Lipidomics for translational skin research: a primer for the uninitiated

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Abbreviations
3Q, triple quadrupole; CMC, carboxymethylcellulose; DESI, desorption electrospray ionisation; ELISA, enzyme-linked immunosorbent assay(s); ESI, electrospray ionisation; FA, fatty acid(s); MALDI, matrix-assisted laser desorption ionisation; MS, mass spectrometry; QToF, quadrupole-time-of-flight; TEWL, trans-epidermal water loss;ToF, time-of-flight; UHPSFC, ultra-high performance supercritical fluid chromatography; UPLC, ultraperformance liquid chromatography; PUFA, polyunsaturated fatty acid(s).
Abstract

Healthy skin depends on a unique lipid profile to form a barrier that confers protection and prevents excessive water loss, aids cell-cell communication, and regulates cutaneous homeostasis and inflammation. Alterations in the cutaneous lipid profile can have severe consequences for skin health and have been implicated in numerous inflammatory skin conditions. Thus, skin lipidomics is increasingly of interest, and recent developments in mass spectrometry-based analytical technologies can deliver in-depth investigation of cutaneous lipids, providing insight into their role and mechanism of action. The choice of tissue sampling technique and analytical approach depends on the location and chemistry of the lipid of interest. Lipidomics can be conducted by various mass spectrometry approaches, including different chromatography and ionisation techniques. Targeted mass spectrometry is a sensitive approach for measuring low-abundance signaling lipids, such as eicosanoids, endocannabinoids, and ceramides. This approach requires specific extraction, chromatography and mass spectrometry protocols to quantitate the lipid targets. Untargeted mass spectrometry reveals global changes and allows analysis of hundreds of complex lipids across a range of lipid classes, including phospholipids, glycerophospholipids, cholesteryl esters and sphingolipids. Mass spectrometry lipid imaging, including matrix-assisted laser desorption ionisation mass spectrometry and desorption electrospray ionisation mass spectrometry, can reveal information about abundance and anatomical distribution of lipids within a single skin sample. Skin lipidomics can provide qualitative and quantitative data on hundreds of biologically-relevant lipid species with different properties and activities, all found within a single skin sample, and support translational studies exploring the involvement of lipids in skin health and disease.
1. INTRODUCTION

The skin is a site of complex and unique lipid metabolism, with lipids essential to cutaneous function and dependent on dietary provision [1, 2]. From fatty acyls to more complex glycerolipids, glycerophospholipids, sphingolipids and sterols, the lipid classes found in skin cover a range of chemistries, and perform niche roles within the organ [3, 4]. Sebum on the skin’s surface predominantly comprises triacylglycerols, wax esters, squalene and free fatty acids; lipid species that help to waterproof the skin, contribute to barrier function, but also support and regulate cutaneous microbiota [5, 6]. An equimolar ratio of ceramides, cholesterol and free fatty acids form the epidermal lipid barrier in the stratum corneum, and are responsible for preventing excessive trans-epidermal water loss (TEWL) and ion permeability [7]. The lipids found in skin cell membranes, predominantly glycerophospholipids, sphingomyelins and cholesterol, play important roles in protein function, cell mobility and cell-cell communications [8-10]. Importantly, membrane lipids also undergo active metabolism to bioactive lipid mediators, including eicosanoids, endocannabinoids and ceramides, which regulate numerous physiological processes in skin homeostasis and inflammation, including immune cell mobility and function [11-13], vasodilation and relaxation [14, 15], and keratinocyte and fibroblast proliferation, differentiation and migration [16-18]. Cutaneous lipid metabolism results in a continuous flux of bioactive lipid species, and the increasing appreciation of the translational role of skin lipidomics has driven developments in lipidomic technologies. In order to obtain the maximum amount of information relevant to skin lipid biology, it is important to use bioanalytical approaches capable of identifying and quantitating across the range of lipids. Here we present a review of the main technology platforms currently used in skin lipidomics, and provide examples of the amount and variety of lipidomic information that can be obtained from skin biopsies using these techniques, including targeted mass spectrometry, untargeted mass spectrometry, and mass spectrometry imaging.

2. SKIN LIPID SAMPLING AND EXTRACTION

Skin lipid analysis requires appropriate lipid sampling, extraction and analysis, which depend on the skin compartment of interest, the chemistry of the target lipids, and the information required, respectively. Examples of different approaches are shown in Figure 1.

2.1. Skin lipid sampling

The choice of skin lipid sampling is determined by the location of the cellular target within the skin. Surface lipids found in sebum or stratum corneum can be captured using non-invasive tape strips. Tapes designed to absorb sebum lipids (e.g. Sebutape® [19]) or remove stratum corneum (e.g. D-Squame® [20]) can be treated with solvents to yield epidermal lipids [21-23]. Tape strips have been used to sample consecutive non-viable stratum corneum layers and provide information on lipid species found at different stratum corneum depth [24], but deeper sampling is needed to examine viable cells. If lipids from the dermis are also required, the most appropriate sampling approach is the full-thickness skin biopsy. Biopsies can be split to allow separate analyses of the different skin compartments, or used for lipid imaging to analyse the full cross-section of the biopsy [25, 26]. A third option is suction
blisters fluid sampling [27]. This involves applying vacuum to the skin to raise a suction blister, followed by aspiration of the blister fluid that contains lipid mediators of primarily epidermal origin [28]. Other types of samples that can be used to study cutaneous lipid biology include the vernix caseosa [29] and sweat [30, 31]. Sample preparation is important, as rapid degradation or metabolism of lipids is possible post tissue-harvesting. Ideally, skin samples (biopsies, blister fluid or tape strips) should be flash-frozen in liquid nitrogen immediately after harvesting. Samples should then be stored at -80 ºC prior to analysis.

