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REVIEW PAPER

Advances in Analyzing the Breast Cancer Lipidome and Its Relevance to Disease Progression and Treatment

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Abstract

Abnormal lipid metabolism is common in breast cancer with the three main subtypes, hormone receptor (HR) positive, human epidermal growth factor 2 (HER2) positive, and triple negative, showing common and distinct lipid dependencies. A growing body of studies identify altered lipid metabolism as impacting breast cancer cell growth and survival, plasticity, drug resistance, and metastasis. Lipids are a class of nonpolar or polar (amphipathic) biomolecules that can be produced in cells via de novo synthesis or acquired from the microenvironment. The three main functions of cellular lipids are as essential components of membranes, signaling molecules, and nutrient storage. The use of mass spectrometry-based lipidomics to analyze the global cellular lipidome has become more prevalent in breast cancer research. In this review, we discuss current lipidomic methodologies, highlight recent breast cancer lipidomic studies and how these findings connect to disease progression and therapeutic development, and the potential use of lipidomics as a diagnostic tool in breast cancer. A better understanding of the breast cancer lipidome and how it changes during drug resistance and tumor progression will allow informed development of diagnostics and novel targeted therapies.

Keywords Breast cancer · Lipidomics · Lipid · Fatty acid

Introduction

Metabolic reprograming is a long-standing hallmark of cancer that encompasses diverse processes involved in energy production, macromolecule biosynthesis and degradation. Abnormally regulated lipid metabolism is relatively understudied compared to other branches of cancer metabolism despite playing a critical role in tumor cell biology. Lipid metabolism is notably altered in breast cancer versus normal breast cells, and is hypothesized to contribute to tumor cell plasticity, therapeutic resistance, and metastasis [1]. However, studies find considerable variation in both lipid anabolic and catabolic pathways, potentially due to intertumoral heterogeneity and the existence of distinct disease subtypes, and thus there is no clear targetable metabolic signature at present.

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Breast cancer is the most common malignancy among women, comprising 30% of newly diagnosed cancer cases in the US [2]. Breast cancer is generally stratified into three main subtypes based on the presence of estrogen receptor (ER) and progesterone receptor (PR) (or hormone receptor (HR) positive), amplification/overexpression of human epidermal growth factor receptor 2 (HER2+), or lack of all three markers [triple negative breast cancer (TNBC)] [3]. Positivity for HR or HER2 initially stratifies patients into anti-endocrine- or HER2-targeted therapies, respectively [4, 5]. Acquired resistance and recurrence occurs in 10–41% of HR+breast cancers depending on grade/stage [6, 7]. Late-stage HR+tumors are treated with inhibitors to CDK4/6 and PIK3CA [8–11]. Treatment of TNBC primarily relies on chemotherapy, with limited options for PARP inhibitors or immunotherapy [12]. Almost all refractory breast cancers eventually develop resistance to second line drugs [13, 14]. Targeting other processes in breast cancer including metabolism has long been thought to hold therapeutic potential. Discovery of the Warburg Effect, or the preference towards anaerobic metabolism in the presence of oxygen, originally highlighted that tumor cell metabolism could be a targetable vulnerability [13, 14]. Unfortunately, development

of anti-cancer drugs targeting metabolic changes has not been successful in breast cancer to date. Lipid regulatory pathways may be effective therapeutic targets, especially in breast cancers where lipid metabolism plays a central role both normal mammary gland and tumor biology.

Lipid metabolism includes the enzymatic biosynthesis, covalent modification, and degradation of fatty acids (FA) and their lipid derivatives. Prior to the use of mass spectrometry to identify organic molecules, technical limitations restricted the study of cellular lipids [15]. The development of electron spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) techniques 30 years ago improved the ability to detect and quantify lipids within biological samples, and the field of lipidomics emerged [16, 17]. In the past decade, these techniques have been further refined and their use has steadily increased in biological research. At present, there are multiple methods for lipid extraction/separation, mass spectrometry, metabolite annotation, and data analysis/normalization, which provides challenges for data sharing and points to the importance of technical "gold standards" [18, 19]. Lipidomics has the potential to provide previously unavailable information on cellular lipidomes and lipid metabolism, which can be combined with proteomic and genomic data to identify novel therapeutic strategies in breast cancer.

