State-of-the-art mass spectrometry imaging applications in biomedical research

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Mass spectrometry imaging has advanced from a niche technique to a widely applied spatial biology tool operating at the forefront of numerous fields, most notably making a significant impact in biomedical pharmacological research. The growth of the field has gone hand in hand with an increase in publications and usage of the technique by new laboratories, and consequently this has led to a shift from general MSI reviews to topic-specific reviews. Given this development, we see the need to recapitulate the strengths of MSI by providing a more holistic overview of state-of-the-art MSI studies to provide the new generation of researchers with an up-to-date reference framework. Here we review scientific advances for the six largest biomedical fields of MSI application (oncology, pharmacology, neurology, cardiovascular diseases, endocrinology, and rheumatology). These publications thereby give examples for at least one of the following categories: they provide novel mechanistic insights, use an exceptionally large cohort size, establish a workflow that has the potential to become a high-impact methodology, or are highly cited in their field. We finally have a look into new emerging fields and trends in MSI (immunology, microbiology, infectious diseases, and aging), as applied MSI is continuously broadening as a result of technological breakthroughs.

Introduction

Mass spectrometry imaging (MSI) is a group of molecular imaging techniques that allow labelled and unlabelled visualisation of molecules in organic and inorganic surfaces such as

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Ron M. A. Heeren obtained a PhD degree in Technical Physics from the University of Amsterdam in 1992. He then led a FOM-AMOLF research group on macromolecular ion physics and biomolecular imaging mass spectrometry in Amsterdam (1995–2014), which focused on new approaches towards high spatial resolution and high-throughput molecular imaging mass spectrometry using secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption and ionization (MALDI). He is currently a distinguished professor at Maastricht University and the scientific director of the Maastricht MultiModal Molecular Imaging Institute (M4I), where he heads the Division of Imaging Mass Spectrometry. Ron Heeren has co-authored over 300 peer-reviewed articles and has been awarded several renowned international and national prizes.

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biological tissue sections. Many technological breakthroughs, especially in matrix-assisted laser desorption/ionisation (MALDI) imaging, have significantly pushed the limits of MSI. Some of these technological advances have increased the sensitivity and specificity using MALDI-2 and ion mobility separation, respectively, provided higher throughput, or improved sample preparation methods. Further examples include the combination of MSI with other imaging modalities and other non-spatial molecular analysis techniques or the implementation of advanced data processing & deep learning for data analysis.

The new possibilities offered by MSI, brought on by the above-described technological improvements, have paved the way for novel applications and hence users. The interest in MSI and the MSI community have consequently grown significantly. MSI has now advanced from a technique uniquely available to MS experts with niche applications to a widely applied tool operating at the forefront of numerous fields ranging from drug development in the pharmaceutical industry, to clinical analyses in hospital settings, and fundamental biochemical discoveries in academia.

This is reflected by an exponentially growing number of publications: a PubMed search, done in August 2023, for the terms “mass spectrometry imaging” and “imaging mass spectrometry” shows that the annual number of publications has grown from ten in 2005 to surpassing 500 after 2020 (Fig. 1).

With a growing field of users, a greater focus must now be put on knowledge-transfer to ensure a continuous, proper utilisation of the strengths of MSI. While reviews exist that focus on knowledge-transfer to ensure a continuous, proper utilisation of the strengths of MSI, most of these reviews are focused on methodological studies, which continue developing the technology.

Benjamin Ballu is a computer scientist by training and entered the field of mass spectrometry imaging (MSI) in 2008 during his PhD at the Helmholtz Zentrum München under the supervision of Prof. Dr Axel Walch. For his postdoc, he joined the group of Dr Liam McDonnell, formerly located at the Leiden University Medical Centre. In 2015, he moved to Maastricht University to fill the position of Assistant Professor for Imaging Bioinformatics at the Maastricht MultiModal Molecular Imaging Institute. His interests in the field of MSI include the integration of MSI with other (imaging) modalities, translational MSI, and the development of novel MSI techniques, which require the interaction of computational and wet-lab-based approaches.

### Oncology

Since its beginnings, biomedical-oriented MSI has been predominantly applied to study the molecular setup of cancerous tissues because it allows, due to its imaging nature, a differentiated analysis of complex cellular samples. Moreover, MSI addresses several other research gaps and clinical needs of cancer (Fig. 2, centre). Initially, the main purpose of MSI was to “extract” the molecular profile of the tumour cells to find...
biomarkers or molecular patterns for staging the disease or follow-up data (Fig. 2D). Over the last few years, it has been realised that the cellular and molecular heterogeneity of the tumour and its direct environment (presence of immune cells or stromal cells) is one of the most important factors for treatment efficacy. Consequently, cancer research focuses now more on intratumour heterogeneity and the cellular and molecular characteristics of the tumour's microenvironment (Fig. 2B), which is reflected in the growing application of spatial omics approaches.

As MSI can be integrated with classical tissue-section based imaging techniques such as optical microscopy, it offers the possibility to study the cellular (by microscopy) and molecular (by MSI) complexity in concert. Moreover, it is the only technique that allows visualising metabolites (Fig. 2A and D), lipids (Fig. 2C), glycans (Fig. 2B), or exogenous small molecules in tissue sections, all molecular classes, which are gaining more attention in oncological considerations. MSI has therefore already given evidence for its broad applicability in the study of cancer, as the selected studies will further demonstrate.

**Novel mechanistic insights in oncology**

MSI detects predictive biomarkers relevant for immunotherapy. MSI has been used extensively in the discovery of predictive biomarkers for cancer treatment response, thereby also revealing biological mechanisms behind a therapy's efficacy. Recently, therapeutic blocking of the PD-1/PD-L1 interaction has been investigated as a new way of reactivating the natural T cell-mediated anti-tumour response. In particular, in non-small cell lung cancer (NSCLC), where tumour cells can express PD-L1 and thereby avoid the adaptive immune system of the patient, this therapy harbours great potential. However,
less than 30% of patients treated with PD-1/PD-L1 blockades will respond, and selective patient stratification is therefore an unmet clinical need.23

In this context, Berghmans et al. showed that overexpression of antimicrobial peptides (AMPs), neutrophil defensins 1, 2, and 3, is associated with a positive response to anti-PD-(L)1 immunotherapy in 25 NSCLC patients.26 Using MALDI-MSI to first visualise differential peptide expressions between tumour- and healthy tissue, they identified three peptides of interest, specifically overexpressed in necrotic tumour tissue. These three peptides-of-interest were identified as neutrophil defensins 1, 2 and 3, on an UPLC-MS3 system. Follow-up in vitro and immunohistochemistry experiments demonstrated that the presence of AMPs is likely to be important for activation of adaptive immunity against cancer cells through the upregulation of IFN-γ and that the degree of neutrophil defensin expression is a prognostic indicator for disease progression. By co-culturing NSCLC tumour cells with immune cells and treating them with neutrophil defensins, they saw a significant increase in IFN-γ expression, which had not been seen in the absence of AMP treatment or in co-cultures with healthy tissue and immune cells. These results suggest that the specific expression-patterns of AMPs have the potential to work as additional biomarkers to predict the patient response to anti-PD-(L)1 immunotherapy for NSCLC.

**MSI provides insights into β-oxidation in cancer.** Metabolic reprogramming in tumours refers to the altered metabolism of cancer cells to help accommodate the increased need for energy to sustain the continuous growth and proliferation of malignant cells. While alterations in the tumour’s energy metabolism are one of its major hallmarks,27 spatial metabolomics studies remain scarce. MSI enables the investigation of metabolic changes in the tumour and its microenvironment.

For instance, Sun et al. utilised a multimodal approach, which combines dual polarity MALDI-MSI with microscopic imaging of histological and immunohistochemical stains, to visualise the reprogramming of carnitine metabolism in breast cancer samples (Fig. 2A).28 Using MALDI-MSI in positive polarity initially, Sun et al. found specifically γ-carnitine & short-chain acylcarnitines to be significantly upregulated in cancer tissues from both xenograft mouse models and 58 human breast cancer samples. Since carnitines play an important role in transporting fatty acids into the mitochondrial matrix via the ‘carnitine shuttle’ as part of the energy production through β-oxidation, these key components of the β-oxidation metabolic pathway were further investigated by MSI and immunohistochemistry. On the one hand, MSI was used to detect short and long chain fatty acids in negative polarity, and on the other hand, immunohistochemistry was used to visualise the expression of several key enzymes (CPT-1A, CPT-2, and CRAT) of the carnitine dependent transport system. All enzymes and fatty acids were found to be significantly increased in the tumour, suggesting that the carnitine shuttle- and β-oxidation pathways are heavily altered, thereby providing novel mechanistic insight and potentially new targets for cancer therapy (Fig. 2A).

**Exceptionally large cohort size**

One of the biggest challenges in clinical MSI concerns the validation of research outcomes due to the mismatch between the high number of dimensions as provided by omics technologies (“curse of dimensionality”) and the low number of samples.29 In MSI, the number of detected ion signals defines the dimensionality and ranges from a few hundreds to several thousands. In contrast to this, the number of human samples for clinically motivated studies rarely surpasses 100 (not considering tissue microarray-based studies).

One of the few exceptions to date is the study by DeHoog et al. who investigated a total of 178 frozen human thyroid tissue samples by DESI-MSI to determine the molecular (mostly lipid) signatures of normal, benign follicular adenoma, malignant follicular carcinoma and papillary carcinoma.10 These signatures were used to build two separate classifiers: one to discern benign patients from follicular carcinomas and one to distinguish benign patients from papillary carcinomas. Importantly, the high number of samples allowed for training and testing of these models on separate and sufficiently large sets of samples, thereby increasing the validity of the achieved prediction accuracies, which were 80% and 93%. Clinical fine-needle aspiration smears from the thyroid of 57 patients were prospectively collected to further validate these findings and demonstrate the translational potential of the results. Applying the two previously tissue-derived classifiers to these cytological samples achieved high accuracies of 93% and 89% to predict if the primary thyroid tissue was benign or malignant, respectively.

Another way to achieve a high number of samples is through the use of tissue microarrays where dozens to hundreds of tissue cores are arranged in one block and can therefore be analysed in the same experimental run (Fig. 2D). Pereira Lopes Gonçalves et al. recently used tissue microarrays to be able to analyse the proteomic content of 1191 human breast cancer samples33 with the aim of establishing machine-learning models for the prediction of the hormone receptor and HER2 status. After splitting the samples into a training (80%) and test (20%) set, different classification models were trained and compared. The k-nearest neighbour algorithms yielded thereby the highest accuracies with >95% accuracy for all classification tasks. While tissue microarrays overestimate the classification performance of clinical reality when samples are measured separately from each other, they offer a great way forward to avoid technical variance between biological replicates and thereby increase sensitivity for more fundamental research questions.

With the advent of more advanced machine and deep learning related studies, it is important to mention that a study’s sample collection should be large enough to allow a split into a training, validation, and test set.32 The first is used to tune the parameters, whereas the test set is used to determine the performance of the final settings of the classifier. It is important to mention that the split of the data should always be on the level at which a classification statement should be done,
Potential high-impact methodologies

Further integration of microscopic imaging and MSI. With the advent of data integration techniques and the rise of deep learning in digital pathology, the question is arising if a combination of quantified microscopic information and mass spectrometry imaging data would improve classification tasks. Oncological research frequently encounters classification tasks.

In that light, Beuque and co-workers have been investigating the potential of integrating MSI and digitalised histology images of oesophageal cancers on a per-pixel level using advanced machine learning. Oesophageal cancer develops over several stages: from Barrett’s oesophagus (BE), over low-grade (LGD) and high-grade-dysplasia (HGD) to cancer. From a clinical perspective it is important to determine the grade and to know which of the dysplasias will progress to cancer.