2.2. Lipid extraction

Lipid extraction primarily relies on solvents to disrupt cellular membranes and solubilise lipids. Solvents are selected based on the chemistry of the lipids being analysed, and vary depending on the polarity of the target lipids (this has been reviewed recently [32]). Whilst tape-stripped stratum corneum and epidermal blister fluid samples can be added directly to solvents [20, 33], biopsy samples require homogenisation to disrupt the tissue and maximize lipid extraction [34]. Some extraction protocols involve further purification of lipids, such as solid-phase extraction, methylamine or alkaline hydrolysis, to remove contaminants and reduce matrix effects that can impede the analysis; this is particularly important when targeting lipid species found in low abundance [35-37]. Lipid imaging approaches (discussed in Section 4) such as matrix-assisted laser desorption ionisation (MALDI) mass spectrometry imaging (MSI) and desorption electrospray ionisation-MSI (DESI-MSI) require no initial extraction steps, as lipids are ionised directly from the tissue sample.

The amount of lipid extracted can be normalised against tissue wet weight, dry weight, sample volume, protein content, phosphate or cholesterol [28, 38, 39]. Most lipid extraction protocols allow the retention of proteins from the sample, for later analysis of protein content. However, consideration must be given to how cutaneous changes might influence the sample protein content. For example, diseased skin may display different levels of cell cohesion compared with healthy skin, resulting in the collection of a different number of cells when using tape strips [40]. In these cases, it may be more appropriate to report total lipids per surface area. Additionally, certain treatments may alter the protein content of blister fluid, so normalization by fluid volume may be more appropriate [41].

3. SKIN LIPID ANALYSIS

Although approaches such as enzyme-linked immunosorbent assays (ELISA) can be used to quantitate some lipid mediators (e.g. prostaglandins), there are limitations of these methods. ELISA can only detect a single lipid species at a time, although are often unable to differentiate between isomeric species, resulting in considerable cross-reactivity [42]. Additionally, discovery of new lipid species is impossible with ELISA, and limited lipid targets are available. Some of the analytical approaches used in lipid analysis, including nuclear magnetic resonance and gas chromatography, can offer some insight into skin lipid biology, but there are limitations in their compound coverage, sensitivity and efficiency [43]. Developments in mass spectrometry and chromatography have accelerated lipidomic analytical capabilities, allowing accurate identification and quantitation of complex mixtures of lipids [44]. Mass spectrometry covers a range of analytical capabilities that can be selected depending on the lipids of interest, relying on the identification of lipids based on...
their ionisation and molecular fragmentation patterns. Although some lipid species are analysed following untargeted shotgun approaches [20], quantitative lipidomics are performed using mass spectrometry paired with chromatography to separate lipid components prior to their analysis, to maximise sensitivity. Complex lipid mixtures found in skin benefit from appropriate separation to improve the analytical sensitivity, minimise matrix effects and ion suppression, and inform peak identification [45]. It is important to consider both the quantity and proportion of lipids present in the skin. For example, aged skin appears to contain lipids in the same proportions as young skin, but overall levels are reduced [46], whereas atopic dermatitis skin demonstrates changes in both the overall level of cutaneous ceramides, and the proportion of different ceramide families [47]. Such detailed investigations require accurate lipid identification and quantitation, and a range of mass spectrometry-based lipidomics approaches are available for consideration.

3.1 Chromatography

Chromatography is used to separate different lipid classes (or lipid species) found in a sample following their extraction from tissue; the choice of chromatography depends on the lipids of interest and their physico-chemical properties. Cutaneous lipids are a diverse family of hydrophobic, low molecular weight compounds, with a range of polarities and considerable structural similarity within each lipid class [48]. These aspects complicate both lipid separation and analysis, and often different approaches will be required to fully interrogate skin lipid biology.

Normal-phase chromatography separates complex lipids based on the functionality of the polar head group, which results in similar retention times for members of the same lipid class, making distinction between lipid isomers extremely difficult, although linear separation by molecular weight is possible [49]. Normal phase chromatography also allows the rapid elution of extremely hydrophobic compounds, such as acylceramides, which is not always possible in reverse-phase chromatography [50]. Reverse-phase chromatography separates lipids based on their fatty acyl components (different chain length and number of double bonds), resulting in co-elution of different lipid classes, but allowing separation of isomers within the same lipid class [51]. Supercritical fluid chromatography is a hybrid of normal- and reverse-phase liquid chromatography, offering separation of molecules with a broad range of polarities in a single chromatographic run [52]. Consequently, this is a useful approach for the untargeted analysis of complex mixtures of skin lipids, including glycerolipids, glycerophospholipids, sterols and free fatty acids, as hydrophilic, hydrophobic, and polar lipid species can be separated based on both their fatty acyl moieties and their polar head groups; this is useful for classes with similar hydrophobicities such as triacylglycerols and cholesteryl esters, which are important in skin [52-56].

3.2. Mass spectrometry

Whether direct infusion or chromatographic introduction is used, there are various mass spectrometry platforms available for lipidomics applications (recently reviewed in [44]). Extracted lipids must be ionised and introduced into the mass spectrometer for analysis. The preferred ionisation method for most lipids is electrospray ionisation (ESI). An electric potential applied to a nebulised sample solution results in a fine spray, forming ions upon desolvation [57, 58]. ESI is considered to be a softer ionisation technique than other popular approaches, and provides a reliable tool for obtaining molecular mass information in
lipidomics [59]. Other common lipid ionisation methods include MALDI and DESI (discussed in Section 4) [44]. DESI is a derivative of ESI, in which spatial distribution of lipids in the sample can be maintained. Lipid ions can be analysed using different detection methods, chosen based on the nature of the analyte and the kind of data required. Typically, tandem mass spectrometry (MS/MS; a combination of two mass selection units) is used to monitor both precursor ions and product ions resulting from precursor fragmentation in a collision cell. Different linear combinations of quadrupoles (Q), time-of-flight (ToF) mass spectrometers, and ion traps generate multiple options for lipidomics analysis (e.g. triple-quadrupoles (3Q), QToFs and ToF-ToFs) [60]. These approaches can provide information about both precursor and product ions, enabling more confident peak identification, and this method of detection is particularly recommended for complex mixtures such as skin lipid extracts.