In this review, we cover recent developments in lipid metabolism and the lipidome in the three main subtypes of breast cancer, and their potential meaning to cancer biologists. We include discussion of various lipidomic methods and data analysis present in the breast cancer literature. Lastly, we highlight the role of breast cancer lipid signatures and their contribution to disease detection, progression, and treatment.

Mammalian Lipid Structures and Functions

Lipids are hydrophobic or amphipathic (having both hydrophobic and hydrophilic regions) small molecules that serve essential functions as membrane components, energy storage, and signaling molecules in mammalian cells. Lipids can be broadly classified into eight categories, seven of which are found in mammals: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, sterols, and prenols (Fig. 1) [20]. These categories can also be subdivided into either nonpolar, neutral lipids or polar (amphipathic) lipids. Hydrophobicity is determined by one or more hydrocarbon chains that vary in chain length and degree of saturation. Some lipids contain headgroups and modifications that provide amphipathic characteristics. In mammalian cells, a family of enzymes termed elongases regulate the length of lipid hydrocarbon tails. Chain length typically ranges from 4–22 carbons; however, chains with > 24 carbons are occasionally present in mammals [21]. Hydrocarbon chain saturation, or the number of double bonds, is regulated by saturase and desaturase enzymes and thus characterized as unsaturated, monounsaturated, or polyunsaturated. The essential linoleic and linolenic acids must be acquired from the diet since mammalian cells lack the desaturase enzymes necessary to produce these FA from their 18-carbon precursors [22]. The degree of lipid saturation impacts properties such as interaction with other organic molecules [23]. The LIPID MAPS consortium devised a lipid nomenclature system for researchers to effectively describe the position and degree of lipid saturation [24].

The main lipid species in cell membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA) and cardiolipin (CL). Phospholipids are amphipathic in nature by containing a polar phosphate and glycerol head group and non-polar fatty acyl chains. The extracellular plasma membrane is a lipid bilayer mainly containing structural phospholipid species: PC, PE, PI, PS, and the phospholipid-precursor, phosphatidic acid. PC is the most abundant phospholipid in eukaryotic cell membranes and accounts for about 50% of total cellular phospholipid mass [25]. PC head groups have a cylindrical geometry which provides a planar shape to lipid bilayers. PE is another abundant membrane phospholipid and contributes to membrane curvature with a smaller conical headgroup geometry. The ratio of PC and PE species within the membrane can impose curvature stress onto the membrane, which is used for budding, fission and fusion [26]. PS is almost exclusively found in the inner cytoplasmic leaflet of the plasma membrane and, when flipped to the outer leaflet, is a signal for apoptosis and platelet activation [27, 28].

Sphingomyelin (SM) and sterols also comprise a large component of the membrane. SM belong to the class of sphingolipids and differ from phospholipids by a long-chain nitrogenous base backbone, termed sphingosine. The saturated (or trans-unsaturated) SM tails allow these species to form longer and narrower cylinders than PC of the same chain length. Consequently, SM can assemble tightly together, a phenomenon sometimes referred to as "lipid-packing", resulting in a more rigid membrane state. Neutral sterols such as cholesterol balance SM structural rigidity and maintain membrane fluidity [29]. Sphingolipids, cholesterol, and the degree of phospholipid hydrocarbon saturation affect overall membrane fluidity [23]. It is hypothesized that lipid bilayers do not exist as a homogenous lipid composition, but rather clusters of dense and fluid areas [30]. Denser areas, referred as lipid rafts, are comprised of packable lipids and clusters of membrane-bound proteins [31]. More fluid