The authors therefore compared the performance of MSI and histology alone versus the combination of both. Not surprisingly, the tissue type (epithelial tissue vs. stroma) could be best distinguished by histology alone (area-under-curve (AUC)\textsubscript{MSI} = 0.89 vs. AUC\textsubscript{histology} = 0.95), whereas the grade (LGD vs. HGD) was best predicted by MSI alone (AUC\textsubscript{MSI} = 0.97 vs. AUC\textsubscript{histology} = 0.85). Moreover, disease progressors could be predicted with 72% accuracy by MSI as compared to 48% accuracy offered by histology alone. Interestingly, and this might be valid for this application only, the combination of the two modalities did not improve the classification accuracies for a single task but they were complementary for different tasks.

Prade et al. furthered this idea in one of their proof-of-concept examples to showcase their developed Spatial Correlation Image Analysis (SPACiAL) workflow, where tissue sections are first analysed by high-mass resolution MALDI-MSI and subsequently aligned to multiple simultaneous immunostainings of the same section. In the referred example, metabolic intratumour heterogeneity in three human gastric cancer samples was studied by the SPACiAL approach. Tumour cells were thereby identified via pan-cytokeratin immunostaining and then, based on parallel immunostaining, classified as either HER2-positive or HER2-negative, which is an important molecular factor in clinical decision-making for advanced gastric cancers. The pixel-accurate annotation allowed a comparison of the metabolic correlation networks for HER2 negative and positive tumour cells, which were found to be centred around glucose 6-phosphate. Depending on the patient, either bioactive lipids such as lysophosphatidylglycerol or carbohydrates, dipeptides, or glycosylamines showed high correlation to glucose 6-phosphate, suggesting high intratumour heterogeneity. The SPACiAL workflow is a prime example of multi-modal imaging using the strengths of MSI to provide objective analysis & in situ metabolomics on intact tissue sections with high spatial resolution. A crucial component is the accurate spatial alignments between the images from the different imaging technologies, especially in their other example, where the authors compare the metabolic networks of pancreatic alpha- and beta cells located in the islets of Langerhans. This requires close-to or factual single-cell resolution.

Single-cell analyses. An increased spatial resolution of both MALDI- and DESI-MSI now allows measurements at either the single cell or the near single cell level. This is important because intratumour heterogeneity is ultimately defined by the number of single tumour cells. One recent proof-of-principle is the study by Bien et al. who presented a workflow to investigate the molecular heterogeneity in cultured cancer and non-cancerous cells by 2 µm MALDI-MSI and optical microscopy. The authors made use of MALDI-2 to increase ion yields to compensate for the loss in sensitivity for the small pixel sizes. Image registration accuracy can suffer from sample preparation between two modalities such as washes and drying steps that might lead to expansion/shrinkage of cells or tissues. Since Bien and co-workers used a dry MSI sample preparation based on sublimation of the matrix on the slide, staining of the cells and image acquisition could be done prior to the MSI experiment using lipid MSI-compatible dyes (Hoechst stain 33342 for cell nuclei and wheat germ agglutinin for cell membranes). The MSI image for image registration was based on an in-source fragment common to many lipids thereby being cell ubiquitous. Single cell segmentation in the optical images was automated using a Python image processing script. This allowed the assignment of MSI lipid spectra to the individual cells where pixels outside of the cell boundaries were disregarded. The potential of lipid-based profiles for the accurate classification of co-cultured cells and describing molecular inter- and intra-culture heterogeneity was demonstrated using machine learning. Interestingly, most misclassified cells showed abnormal morphological features, pointing towards the necessity to include this information also into the dataset.

This was done by the study of Rappez et al., who correlated the single cell metabolic profiles with both morphometric features and spatial context features of the cell like the number and type of neighbours. While these examples demonstrate that single cell studies by MSI on cultured cells are routinely possible, single cell studies in tissues remain scarce. One reason for this is the yet labour-intensive task of correlating the high-spatial resolution molecular images gained from MSI with the histological tissue stains in a sufficiently highly accurate manner.

An exception to this is the study by Ščupáková et al. who have developed a semi-automated workflow to correlate 10 µm lipid MALDI-MSI data of tissues to the high-resolution histological image of the same sample. The image registration builds upon an initial manual control-point based registration followed by an individualised image processing to emphasise common morphological features to guide an automatic intensity-based fine-tuning of the registration. This reduced the
Critical Review

and N-linked glycans must be detached from proteins via enzymatic digestions; free glycans can be directly measured. One prime study that exemplifies this potential is the study by McDowell et al. who studied 151 N-glycan structures in human pancreatic ductal adenocarcinoma (PDAC; 53 patients on tissue microarrays and 13 on whole-block samples) and healthy pancreatic tissues. To comprehensively study N-glycans and their synthesis or modification, the authors added many additional experimental steps such as the derivatization of sialic acids, the digestion by endoglycosidase Endo F3, and immunostaining of the lectin PHA-E and GSL-II to be able to distinguish isomeric glycan configurations.

Using this approach, the authors were able to link several N-glycan structures to histological features in pancreatic normal and cancerous tissues such as the islets of Langerhans, necrotic, primary tumour, tumour stroma, and tumour margin regions (Fig. 2B). Moreover, they were able to relate a set of N-glycan structures to the presence of carbohydrate-based PDAC markers (CA19-9 and sTRA) in the tissues, which were visualised using immunostaining. Compared to these, the MSI-detected glycan profiles significantly improved the predictive value of the antigen-based markers from an AUC of 0.72 to 0.94. This analysis also indicated that glycans with a terminal GlcNAc or GalNAc could be higher in tumours, which was assessed by fluorescence staining of selected lectins which are able to recognise specific carbohydrate motifs. The lectins confirmed the expression of the epitopes in cancer cells and revealed different tumour-associated staining patterns between glycans with bisecting GlcNAc and with terminal GlcNAc.

All the above findings showcase the unique capability of MSI of N-linked glycans to propose new valuable biomarkers and to serve at the same time as potential assays for these markers.

Highly cited studies

The most highly cited study in MSI-oncology has been published by Sans et al. in the journal Cancer Research in 2017 and it is about cancer subtyping using molecular patterns provided by MSI in combination with multivariate classification models.

In their study, Sans et al. used DESI-MSI to identify patterns of metabolites and lipids that have the diagnostic potential for accurately subtyping serous ovarian cancers as high-grade serous carcinoma (HGSC) or borderline ovarian tumours (BOT) as compared to normal ovarian tissue samples. Imaging in both positive- and negative ion modes, the researchers were able to identify specific molecular profiles for both the stromal-confined BOT and the more aggressively invasive HGSC, as well as necrotic surrounding regions, which are associated with HGSC. These findings were validated by subsequent H&E-stained images. Furthermore, the MSI data were used to build a set of at least absolute shrinkage and selector operator (Lasso) classification models that could discriminate normal from HGSC and BOT with 96.2% accuracy and HGSC from BOT with 93% accuracy. Interestingly, the Lasso model misclassified three BOT samples, which after re-evaluation by pathology showed features that are not characteristic of the BOT molecular model, including increased invasiveness and extensive micropapillary growth. In general, Sans et al. used the altered abundances of a diverse group of metabolites and lipids, found using DESI-MSI, to help and improve the diagnosis and classification of serous ovarian cancers, thereby showcasing how MSI can be integrated into the clinical workflow of oncological tools.

Unique never seen application

A unique, so far never seen application, which MSI enables, is the spatial and multiplex detection of carcinogenic exogenous compounds in tissues and their relation to the patients’ clinical outcome. Kunzke et al. impressively demonstrated the clinical value of the in situ chemical analysis of black carbon particles in 330 primary resected squamous cell carcinomas of the lung. Using high mass resolution MALDI-MSI, the authors detected eleven exogenous compounds in and nearby the pigments: five polycyclic aromatic hydrocarbons (PAHs), three tobacco-specific nitrosamines, two aromatic amines, and one organohalogen. The previously introduced SPACiAL approach was used to define tumour and stromal areas using multiplex immunohistochemistry. This enabled a differentiated analysis for both regions, which revealed several significant correlations between the carbon-bound compounds and smoking behaviour, DNA damage, T-cell infiltration, PD-L1 expression, tumour staging, and overall survival. As expected, higher levels of carbon-bound compounds came along with higher hazard ratios for survival except for NNAL-N-glucuronide and dibenz(a,h)anthracene, which were significantly linked to a favourable outcome when present in the stroma. The authors speculate that the favourable effect of NNAL-N-glucuronide might be related to the fact that this compound is the detoxified version of one of the tobacco-specific nitrosamines through glucuronidation. Conversely, all exogenous substances associated with poor survival are related to tobacco smoke, confirming the significance of this lifestyle factor for prognosis.

In addition, many other endogenous metabolites were detected in this study: 133 within tumour cells and 159 within the stroma. Spatial correlation analysis between the exogenous and endogenous compounds revealed different pathways and metabolic processes associated with the different exogenous compounds. In tumour, N-hydroxy-MeIQx was found to be
associated with altered lipid and glutathione metabolism, whereas in stroma, PAHs and tobacco-specific nitrosamines were co-localised to metabolites of the amino acid and nucleotide metabolism. This study showed that the MSI-based approach expanded the knowledge of known exogenous carcinogens found in anthracotic pigments of the lung in terms of their location-dependent (tumour/stroma) clinical impact and surrounding metabolism.

**Neurology**

There is no better example of the importance of the compartmentalisation and spatial arrangement of cells and their molecular setup than in the central nervous system, especially in the brain, where specific cognitive functions or diseases are associated with certain areas, cell types, and molecules.

MSI brings a novel perspective on brain research where molecular distributions and mechanisms in brain sections can be related to brain regions and therefore to cognitive functions or diseases of the brain (Fig. 3). MSI has therefore been applied since its inception for more detailed analysis of complex structures, functions, and pathologies within the field of neurology including stroke, migraine aura, neuroinflammation, Parkinson’s disease, traumatic brain injury, Huntington’s disease, multiple sclerosis, and extensively to study Alzheimer’s disease.

**Novel mechanistic insights into Alzheimer’s disease**

Alzheimer’s disease (AD) is the most common and societally impactful neurodegenerative disease and as such, it is also one of the most researched diseases within neurobiology. Deposition of β-amyloid (Aβ) as plaques in the brain is a consistent feature of AD and post-mortem control for the presence of Aβ is the only way to diagnose the disease with certainty, to this day. While Aβ plaque formation (alongside hyperphosphorylated tau protein) is recognised as one of the main

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**Fig. 3** MSI facilitates unique insights into neurological research. (A) 3D MSI model of Aβ-plaques of various lengths reveals multiple options for plaque-architecture based on the Aβ-peptide length. (B) MALDI-MSI peptide imaging allows for the visualisation of different Aβ-peptide species in the same tissue section. (C) Region-specific MSI of multiple neuropeptides in a single tissue section. (D) MSI at multiple time points reveals local changes in the lipid metabolism of cholesterol CE, throughout the brain, after TBI. (E) Multimodal application of high-resolution MSI (10 µm) and IHC allows for precise correlation of lipid imaging with Aβ-plaques. (F) Alternative sample preparation techniques allow MALDI-MSI to visualise and quantify neurotransmitters in different brain regions. Figures were reprinted and/or adapted with permission from ref. 65 (Copyright 2020 American Chemical Society), ref. 55 (CC BY 4.0), ref. 51 (Elsevier 2020), ref. 71 (Elsevier 2016), ref. 61 (Copyright 2017 American Chemical Society), and ref. 67 (Elsevier 2014) for (A), (B), (C), (D), (E), and (F), respectively. Parts of the figure were created using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.
pathological hallmarks of AD, the lack of capable imaging modalities that could visualise this process has been a major roadblock for our understanding of AD pathogenesis.