3.2.1. Targeted mass spectrometry

Targeted mass spectrometry can be used to investigate a particular lipid family or pathway of interest. Molecular fragmentation patterns can be identified for individual lipid species, and, if the analysis includes separation by chromatography, retention times can provide extra identifying information. Additionally, if lipid standards are available commercially or can be synthesised, calibration lines can be constructed to allow accurate quantitation. A discrete list of lipid species can thus be identified and quantitated from a complex lipid extract. The most common approach for targeted quantitation is with multiple-reaction-monitoring on 3Q mass spectrometers owing to the high degrees of sensitivity and specificity.

This approach has been used to generate information about bioactive lipid mediators in skin, including eicosanoids and related species, endocannabinoids and other N-acyl ethanolamines [28, 34, 61-63]. As many of these pathways and lipid classes have been well elucidated, individual species can be analysed using a targeted approach, and, by limiting the range of lipids being analysed, sensitivity is maximised, which is important given that these lipids are usually present in low abundance [42]. Another set of cutaneous lipids that can be analysed using targeted lipidomics is the ceramide family, a group of lipids that are essential components of the epidermal lipid barrier [64]. Although ceramides are found throughout the body, the skin contains unique acylceramides with an additional linoleic acid sub-group, and these are formed within the epidermis [65-67]. Ceramides have been implicated in diseases of epidermal barrier dysfunction, including psoriasis [68], atopic dermatitis [47] and ichthyosis [69], and so are of clinical interest. The analysis of ceramides has progressed from simple, thin-layer chromatography, which provides total ceramide content or analysis of the ceramide classes found in skin lipid extracts, including the complex acylceramides, through qualitative identification of individual species, to accurate identification and quantitation or semi-quantitation of more than 320 ceramide species using multiple-reaction-monitoring by MS/MS [23, 47, 50, 64, 70-74].

Preliminary targeted analysis of epidermal and dermal ceramides performed by assays developed in our group (Supplementary-S1) reveals differences in the profiles of the two skin compartments (Fig. 2D). The most obvious difference is the absence of acylceramides (CER[EOH], CER[EOS] and CER[EOP]) in the dermis, as these are only found in the stratum corneum. Due to the limited availability of standards representing each ceramide class, we performed relative semi-quantitation using a 43-carbon CER[NS] as internal
standard. However, this does not account for differences in ionisation efficiencies in different lipid classes, and as class-specific deuterated standards, including standards representing the very non-polar acylceramides, continue to become commercially available, ceramide quantitation will become more accurate. Importantly, a similar targeted approach can be used to analyse a greater range of bioactive lipid species with signalling roles in skin health and disease, including eicosanoids and endocannabinoids [28, 34, 42]. However, this analysis necessitates more sample as the extraction protocols of low-abundance species, such as the eicosanoids, requires a different extraction protocol optimised for this lipid class [28, 34]. Such low-abundance, short-lived lipid mediators are unlikely to be identified by untargeted analyses or lipid imaging, so targeted mass spectrometry remains the most appropriate platform for their analysis. Commercial availability of synthetic standards and deuterated internal standards permits the use of calibration lines for quantitation, and the resulting assays can reveal disease- or treatment-induced changes in specific lipid pathways [33, 34, 73].

3.2.2. Untargeted mass spectrometry

Untargeted lipidomics can provide a useful and rapid approach to identify global changes in lipid profiles, as well as discover novel lipid species. Although restrictions include limited identifications due to the large number of lipid species being analysed, and reduced sensitivity, hundreds of lipid species can be identified and semi-quantitated in one analytical run [45, 75]. One of the simplest and fastest data acquisition modes for untargeted lipidomics is MS$^E$, which involves detection of molecules within a given mass-to-charge ratio ($m/z$) range applying alternating low and high collision energies, using hybrid mass spectrometers such as Q-ToF that provide accurate mass information to allow the identification of co-eluting lipid analytes. In this approach, fragment ions are formed in an untargeted manner, revealing precursor and product ions that can be matched by retention time, enabling lipid identification. The approach is useful for lipids such as glycerolipids, glycerophospholipids, and sterols. These classes of lipids are complex and contain multiple fatty acids, with different combinations resulting in numerous different species. Some lipid classes have characteristic fragmentation patterns (typically common head groups) which aids identification if different lipid classes co-elute.

Untargeted UHPSFC/ESI-QToF analysis of human skin biopsies performed by our group (Supplementary-S2) reveals a huge array of lipid species, including triacylglycerols, diacylglycerols, cholesteryl esters, phospholipids including phosphatidylcholines and phosphatidylethanolamines, and sphingomyelins. The most abundant species were identified and semi-quantitated using class-specific deuterated internal standards, and normalised against tissue protein content. Totals for each lipid class in this preliminary study are shown in Fig. 2C, revealing differences in the lipid profiles between the dermis and epidermis. This approach can identify global lipid changes in skin diseases, and may be particularly useful in biomarker discovery.

4. MASS SPECTROMETRY LIPID IMAGING

The mass spectrometry approaches described above rely on the extraction of lipids from tissue prior to analysis. However, this means that detailed anatomical information about the
location of lipids or lipid changes within skin is lost upon homogenisation of the tissue. Another option for skin lipid analysis is mass spectrometry imaging (MSI). This allows the direct sampling of lipid ions from the skin tissue, without prior extraction, so that lipids can be analysed in situ [76]. While direct ionisation techniques such as MALDI and DESI can be used for homogenised samples, they have also proved useful in providing spatial distribution information through skin imaging analysis [26, 77-80]. A full-thickness skin biopsy can be sectioned onto a glass slide, and by placing the sample on a moving stage, the mass spectrometer samples ions as it performs spot-to-spot analysis, or rasters, across the skin tissue, allowing reconstruction of an image of the full-thickness skin representing ion intensities [81]. For each pixel with a given x, y coordinate, a mass spectrum is recorded with information on m/z and ion abundance. By selecting an ion of interest and extracting the data for each pixel, a chemical map of the tissue can be built up, showing the distribution and abundance of that lipid molecular ion across the sample [82, 83]. This provides information on the different skin compartments in one image, revealing anatomical lipid distribution, as well as allowing semi-quantitative comparison of lipid intensities between samples, and the MSI data can be co-registered with the same or a consecutive section that has undergone histochemical staining [26, 77]. The size of the pixels and the ions sampled depends on the ionisation approach, and MALDI and DESI offer different advantages.