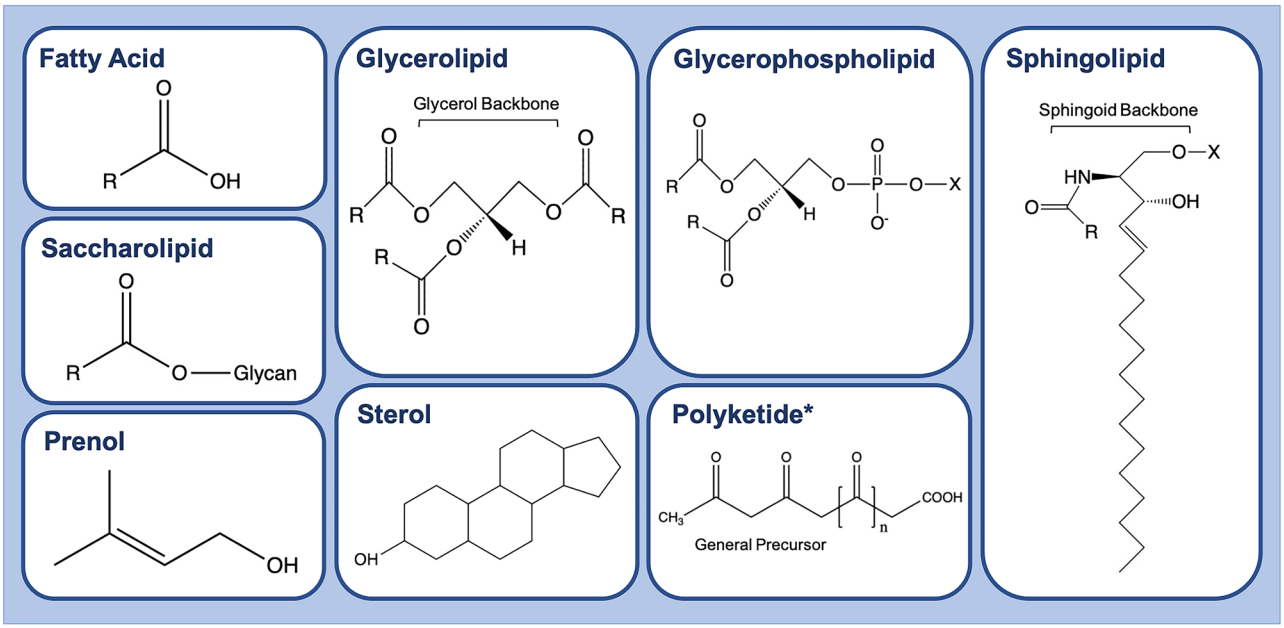

Fig. 1 Lipid Classes. The eight lipid classes: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides. Fatty acids and fatty acyls (activated fatty acids) are the simplest lipid category that serves as building blocks for complex lipids; includes eicosanoids, docosanoids, fatty alcohols, fatty aldehydes, fatty esters, fatty amides, fatty nitriles, fatty ethers, and hydrocarbons. Glycerolipids are neutral lipids containing a glycerol backbone; includes monoacylglycerol (MG), diacylglycerol (DG), and triacylglycerol (TG) species. Glycerophospholipids are membrane lipids that contain a phosphodiester linked to a hydroxyl group of glycerol and are differentiated by the type of moieties (X) esterified to the phosphate. Sphingolipids contain a sphingoid base backbone and vary by polar moieties (X) esterified to the backbone. Sterols contain a common steroid core of a fused four-ring structure with a hydrocarbon side chain and an alcohol group, cholesterol being the most common and functionally important for membrane integrity. Prenol lipids consist of one or more 5 carbon prenol derivatives that can link in chain or ring-like structures. Saccharolipids generally consist of fatty acids directly esterified to a sugar. (R) represents hydrocarbon chain at an arbitrary length. (n) represents repeating carbonyl components

membrane areas contain unsaturated phospholipids as their non-linear acyl tails prevent tight "lipid-packing" interactions [32]. The plasma membrane also contains structures involved in endocytosis such as caveolae, which studies have found are rich in SM [33]. Most intracellular organelles also contain lipid bilayers, including the endoplasmic reticulum, golgi, nucleus, mitochondria, and lysosomes. Their membranes consist of PC, PE, PI, and some cholesterol which result in a dynamic flexible interface. The endoplasmic reticulum is the primary location of phospholipid, glycerolipid, and cholesterol synthesis. The golgi membrane closely resembles the endoplasmic reticulum membrane and contains similar lipid species with increased SM and PS content [34]. In mammalian cells, the golgi is the main producer of complex sphingolipids like SM, glucosylceramide (GlcCer), and lactosylceramide (LacCer), although, Cer from the endoplasmic reticulum is required for golgi-mediated sphingolipid metabolism [35, 36]. Nuclear membranes are also enriched in PC, PE, PI and cholesterol which contribute to flexible membrane dynamics. While there are fewer studies on eukaryotic nuclear and nucleolar membrane compositions, they are thought to be similar to the endoplasmic reticulum [37]. The mitochondria are unique in having two membranes separating spaces with different pH. Unlike the plasma membrane, mitochondrial membranes are composed of about 15% CL and low levels of sphingolipids and cholesterol [38]. CL plays an essential role in regulating mitochondrial transporter function and mitochondria-organelle interactions [39, 40]. Lysosomal lipid membranes are low in cholesterol and high in sphingolipid content [41]. Lysosomes are also involved in lipid trafficking, specifically cholesterol and exogenous triacylglycerol (TG), sterols, and phospholipids from endosomes [42].