**MSI for the study of Aβ peptides in AD samples.** The precursor to Aβ peptides is the amyloid precursor protein (APP), which is cleaved by either β- or γ-secretases to create Aβ peptide alloforms ranging in size from 36 to 43 amino acids. In a study by Kakuda et al., MALDI-MSI has shown selective spatial sequestering of differently sized Aβ peptides in different brain regions.55 Comparing human post-mortem brain tissue from AD patients with age-matched healthy controls, the authors found that Aβ1−36 to Aβ1−41 were predominantly deposited in leptomeningeal blood vessels, whereas Aβ1−42 and Aβ1−43 aggregated in the cerebral parenchyma (Fig. 3B). Since this study was the first to detect the presence of Aβ1−41 in human brains, the validity of this result was corroborated by supplemental immunohistochemically stained tissue images. As a mass spectrometry-based method, MSI can detect molecular characteristics that are invisible to antibody-based methods. For instance, the researchers observed that N-terminally truncated Aβ peptides had largely similar spatial distributions as full length Aβ1−40 and Aβ1−42, except for N3pE-Aβ42 which was not only detected in the parenchyma, but also in the blood vessels. This was unique for MSI, since the N-terminal specific antibodies for Aβ1−42 cannot distinguish between full length Aβ1−42 and N3pE-Aβ42. It has also been shown that the single amino acid difference between Aβ1−41 and Aβ1−42 causes large changes in spatial distributions, suggesting that the C-terminus, rather than the N-terminus, is important for the dynamics of the Aβ peptide. This theory was validated in an in vitro aggregation assay using thioflavin T, which demonstrated that Aβ1−42 aggregates faster than Aβ1−40 and Aβ1−41 over a 24 h period.55 This study provided new insights into the spatial location and aggregation abilities of different Aβ peptide alloforms, which could not have been identified with conventional imaging techniques.

**MSI for the study of AD-plaque-associated lipids.** Likewise, MSI constitutes a unique technology to study lipid distributions in tissues.36 This is especially interesting since it is known that lipids play an important role in AD57 and MSI can therefore offer an edge to identify lipid alteration associated with Aβ deposits.58

In that sense, Kaya and co-workers, in a series of publications, provided insights into the lipidic composition of Aβ plaques using a set of novel multimodal imaging workflows, which included high-spatial resolution MSI as the central modality. All these workflows are based on initial lipid detection followed by a subsequent imaging experiment on the same tissue section, such as MSI of proteins59 or again MSI of lipids but in another polarity,60 immunohistochemistry,61 or fluorescence imaging62 to identify lipids that co-localise with Aβ aggregates.

Utilising this multimodal MSI-centred workflow, Kaya and colleagues investigated brain sections from tgSwe,63 tgArcSwe59,60 and tgAPPSwe62 AD mice models and found strong co-localisation of ceramides, phosphatidylinositol, lysophosphatidylcholines, and phosphatidylcholines with Aβ1−37, Aβ1−38, and Aβ1−40 peptides (Fig. 3E). Moreover, they also demonstrated that sulfatides (ST 24:0) were absent in Aβ plaques.63 All these lipids were confirmed using MS/MS and are thought to contribute to the pathology of AD through either oxidative stress, demyelination or altered cell homeostasis.

**MSI for the study of kinetics of Aβ peptides.** Besides allowing obtaining spatial molecular information that has so far been inaccessible to other imaging modalities, MSI also offers the possibility to follow metabolite kinetics in tissues through stable isotopic labelling.64 In the context of AD, Michno et al. used this approach to follow Aβ plaque formation through isotopic labelling of Aβ peptides in an AD mouse model (APPNL−G−F).64 By utilising a set of three different PULSE/CHASE 15N-based protein diet schemes, the authors were able to follow Aβ deposition in a time- and space-dependent manner as measured by MALDI- and NanoSIMS imaging. Based on the distributions and ratios of labelled vs. unlabelled Aβ peptides, they found that the less-labelled Aβ1−42 was primarily localised in the plaques’ core which suggests that dense core formation represents the earliest seeding event in extracellular plaque deposition in APPNL−G−F mice. Following the same PULSE/CHASE schemes, the degree of N-label incorporation proved concordantly that plaque pathology starts with Aβ1−42 deposition and plaque formation in the cortex, followed by the development of plaque deposits in the hippocampus. Moreover, this study showed that the formation and plaque deposition of aggregation-prone, C-terminally truncated Aβ1−38 take place later than Aβ1−42 deposition.64 In conclusion, this study demonstrates impressively how spatio-temporal mechanistic insight into amyloid pathology can be obtained using stable isotope labelling in combination with MSI.

**Potential high-impact methodologies**

**MSI-based automatic annotation and 3D MSI of Aβ plaques.** The observation that Aβ1−42 was primarily localised at the plaque’s core has recently been confirmed by another MSI study conducted by Enzlein et al.65 who automated the detection of all single plaques in an MSI dataset by dichotomising a series of Aβ-specific MSI images using individual intensity thresholds. The binary images (1 for plaque, 0 otherwise) then underwent image processing to detect and label every plaque as an individual object. This enables the extraction of different parameters from every plaque, such as its size, molecular composition, and spatial location in the brain. Applying this method to several MALDI-MSI datasets acquired at 20 µm lateral resolution from two different AD mouse models (APP PS1 and APP NL-G-F; Aβ-peptides of the latter carry the Arctic mutation) allowed comparison of the plaque populations between these biological groups. Amongst the many findings, it was concluded that plaques in APP NL-G-F mice were more heterogeneous in their Aβ-composition than plaques in APP PS1 mice, and that larger plaques are characterised by more Aβ-peptide diversity than smaller ones. Conversely, plaques containing Aβ1−38 were found to be consistently enlarged.

Moreover, a 3D-MALDI-MSI experiment was performed on an APP NL-G-F mouse to study the plaque’s volumetric compo-
publication in more detail. Data were obtained from nine consecutive brain sections and volumetrically reconstructed using M2aia software. The analysis of the 3D dataset revealed that the core and outer shell of larger plaques were mainly composed of \( \text{A} \beta 1-42 \text{Arc} \) and \( \text{A} \beta 1-38 \text{Arc} \), respectively (Fig. 3A). When comparing the single-plaque statistics of the 2D data with the data from the 3D model, several differences could be observed. For instance, both \( \text{A} \beta 1-42 \text{Arc}/\text{A} \beta 1-38 \text{Arc} \) ratios and the number of small plaques were underestimated in the 2D data as compared to the volumetric MSI data. This indicates that a 3D model represents a better estimate in MSI studies when dealing with many sparse and small objects, such as those encountered in AD in the form of amyloid plaques.

**MSI for the study of neurotransmitters.** Neurotransmitters are small-molecule messengers that facilitate and modulate signalling between neurons in the brain. Under healthy conditions, the signals propagated between specific brain regions make up complex and highly regulated networks of neurotransmitters and their metabolites. However, these networks are almost always disrupted as a hallmark of most neurological disorders and this manifests itself as the cognitive deficits experienced. Thus, understanding how the neurotransmitter networks are altered in disease, with regard to abundance and spatial distribution, can help reveal the molecular mechanisms driving neurological diseases. Previous MSI methods allowed for visualisation of some neurotransmitters and metabolites (Fig. 3F). However, downstream metabolites dominated by the monoamine oxidase (MAO) enzyme are not detected, which limits the applicability of the method, as dysregulation of MAO is an important factor in many psychiatric disorders. With this in mind, Shariatgorji et al. developed a novel reactive matrix for mapping neurotransmitter networks, including precursors and metabolites involved with MAO, using MALDI-MSI. The matrix, methylated 4-(anthracen-9-yl)-2-fluoro-1-alkylpyridin-1-ium (FMP-10), was designed to selectively target primary- and phenolic amine groups to facilitate the imaging of catecholaminergic and serotonergic signalling networks. By incorporation of a reactive fluoropyridinium moiety, for charge tagging, and a conjugated chromophore domain, for improved laser desorption, FMP-10 improved the limit of detection for select neurotransmitters from 10 to 500 times. Applied to both rat- and human brain samples, derivatization with FMP-10 improved the signal-to-noise of dopamine and 5-HT to an extent that allowed for imaging of these neurotransmitters in areas of previously undetectable concentrations, and with a spatial resolution down to 10 µm. This development in sample preparation has enabled the use of MALDI-MSI to map neurotransmitters within specific brain regions, to help shed light on the underlying molecular mechanisms of neurological diseases.

**Highly cited studies**

In recent years, the role of lipid metabolism and signalling has been more heavily researched, especially in the field of neurology, as lipids make up more than 50% of the weight of a brain. Traumatic brain injury (TBI) occurs as a sudden injury to the head that causes temporary or permanent damage to the brain structure and function, and previous publications suggest that altered lipid metabolism is a driving force in the pathophysiology of TBI. The pathophysiology of TBI takes on two stages: primary injury, which is the direct tissue damage resulting from the injury, and secondary injury, which is driven by the molecular changes initiated caused by the primary injury, and includes inflammation, cerebral ischemia and oedema often leading to neuronal apoptosis. While MSI has greatly expanded the field of lipidomics by providing the necessary tools to both characterise lipid species and map their spatial location in tissues, sample preparation is continuously being optimised for lipid imaging. Traditional “wet” matrix application techniques have the potential downside of causing analyte extraction or delocalisation and one workaround to this is by using “dry” matrix application techniques, such as the implantation of metal clusters. With this background, Roux et al. developed a method for incorporating silver nanoparticles into tissue sections, which when imaged with MALDI-MSI, showed the distinct distribution of lipid species in brain tissue from a controlled cortical impact TBI rat model. The animals were sacrificed at three time points (day 1, 3 and 7) after TBI to follow the pathology progression associated lipid changes in the brain. This allowed imaging and identifying 93 lipids using MS/MS, including ceramides (CERS), diacylglycerols (DAGs), cholesterol esters (CEs), galactosyl ceramides (GALCERS), phosphatidylcholines (PCs), cholesterol (CHL), sphingomyelins (SMs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs) and sulfatides (STs). Interestingly, at one day post-injury, CERS were the only lipid species to show an increased concentration around the injury site, while on day three post-injury CERS, CE, SMs and DAGs were all significantly increased. On day seven post-injury, CER, CE and SM concentrations had decreased again, while DAG expression was completely absent from the injury site (Fig. 3D). The changes observed for SM and CER expression might be associated with the SM/CER signalling pathway of apoptosis. When looking closer at individual sphingolipids, low mass SM and CER (≤C16), localised exclusively in the ventricles of control animals or at the injury sites of TBI animals, were both significantly upregulated only on day 3 post-injury. The presence of these low mass SM and CER species, normally only present in blood, suggests that the integrity of the blood-brain barrier could be compromised and thereby help drive the secondary injury through recruitment of inflammatory stimulating cells. In conclusion, Roux and colleagues have presented a novel method of sample preparation for visualising lipid species in brain tissue using MALDI-MSI and provided new insight into the lipid pathophysiology of TBI.

**Cardiovascular disease**

Cardiovascular disease (CVD) is a collection of diseases that target the heart or the blood vessels and is currently the leading cause of death in the developed world. The most...
common types of CVD are stroke and coronary heart disease, both of which are mainly caused by atherosclerosis.