4.1. MALDI

MALDI-MSI involves coating the tissue with a matrix that promotes ionisation of the lipid species [84, 85], and has been used to study lipid changes in skin and skin equivalents [26, 77, 78]. MALDI-MSI can provide very good spatial resolution depending on the laser spot size on the surface, with small pixel sizes of 15 µm obtainable using commercial systems [86], but analysis requires special consideration of sample preparation, including matrix application, which depends on factors such as the target ions (recently reviewed in [87]). An important consideration of matrix selection is the spectrum of matrix-derived ions and whether these interfere with the compounds of interest. Examples of matrices that are used for lipid analysis include α-cyano-4-hydroxy-cinnamic acid (CHCA) and 9-aminoacridine (9AA) [88]. CHCA typically produces ions below m/z 500 [89], so these do not interfere with most lipid species of interest in skin, including phospholipids and acylglycerols [85]. 9AA is commonly used in negative mode, and produces low m/z ions (majority below m/z 300) [90], which would not interfere with lipids typically identified in negative mode, including phosphatidylglycerols and phosphatidic acids.

4.2. DESI

DESI allows ionisation of lipids directly from skin tissue under ambient conditions by directing an electrically-charged fine spray of an appropriate solvent at the tissue sample, and sampling the ionised lipids directly into the mass spectrometer [91]. Unlike MALDI-MSI, no matrix is required, so skin tissue simply requires sectioning prior to analysis. DESI-MSI has proven capabilities in imaging of lipids across tissues [92-94], but until recently has lacked the spatial resolution to analyse intricate tissues such as skin, with typical pixel sizes around 100 µm [82, 83, 86].

4.3. Skin biopsy analysis
Untargeted lipid imaging analysis by MALDI-MSI (Supplementary-S3) and DESI-MSI (Supplementary-S4) performed by our group provides examples of the images that can be produced. MALDI-MSI was performed at a pixel size of 15 µm, and the image generated of an example ion shows varied distribution across the tissue (the m/z 739.5 ion in positive mode (Fig. 2A)), with increased intensity in the epidermis. DESI-MSI analysis was performed at 40 µm pixel size (Fig. 2B), allowing analysis of the whole skin sections in the same time-frame, although with less spatial resolution than MALDI-MSI. The image generated shows the distribution and intensity of a different ion (m/z 789.5 in positive mode) in three skin samples, again with increased abundance in the epidermis. Due to differences in ionisation and adduct formation in the two techniques, different lipid ion profiles are often identified, making direct comparison difficult. However, the two approaches may provide complementary information on skin biology. These examples were chosen from untargeted analysis because of clear differences across the tissue in this preliminary study, but targeted approaches examining specific lipids of interest can also be performed using both MALDI-MSI and DESI-MSI.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Lipidomics analysis of skin can provide a vast amount of information from limited tissue samples, including bioactive lipid mediators, structural lipids, and lipid imaging. Lipid imaging and untargeted analysis may not be able to detect low abundance short-lived mediators such as eicosanoids, which are easily detectable by UPLC/ESI-MS/MS, whilst targeted analysis may miss important changes in unknown lipids. To obtain a full appreciation of the skin’s lipid profile, it may be necessary to combine several approaches. Indeed, DESI-MSI and MALDI-MSI studies could be targeted to analyse a selection of lipids identified through UPLC/ESI-MS/MS or UHPSFC/ESI-QToF, to investigate whether their distribution throughout the skin changes as well as their abundance, whilst lipid ions identified through lipid imaging could be followed up with more targeted analysis for improved sensitivity. Lipid analysis can reveal diverse information about cutaneous lipid biology, from mechanistic insight into inflammatory skin conditions such as sunburn [61, 62] and irritant dermatitis [34], to the effects of topical treatments and nutritional supplementation on the skin lipidome [33, 95-99], revealing biomarkers, unravelling complex signalling pathways, and identifying potential therapeutic targets. As mass spectrometry technology continues to improve, resulting in increased mass accuracy, sensitivity, spatial resolution and more, we expect skin lipidomics to become a routine consideration in the investigation of cutaneous homeostasis and inflammation.
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Author contributions

AN, MM and CEMG were responsible for the study design. MMK coordinated volunteer recruitment and biopsy sampling and performed lipid analysis. ACK, EAJ, PJH and MT performed lipid imaging analysis. AK analysed lipid data and drafted the manuscript. All authors performed critical revision of the manuscript.

Conflicts of interest

EAJ, PJH, MT and MM are employees of Waters Corporation.
Figure legends

Figure 1. Schematic representation of skin lipidomics workflow. Several mass spectrometry-based lipidomics approaches are available for the investigation of the skin lipidome. A combination of these platforms can reveal the most information about skin lipids. 3Q, triple-quadrupole; IS, internal standards; RPC, reverse-phase chromatography; UHPSFC, supercritical fluid chromatography; ESI, electrospray ionisation; MALDI, matrix-assisted laser desorption ionisation; DESI, desorption electrospray ionisation.