The two other essential lipid functions are providing metabolic fuel and acting as signaling molecules. Lipids are stored in droplets in the cytosol, composed mainly of neutral lipid species: TG, diacylglycerol (DG), and cholesterol species. Lipid droplets are formed off the endoplasmic reticulum where newly synthesized TGs can be readily packaged by the perilipin (PLIN) family of 5 proteins [43]. This process is particularly important for alveolar cells in the lactating mammary gland where lipids obtained via de novo synthesis and diet feed into TG synthesis for milk-fat globule production [44]. In times of nutrient deprivation,

cells can initiate hydrolysis of TG and DG from lipid droplets, releasing FAs from glycerol for degradation by fatty acid oxidation (FAO). Not all lipid droplet components are broken down for energy. DG serves as a lipid messenger activating protein kinase C (PKC) and intracellular Ca^{2+} release [45]. DGs also trigger the translocation of protein kinase D which catalyzes the formation of secretory vesicles [46, 47]. Phosphorylation of DG or hydrolysis of phospholipids results in phosphatidic acid, another multifunctional lipid second messenger. Phosphatidic acid has been shown to attenuate hippo pathway signaling through lipid-protein interference [48]. Although PI is a membrane component, its phosphorylation plays a role in PI3K signaling and AKT activation, the most frequently mutated pathway in HR + breast cancer [49]. Additionally, Cer synthesized at high levels triggers a cellular apoptotic program through JNK and p38 signaling [50]. Prostaglandins and other eicosanoids are involved in immune cell signaling and inflammation through a variety G-protein coupled receptors. The functions described here give a brief overview of the fundamental role lipids play in most cellular processes.

Current Analytical Methods for Lipidomics

The collective lipid content in a cell is termed the lipidome. Lipidomics is the study of the lipidome through identification and quantification of lipid analytes within a given sample. The term "lipidomics" emerged in the early 2000s when mass spectrometry (MS)-based methods were optimized for lipid identification studies. In the past decade, lipidomics have been conducted for the study of human diseases such as cancer. Technological advancements in MS now allow different analytic coverage (global/untargeted or targeted lipidomics) and has broadened the scope of lipid research. There are several detailed technical reviews on lipidomics [51–53]; here we give a brief overview of current analytical methods and their application towards breast cancer research.

The general workflow of lipidomic experiments typically involves sample preparation, MS acquisition, and data processing (Fig. 2). Liquid chromatography (LC)-MS is the most common technique which utilizes columns for analyte separation before MS detection and can be used for targeted or untargeted approaches. Direct-infusion techniques such

