a fast and reproducible analytical technique. This technique has the major advantage of providing a qualitative and quantitative analysis at the same time.

2.3. MS-based metabolomics

MS has been defined by the International Union of Pure and Applied Chemistry (IUPAC) as "the study of systems by a process of forming gaseous ions, with or without fragmentation, which are then characterized by their mass/charge ratios (m/z) and their relative abundances". The studied species must be charged particles (ions) because mass separation relies on the movement of these particles under the influence of electric and/or magnetic fields. High vacuum conditions, where the mean free path of charged particles is sufficient, are required to ensure that ions mostly travel without collisions [23]. Nonetheless, ion separation by m/z can be also assessed in field free regions (e.g. time-of-flight analyzer) [24]. The term MS encompasses a wide range of technologies, which differ in principles and performances [8,23].

MS-based metabolomics usually requires a prior step of sample preparation. This step may have several objectives; e.g. to extract analytes from a complex biological matrix (as liquid-liquid or solid-phase extractions), to make analytes more volatile and/or less polar (derivatization) or to pre-concentrate the analytes to enhance their detection limits [25].

Four major steps can be described for analysis by MS: (1) a sample introduction system (chromatographic system or insertion probe) introduces compounds to be analyzed in the mass spectrometer; (2) ions are produced from neutral sample molecules in an ionization source; (3) ions are then separated according to their mass-to-charge ratio (m/z) in a mass analyzer (4); an ion detector enables determination of ion abundance (Fig. 1). These steps take place with or without fragmentation of ions depending on the type of method [23,26–28].

2.3.1. Introduction methods

Direct-infusion MS of crude mixtures without prior chromatographic separation has been described as very susceptible to ion suppression (which occurs when ionization capacity is overcome by large quantities of analytes or background ions) [8,29]. Despite improved ionization techniques, direct injection of the sample in the mass spectrometer is less common than prior chromatographic separation of the analytes especially for a complex matrix as biological samples.

Thus, hyphenated techniques combining chromatography separation with MS detection are widely used in the metabolomics field. Chromatographic separation of metabolites reduces matrix effects, separates isomers, provides additional data for metabolite characterization and enhances quantification. Usual chromatography methods in metabolomics are gas chromatography (GC), high-performance liquid chromatography (HPLC or LC) and less commonly capillary electrophoresis (CE) [8,19].

2.3.1.1. Gas chromatography. The basic principle of GC involves the volatilization of the sample heated in an injector and the separation of its components in a heated hollow tube (column). The difference in volatility and polarity of the compounds is responsible for differences in distribution between the carrier gas (hydrogen or helium) and a stationary phase (coating of the column). A component that spend little time in the stationary phase elute more quickly. This technique