Figure 2. Lipidomic analysis of skin by multiple approaches. 6 mm skin half-biopsies (n=3) were embedded in carboxymethylcellulose and sectioned onto glass slides (10 µm thickness sections) for MALDI-MSI (A) and DESI-MSI (B). Remaining unsectioned tissue was washed, divided into dermis and epidermis, and analysed by UHPSFC/ESI-MS/MS for structural lipids (C) and UPLC/ESI-MS/MS for ceramides (D). A) Matrix assisted laser desorption ionisation mass spectrometry imaging (MALDI-MSI) of skin lipids. Skin sections were analysed by MALDI-MSI at 15 µm pixel size. Representative image shows the ion m/z 739.5 in positive mode, reconstructed by extraction of the ion from the mass spectrum at each pixel. B) Desorption electrospray ionisation mass spectrometry imaging (DESI-MSI) of skin lipids. Skin sections were analysed by DESI-MSI at 40 µm pixel size. Representative image shows the ion m/z 789.5 in positive mode, reconstructed by extraction of the ion from the mass spectrum at each pixel. C) Structural lipids in skin samples. Structural lipids of the epidermis and dermis were analysed by UHPSFC/ESI-MS/MS. The most abundant lipids were identified using in-house algorithms and lipid databases, including LIPID MAPS, and semi-quantitated against class-specific internal standards. Lipid data are shown as total µg/mg protein per lipid class, mean ± SD, n=3 (LPC, lysophosphatidylcholines; PE, phosphatidylethanolamines; SM, sphingomyelins; PC, phosphatidylcholines; CHL, cholesterol; CE, cholesteryl esters; DAG, diacylglycerols; TAG, triacylglycerols). D) Ceramide analysis of skin samples. Ceramides of the epidermis and dermis were analysed by UPLC/ESI-MS/MS and multiple-reaction-monitoring, and semi-quantitated using internal standards. Ceramides are presented as total pmol/mg protein per ceramide class, mean ± SD, n=3. Ceramide classes are named according to the combination of fatty acid (N, non-hydroxy; A, alpha-hydroxy; EO, ester-linked omega-hydroxy) and sphingoid base (S, sphingosine; DS, dihydrosphingosine; H, 6-hydroxysphingosine; P, phytosphingosine).

Supplementary files

Supplementary-S1: Ceramide analysis
Supplementary-S2: Structural lipid analysis
Supplementary-S3: MALDI-MSI analysis
Supplementary-S4: DESI-MSI analysis
References

Figure 1.

**Mediator Lipidomics**
- Eicosanoids
- Endocannabinoids
- Ceramides

- Tape strip/blister fluid/biopsy
- Liquid/liquid and solid phase extraction
- RPC to separate lipid analytes
- ESI
- MRM on 3Q-MS (unique fragmentation pattern and retention time)
- Quantitation with IS and calibration lines
- Quantitative differences in lipids/pathways following disease/treatment

**Structural Lipidomics**
- Glycerolipids
- Phospholipids
- Sterols
- Free fatty acids

- Tape strip/blister fluid/biopsy
- Liquid/liquid extraction
- UHPSFC to separate lipid classes
- ESI
- MS² analysis on Q-ToF-MS (precursor and product ion information)
- Database search to identify lipids
- Semi-quantitation with IS
- Qualitative and semi-quantitative differences in lipids/pathways following disease/treatment

**Lipid Imaging**
- Glycerolipids
- Phospholipids
- Sterols
- Free fatty acids

- Biopsy
- Embed sample and section onto glass slides
- Apply matrix
- MALDI
- DESI
- MS analysis on Q-ToF MS
- Database search to identify lipids
- Lipid changes across tissue and semi-quantitative differences between samples

**Skin sample**
Supplementary-S1 – ceramide analysis

Materials

Extraction solvents were obtained from Fisher Scientific (Loughborough, UK). LC/MS-grade chromatography solvents were obtained from Sigma (Gillingham, UK). Ceramide/Sphingoid Internal Standard Mixture I was obtained from Avanti Polar Lipids (Alabaster, USA).

Sample preparation

Skin biopsies (6 mm diameter) were obtained with informed written consent from volunteers at The Salford Royal Hospital with full ethical approval from a local ethics committee and according to the Declaration of Helsinki. Punch biopsies (6 mm) were cut, bisected, snap-frozen in liquid nitrogen, and stored at -80°C prior to analysis. Dermis and epidermis of half-biopsies were separated by scalpel and lipid extraction was performed according to the modified Folch lipid extraction procedure[1]. 3 mL ice-cold 2:1 (v/v) chloroform:methanol was added to the sample with 50 pmol ceramide internal standard cocktail (along with 50 µL deuterated lipid cocktail for glycerolipid/glycerophospholipid analysis, described in Supplementary-S3). Tissue was homogenised using a blade homogeniser (X 10/25 drive with 10 mm diameter shaft, set at a speed of 11 kHz; Ystral, Ballrechten-Dottingen, Germany) on ice [2, 3]. Homogenates were incubated on ice for 90 min to allow solvent lipid extraction. Next, 500 µL of ice-cold, purified water was added to the vial and centrifuged. The organic layer was removed, separated into two equal aliquots (one for glycerolipid/glycerophospholipid analysis) and the ceramide aliquot was dried under nitrogen, before reconstitution in 150 µL methanol with 0.1 % formic acid and storage at -20 °C until analysis. The protein layer (above the organic layer) was kept for analysis of protein content.

UPLC/ESI-MS/MS conditions

Analytes were separated using reverse-phase chromatography on a C8 column (Acquity UPLC BEH, 1.7 µm, 2.1 x 100 mm; Waters, Wilmslow, UK) using a gradient of water (with 0.1% formic acid) and methanol (with 0.1% formic acid) as previously described [4]. Ceramides were identified on a Xevo TQ-S (Waters, Wilmslow, UK) using multiple reaction monitoring in the positive ion mode (Fig S1-3). Since standards are not yet commercially available for each individual ceramide compound, relative quantitation of ceramides was performed using internal standards.

Protein content analysis

The protein content of the skin samples was analysed using Bio-Rad Protein Assay II (Bio-Rad, Hemel Hempstead, UK).
Figure S1. Representative chromatograms of CER[EOH] ceramides in healthy epidermis. Acylceramides from the CER[EOH] family were extracted from healthy epidermis samples and analysed by UPLC/ESI-MS/MS. Individual ceramide species were identified by their unique fragmentation pattern and retention time.
Figure S2. Representative chromatograms of CER[EOP] ceramides in healthy epidermis. Acylceramides from the CER[EOP] family were extracted from healthy epidermis samples and analysed by UPLC/ESI-MS/MS. Individual ceramide species were identified by their unique fragmentation pattern and retention time.
Figure S3. Representative chromatograms of CER[EOS] ceramides in healthy epidermis. Acylceramides from the CER[EOS] family were extracted from healthy epidermis samples and analysed by UPLC/ESI-MS/MS. Individual ceramide species were identified by their unique fragmentation pattern and retention time.