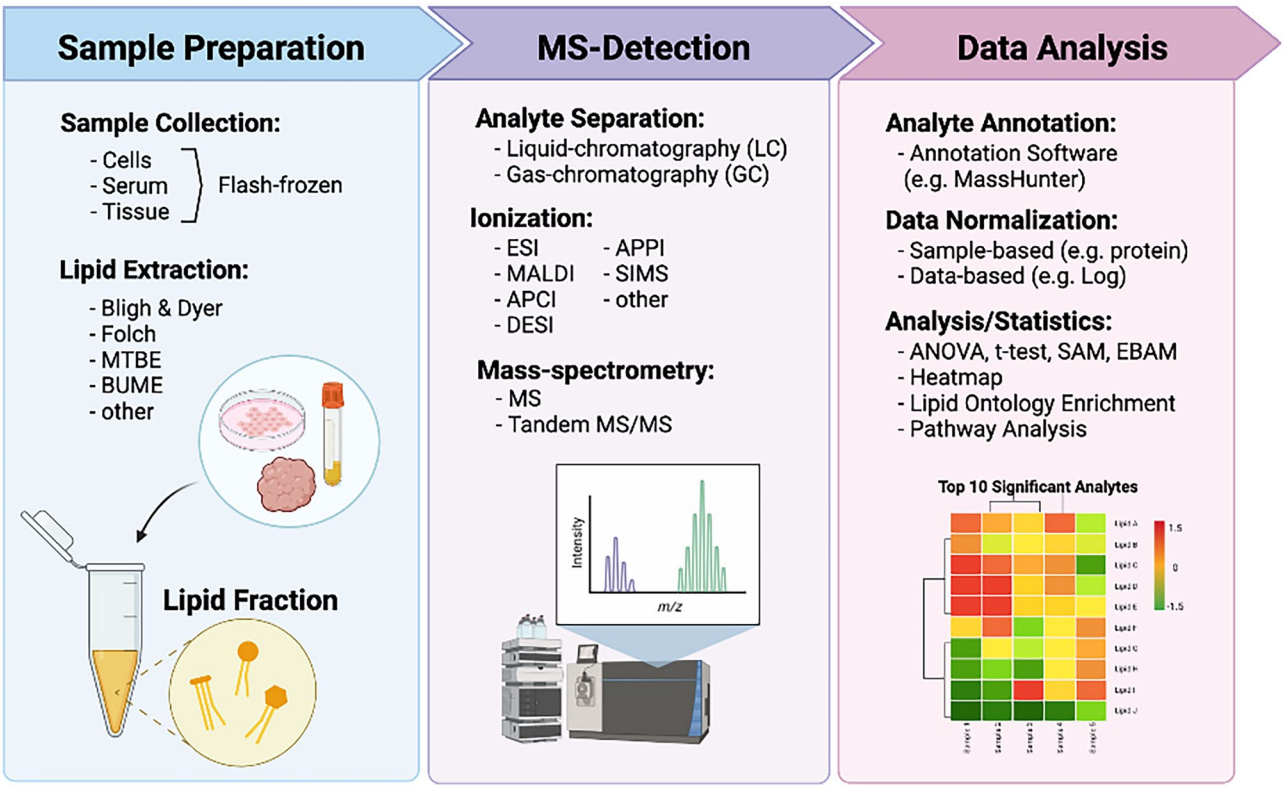

Fig. 2 General Workflow of Lipidomics for Breast Cancer Research. Three main steps to lipidomic analysis include sample preparation, MS-Detection, and Data Analysis. MTBE = methyl tert-butyl ether, BUME = butanol/methanol, ESI = Electrospray ionization, MALDI = Matrix assisted laser desorption ionization, SIMS = Secondary ion mass spectrometry, APCI = Atmospheric Pressure Chemical Ionization, APPI = atmospheric pressure polarization ionization, DESI = Desorption electrospray ionization, MS = Mass spectrometry, ANOVA = Analysis of variance, SAM = Significance Analysis of Microarrays, EBAM = Empirical Bayes Analysis of Microarrays

as shot-gun lipidomics take advantage of the chemical properties of lipids for lipid identification and allow for direct use of samples with minimal preparation. Most lipidomic techniques utilize extracts prepared from biological samples (i.e. cell lines, tissues); however, MS imaging can analyze whole tissue slices (e.g. MALDI MS-imaging). Sample extraction isolates the lipid fraction of a biological sample for MS analysis. A few common extraction methods include the Modified Bligh and Dyer, Modified Folch, Methyl tertiary-butyl ether (MTBE), and butanol-methanol (BUME) methods, each varying in solvent and solvent ratios. Unfortunately, there is not a single extraction method that captures all lipid species with high recovery percentage, yet each of the following liquid–liquid extraction methods have their advantages and disadvantages. Modified Bligh and Dyer method uses chloroform/methanol/H_2O (1:1:0.9, v/v/v) for extraction of small biological samples (< 50 mg of tissue) and traps lipids in the chloroform phase [54]. The Modified Folch method is similar to Bligh and Dyer and uses chloroform/methanol (2:1, v/v) for biological tissue extraction (~ 100 mg), then water or 0.9% NaCl (0.2 volume) is added to wash extracts [55]. This method was designed to improve capture from lipid-rich samples which may otherwise be excluded using the Bligh and Dyer method. The MTBE method uses MTBE/methanol/water (5:1.5:1.45, v/v/v), trapping total lipids in the top MTBE fraction [56]. The benefit of this method is that uses fewer toxic solvents and is more feasible for high throughput or automated set ups. The BUME method uses a volume of butanol/methanol (3:1, v/v) and a small aqueous phase volume. An equal volume of heptane/ethyl acetate (3:1, v/v) is then added followed by an equal volume 1% acetic acid [57]. This method is proposed to reduce water-soluble contaminants that may be found in the previously described methods. The methods described each use organic systems for "wide net" lipid analyte capture and are used for both targeted and untargeted analyses [58]. The choice of extraction method for targeted analyses is dependent on the subset of lipids in question. Neutral lipid species with higher hydrophobicity are best captured by methods with nonpolar solvents such as cyclohexane or toluene. Intermediate polar lipids such as sphingolipids or phospholipids are best extracted with polar solvents like chloroform or MTBE [58]. It is important to note that MALDI lipidomics require little to no sample preparation and was recently discussed in a detailed technical review [59]. After extraction, additional steps may be necessary depending on the type of MS being conducted. For direct-infusion based approaches, it is important to simplify sample extracts since no chromatography separation is applied prior to MS analysis [52]. This can be achieved through physical (phase separation) or chemical approaches (base hydrolysis) to enrich low abundance lipids [60, 61]. Lipid derivation is another option that chemically tags specific functional groups on lipids and can aid in MS identification [61]. The benefit of these steps is that they increase detection of target analytes (i.e. sphingolipids) in low abundance samples without the use of columns for analyte separation for direct-infusion approaches such as shot-gun lipidomics.