Atherosclerosis is a disease in which an arterial wall develops lesions and fat-containing atherosclerotic plaques (Fig. 4B). This build-up takes place over decades and can potentially lead to the rupture of the plaque. This releases a thrombus, which can clot an artery, thereby leading to a stroke or a myocardial infarction. Not every plaque will rupture, which can be due to the complex cellular and morphological setup (e.g. calcifications, necrosis, lipid accumulations, presence of macrophages, tissue architecture, etc.)\(^7^4\). MSI coupled with microscopy hence constitutes an attractive tool to investigate these plaques by being able to spatially resolve the histological and molecular components of the plaque. This has initially been done by several secondary ion mass spectrometry (SIMS) imaging studies focusing on the lipid content of atherosclerotic tissues.\(^7^5\)-\(^7^7\)

Novel mechanistic insights into atherosclerosis

The inflammatory state of the lesion is partly driven by lipid pro-inflammatory molecules such as leukotrienes and prosta-

![Fig. 4](https://example.com/figure4.png)

**Fig. 4** Mass spectrometry imaging (MSI) in cardiovascular research of atherosclerosis. (B) Atherosclerosis is characterised by the thickening of the intima layer of blood vessels due to inflammation and lipid accumulation, in the worst case leading to the release of a thrombus with the chance of heart attack or stroke. This disease develops over the years and in different stages. (A) Hence, SIMS-MSI has been used to characterise the lipid content of human coronary arteries with different severities. (C) 3D MALDI-MSI characterised the volumetric lipid content of plaques since plaques are not homogenous along the blood flow direction. (D) MALDI-MSI revealed cell-type specific changes in lipids in neurologically symptomatic patients with carotid atherosclerosis. Figures were reprinted and/or adapted with permission from ref. 75 (Elsevier 2015), Wikimedia Commons (Npatchett, CC BY 4.0), ref. 84 (WILEY 2016), and ref. 79 (CC BY 4.0) for (A), (B), (C) and (D), respectively.

glandins, to which free arachidonic acid and lysophosphatidylcholine (LPCs) are important precursors. Recently, it has been discovered that LPC-acyltransferase-3 (LPCAT3) can regulate the levels of free arachidonic acid and LPC through conversion to arachidonyl-PC. For this reason, Tanaka *et al.* decided to investigate the correlation between LPCAT3 expression and atherosclerosis disease progression using MALDI-MSI in human and mouse atherosclerotic tissue samples.\(^7^4\) A total of twelve lipid signals corresponding to either LPCs or PCs were identified, where LPC and arachidonyl-PC showed a complementary distribution in the tissue sections. While LPC increased in intensity as the disease progressed from stage I to IV, arachidonyl-PC showed an anti-correlation with disease progression. In line with this, the expression levels of LPCAT3, as determined by immunohistochemistry, were found to decrease with disease progression, by up to 72% in stage IV tissue samples. Thus, these results corroborate the progression of atherosclerosis through dysregulation of LPCAT3. Moreover, the spatial information provided by MSI was able to localise the consequence of this altered expression, namely the accumulation of arachidonyl-PC, to vascular smooth muscle cells (VSMCs). This gives evidence for the need to distinguish and analyse distinct regions within the plaque to understand the underlying pathophysiology of the disease.

Large cohort size in atherosclerosis

This was further substantiated by Greco *et al.* who used MALDI-MSI to study local lipid alterations in atherosclerotic plaques from six human subjects with asymptomatic or symptomatic clinical outcomes (Fig. 4D).\(^7^9\) While six patients do not seem like a large cohort size, it is the largest so far in that field with follow-up data that have been analysed by MSI. The MSI measurements were co-registered with several fluorescent images and the conventionally stained bright-field microscopy image to annotate the functionally important regions in each plaque. This resulted in seven defined regions in each sample: lipid-necrotic core, calcification, collagen-rich area, haemorrhage, macrophage-rich area, and inner and outer VSMCs. Unsupervised analyses across all patient samples, in the form of principal component analysis and *k*-means clustering, confirmed on the one hand the compositional similarity in lipid profiles between histologically similar regions but on the other hand also revealed visual clusters that are specific for symptomatic and asymptomatic plaques, in particular in the inner VSMCs and in macrophage-rich regions. This supports previous findings, which indicate that macrophages are essential players in the inflammation of atherosclerosis as foam cells.\(^8^0\) Supervised regression then revealed the location and trend of many lipid species in symptomatic plaques such as increased sphingomyelins in macrophage-rich regions and reduced PC levels in the lipid-necrotic core. This was in accordance with previous results as SMs have been thoroughly investigated in atherosclerosis.\(^8^1\)

Potential high impact technologies

3D MSI of an atherosclerotic plaque

Another way in which MSI can fundamentally further our understanding of athero-
sclerosis is with three-dimensional (3D) MSI. 3D-MSI is unique in that it facilitates analysing and visualising the molecular information based on the sample’s volumetric structure. In MALDI-MSI, this is achieved by first measuring several consecutive sections of the same sample and then digitally stacking the resulting 2D-MSI datasets to a volume using image registration methods. The ability to investigate the 3D architecture of atherosclerotic plaques is important since the plaque grows asymmetrically mainly due to the asymmetrical forces exhibited by the directional blood stream. In this context, 3D-MSI can thereby help to unravel the molecular composition of atherosclerotic plaques by taking into consideration this volumetric variability. Patterson et al. impressively demonstrated how atherosclerotic plaques measured by MALDI-MSI can be volumetrically analysed to capture changing lipid distribution patterns through the depth of the plaque (Fig. 4C). Therefore, 118 plaque-containing sections sliced from a human carotid artery sample were measured by MALDI-MSI for lipids. This resulted in a total depth description of 1.18 cm. Using image registration followed by a 3D-spatially aware segmentation algorithm, the plaque was clustered into five segments: two segments in the interior region, facing the blood flow in the artery, two segments in the larger middle/core region, and one segment in the outer region facing the arterial wall. Interestingly, as stenosis increased at different lengths of the plaque, different segments and their characteristic lipids became more dominant in intensity. For the interior region, phosphatidylethanolamine (16:0/20:4) increased with increased stenosis, whereas the middle part of the plaque showed the largest increase in physical area with increased stenosis, due to increased signals of lysophosphatidylcholines. While the study shows that 3D-MALDI-MSI is an extensive endeavour due to the measurement of many sections and their (semi)-manual reconstruction, it confirms again that 3D-MSI can provide valuable information that might be missed in 2D.

**MSI for the quantification of MRI-contrast agents in atherosclerosis.** Nevertheless, 3D-MSI remains a highly invasive imaging approach, as compared to, for instance, magnetic resonance imaging (MRI). The latter is, for example, used in atherosclerosis for the quantification of scar formation after myocardial infarction. Monitoring the healing process of an infarct is an important aspect of preserving cardiovascular function as the uneven formation of the extracellular matrix may result in heart failure. While MRI can visualise this process using extracellular matrix-specific contrast agents (such as gadofluorine P), most in vivo technologies are currently unable to quantify the absolute amount of contrast agents in tissue.

Recently, it has been shown that MSI can complement MRI by the spatial quantification of the gadolinium agents directly in tissue with high spatial resolution. In that line, Lohöfer et al. presented a novel multimodal imaging workflow, which involves MRI, MALDI-MSI, and LA-ICP-MSI, to assess and quantify gadofluorine P distributions during infarct healing in a mouse model of myocardial infarction. To visualise scar formation, animals were imaged and sacrificed at 1 week and 6 weeks post-infarction using MRI and MSI, respectively. The infarcted myocardium showed significantly increased $R_1$ values after gadofluorine P injection at 6 weeks, compared to week 1 and this increase in signal was corroborated by an increased concentration of gadofluorine P in the infarct scar as directly measured by MALDI-MSI and indirectly quantified via the gadolinium element by LA-ICP-MSI. This change was not observed in healthy myocardium. The accumulation of gadofluorine P at the infarcted region was correlated to increased extracellular matrix synthesis as revealed by Elastica-Van-Gieson staining. Interestingly, both the increased $R_1$ values of the MRI and gadofluorine P concentrations from MALDI-MSI at week 6 correlated with the function of the left ventricle, as measured by an increased ejection fraction at week 6 compared to week 1. Thus, with this novel combination of techniques, Lohöfer and colleagues managed to quantify the accumulation of gadofluorine P using MALDI-MSI and LA-ICP-MSI, which has the potential to calibrate MRI signals for quantitation, thereby aiding the process of more precisely tracking myocardial healing and treatment response.

**Highly cited studies**

Another interesting application using secondary ion mass spectrometry (SIMS) has been reported in a highly cited publication by Vujic et al. In this study, multi-isotope imaging mass spectrometry (MIMS) has been used for studying cardiomyocyte proliferation based on the measurement of stable isotope tracers of DNA synthesis. The quantification of the regeneration of cardiomyocytes in the adult heart has long been a topic of interest, as the loss of cardiomyocytes increases the risk for heart failure.

The advantage of the MIMS approach over other methods is that it offers high-resolution quantification of prospectively administered, non-radioactive stable isotope tracers, which do not interfere with biochemical reactions. More precisely, Vujic et al. used MIMS to measure exercise-induced cardiomyogenesis by monitoring $^{15}$N-thymidine incorporation, as a measure of DNA synthesis. Therefore, healthy mice and mice after induction of myocardial infarction underwent voluntary running exercise in a wheel with a continuous infusion of $^{15}$N-thymidine. After eight weeks, exercised mice showed a significantly higher number of $^{15}$N-thymidine positive cardiomyocytes compared to control mice. This increase in $^{15}$N-thymidine positive cells was accompanied by angiogenesis as shown by immunohistochemistry. The MIMS approach also revealed that in mice suffering from myocardial infarction cardiomyogenesis was significantly more abundant in the border region as compared to the peri-infarct region.

This study demonstrates how MSI can use mass spectral information of isotopes to provide valuable information on biological processes such as mitosis.

**Endocrinology**

Endocrinology is the study of hormonal signalling in the body and thus endocrine disorders are conditions in which hormo-
nal signalling is in imbalance, often due to genetic factors, external factors, or tumour formation near the hormone-producing glands. Hormones are produced by glands and within the glands by specialised hormone-producing cells. An imaging-based and/or mass spectrometry-based approach is therefore sensible.

**High impact methodology in MSI of steroid hormones**

Many steroid hormones have isomeric structures, which poses a challenge to conventional MSI since these hormones have identical molecular weight and composition. As these different isomeric steroids have very different functions, being able to visualise and discriminate between them is of utmost importance.

A MALDI-MSI study has addressed this issue and shows how structural isomers of steroids can be distinguished by using on-tissue chemical derivatization and tandem MSI. Here, Takeo et al. enabled the ionisation of the steroids by Girard’s T reagent derivatization and combined it with the chemical specificity provided by ion-trap-based MS³ to visualise and distinguish aldosterone and cortisol from their structural isomers, cortisone and 18-hydroxycorticosterone, respectively. Most importantly, while the MALDI-MS analysis and MS² fragmentation patterns resulted in near identical spectra from the structural isomers, MS³ analysis showed molecule-specific fragmentation patterns. For example, the MS² peak at m/z 415 showed a transition to m/z 397 and to m/z 385 corresponding to the loss of water from aldosterone and the loss of CH₂O from cortisone, respectively. The capabilities of this MS³-based approach were then used to investigate aldosterone accumulation in the adrenal glands of sodium-deficient diet fed rats, which is known to increase aldosterone production. Furthermore, by imaging healthy human adrenal glands, the researchers found that aldosterone, as well as the hybrid steroids 18-oxocortisol and 18-hydroxycorticosterone accumulated in aldosterone-producing cell clusters rich in the enzyme CYP11B2, while cortisol was absent in these cells. Based on these findings, the authors deduced that 18-oxocortisol and 18-hydroxycorticosterone are converted from cortisol by CYP11B2 through a rapid mechanism as soon as the aldosterone-producing cell clusters internalise cortisol. Thus, Takeo et al. created an MS³-based imaging workflow that allows the discrimination of structural isomers of steroids on tissue. This high impact methodology paves the way for investigating and imaging steroids in hormone producing organs such as the adrenal gland.