References

Supplementary S2 – structural lipid analysis

Materials

Methanol, ethanol, 2-propanol, chloroform (all HPLC grade), and ammonium acetate were purchased from Sigma Aldrich (Gillingham, UK). Deionised water was prepared with Elga Water Purelab Flex purification system. Liquid CO₂ CP grade (99.995%) was supplied by BOC (Guildford, UK).

Deuterated lipid standards representative of 12 lipid classes (CE, cholesteryl esters; Chl, cholesterol; SM, sphingomyelins; PC, phosphatidylcholines; LPC, lysophosphatidylcholines; PE, phosphatidylethanolamines; TAG, triacylglycerols; DAG, diacylglycerols; PG, phosphatidylglycerols; PA, phosphatidic acids; LPE, lysophosphatidylethanolamines; FFA, free fatty acids) were used for UHPSFC/ESI-MS development, lipid identification and semi-quantitation: 15:0 cholesteryl (d7) ester (CE-d7), cholesterol-d7 (Chl-d7), 16:0-d31 sphingomyelin (SM-d31), 16:0-d31-18:1 phosophatidylcholine (PC-d31), 26:0-d4 lysophosphatidylcholine (LPC-d4), 16:0-d31-phosophatidylethanolamine (PE-d31), 17:0-17:1-17:0-d5 triacylglycerol (TAG-d5), 18:1-d5 diacylglycerol (DAG-d5), 16:0-d31-18:1 phosophatidylglycerol (PG-d31), 16:0-d31-18:1 phosophatic acid (PA-d31), 18:1-d7 lysophosphatidylethanolamine (LPE-d7), and palmitic acid-d3 (FFA-d31).

A deuterated lipid cocktail was prepared using individual deuterated lipid standards at final concentrations comparable to the most abundant species of that class found in skin. Final concentrations in the cocktail were: 100 µg/mL (FFA-d31, CE-d7, PC-d31, PE-d31, TAG-d5, LPC-d4,SM-d31, DAG-d5, LPE-d7); 200 µg/mL (Chl-d7); 300 µg/mL (PG-d31, PA-d31).

Sample preparation

Skin biopsies (6 mm diameter) were obtained with informed written consent from volunteers at The Salford Royal Hospital with full ethical approval from a local ethics committee and according to the Declaration of Helsinki. Punch biopsies (6 mm) were cut, bisected, snap-frozen in liquid nitrogen, and stored at -80°C prior to analysis. Dermis and epidermis of half-biopsies were separated by scalpel and extraction was performed according to the modified Folch lipid extraction procedure [1]. 3 mL ice-cold 2:1 (v/v) chloroform:methanol was added to the sample with 50 µL deuterated lipid cocktail (along with 50 pmol Ceramide/Sphingoid Internal Standard Mixture I, Avanti Polar Lipids, Alabaster, USA for ceramide analysis, described in Supplementary-S1). Tissue was homogenised using a blade homogeniser (X 10/25 drive with 10 mm diameter shaft, set at a speed of 11 kHz; Ystral, Ballrechten-Dottingen, Germany) on ice [2, 3]. Homogenates were incubated on ice for 90 min to allow solvent lipid extraction. Next, 500 µL of ice-cold, purified water was added to the vial and centrifuged. The organic layer was removed, separated into two equal aliquots (one for ceramide analysis) and the glycerolipid/glycerophospholipid aliquot was dried under nitrogen. The lipid extract was reconstituted in 1 mL 1:1 (v/v) chloroform-2-propanol and stored at -20 °C until analysis.

UHPSFC/ESI-MS conditions

Lipid analytes were separated by ultra-high performance supercritical fluid chromatography (UHPSFC) [4] on an Acquity UPC² instrument (Waters, Wilmslow, UK) using an Acquity
UPC² Torus 2PIC column (100mm x 3mm x 1/7µm, Waters, Wilmslow, UK) under the conditions outlined in **Table S1** and using the gradient described in **Table S2**.

**Table S1: UHPSFC conditions**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow rate</strong></td>
<td>1.5 µL/min</td>
</tr>
<tr>
<td><strong>Injection volume (positive mode)</strong></td>
<td>2 µL</td>
</tr>
<tr>
<td><strong>Injection volume (negative mode)</strong></td>
<td>7 µL</td>
</tr>
<tr>
<td><strong>Column temperature</strong></td>
<td>60 ºC</td>
</tr>
<tr>
<td><strong>Active back pressure regulator</strong></td>
<td>1800 psi</td>
</tr>
<tr>
<td><strong>Injector needle wash</strong></td>
<td>100 % methanol</td>
</tr>
<tr>
<td><strong>Mobile phase A</strong></td>
<td>100 % liquid CO₂</td>
</tr>
<tr>
<td><strong>Mobile phase B</strong></td>
<td>99:1 (v/v) methanol:water with 30 mM ammonium acetate</td>
</tr>
<tr>
<td><strong>Make-up solvent</strong></td>
<td>99:1 (v/v) methanol:water with 30 mM ammonium acetate</td>
</tr>
<tr>
<td><strong>Make-up solvent flow-rate</strong></td>
<td>0.25 µL/min</td>
</tr>
</tbody>
</table>

**Table S2: UHPSFC gradient**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>10</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>99</td>
<td>1</td>
</tr>
</tbody>
</table>

The UHPSFC instrument was coupled to a Synapt G2 High Definition Q-ToF Mass Spectrometer (Waters, Milford, MA, USA) with electrospray ionisation. The mass spectrometer was used in high-sensitivity mode according to conditions outlined in **Table S3** in positive mode (CE, Chl, TAG, DAG, PC, LPC, SM, PE, LPE) or negative mode (PG, PA, FA).