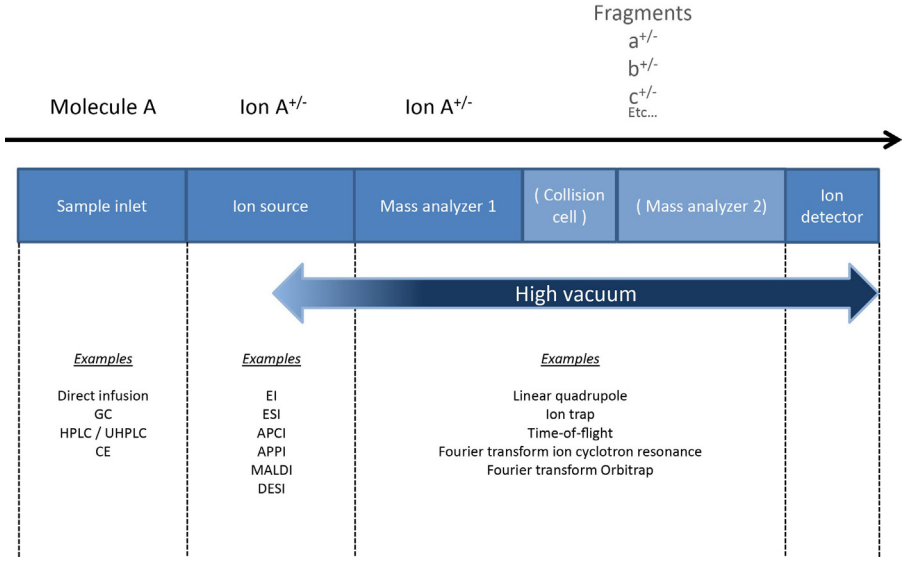

Fig. 1. General scheme of a mass spectrometer. GC: gas chromatography; HPLC: high-performance liquid chromatography; UHPLC: ultra-high pressure liquid chromatography; CE: capillary electrophoresis; EI: electron impact; ESI: electrospray ionization; APCI: atmospheric-pressure chemical ionization; APPI: atmospheric-pressure photoionization; MALDI: matrix assisted laser desorption; DESI: desorption electrospray ionization.
Schéma général d'un spectromètre de masse. GC : chromatographie en phase gazeuse ; HPLC : chromatographie liquide haute performance ; UHPLC : chromatographie liquide ultra-haute pression ; CE : électrophorèse capillaire ; EI : impact électronique ; ESI : ionisation électrospray ; APCI : ionisation chimique à pression atmosphérique ; APPI : photo-ionisation à pression atmosphérique ; MALDI : matrix assisted laser desorption *; DESI :* desorption electrospray ionization.

is suitable for compounds, which can be vaporized without decomposition and is also widely used with volatile-rendered compounds using derivatization however, extending the analysis time [29–31].

2.3.1.2. Liquid chromatography. In LC (or HPLC), the mobile phase is a liquid flowing through a column packed with chemically derivated beads forming the stationary phase [29]. LC is considered more versatile than GC and is suitable for separating metabolites independently of their polarity or volatility [20,27,32]. Metabolite separation in LC is determined not only by their physicochemical properties (e.g., hydrophobicity, charge, size) but also by their affinity with the stationary phase [29]. LC has benefited from many improvements among which should be mentioned ultra-high pressure liquid chromatography (UHPLC) and capillary LC. Speed, resolution and sensibility have been increased in available UHPLC systems by reducing packing-particle diameter from 5 µm to less than 2 µm, operating at pressures within the 6000–15,000 psi range. With the capillary chromatography, size reduction of the column is accompanied by the decrease in flow rate, which also increases the sensitivity [5,8,20,32–34].

2.3.2. Ionization source

To be analyzed in a spectrometer, a molecule must be in the form of gas-phase ions (i.e., in a charged state within a high vacuum) [27,29]. This is quite straightforward when using GC because molecules are already in gaseous form by the time they reach the mass spectrometer. There are several techniques available for ionization in GC-MS and LC-MS devices but the coupling is more problematic for LC-MS because gas-phase ions must be produced before entering the MS [27].

Internal energy transferred during the ionization process is an important characteristic of an ion source. Very energetic ionization techniques cause extensive fragmentation yielding a characteristic mass spectrum. Softer techniques produce ions of the molecular species [27]. The most often-used ionization methods in the field of metabolomics are electrospray ionization (ESI–soft ionization method) and electron impact (EI) ionization (EI–hard ionization) [19]. EI and ESI are widely used in GC-MS and LC-MS, respectively. Although other types of ionization sources are used in metabolomics studies, only these two types of ion sources will be discussed here.

When working conditions (e.g., electron energy, temperature and pressure) are kept constant spectra obtained by EI exhibit good reproducibility. This feature enables not only a straightforward comparison between spectra coming from different manufacturers but also the establishment of useful spectral libraries for untargeted metabolomics [28]. These libraries are available only for volatile and thermally stable compounds as far as EI is only implemented in GC-MS spectrometers [27].

The analytes (in solution) are introduced by nebulization as charged droplets into an ESI source, ultimately leading to the formation of gas phase ions. Ions are produced at atmospheric pressure and focused through vacuum pumping stages [27,33]. ESI can be used for metabolites as well as for larger molecules like peptides and proteins. Therefore, ESI is the most commonly used technique in LC-MS ‘‘omics’’ studies including metabolomics [19,33,35].

2.3.3. Mass detectors

To date there is an overwhelming amount of available detectors for MS and the reader is referred to specialized textbooks for detailed information concerning each detector. Even when all detectors measure a mass-to-charge ratio (m/z) for gas-phase ions they have different performances according to mass range limits, analysis speed, mass accuracy and mass resolution [27,29,35].

2.3.3.1. Ion trap. An ion trap uses a RF quadrupolar-oscillating field to trap ions in two or three dimensions: 2D or 3D ion traps. Ions of different masses are trapped on a 3D or 2D trajectory and expelled successively according to their mass-to-charge ratio so as to obtain a spectrum [27].