**Mechanistic insights in endocrinology**

**MSI of endocrine tumours.** In that context, adrenocortical carcinoma (ACC) is characterised by an altered expression of steroid- and sex hormones caused by an abnormal growth of cancer cells on the outer layer of the adrenal gland. Since 40% of the tumours do not show the known genetic driver, there is a need to investigate the molecular aetiology, onset, and progress of ACC. Steroid hormones and steroid sulphates have previously been investigated in the healthy adrenal cortex by Sun et al. using MALDI-MSI. The same authors have consequently extended their approach to assessing sulfated steroid hormone levels and associated regulators in ACC patients. A total of 72 formalin-fixed, paraffin-embedded ACC tissue samples were measured on a high-resolution FT-ICR-MS in negative ion mode, which resulted in the detection of 1141 metabolite signals, of which five corresponded to sulfated steroids: cholesterol sulfate, pregnenolone sulfate, estradiol-17β 3-sulfate (E2S) and estrone 3-sulfate (E1S), as well as the di-sulfated estradiol-17β 3,17-disulfate (E2S2). The abundances of E1S and E2S strongly correlated in all measured samples and high levels of these were indicative of a favourable long-term outcome. In contrast, E2S2 was only detected in six out of the 72 samples and these patients showed a significantly poor prognosis. Hierarchical cluster analysis of the patients suggested three molecular phenotypes: one low in E2S abundance, one high in E2S abundance, and one where E2S2 is detectable. Between the three phenotypes, amino sugar, nucleotide sugar and purine metabolism differing, suggesting a sulfated steroid mediated metabolic reprogramming. Moreover, electron microscopy showed that the E2S2-positive phenotype samples were characterised by the presence of multiple membrane-delimited vacuoles containing an amorphous material.

To further expand the picture of the sulfated steroid pathway, immunohistochemistry was performed to visualise the distributions of the sulfotransferases SULT1E1, SULT2A1, and SULT2B1, and the steroid sulfatase (STS), all of which catalyse the metabolism of steroid hormones. In accordance with the MALDI-MSI experiments, high levels of all sulfotransferases correlated with a positive overall survival, and curiously, high levels of STS were also associated with a positive overall survival. The association of SULT2A1 with prognosis was validated on two externally published data sets, strengthening the findings. With these findings, Sun et al. demonstrated how MALDI-MSI can be used to detect novel prognostic phenotypes in ACC patients based on the otherwise inaccessible spatial information of steroid hormones, thus providing novel mechanistic insights with translational potential.

**MSI of diabetes.** Type 2 diabetes is the most common type of endocrine disorders, driven by the increasing obesity worldwide. Despite the extensive research in this field, the underlying mechanisms leading to β cell dysfunction and subsequent impairment of insulin metabolism remain largely unknown. Isolation and culturing of β cells or entire islets of Langerhans (LHIs) are the most extensively used model systems for investigating diabetes, which lack the capability to mimic the complex natural environment surrounding β cells in vivo. MALDI-MSI therefore provides a unique opportunity to investigate the pathophysiology of type 2 diabetes in situ and analyse the disease-related metabolic changes that lead to dysfunction of β cells.

Aichler et al. generated comprehensive in situ omics data by imaging both the peptide hormones insulin and glucagon, as well as >500 metabolites in 263 LHIs from different mouse models (pre-diabetic, diabetic and non-diabetic) and...
54 human LHIs (healthy control and type 2 diabetic) using multimodal MALDI-MSI. The LHIs from two mouse models were analysed with MALDI-MSI to compare the metabolic profile between the different disease stages in relation to type 2 diabetes progression. They found 187 pre-diabetic specific metabolites and 317 diabetic specific metabolites common to both mouse models. As these metabolites were in both mouse models of type 2 diabetes but not in healthy controls, they were considered genetically independent and representative alterations to the metabolome of pre-diabetic- and diabetic mice. Conducting metabolic pathway analysis on the MSI data, the researchers found that acylcarnitines had strong correlations with numerous pathways and were more abundant in LHIs isolated from type 2 diabetic patients compared to healthy controls, indicating an involvement in disease progression. Based on further in vitro experiments, in which cultured ß cells were treated with acylcarnitines, the authors found that administration of acetylcarnitine increased insulin secretion, while stearoylcarnitine impaired insulin synthesis through inhibition of the energy metabolism, oxidative phosphorylation and Krebs cycle. N-Acyl taurines are another class of molecules that were found in the LHIs for the first time and showed multiple strong correlations in the pathway analysis, indicating an important role in the disease. When comparing mouse models with human LHIs, only N-acyl palmitoyl was detected in human samples. With similar in vitro studies of isolated ß cells, N-acyl palmitoyl was found to increase insulin secretion. Thereby, Aichler et al. used the spatial and quantitative information from MALDI-MSI to propose a novel mechanism of imbalance between insulin production and secretion leading to ß cell dysfunction in type 2 diabetes.

**Rheumatology**

Rheumatic diseases affect joints, muscles and bones and therefore the research into this group of diseases focuses on the study of bone, muscle, and cartilage tissue for which the sample preparation is particularly challenging. One example is bone sectioning, which usually requires a decalcification step, and another challenge is the high degree of collagen in cartilage, which blocks the access of enzymes to the proteolytic enzymes needed for proteomic investigations. This has impaired a broader application of MSI in the field of rheumatology.

**Highly impact methodology**

With this background, most studies include methodological developments with the aim to enable or optimise MSI-based investigations of these challenging tissues.

One successful example of this field is the work by Barré et al. who were the first to detect a drug with MSI in cartilaginous tissue. The investigated drug, triamcinolone acetonide (TAA), is a compound that produces relief of pain and inflammation in osteoarthritis (OA). OA is the most common rheumatic disease and is characterised by cartilage erosion in the joints, which is mediated by inflammatory processes. Since TAA is a corticosteroid that can have systemic consequences, understanding and quantifying the local accumulations of TAA in the target and other tissues is of utmost importance. While MSI offers many advantages for such a task (see section on drug imaging), its success depends on the ionisation efficiency of the target compound. Unfortunately, TAA is not easily ionisable. Using either chemical derivatization, or in a subsequent study MALDI-2, the authors were able to increase the signal of TAA by 1–2 orders of magnitude within the cartilage.

The authors made use of the currently most common approach in MSI for the quantitation of exogenous compounds to enable quantitation of TAA. This involves a deuterated analogue of TAA being sprayed homogeneously over the target tissue to normalise the TAA signal. The authors also demonstrated that the use of an internal standard is very important to account for the varying ionisation efficiency of TAA in the different cartilage zones. Combining the derivatization with the quantitation approach, Barré and co-workers found that TAA homogeneously distributed in TAA-incubated cartilage tissues from three OA patients in amounts that range 3.7 ng µL⁻¹ to 22.2 ng µL⁻¹. In summary, the works by Barré et al. demonstrate the potential to monitor drugs even in challenging tissues such as cartilage in the context of OA.

**Mechanistic insights in rheumatology**

In that respect, MSI has also been used in the search for novel therapeutic agents in OA. Since defective chondrogenic differentiation strongly compromises cartilage regeneration, obtaining more molecular knowledge on chondrogenesis in OA could provide novel therapeutic targets. Rocha et al. therefore used MSI to complement SILAC-based proteomics by spatially resolved metabolomics of human OA-derived cell cultures undergoing chondrogenesis. Multipotent stromal cells were isolated from the bone marrow of 11 healthy and 13 OA patients and cultured with standard and SILAC nutrients, respectively. During the subsequent 3D cell culture, chondrogenic differentiation was initiated resulting in tissue-like dense cell-pellets (also referred to as micromasses), which were harvested after two (undifferentiated phenotype) and 14 (differentiated phenotype) days. Immunohistochemistry confirmed the increased chondrogenesis over time and the pathological phenotype of chondrocytes in the OA samples after two weeks.

Comparing three OA micromasses from day 2 and day 14 by SILAC-based quantitative proteomics revealed 33 proteins that are elevated during chondrogenic differentiation. Since these proteins are involved in several metabolic processes, high-mass resolution MALDI-MSI of metabolites was performed for the first time on control (n = 3) and OA (n = 5) micromasses. The authors found that healthy and OA samples exhibited similar metabolic profiles for day 2, but not for day 14. In OA samples, 35 metabolites showed significant variation between days 2 and 14.

In an unprecedented fashion, the study then combined the lists of modulated proteins and metabolites from both, SILAC-MS and MSI, to enable an integrative pathway analysis.
This revealed, amongst others, the UDP-glucuronic acid interconversion pathway to be significantly altered during chondrogenesis in OA. The spatial metabolite levels as well as the enzyme changes, as detected by quantitative proteomics, integrated logically and congruently, and enabled us to obtain an almost complete picture of the spatial- and time-related changes for these pathways. For instance, the levels of GlcNAc and UDP-GlcNAc were significantly decreased in the micromass cores in OA samples after 14 days, indicating a down-regulation of the pathway during chondrogenic differentiation. Since it is known that amino sugar nucleotides play an important role in cartilage repair and the prevention of inflammation, the UDP-glucuronic acid interconversion pathway could serve as a therapeutic target for enhancing cartilage restoration and reducing inflammation in OA.

Large study cohort

Another anatomical component of the joint that is commonly affected by inflammation in OA is the synovial membrane. Rocha et al. investigated the role of lipids and metabolites in synovitis during OA, rheumatoid arthritis (RA), and psoriatic arthritis (PA) as compared to healthy metabolites in synovitis during OA, rheumatoid arthritis, and psoriatic arthritis (PA) as compared to healthy synovium using MALDI-MSI in a later study. The study included an extensive number of samples since synovial samples were obtained from 13 OA, 6 RA, 12 PA patients, and 10 healthy donors, as well as every patient was measured in duplicate.

52 lipids species were found with differential abundances between OA and control synovial membranes. Their spatial distributions exhibited correlations with anatomical and histological features of the samples, e.g., phosphatidylcholine (18:1_18:1) was predominantly observed in the hypertrophic lining layer of the OA synovium and phosphatidylserine (18:0_20:4) in areas rich in blood vessels and diffuse inflammatory cell aggregates.

Furthermore, OA lipid profiles were compared to the profiles of other inflammatory arthropathies. 15 lipids showed significant variation with increased levels of several phosphatidicholines and sphingomyelins and reduced levels of phosphatic acids and phosphatidylethanolamines in OA as compared to RA and PA. Some of these lipids are known to be involved in inflammation processes whereas others are not. Hence this study does not only advance the understanding of the pathogenic mechanisms of OA, but the lipid patterns have also the diagnostic potential to differentiate OA from other inflammatory joint diseases.

Drug imaging

Most of the mass spectrometry imaging applications are in the field of pharmaceutical research, contributing almost 30% of all MSI studies in the last decade (Fig. 1). The applications of MSI in this field are vast and diverse, but can be categorised into studies of pharmacokinetics, pharmacodynamics, or methodological advances for drug imaging (Fig. 5).

MSI for pharmacokinetics

Pharmacokinetics is about what the organism does to the drug, i.e., how the body distributes and metabolises the drug. As a label-free and multiplexed imaging technique, MSI offers advantages over traditional drug imaging technologies, such as autoradiography, namely the simultaneous detection of one or more drugs, including their metabolites. MSI has hence mostly been used to investigate the spatial distribution and metabolism of drug compounds in tissues.

Drug distribution and efficacy. An example of such a study has been recently published by Strittmatter et al., who – utilising different MSI modalities – investigated the localisation of the anticancer drug gemcitabine and its active metabolites to assess its local efficacy. In rapidly dividing cells, such as cancer cells, gemcitabine is metabolised and phosphorylated, allowing it to act like a nucleoside analogue that causes irreparable DNA damage and eventually apoptosis. Gemcitabine is mainly used for the treatment of pancreatic cancer and due to the relatively high proportion of non-cancerous cells in pancreatic tumours, knowledge about the distribution of both pro-drug and its metabolites is essential.