Ionization is the next step following lipid extract preparation. The type of ionization depends on whether direct infusion MS (shotgun lipidomics) or chromatography-based MS (LC-based lipidomics) is being performed. It is important to note that MS imaging requires ionization as well. The two most popular ionization techniques are ESI and MALDI. ESI is a soft ionization technique that uses an electrospray produced from a strong electric field applied to a liquid passing through a capillary. This results in a fine aerosol from which ions are formed by desolvation [16]. MALDI is also a soft ionization technique but allows analysis of larger and labile molecules like peptides, proteins, and lipids. This technique is useful for MS imaging of tissue and establishes a matrix for analytes that absorbs energy at the wavelength of the laser. As the pulsed laser hits analytes, this triggers ablation and desorption from the matrix which facilitates analyte ionization [17]. Other popular ionization techniques include Atmospheric Pressure Chemical Ionization (APCI), Atmospheric Pressure Polarization (APPI), Secondary Ion Mass Spectrometry (SIMS), and Desorption ESI (DESI) which have been reviewed in detail [62]. Ion mobility is an optional step that furthers ion separation according to their charge shape and size [63]. Following ionization and ion mobility, full MS or tandem mass spectrometry (MS/MS) is performed depending on whether global or targeted analysis is desired. After MS analysis, the data is represented as MS spectra, MS/MS spectra, ion chromatogram, or images (MS-imaging).

Following data acquisition, spectral MS data undergoes deisotoping to remove spectral complications from the presence of isotopic clusters. This allows for easier mass identification and analyte annotation by lipidomics software. Lipidomic software match molecular masses to lipid identifiers specific to comprehensive databases such as LIPID MAPS, SWISS LIPIDS, Chemical Entities of Biological Interest (ChEBI), KEGG compound database, or human metabolome database (HMBD) [24, 64, 65]. Once qualitative and quantitative data are acquired, the results are further processed for bioinformatic analysis. There are many free online analysis tools available to apply statistical calculations and most accept raw spectra (mzML, mzXML or mzData) or MS peak intensities (e.g. Metaboanalyst) [66]. A key aspect to consider in data analysis is method of normalization. Currently, there lacks a "gold standard" method for lipidomic data normalization; however, there are several accepted methods in the literature (discussed in [19]). Data normalization can include both sample-based (e.g. sample protein,) and data-based (e.g.

Log transformation) methods. Both are easily applicable to spectral data on the mentioned online analysis platforms; however, it is important to clearly document which methods are applied and make raw data publicly available at publication. Down-stream analysis of lipidomics data include pathway or enrichment analyses. These analyses are currently better suited for genetic data; however, new online tools tailored to lipids, such as Lipid Ontology Enrichment Analysis (LION) or Lipid Pathway Enrichment Analysis (LIPEA), have been recently developed [67, 68]. With the lipidomic analysis tools available today, we can conduct statistical comparisons between samples of normal and diseased states, calculate disease specific pathway enrichment, and assess global impacts of lipid metabolic networks as a consequence of disease. There is a growing application of lipidomics to biological research.