2.3.3.2. Quadrupole. A quadrupole analyzer is made of four cylindrically or hyperbolically shaped rod electrodes extending in the z-direction parallel to each other. Positive ions entering the space between the rods in z-direction are drawn towards the negative charged rod. In the same way, negative ions will be drawn toward the positive charged rod. If the sign of the potential, which is composed of a DC and a RF component, switches before ion discharges itself on the rod, the ion will deviate on the x- and y-directions. Ions are separated based on the stability of the trajectories in oscillating electric fields according to their mass-to-charge ratio [27,28].

2.3.3.3. Time-of-flight (TOF). Ions are accelerated by an electric field and then separated according to their velocities in a free-field region (flight tube of known length). Ion drift-time depends on mass-to-charge ratio: lighter ions arrive earlier at the detector than the heavier ones. Mass resolution has been enhanced in today’s TOF analysers by lengthening the flight tube or by adding a reflectron. A reflectron creates a retarding electric field that acts as an ion mirror sending ions back through to the flight tube [27,28].

2.3.3.4. Tandem MS. Tandem mass spectrometry or MS/MS involves at least two stages of mass analysis. MS/MS experiments are conducted in two separate steps either in space or in time. Methods involving two steps separated in space (beam-based MS/MS) rely on the coupling of two physically distinct mass analyzers separated by a collision cell. The first analyzer isolates among ions produced by the source a precursor ion, which undergoes a fragmentation in a collision cell. Fragments called ion products are then assessed by a second analyzer [24,27,35]. The most common MS/MS instruments are triple quadrupole (QqQ–with q corresponding to a quadripolar or hexapolar cell collision) and quadrupole-time-of-flight (Q-TOF) mass analyzers. These analyzers can be operated in different scan modes according to the objectives of the study (e.g. metabolite identification or quantitation).

2.3.3.5. High resolution MS. HRMS is a prerequisite for untargeted metabolomics and the discovery of new metabolites or biomarkers. A large number of data is commonly obtained from analytical platforms based on MS. Comparative metabolomics studies provide usually a huge amount of spectra. For this reason the selection of the instrumentation is an important issue since it is a key factor regarding the number of peaks detected and their sensitivity/specificity. Because of their high resolution, mass accuracy, and full-spectrum acquisition capabilities, high resolution (HR) mass analyzers such as Orbitrap, TOF, or hybrids analyzers like quadrupole-time-of-flight (QTOF) and quadrupole Orbitrap (Q Orbitrap) are adequate to develop metabolic profiling methods in complex biological matrixes e.g. biofluids or tissue extracts [20,36,37]. Additionally less more developed for the moment but increasing year after year, the expensive very high resolution Transform Fourier induced cyclotron resonance (FT-ICR) mass spectrometer that allows the identification of metabolites with the highest mass accuracy and determination of the putative structure are excellent tools for this purpose as well.

2.4. Intact tissue metabolomics

2.4.1. High resolution magic angle spinning (HR-MAS) NMR

Metabolomics assisted by NMR spectroscopy has started at the beginning of the eighties with the first application of C.E. Mountford [38]. The authors used RMN liquid techniques directly on biopsy sample. This experiment highlighted a major loss of resolution due to pseudo-solid state of sample. In fact inside tissues samples, metabolites were immobilized which involved interaction in space (dipolar interaction) and magnetic susceptibility. In order to obtain a better resolution, a NMR technique for solid state [39] has been used for biopsy. This experiment allows working on magic angle (54.7°) with sample rotation in order that average interaction. NMR-HRMAS, is an advanced technology generating a quality spectra similar to liquid spectra [40]. This experiment is often used for biopsy analysis [41] involving sample preparation, which is easy and fast to limit bias preparation.