Pancreatic ductal adenocarcinoma (PDAC) samples were extracted from a mouse model, treated with gemcitabine and imaged with both DESI- and MALDI-MSI in order to be able to detect the pro-drug, all three phosphorylated active metabolites (dFdCMP, dFdCDP and dFdCTP), and its un-phosphorylated inactive form (Fig. 5A). The researchers found that gemcitabine distributed heterogeneously throughout the tumour tissue with markedly higher abundance in tertiary lymphoid structures. While the heterogeneous distribution of the pro-drug was similar to that of the active metabolites, these metabolites showed no higher abundance at the lymphoid structures, showing that pro-drug abundance should not be used to indicate drug activity. While the detection of active metabolites is an indicator of efficacy, a phenotypic assessment of the local efficacy of gemcitabine was performed by evaluating DNA damage through the fluorescence staining of γH2AX in adjacent sections. Co-registration of MSI and immunofluorescence revealed the highest levels of γH2AX in areas where dFdCMP, dFdCDP and dFdCTP were also located. These co-localised markers also correlated with markers of cell proliferation, the main target of gemcitabine action. The researchers have utilised the strengths of MSI in this study to show that knowledge about pro-drug abundance is not adequate to infer information about drug metabolism at the site of action.

Another example of MSI being used to relate a drug’s distribution to the phenotypic drug efficacy has been presented by Grüner et al., who examined the distribution of erlotinib, a small-molecule inhibitor, in a genetically engineered PDAC mouse model. The clinical benefit of erlotinib in combination with other chemotherapeutic agents is minimal and is thought to be due to poor drug delivery in desmoplastic tumours. In line with this, the authors observed, using MALDI-MSI, a lower relative abundance of erlotinib in tumour areas as compared to healthy tissue directly adjacent to the
Most importantly, overlay and digital image analysis of the H&E-stained microscopic images with the MSI images revealed that the total percentage of erlotinib found in the glandular complexes correlated positively with the survival time of periodically treated PDAC mice. In other words, the effect of erlotinib depends on its presence in specific cell types.

The presented two studies display how MSI can be used to gather information regarding a drug’s efficacy by providing spatial information on both the pro-drugs and metabolites as well as linking the localisation of the drug/metabolite to local and global phenotypic factors of efficacy like alterations in the tissue or survival time.

**Drug imaging and adverse effects.** Besides drug efficacy, knowledge of the spatial distribution of a drug and its metabolites can also aid in the understanding of potential adverse effects associated with drug treatments.

L-DOPA is currently the most effective drug for symptomatic treatment of Parkinson’s disease, as it is converted into dopamine (DA) in the brain, thereby compensating the degeneration of dopaminergic neurons in the striatum, as is seen in Parkinson’s disease. However, long-term treatment can lead to L-DOPA induced dyskinesia (LID), which involves debilitating involuntary movement. Using the previously described method based on a reactive matrix for the detection of neurotransmit-
tiers and their metabolites, Fridjonsdottir et al. used FTICR MALDI-MSI to investigate the localisation of L-DOPA and its main metabolites across 18 different brain regions in a nonhuman primate model of Parkinson's disease to better understand the pathophysiology of LID. A comparison of the distribution of L-DOPA in the brains of nondyskinetic (non-LID) and dyskinetic (LID) animals, revealed that L-DOPA and its metabolite 3-OMD was increased in all 18 brain regions of LID animals. Likewise, DA was also significantly increased in the brains of LID animals except in the caudate nucleus and putamen of the striatum, while 3-MT, the main metabolite of DA, was increased in 13 out of 18 regions. Furthermore, linear regression correlation analysis in the LID group revealed a linear relationship between L-DOPA and 3-OMD in all brain regions and a linear relationship between L-DOPA and DA in all regions except the striatum, at elevated L-DOPA abundances. These findings used the unique spatial information provided by MSI to indicate that extrastriatal regions in LID have a dysregulated metabolism of L-DOPA and are unable to regulate the formation of DA, leading to elevated DA abundance, at high concentrations of L-DOPA.

Drug imaging in human samples. In the early phases of pharmacological research, it is common to use animal models. MALDI-MSI has also been used to localise and quantify anticancer drugs in human samples only recently. This is only possible when the tissue samples have been obtained with appropriate ethical approval and with most of the drug still being in the organism i.e., with drugs that have long clearance times.

The long half-life of imatinib (16–18 h) enabled Sammour et al. to investigate its distribution in gastrointestinal stromal tumours (GISTs) and matched normal tissues obtained from 27 patients who received imatinib just prior to surgery. Imatinib was quantified using a dilution series on a separate tissue section and deuterated imatinib for normalisation (see chapter on Methodological advances for drug imaging). Before quantifying the drug across all tissues, the authors investigated some methodological aspects. First, they observed nonlinear responses in the calibration curve due to hypothesised non-optimal matrix-analyte-ratios and hence suggested the use of nonlinear regression. Second, they found that different MSI methods largely concurred with the gold standard ESI-MS but generally underestimated its values: 22% for MALDI-TOF-MSI and 13% for MALDI-FTICR-MSI. This was attributed to the full volumetric exploitation of the tissue by ESI-MS as compared to MALDI-MSI, which depends on the vertical extraction efficiency of the drug from the tissue section.

Looking at all clinical tissues, MALDI-TOF-MSI revealed the absence of imatinib, defined as signal/noise < 3, in most samples, except in the normal liver. The drug seemed unable to penetrate the GIST liver metastases despite its high concentrations in the surrounding normal hepatic areas (Fig. 5B). This was found independent of the tumour samples' somatic mutation statuses, which are typically linked to impaired tumour penetration. Furthermore, N-desmethylimatinib, the main metabolite of imatinib, as well as imatinib signals based on sodium and potassium adducts also showed highest intensities outside of the tumour area, thereby ruling out metabolisation or MALDI effects responsible for the low drug levels in GIST. Finally, Haem B, which is a marker for vascularisation, and which can also be seen with MALDI-MSI, showed that the tumour core had significantly reduced vascularisation as compared to the surrounding normal tissue (Fig. 5B). This is in line with reports by third parties that observed a GIST's vascularity to decreases upon treatment with imatinib. Since this study has been performed in human samples, the authors finally propose the use of MALDI-MSI as a post-surgical tool to evaluate a drug's efficiency.

Cassette dosing. MSI can be applied in pharmacodynamics studies for imaging multi-drug schemes (cassette dosing) due to its virtually unlimited multiplex capability.

In respiratory diseases, inhaled drug delivery is used to achieve specific access to the drug target sites while minimising systemic exposure and thereby increasing the risk of off-target or adverse effects. Due to the limited data available on drug retention in the lungs after inhalation compared to alternative delivery methods, Hamm et al. investigated this for three different classes of drugs. Using middle- (70 µm) and high- (30 µm) spatial resolution DESI-MSI, distributions of salmeterol, salbutamol and fluticasone propionate (FP) were imaged in whole-lung sections obtained from rats after inhalation of all three drugs as compared to intravenous (IV) administration of deuterated versions of the cassette. Drug distributions were measured 2 and 30 minutes after administration and all three drugs were detectable in the lungs already after 2 minutes. Using the relative standard deviation of signal intensities as an indicator of distribution heterogeneity, inhalation resulted in more heterogeneous distribution patterns with a higher presence of salmeterol and salbutamol in bronchiolar regions whereas IV resulted in overall more homogeneous distribution patterns with a tendency to alveolar tissue regions. This was also true for the 30 minutes time point, however at significantly lower intensities. Finally, lung targeting factors were calculated by normalising the per-region MSI intensities with the drugs' plasma concentrations. This showed for instance for salmeterol that, at equal plasma concentration, inhalation leads to a ~40× higher presence at the target site as compared to systemic dosing. With this, the researchers have described the advantage of inhalation over IV dosing.

MSI for pharmacodynamics

Pharmacodynamics studies, in contrast to pharmacokinetics, explore the effect of the drug on the target tissue, organ, or whole organism where MSI can be used to look at drug-induced spatial concentration changes of endogenous molecules (Fig. 5). The molecular class that is being investigated by MSI can be either the direct target molecular class of a drug (Fig. 5D) or more downstream changes in the molecular setup of the cells (Fig. 5C).

Effects on target molecules. One example of the first is the study by Ait-Belkacem et al., who investigated the spatial con-
centration of tryptophan (Trp) and kynurenine (Kyn) upon IDO1 inhibition. IDO1 is involved in the immune response homeostasis by converting tryptophan (Trp) into kynurenine (Kyn) and hence IDO1 inhibitors are being investigated as stimulators of anti-tumour immune responses.\textsuperscript{111} Murine mastocytoma xenografts with low and high IDO1 expression were investigated by MALDI-FTICR imaging to mimic IDO1 inhibition. To facilitate absolute quantitation of Trp and Kyn, deuterated versions of these (Trp-d5 and Kyn-d4) were sprayed onto the sections as well as spotted in different concentrations as calibration curves on adjacent liver tissue sections (see the section on quantitation below). Importantly, lower and upper limits of quantitation and the limit of linearity were defined for each calibration curve. The calculated quantities by MSI were validated by the golden standard in the field, namely LC-MS.

As expected, high Kyn/Trp ratios were observed in mice with high IDO1 expression and \textit{vice versa}. This was also true for another four downstream metabolites of the tryptophan pathway. Also, the spatial IDO1 expression – which varied within tumours, and which was determined by immunohistochemistry and overlaid with 30 µm MSI – was positively correlated with higher Kyn intensities. This allowed making relationships between local IDO1 expressions and local tumour cell metabolic phenotypes, e.g., IDO1-positive tumour areas were characterised not only by low Trp levels but also by low glucose levels.

Another class of small-molecule inhibitors are histone-deacetylase inhibitors (HDACis) which constitute the most advanced class of epigenetic drugs by blocking the deacetylation of acetylysine modifications on histone N-terminal tails.\textsuperscript{112} Munteanu \textit{et al.} investigated if MALDI-MSI can be used to \textit{in situ} monitor the effect of different HDACis, represented as an increase of acetylation on histones H4, in leukaemia suspension cells and different cancer mouse models.

Amongst these, a genetic mouse model of gastric cancer was treated with the HDACi panobinostat intraperitoneally and vehicle control for 1 and 4 hours. While vehicle-treated mice were characterised by hypoacetylation of H4 in tumour areas, tumour areas of treated mice showed a stepwise and time-dependent increase in acetylated H4 species. Interestingly, hyper-acetylation was minimal in tumour-surrounding connective tissue.

Both studies show that MSI provides a powerful tool to spatially assess the drug’s effect on target molecules which, e.g., in cancer research, could be helpful to relate a drug’s efficacy to the spatial clonality/heterogeneity of a tumour.

\textbf{Effects of therapy on surrogate molecules.} Although the direct target molecules of a pharmacological intervention might not be known, research, including MSI studies, have looked for changes in other endogenous molecular classes that correlate for instance with therapy efficacy. It is therefore important to distinguish if one wants to investigate the molecular constitution of the target tissue \textit{before} exposure to the pharmaceutical compound, e.g., to predict the response of the tissue/organism to the drug,\textsuperscript{74} or \textit{after} administration to study the drug-induced changes in the original molecular constitution.

In line with the latter, Shen \textit{et al.} have recently used MALDI-MSI to investigate non-small cell lung cancer tissue samples for metabolic changes after neoadjuvant chemotherapy.\textsuperscript{113} Neoadjuvant therapy is administered before surgical intervention in order to reduce the tumour size and thereby facilitate its easier removal. While this strategy generally improves the survival time in patients, not all patients benefit equally from this therapy. Hence, the authors aimed at finding tissue-specific metabolic profiles that relate to the survival time in 88 lung cancer patients who received platinum-based chemotherapy \textit{prior} to resection.