Breast Cancer Lipid Signatures in Cell Lines and Tumors

Advances in lipidomics technologies has led to an increase in the number of lipid studies focusing on breast cancer within the past 5 years. In 2020, a PubMed search query for "lipidomics" returned 1,560 articles, and adding "breast cancer" reduced the list to 28 publications. Here we will highlight several recent research studies utilizing breast cancer research lipidomics (2012–2021) that we found insightful to the scope of this review. These fall into two general categories: studies conducted in breast cancer cell lines versus those conducted in clinical breast tumor specimens. A summary of results from the studies discussed in this section can be found in Table 1.

Table 1 Significant Identified Lipids in Breast Cancer. Increased and decreased lipid species compared to MCF10A cells, normal adjacent tissue, or healthy patient serum for breast cancer cell lines, tumor tissue, breast cancer patient serum, respectively

HR + Breast Cancer		
T47D Cell Line	Up	PE(32:2), PE(36:5), PE(38:0), DG(32:0), DG(34:0), LPC(16:0), LPC(18:0)
	Down	NR
MCF7 Cell Line	Up	TG(46:1), TG(46:2), TG(48:2), TG(50:2), TG(50:3), TG(52:2), TG(52.3), TG(54:3), PC(28:0), PC(28:1), PC(30:1), PC(40:2),PE(32:2), PE(36:5), SM(44:1), MUFAs
	Down	NR
CAMA-1 Cell Line	Up	PC(28:0), PC(28:1), PC(30:1), PE(P34:1/O-34:2), PE(P34:1/O-34:3), PE(P32:1), PE(36:3), PE(38:0), SM(44:1), SM(44:2), DG(32:0), DG(34:0)
	Down	SM(32:1)
Tumors		NR
Serum		NR
HER2 + Breast Cancer		
SK-BR-3 Cell Line	Up	TG(40:0), TG(40:1), TG(42:0), TG(42:1), TG(44:0), TG(46:1), TG(46:2), PC(28:0), PC(28:1), PC(30:1), PE(P-32:1), PE(32:2), PE(36:5)
	Down	PE(P-36:4), PE(O-34:2), PE(P-38:4), PE(O-38:5)
Tumors	Up	PC(16:1), PC(32:2)
	Down	NR
Serum		NR
Triple Negative Breast Cancer		
MDA-MB-231 Cell Line	Up	PC(34:0), PC(O-34:0), DG(32:0), DG(34:0), LPC(18:0), PUFAs
	Down	PE(P-34:1/O-34:2), SM(32:1)
MDA-MB-436 Cell Line	Up	PC(34:0), PC(O-34:0), SM(34:2), DG(32:0), DG(34:0), LPC(16:0), LPC(18:0)
	Down	PE(P-34:1/O-34:2)
Tumors	Up	PC(32:1), PC(30:0), PC(32:0), PE(36:1)
	Down	NR
Serum	Up	PC(32:1), Cer(43:1), stearic acid
	Down	NR
General Breast Cancer versus Normal		
Tumors	Up	PC(34:1), PC(32:0), PC(34:1), SM(d18:1/16:0), PE(P-16:0/22:6), PS(38:3), Free FAs
	Down	NR
Serum	Up	PC(32:1), Total TGs
	Down	NR
Recurrent versus Non-recurrent Breast Cancer		
Tumors	Up	PC(32:1), PC(30:0)
	Down	NR

NR None reported

Established cell lines models provide the most feasible models for querying lipid profiles under baseline and manipulated states. Three studies compared lipid profiles of breast cancer subtypes. Eiriksson et al. was the first study to conduct untargeted LC–MS lipidomics on a panel of seven widely used cell lines: non-tumorigenic MCF10A cells, and ER + (MCF7, T47D, CAMA-1), TNBC (MDA-MB-231, MDA-MB-436), and HER2 + (SK-BR-3) breast cancer cells [69]. Based on their analysis, they concluded that breast cancer cells (with one exception) have lipid profiles distinct from MCF10A cells, and that each breast cancer subtype had a distinct lipidome. SK-BR-3 cells displayed the most distinct lipid profile with a relative abundance of TGs (less than C46) versus MCF10A and the other breast cancer cell lines. This could potentially be due to the high rate of de novo FA synthesis reported in HER2 + breast cancers [70]. ER + MCF7 and T47D cells contained similar lipid signatures with a notable increase of PE(32:2) and PE(36:5) compared to MCF10A cells while CAMA-1 cells showed minimal difference. MDA-MB-231 and MDA-MB-436 TNBC cell lines exhibited an abundance of medium chain PC species (C < 40) and saturated DG(32:0) and DG(34:0) species versus MCF10A cells. Therefore, TG and PC abundance may serve as a key lipid profile indicator of breast cancer subtype based on elevated de novo FA and PC synthesis observed in HER2 + and TNBC, respectively. It is important to note these studies were conducted in the absence of steroid hormone treatments in ER + breast cancer cell lines. Future studies in the presence of hormones (i.e. estrogen and progesterone), and endocrine therapies (i.e. tamoxifen) will be important for deciphering the lipidome in ER + breast cancer cells.