**Table S3: Mass spectrometer conditions**

<table>
<thead>
<tr>
<th>Mass range</th>
<th>m/z 50-1100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Capillary voltage</strong></td>
<td>2.5 kV</td>
</tr>
<tr>
<td><strong>Sampling cone</strong></td>
<td>20 V</td>
</tr>
<tr>
<td><strong>Source offset</strong></td>
<td>90 V</td>
</tr>
<tr>
<td><strong>Source temperature</strong></td>
<td>150 ºC</td>
</tr>
<tr>
<td><strong>Drying temperature</strong></td>
<td>500 ºC</td>
</tr>
<tr>
<td><strong>Cone gas flow</strong></td>
<td>0.8 L/min</td>
</tr>
<tr>
<td><strong>Drying gas flow</strong></td>
<td>1.7 L/min</td>
</tr>
<tr>
<td><strong>Nebuliser gas flow</strong></td>
<td>4 bar</td>
</tr>
<tr>
<td><strong>Lock-spray</strong></td>
<td>Leucine enkephalin (m/z 556.6)</td>
</tr>
<tr>
<td><strong>Calibration reference</strong></td>
<td>Sodium formate</td>
</tr>
<tr>
<td><strong>Acquisition mode</strong></td>
<td>MS⁺</td>
</tr>
<tr>
<td><strong>Low collision energy</strong></td>
<td>4 V</td>
</tr>
<tr>
<td><strong>High collision energy</strong></td>
<td>15-30 V ramp</td>
</tr>
</tbody>
</table>
Method Validation

The reproducibility of peak area was determined from 10 consecutive measurements of lipid cocktail. The reproducibility of retention times was calculated as a standard deviation from 10 consecutive injections of the standards.

Lipid identification and nomenclature

Data processing was carried out using a data processing algorithm. Lipid identification of the most abundant species was performed and confirmed through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Representative spectra of different lipid classes are shown in Fig. S1-5, including identification of the most abundant species. The total number of species identified in each lipid class is shown in Table S4.

Table S4. Number of lipid species of each class identified in human dermis and epidermis

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Epidermis</th>
<th>Dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl esters</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>Phosphatidylcholines</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Sphingomyelins</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Phosphatidylethanolamines</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Semi-quantitative analysis

Lipids were semi-quantitated based on mass spectra peak areas and those of the lipid class standards. Following tissue homogenisation and extraction of the lipids from the biopsies, the protein precipitate was collected and the protein content measured using Bio-Rad Protein Assay II (Bio-Rad, Hemel Hempstead, UK). Lipid concentrations were calculated in µg/mg of protein.
Figure S1. Representative spectra of triacylglycerols in epidermis and dermis. Individual triacylglycerol (TAG) species were identified through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Epidermis and dermis show differences in terms of ions present and relative abundances.
Figure S2. Representative spectra of diacylglycerols in epidermis and dermis. Individual diacylglycerol (DAG) species were identified through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Epidermis and dermis show differences in terms of ions present and relative abundances.
Figure S3. Representative spectra of phospholipids in epidermis and dermis. Individual phospholipid species including sphingomyelins (SM) and phosphatidylcholines (PC) were identified through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Epidermis and dermis show differences in terms of ions present and relative abundances.
Figure S4. Representative spectra of cholesteryl esters in epidermis and dermis. Individual cholesteryl ester (CE) species were identified through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Epidermis and dermis show differences in terms of ions present and relative abundances.
Figure S5. Representative spectra of free fatty acids in epidermis and dermis. Individual free fatty acid (FFA) species were identified through fragmentation patterns, using the LIPID MAPS database and in-house information and publications. Epidermis and dermis show differences in terms of ions present and relative abundances.
Supplementary references

Supplementary-S3 – Matrix assisted laser desorption ionisation mass spectrometry imaging (MALDI-MSI)

Materials

All organic solvents, MALDI matrices and associated additives were supplied by Sigma Aldrich (Dorset, UK). All organic solvents were of HPLC grade. Deionised water was prepared with Elga Water Purelab Flex purification system.

Sample preparation

Skin biopsies (6 mm diameter) were obtained with informed written consent from volunteers at The Salford Royal Hospital with full ethical approval from a local ethics committee and according to the Declaration of Helsinki. Punch biopsies (6 mm) were cut, bisected, snap-frozen in liquid nitrogen, and stored at -80°C prior to analysis. Half-biopsies were embedded in 2 % (v/v) carboxymethylcellulose (CMC), sectioned (10 µm thick) onto glass slides, and stored at -80°C prior to analysis. For analyses in positive ionisation mode, ultra-pure α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used as the MALDI matrix. Here, the matrix was dissolved in a solution of 70% ethanol, 30% water, 0.1% TFA, to a final concentration of 5 mg/mL. An equimolar amount of aniline was then added to the solution before a 5 minute sonication step. The aniline is used to limit the adduction of CHCA, reducing the related signals observed in the mass spectra and as such improving the visualisation of lipid related ions [1]. For analyses in negative ionisation mode, 9-aminoacridine (9AA) was used as the MALDI matrix, and was dissolved in a solution of 80% ethanol, 30% water. In every case, matrix solutions were sprayed onto the sectioned skin using a Suncollect™ (SunChrom, Friedrichsdorf, Germany), automated spraying device to give a homogenous coating.

MALDI-MSI conditions

All imaging acquisitions were set up in High Definition Imaging (HDI) informatics, version 1.4. Slides were scanned using a standard flat-bed scanner (Epson). Images were acquired in both positive and negative ionisation modes on a Synapt G2-Si (Q-TOF-MS) (Waters, Wilmslow, UK).

A modified MALDI source using a custom acquisition script to allow higher acquisition rates was utilised. An Nd:YAG solid state laser was used with a firing rate of 1000Hz. Data were acquired at approximately 20 pixels per second in a continuous raster mode. The custom script utilised the Waters Research Enabled Software (WREnS) platform to allow control of the source, bypassing elements of the standard instrument control. The modified MALDI source accommodates a larger plate capacity with positions for up to 3 slides. This allowed for all samples in this experiment to be loaded and acquisitions queued and initiated without further user intervention. Additionally, the laser geometry of this source has been adjusted to allow for a laser focus of between 15-20 µm. Consequently, this allowed for MALDI-MS images to be acquired at a spatial resolution (and final pixel size) of 15 µm.