The samples were provided as tissue microarrays, which are formalin blocks that contain many small tissue punches from many different samples. This removes variance due to inter-slide sample preparation and hence facilitates a better comparison of the samples. MSI experiments were performed on a high-mass resolution (FTICR) instrument, which resulted in the detection of >5000 molecular features, and at a spatial resolution of 50 micrometres, which due to a diameter of 600 micrometres per punch, resulted in about 100 MSI pixels per sample. The authors assigned these pixels, based on the previously established and described SPACIAL workflow,\textsuperscript{36} to either tumour stroma or actual tumour cells. Machine learning was then used to train metabolic classifiers separately for these two histological entities with the aim to distinguish short- from long-term survivors (dichotomised based on the median survival time).

Because it is important to always compare new classifiers with the clinical standard, the authors performed a multivariate Cox regression analysis that demonstrated that these new metabolic classifiers were independent of major clinico-pathological parameters, including TNM classification and pathological response assessment. Moreover, both tumour and stroma metabolic classifiers were able to significantly stratify additional prognostic groups within responders and non-responders.

This study shows that the metabolic constitution of the tumour, after neoadjuvant therapy, contains important information for the long-term response of the patient independent of TNM staging and microscopic response evaluation. Key metabolites in the unfavourable survival group could be new interesting drug targets.

\textbf{Toxicological effects of drugs.} The uptake of drugs usually comes along with a detoxification process that predominantly takes place in the liver and kidneys.

MSI has therefore, for instance, been used by Kampa \textit{et al.} to study amitriptyline-related hepatic injuries reflected as metabolic changes (Fig. 5C).\textsuperscript{114} Amitriptyline is an antidepressant and prolonged use can cause liver damage through lipidosis. Three rats for each control and high-dose group, which received amitriptyline for nine days, have undergone MSI. The authors used 2,5-dihydroxybenzoic acid (DHB) as a matrix to detect the drug and its metabolites as well as the potential effects of the treatment on lipids, whereas 1,5-diami-
nonaphthalene (DAN) hydrochloride has been used to detect alterations in bile acids.

While amitriptyline has been detected at homogeneous levels in the liver samples of dosed rats, the overall levels of its active metabolites varied strongly between rats. When looking at the effect on endogenous metabolites, nine phosphatidylcholine lipids were found to be upregulated in the hepatic lobules, and two bile acids were downregulated in the bile ducts of the amitriptyline treatment group.

While this study investigated the molecular changes of drugs that were deliberately ingested by the organism, MSI has also been used for toxicological studies where the effect of environmental pollutants was studied.\(^{115}\)

**Methodological advances for drug imaging**

**Improving the detectability of drugs.** Mass spectrometry imaging of drugs is mainly struggling with the detectability of the compounds. This is in most cases related to the low in situ concentrations, fast metabolic turnover, and ion suppression coupled with unfavourable chemical properties of the compound for its ionisation.

Low in situ concentrations are an unchangeable matter of fact since studies with animal models or humans are cleared with a maximum dosage amount. To better deal with the issue of a fast metabolic turnover, some studies have performed an initial time profiling where the subsequent work continues with the time points with the highest drug signal.\(^{106}\) This leaves most of the methodological efforts on improving drug signals in MSI with an increase of ionisation efficiency.

Ion suppression by endogenous molecules in the tissue is another damper of a drug’s signal intensity. As shown before for other molecular classes,\(^{116}\) it is therefore important to remove these endogenous molecules through a series of washes. Chen et al. investigated this possibility for central nervous system drugs.\(^{117}\) They developed a protocol which included washing the tissue sections in an ammonium acetate solution, then incubation with trifluoracetic acid vapour, and a final rinse in n-hexane before MALDI-MSI. This increased the drugs’ intensities by 4.7- to 31.5-fold as compared to untreated tissue sections.

Recently, MALDI-2 has been introduced for MALDI-MSI to increase the ion yield during the ionisation process using a second laser.\(^{2}\) This laser fires with a small delay to the MALDI laser and in parallel to the sample stage into the desorption gas phase plume. Barré et al. investigated the effect of MALDI-2 on the signal intensity improvement of ten different drug compounds.\(^{98}\) They found that MALDI-2 increased the signal intensities for seven out of the ten drug compounds by up to two orders of magnitude, however for the sake of a higher chance of fragmentation of some of the molecules. The use of MALDI-2 hence harbours huge potential for increasing sensitivity in MALDI-MSI, since it does not require any additional sample preparation steps, unlike derivatization.

Derivatization through chemical modification of the drug is another way to increase its ionisation efficiency.\(^{118}\) Merdas and co-workers have recently exemplified the usefulness of chemical derivatization for the detection of Acetaminophen in the whole-body sections of dosed rats by MALDI-MSI, where the derivatization agent is sprayed on the tissue before matrix application.\(^{119}\)

While they found that the signal intensity of Acetaminophen and several of its metabolites increased >20-fold with 2-fluoro-1-methylpyridinium p-toluene sulfonate (FMPTS) in combination with the matrix CHCA, several other metabolites were best detected without derivatization and/or with other matrices such as DHB or 9-aminooacridine. Then they optimised the concentration of FMPTS, which involves identifying the minimal concentration that still provides the highest intensity.

Moreover, they also spiked isotopically labelled standards of Acetaminophen and some of its metabolites into the MALDI matrix. This not only facilitated the localisation of Acetaminophen in the whole rat but also showed that on-tissue derivatization is compatible with absolute quantification, which is another major research area of MSI.

**Quantitation.** MSI is a semi-quantitative technique, but pharmaceutical research legally needs to determine the absolute concentrations in the target tissue. The development of approaches for the absolute quantitation of drugs or their metabolites by MALDI-MSI has therefore had a lot of attention in the field.\(^{120}\) Several different strategies have therefore been developed over the last 15 years, which incorporate one or both of the following components: the normalisation of the drug signal and the setting-up of a calibration curve. The excellent tutorial review by Rzagalinski and Volmer summarises these approaches comprehensively.\(^{121}\)

Nowadays, three different methods have established themselves: the dilution series model, the tissue-extinction-coefficient model, and the mimetic tissue model,\(^{122}\) of which the first is the most used. In this strategy, the dosed tissue is accompanied by the slide with a control tissue on which a dilution series of the drug is spotted. Both the dosed and the control are then covered with a layer of internal standard (which is usually a structural analogue of the drug). All drug signals, including the signal from the calibration spots, are then normalised by the signal of the internal standard.

While the method is now widely accepted in the field of MSI, the availability of validation procedures for such bioanalytical methods is the next urgent need due to the embedding of this approach in pharmaceutical research and development. Källback and co-workers have provided a blueprint for such a validation that addresses the FDA requirements of selectivity, accuracy and precision, recovery, and stability.\(^{123}\) Using MALDI-MSI to locate citalopram in mouse brains as a use case, the authors demonstrated that the dilution series model yielded similar results to those from the reference method (LC-MS/MS) for both mass analyser types, TOF and FTICR, and that FDA criteria were within the limits.

**Multimodal imaging and digital image analysis in MSI of drugs.** Integration of the MSI images with histological information, as provided by light microscopy, for a better interpretation of the molecular images has ever since been part of good
MSI practices. Nowadays, MSI is being spatially integrated with many other ex vivo and in vivo imaging techniques. In addition, the digitalisation of images from other imaging techniques, such as optical microscopy, has opened new means for the interpretation of MSI datasets, also in the context of pharmacological MSI studies.

An older bimodal study by Huber et al. gives an example of this, where MALDI-MSI was used to study the distribution of several anti-cancer drugs in the spatial context of the vascularisation of xenograft tumours as indicated by immunohistochemistry (IHC) against CD31. Registration of the MSI drug image with the IHC image allowed a direct spatial comparison of the vessels’ locations with the drug distribution. Digital image analysis then revealed a correlation of the drug intensity with the blood vessel density and a steady decay of the drug’s signal intensity with an increase in the distance to the blood vessels.

A recent trimodal study by Strittmatter et al. integrated DESI-MSI with imaging mass cytometry targeting 27 antigens and light microscopy. DESI-MSI was used to follow the pharmacological MSI studies. Moreover, due to the high specificity of MSI data have also been compared to in vivo imaging data, for instance in the study by Jacobsen et al., who used DESI-MSI to visualise and quantify the PET tracer Cimbi-36 in rat brain. Rats were dosed with Cimbi-36 and euthanised after 5, 10, 15, 30, 60 and 120 min of intraperitoneal injection. The distributions and pharmacokinetics of Cimbi-36 were compared between ex vivo MSI, in vitro autoradiography, and in vivo PET. Autoradiography and MSI images confirmed the accumulation of the tracer in receptor-rich regions of the brain, e.g., in the prefrontal cortex, which could not be spatially resolved by PET. Moreover, due to the high specificity of MSI based on MS/MS measurement, it was confirmed that PET and autoradiography were actually detecting the parent compound and not any metabolite of it that still carries the radioactive label. This might also be the reason why the MSI signal of the parent drug peaked at 15 min and was eliminated at 120 min, whereas PET still detected the label at 120 min. This exemplifies the added value of integrating ex vivo unlabelled techniques such as MSI as validation tools into tracer developments.

An important aspect of the integration of ex vivo (usually 2D data) with in vivo (usually 3D) imaging data is the increased challenge for image registration due to the lack of direct spatial correspondences. Abdelmoula et al. addressed this image processing issue in a use case of in vivo MRI and 3D MALDI-MSI of erlotinib in mouse glioblastoma. First, the dimensionality of each MSI section was reduced by the use of t-SNE to obtain a single 2D t-SNE score image per section that retains most of the information. Then image registration between MRI and the t-SNE score images was performed in a slide-to-slide fashion, where the MRI dataset acts as a volumetric reference to preserve the original tissue shape while non-linearly warping the individual t-SNE score images. This finally allowed visualising the distribution of the anti-cancer medicine erlotinib within the 3D anatomical features of the brain tumour as provided by MRI.

Emerging applications of MSI in biomedical sciences

Mass spectrometry imaging is expanding also to other fields in biomedical sciences such as immunology and microbiology, everywhere where cells change their phenotype and metabolic state dynamically based on the spatial conditions of their microenvironment. This applies certainly – and maybe more than to any other cell – to immune cells.

Immunology

While only a few studies have been published so far on this matter, it has already become clear that MSI, providing the metabolic information on the immune cells, needs to be complemented with the established catalogue of immunostaining-based phenotypic categorization of immune cells. An example of this is the recent publication by Goossens et al., which exemplifies how MSI can be integrated with multispectral immunohistochemistry to correlate the different phenotypes of macrophages with their lipidome.

Studying the immune system has very practical implications due to the many novel immune therapies, especially in the field of oncology. In this line, Graziano et al. used MSI to determine adenosine levels and distributions in a mouse model of pancreatic cancer. Mice were exposed to immune therapy in combination with the inhibition of the adenosine receptor Adora2a of protumorigenic M2 macrophages, thereby blocking the in vivo formation and function of extracellular adenosine, as shown by MSI. This was found to slow down tumour growth and reduce the metastatic burden.

Studies on immune cells using MSI also evidence the need for a high spatial resolution to distinguish infiltrating immune cells from their surrounding cells (Fig. 6). An example of this is the study by King et al. who used DESI-MSI to find long-chain polyunsaturated lipids to be increased in responsive tumours of different mouse models upon anti-PD-1 immune therapy. While it was observed that the predictive lipid profile was more intense in tumours with dense regions of immune infiltrates, a resolution of 150 μm does not allow assigning the profiles to either the tumour cells or the infiltrating immune cells (Fig. 6).