2.4.2. MS imaging

Understanding the complex biochemical processes that occur within living biological systems requires not only the elucidation of the molecular entities involved in these processes, but also their spatial localization and distribution within the organism. In line with that, a technique called imaging mass spectrometry has recently emerged. This new technology enables us to determine spatiotemporal distribution of biological molecules present in tissue slices by direct ionization and detection without the need of previous time-consuming molecule derivatization. This technique and the word mass spectrometry imaging (MSI) was first coined by the pioneer work of Caprioli's group in 1997 [42]. A simple interrogation in Pubmed with the words ''mass spectrometry imaging'' (or ''mass spectrometric imaging'' or ''imaging mass spectrometry'') in December 2015 gives about 1400 hits of publications, indicating how fast this technique has growing this last decade (Fig. 2). MSI has found a place as major scientific fields and is applied on all type of tissue from all types of living organisms (human, animals, plants, and microbes). MSI is now widely used for in situ imaging of endogenous or exogenous molecules such as proteins, peptides, lipids, metabolites, drugs, pesticides and their fragments or conjugates. MSI is a great new tool for pathological analysis and the investigation of disease mechanisms. To date, MS hyphenated chromatography techniques such as LC-MS or GC-MS has been known to be a conventionally used strategy for metabolomics [43–45]. However, these methods have a major drawback in the analysis of tissue samples because of the requirement of metabolite extraction, which causes the loss of information on the spatial localization of the metabolites. In contrast, imaging techniques capable of determining the spatial localization of molecules have revolutionized our approach to diseases by allowing us to directly examine the pathological process, thereby giving us a better understanding of the pathophysiology (Fig. 3). MSI is therefore a promising technique that could be used for biomarker discovery with additional spatial information. Three main ionization techniques are used nowadays in MSI. The most popular technique currently used in MSI is undoubtedly matrix assisted laser desorption (MALDI), and in less extend nanostructure-initiator MS (NIMS) which could be considered as an improved Direct ionization on silicon (DIOS) method [46,47] and Desorption electrospray ionization (DESI), developed by Cooks et al., 2004. MALDI MSI is the most popular technique coupled to HRMS with a spatial resolution of up to 5 µm. Additionally MALDI MSI takes benefit from the recent progress that have been made in the development of new matrices more specific for a particular class of metabolites e.g. recently, 9-aminoacridine (9-AA) was reported as a suitable matrix for

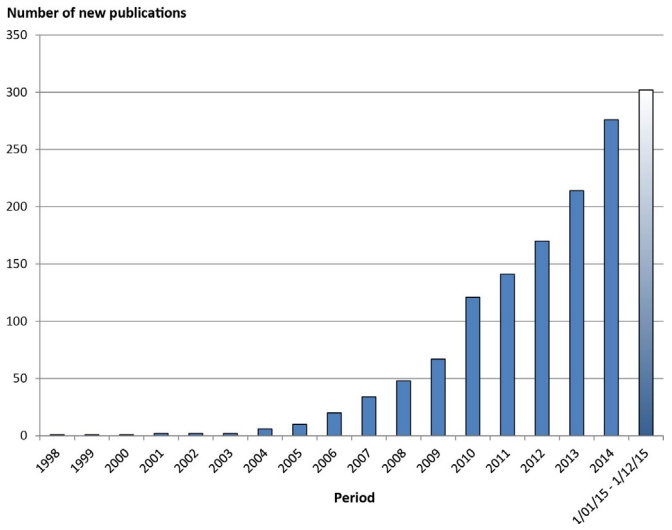

Fig. 2. PubMed search results using ''mass spectrometry imaging'' or ''mass spectrometric imaging'' or ''imaging mass spectrometry'' as the keywords.
Résultat de la recherche dans PubMed avec les mots-clés « mass spectrometry imaging *» ou «* mass spectrometric imaging *» ou «* imaging mass spectrometry *».*