Supplementary references

Supplementary-S4 – Desorption electrospray ionisation mass spectrometry imaging (DESI-MSI)

Materials

All organic solvents and water used were of HPLC grade and supplied by Sigma Aldrich (Dorset, UK). Any additives used in the investigation of optimum conditions were also supplied by Sigma Aldrich and the solutions prepared fresh on the day of analysis.

Sample preparation and sampling optimisation

As discussed in the main article, the DESI-MSI technique requires no specific preparation of the tissue sections prior to analysis. Fresh frozen sections of the tissue are collected at a sample dependent thickness between 10 and 30 µm (typically 12 µm) and stored at -80°C until required. Prior to analysis the samples are warmed to room temperature, if the samples have a high aqueous content this can be done in a vacuum desiccator, and then placed onto the sampling stage of the DESI source.

Skin biopsies (6 mm diameter) were obtained with informed written consent from volunteers at The Salford Royal Hospital with full ethical approval from a local ethics committee and according to the Declaration of Helsinki. Punch biopsies (6 mm) were cut, bisected, snap-frozen in liquid nitrogen, and stored at -80°C prior to analysis. Half-biopsies were embedded in 2 % (v/v) carboxymethylcellulose (CMC), sectioned (10 µm thick) onto glass slides, and stored at -80°C prior to analysis.

DESI-MSI

The DESI-MSI process involves a focussed spray of electrostatically charged droplets impacting upon a surface. The composition of the solvent used is selected to favour the process of dissolving and ionising the molecular species of interest. For the lipid analysis described here, high methanol content such as 98:2 (v/v) methanol:water was found to be most suitable. Other molecular classes such as peptides may be most successfully analysed with acetonitrile and higher aqueous percentage, the addition of organic acids or bases have also been used to aid in the ionisation process [1].

During the analysis the surface is wetted by the spray, forming a thin liquid film into which species are solubilised and then ejected from the surface as secondary droplets, due to the continued gas flow. These secondary droplets will contain molecules from the surface, many of which will be ionised due to the charge imparted on the primary droplets. An inlet capillary which is attached to the first vacuum region of the mass spectrometer draws the droplets from above the surface, and through evaporation of the solvent will present molecular ions to the mass analyser.

By moving the sample under this spray and recording the co-ordinates, chemical maps of the surface can be reconstructed that can be retrospectively interrogated. Using the time-of-flight mass analyser, all molecules that are collected and ionised by the spray will be detected, there is no need for prior knowledge of the molecules of interest and no labels are
required. The trade-off is that the data sizes are large and typically multivariate analysis techniques are required to draw out the important information.

Conventionally, DESI-MSI is performed by moving the sample horizontally under the spray to collect a linescan, and then the sample is moved down one y-pixel step size, and the next linescan is conducted. As the sampling is a continuous event the spray is always on, the size of the pixels in the x-direction is dependent upon the rate of the mass spectrometer scanning and the velocity of the sampling stage. If the stage is moving at 100 µm/s and the mass spectrometer collects 1 scan per second then each pixel in the image will have an x pixel size of 100 µm. If the stage is moving at 1mm/ s and the mass spectrometer has a scan time of 0.1s then the x-pixel length will still be 100 µm, and experiment will be conducted 10 times quicker, though each pixel will be the accumulation of ten times less ion signal. If the species of interest are abundant then this will not be a problem, however for lower concentration molecules (or those with a lower ionisation efficiency) then longer experiments may present more meaningful results. It is typical to set the distance in between each linescan (the length of the y-pixel) to be the same as the x pixel length, therefore in the examples above the stage would be moved 100 µm on the y axis each time.

Data viewing and analysis

A single MSI experiment can contain tens or hundreds of thousands of pixels, with each one containing a whole mass spectrum, and as such it is not uncommon for one image to contain 10 GB or more of data. In order to make this manageable, the data is processed by combining all spectra and carrying out a peak picking routine. The user chooses how many peaks to keep for visualising, and these peak areas are extracted to form the final format which is a matrix of co-ordinates, m/z values and intensities. For the Waters data this can be viewed with the High Definition Imaging (HDI) 1.4 software. Alternatively, there are a number of software packages that can accept the standard imzML file format, to which all data can be converted. In this manuscript the data was converted to the imzML format and then imported into the SCiLS Lab software (SCiLS, Bremen, Germany). This package offers a set of multivariate analysis tools to compare regions of interest drawn by the user (e.g. epidermis vs dermis) or to perform unsupervised segmentation of the data based on the chemical profile of the pixels. Further tools then allow the significance of differentiating ion species using hypothesis testing and received operating characteristic curves. When multiple samples are to be compared, such data handling approaches are invaluable.

Mass spectrometry analysis

The DESI-MSI experiments were performed on a Xevo-G2-XS Q-ToF instrument (Waters, Wilmslow, UK) with a Prosolia (Indianapolis, US) 2D DESI source. The analyses were conducted in both positive and negative ion mode. The final DESI conditions chosen were 98:2 (v/v) methanol:water solvent flowing at 2µL/min with a nebulising gas of 6 bar N₂. The inlet capillary to sprayer distance was 6mm, 75° sprayer to surface angle, 5° inlet capillary collection angle and a sprayer to surface distance 1mm. In order to maximise ion intensity yields a heated inlet capillary was constructed and operated at approximately 450°C powered by a 20V DC power supply. A 2.5mL Hamilton syringe and a Harvard 11 Elite (Harvard Scientific) syringe driver system were used to supply the solvent flow, with operating times of approximately 20 h between refilling.
Experiments were set up by first collecting an optical snapshot of the slide on the stage using a Logitech C920 webcam, and registering this in the HDI software. Regions to be analysed can be drawn on this optical image and used to set up the experiment. Additional parameters also defined at this stage are the polarity to be analysed, the pixel dimensions required and the rate of sampling for the experiment.

References