Lastly, the study of immune cells is also inherently linked to the topic of infections as the study of Guerrini et al. exemplifies, in which the authors used MALDI-MSI to determine...
is important to note that accumulations of immune cells around the pathogen. Examples are infection foci which are interactions (Fig. 6) or if surrounding host tissue is included in the read-out. Infection foci are interactions between the host and the pathogen at specific places in the host or the pathogen interface. MSI studies with MALDI-MSI, it is important to note that–similar to the case of immune cells–the spatial resolution of MSI determines if the molecular state of the pathogen itself can be read out exclusively (Fig. 6) or if surrounding host tissue is included in the read-out. Examples are infection foci which are interactions between the host and the pathogen at specific places in the host organism such as granulomas, which are localized accumulations of immune cells around the pathogen.

Eukaryotic infections. MSI has been used on two occasions to study hepatic granulomas caused by parasite infections of tropical diseases.

Von Bülow et al. investigated the effect of Schistosoma mansoni eggs on the liver tissue of infected hamsters. The eggs of this worm-like species are the driver of host morbidity since they obtain their nutrition from the host tissue. MSI offers a useful tool to shed light on these in situ mechanisms of this parasite–host interaction which is not yet understood. Using 10 µm MALDI-MSI the authors were able to identify specific phospholipids that were enriched in the Schistosome eggs (up to 200 µm in length) but exhausted in granulomas and the surrounding liver parenchyma, thereby strengthening the evidence of uptake of these molecules from the host. Overall the study delivered evidence and valuable insights into the metabolic reprogramming of the host tissue, ultimately leading to its own damage.

In a similar fashion, Kloehn et al. used MALDI-MSI of Leishmania mexicana infected mice to describe the metabolic activity of dermal granulomas and surrounding tissues. Leishmania are intracelular parasites and hence not resolvable by MSI. The use of in vivo heavy water labelling in combination with MSI allowed quantifying the metabolic turnover rate of parasite and host-specific lipids. Using this approach, the authors were able to localize metabolically active and quiescent parasite-rich regions. MSI also allowed to simultaneously image the administered first-line drug miltefosine in the tissues which revealed high accumulation of the drug in macrophage-rich and metabolically active lesions and reduced accumulation in metabolically quiescent and fibrotic regions. The authors suggest that these hibernating parasites in cured lesion tissue could be responsible for therapy failure.

Bacterial infections. For bacterial infections, Scott et al. used MALDI-MSI to map and temporally follow the pathogen-exclusive lipid A in murine spleen infected with Francisella bacteria, which can cause the very lethal disease of tularemia. While lipid A was found to increase significantly after 36 hours of infection, other endogenous lipids were found to change after that time point, such as the inflammatory phosphatidylinositol 38:4 (SAPI). The authors also demonstrated that tissue destruction and not the immune response causes the decrease of SAPI and that 3D-MSI is a valuable tool to clearly define the host–pathogen interface.

Compared to the very seldom infections of humans by Francisella bacteria, Salmonella infections are very common and one of the major causes of human gastroenteritis. Similar to the examples above, they cause the formation of granulomas in the spleen and liver and have the capability of influencing infected and surrounding immune cells to their benefit. For this reason, Strittmatter and co-authors used DESI-MSI and imaging mass cytometry: the latter targeting major immune cell antigens and one to locate Salmonella bacteria, and the first to study the metabolic effects of Salmonella Typhimurium infection on infected mouse liver tissue. This allowed relating the presence of Salmonella, be it intra-cellular or in the vicinity, with the host immune cell phenotype or immune cell composition of the tissue, respectively, and to find metabolic features of infection.

Finally, MSI has been used by a research group of the U.S. Army Medical Research Institute to find serum biomarkers indicative of Burkholderia mallei infection reasoning that changes in the tissue proteome caused by the infection should be reflected on a circulating fluid level. Green monkeys were infected through inhalation and MSI found the bacterial protein GroEL to be highly upregulated in infected regions of the lung and skin. Using protein arrays for serum analyses, it was found that GroEL was 10–100 times increased in infected subjects compared to control proteins from another pathogen. Additional LC-MS based analyses of infected regions identified the host protein calprotectin, which could be validated as a complementary marker of active infection in sera of different infected animals.
Viral infections. Studies on viral infections are still very rare and have until now mainly focused on investigating cancers that are caused by oncogenic viruses, as summarized in the review by Bertzbach *et al.*139

Nevertheless, the recent COVID-19 pandemic has led to one publication by Ackermann *et al.* who used MALDI-MSI to obtain information on predominant metabolic processes in lung tissues of COVID-19 patients as compared to common forms of fibrosing interstitial lung diseases.140 Spatial metabolomics data revealed most differential metabolites being upregulated in control tissues and being part of the tryptophan metabolism, which is involved in inflammation and immune responses.

Microbiology

Metabolic interactions of co-cultured microbial communities or their exposure to antibiotics are spatial events that can be spatially assessed with MSI.

As a first example, the pioneering Dorrestein lab in that field has used MSI to study the metabolic exchange of co-cultured *Pseudomonas aeruginosa* and *Bacillus subtilis*. Surprisingly they found several lipopeptide tails of surfactins, which are antibacterial lipopeptides produced by *B. subtilis*, to be spatially located in the close vicinity of *P. aeruginosa* and not located in monocultures of both bacteria, pointing towards the interaction of both bacteria via these molecules. Subsequent motility experiments, where *P. aeruginosa* cultures were exposed to pure surfactins, surfactin-deficient *B. subtilis* strains, and purified surfactin degradation products, demonstrated that *P. aeruginosa* is attracted by the presence of surfactins and repelled by the degraded lipopeptidic tail of surfactins.

As also shown by this example, MSI enables correlating the spatially discrete phenotype of microorganisms with their actual chemotype, thereby helping in identifying the mechanisms of antibiotic resistance of resistant subpopulations.141 Due to their protective role, analysing the spatial structure and generation of biofilms on medical implants by MSI is gaining interest, too.142,143

The recent review by Feucherolles and Frache comprehensively summarizes the developments of MSI in the field of microbiology.144

Conclusion

In this review, we tried, to the best of our knowledge, to give recent (and in some cases where not otherwise possible older) examples that can serve research groups entering the field of mass spectrometry imaging an applied reference framework.

We decided to look for suitable examples in the major six fields of application of MSI (Fig. 1). While we additionally highlighted a few emerging fields, such as immunology, infectious diseases, and microbiology, we understand that, due to this decision, we might have missed to report other interesting areas of application of MSI in biomedical sciences (developmental biology, hepatology, nephrology, etc.). Some applications of MSI can also be associated with the field of aging, one of the big challenges of the next decade for Western societies. While aging is an umbrella term covering most of the fields in this review (cancer, neurodegenerative diseases such as Parkinson’s and Alzheimer’s, endocrinology such as diabetes type 2, and rheumatology such as arthrosis), some MSI studies deal with other aspects of aging. In these studies, MSI has been, for instance, used to study eyesight degeneration,145,146 for visualizing oxidative stress in the brain,147 or to investigate skin aging.148,149

Other expanding areas of MSI – beyond biomedicine and which address urgent global challenges of the next decade (climate change and agriculture/food production) – are its application in plant biology and food analysis.

Microplastics are not only a worrying contaminant of the oceans but for nature in general and consequently also of our food chain. The location of microplastics in seafood mussels has therefore been recently investigated by Pedersen *et al.* with MALDI-MSI.150 With respect to agriculture and a changing climate, plants are nowadays exposed to more extreme weather and environmental conditions. MSI has hence been used to study the metabolic changes in plants upon exposure to excessive salt stress.151

While these are interesting developments outside of the scope of this review, we might not only have missed to report studies from other biomedical fields, but also from the fields that we selected since we have particularly looked for publications that excel in one particular category, *e.g.*, as a high impact methodology. With respect to the latter category and the fact that we focused on six areas of application only, the picture of methodological developments in MSI is certainly incomplete. As can be seen from Fig. 1, we estimate that 30–40% of the publications in MSI are dedicated to methodological developments. Some specific methodological developments that we have missed to report are the use of MALDI-MSI for multiplex labelled immunohistochemistry as an alternative to mass cytometry using photo-cleavable mass tags on antibodies152 and ion mobility as additional dimensions of separation of compounds,153 which have both experienced revivals in MSI. In our opinion, the most promising overall technical development, however, is the integration of MSI with other imaging and non-imaging (omics) modalities combined with advanced data science techniques. Incorporating MSI into a family of spatial omics techniques, thereby covering the spatial metabolomics part, has the potential to become a magic bullet for biomedical research.

Finally, we want to stress that the focus on selected areas and predefined categories does not lower the quality of the many other scientific works performed with MSI, which were not mentioned in our review. It does, however, imply that our review does not constitute a fully comprehensive overview of the whole field of MSI. We hence want to refer the reader to the many reviews that focus on and cover specific aspects of the large field of MSI, such as unsupervised data analysis13 or the imaging of glycans154 and other molecular classes.
We hope the reader finds our review useful and assists in the developments and innovations throughout the field of mass spectrometry imaging with new ideas and research applications.

Author contributions

KKK: conceptualization, writing – original draft, and visualization; RMAH: writing – review & editing; BB: conceptualization, writing – original draft, and visualization.

Conflicts of interest

There are no conflicts to declare.

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References

28 C. Sun, et al., Mass spectrometry imaging-based metabolomics to visualize the spatially resolved reprogramming of carcinitine metabolism in breast cancer, Theranostics, 2020, 10(16), 7070–7082.


36 V. M. Prade, et al., De novo discovery of metabolic heterogeneity with immunophenotype-guided imaging mass spectrometry, Mol. Metab., 2020, 36, 100953.


56 T. Zullig and H. C. Kofeler, High Resolution Mass Spectrometry Imaging at 10 μm Reveals Spatial Lipid and...
Critical Review


68 M. Shariatgorji, et al., Comprehensive mapping of neurotransmitter networks by MALDI-MS imaging, Nat. Methods, 2019, 16(10), 1021–1028.


74 J. L. M. Bjorkgren and A. J. Lusis, Atherosclerosis: Recent developments, Cell, 2022, 185(10), 1630–1645.


76 S. Mas, et al., Local non-esterified fatty acids correlate with inflammation in atheroma plaques of patients with type 2 diabetes, Diabetes, 2010, 59(6), 1292–1301.


86 F. Löhöfer, et al., Molecular imaging of myocardial infarction with Gadofluorine P - A combined magnetic resonance and mass spectrometry imaging approach, Heliyon, 2018, 4(4), e00606.


91 N. Sun, et al., Prognostic Relevance of Steroid Sulfation in Adrenocortical Carcinoma Revealed by Molecular


M. Nishidate, *et al.*, Applications of MALDI mass spectrometry imaging for pharmacokinetic studies during...


126 N. Strittmatter, et al., Multi-modal molecular imaging maps the correlation between tumor microenvironments and nanomedicine distribution, Theranostics, 2022, 12(5), 2162–2174.


131 P. Goossens, et al., Integrating multiplex immunofluorescent and mass spectrometry imaging to map myeloid heterogeneity in its metabolic and cellular context, Cell Metab., 2022, 34(8), 1214–1225.


133 V. Guerrini, et al., Heterogeneity of foam cell biogenesis across diseases, bioRxiv, 2023, DOI: 10.1101/2023.06.08.542766.


140 M. Ackermann, et al., The fatal trajectory of pulmonary COVID-19 is driven by lobular ischemia and fibrotic remodelling, EbioMedicine, 2022, 85, 104296.


144 M. Feucherolles and G. Frache, MALDI Mass Spectrometry Imaging: A Potential Game-Changer in a Modern Microbiology, Cells, 2022, 11(23), 3900.


151 C. Keller, et al., Comparison of Vacuum MALDI and AP-MALDI Platforms for the Mass Spectrometry Imaging