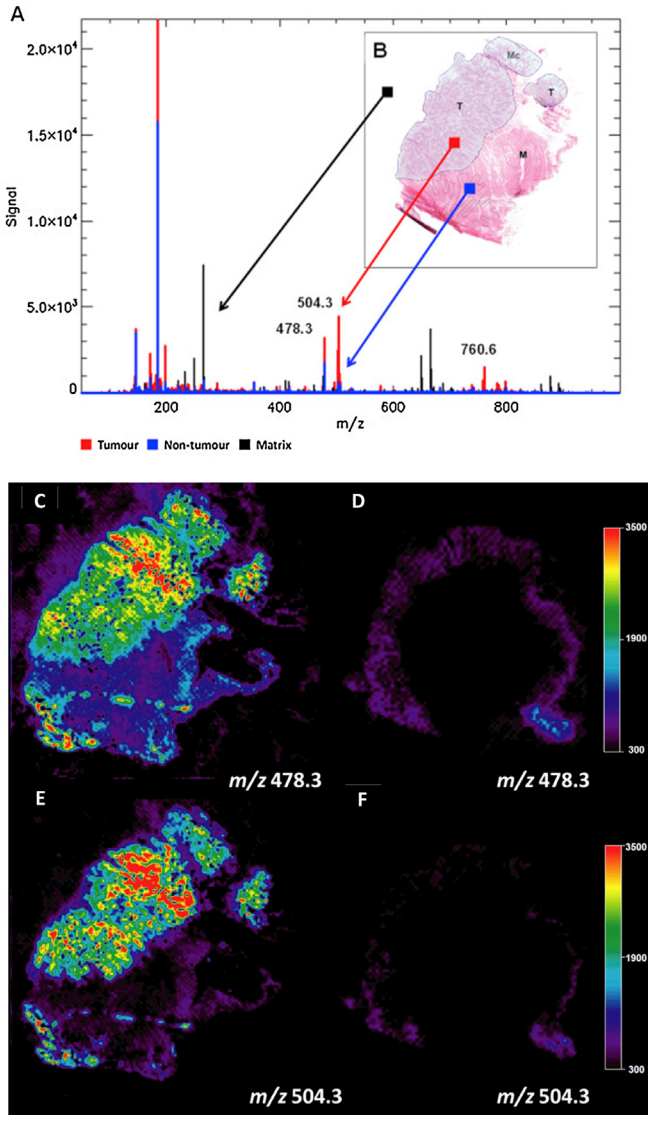

Fig. 3. Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-MSI): intact fresh frozen sections of colorectal cancer tissue analysis with simultaneous localization and quantification of lipids in different histological regions of interest. A. Overlapped MALDI spectral profiles for cancerous tissue (red), non-cancerous tissue (blue) and from regions outside the margin of the tissue section (black, matrix only). B. Digitized image of the corresponding haematoxylin and eosin stained section for this sample with morphological regions of interest defined by a solid line (Mc: mucosa, T: tumour tissue, M: muscle). C, D, E, F. MALDI-MSI ion images revealing the distribution of *m/z* 478.3 (LysoPC(16:0); images C and D) and *m/z* 504.3 (LysoPC(18:1); images E and F) in cancer-bearing (centre of tumour; images C and E) and non-cancer-bearing (healthy mucosa 10 cm from the tumourmargin; images D and F) tissue sections. These ionic species are seen to be specifically over-expressed in cancerous regions with little expression evident in healthy tissue.

Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-MSI) : analyse de coupes intactes fraîches congelées de tissu de cancer colorectal avec localisation et quantification simultanées des lipides dans différentes zones histologiques d'intérêt. A. Profils spectraux MALDI se chevauchant des tissus cancéreux (rouge), des tissus non cancéreux (bleu) et de régions en dehors de la zone de la coupe de tissu (noir, matrice uniquement). B. Image numérisée de la coupe correspondante colorée par l'hématoxyline et de l'éosine pour le même échantillon avec les régions morphologiques d'intérêt délimitées par une ligne continue (Mc: muqueuse, T : tissu tumoral, M : muscle). C, D, E, F. Images MALDI-MSI d'ions révélant la répartition des ions m/z 478,3 (LysoPC (16: 0) ; images C et D) et des ions m/z 504,3 (LysoPC (18: 1) ; images E et F) sur des coupes incluant du tissu cancéreux (centre des tumeurs ; images C et E) et sur des coupes exemptes de tissu cancéreux (muqueuse saine à 10 cm de la marge de la tumeur, images D et F). Ces espèces ioniques sont repérées comme spécifiquement surreprésentées dans les régions cancéreuses avec une faible présence dans les tissus sains.

Adapted from reference [51].

low-molecular-weight metabolite analysis [48]. Lipids became the first targets for MSI studies of endogenous metabolites because the m/z range of most lipid molecules was more than 700. Lipids are also abundant in tissues (e.g., more than 60% dry weight of brain tissue) and are easily ionized because of the presence of a polar head [49,50]. However, the application of MSI to the study of endogenous metabolites has received considerable attention because metabolites are the result of the interactions of a system's genome with its environment and a total set of these metabolites represents more closely the phenotype of an organism under a given set of conditions.

Disclosure of interest

The authors declare that they have no competing interest.

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