The process of epithelial-mesenchymal transition (EMT) is associated with metabolic changes yet studies on lipid metabolism in EMT are lacking. To investigate this, Giudetti et al. measured proteomic profiles via LC–MS and lipid profiles using GC/MS and NMR in MCF7 and MDA-MB-231 breast cancer cells as models of epithelial-like and mesenchymal-like cells, respectively [71]. By proteomic analysis, MDA-MD-231 cells had reduced expression of lipogenic enzymes compared to MFC7 cells (i.e. FASN, ACC, ACLY). Lipidomic analysis found that MDA-MB-231 cells exhibited increased levels of PUFAs, most notably PI(38:4), and cholesterol compared to MCF7 cells. Conversely, MCF7 cells displayed a higher percentage of monounsaturated fatty acids (MUFAs) which the authors suggested could be due to their observation that stearoyl-CoA desaturase (SCD) is overexpressed in MCF7 versus MDA-MB-231 cells. SCD has also been found to be elevated in ER + versus TNBC clinical samples [72]. While this study identified potentially interesting changes in lipid use during EMT, a limitation is the inclusion of a single cell line of each type. These observations merit extension to additional breast cancer cell lines of various subtypes, as well as cells that have been induced to undergo EMT which would identify EMT-induced changes in the same genetic background.

One study investigated lipid changes associated with metastatic potential. Nishida-Aoki et al. compared lipidomic profiles between parental MDA-MB-231 cells and two syngeneic sublines selected for high (D3H2LN) vs low (D3H1) lymph node-metastatic potential [73]. Lipidomics were conducted on the three cell lines in addition to their secreted extracellular vesicles. D3H2LN vs parental and D3H1 cells had increased abundance of LPE, SM, PA, and hexosylceramide (HexCer) in their lipidome, and it was hypothesized the relative percent changes in these lipid species may be associated with metastatic potential. Both D3H2LN cells and EVs had significantly increased saturated DG(14:0/22:0) vs parental and D3H1 derived EVs, which could potentially activate PKD/PKC signaling in surrounding endothelial cells to promote angiogenesis. However the authors found no significant difference in the ability of EVs from either subline to activate PKC and thus the implication of saturated DG(14:0/22:0) is still undetermined [74].

The extracellular microenvironment plays a significant role in influencing tumor cell metabolism. Several studies measured the impact of specific environmental stressors on the cellular lipid landscape. Enhanced glycolysis is a common feature of cancer cells and results in an acidic tumor microenvironment from the production of lactate [75]. Urbanelli et al. determined the effect of microenvironment acidification (pH 6.5) on the lipid profiles of several cancer cell lines including MCF7 [76]. Under acidic vs baseline pH conditions, MCF7 cells decreased PC chain saturation and increased elongase and desaturase enzyme expression. These data insinuate a protective effect of longer, unsaturated phospholipid remodeling against acid pH that requires further study. Nutrient deprivation can also occur in specific tumor microenvironments. For example, methionine (Met) is an essential amino acid required for cancer cells to grow under in vitro conditions and is also important in lipid biosynthesis due to the requirement for S-adenyl-methionine [77]. Borrego et al. assessed the impact of Met stress (deprivation) on the cellular lipid composition of TNBC MDA-MB-468 cells and a Met stress insensitive derivative, MDA-MB-468res-R8, under control and Met-stress conditions [78]. There was a rapid and extensive decrease in lipid abundance, except for unsaturated TGs, in Met-dependent MDA-MB-468 cells that was not observed in Met-res cells, and there was an associated increase in cytoplasmic lipid droplets reflecting an overall increase in neutral storage lipids. Replacement of Met with its metabolic precursor, homocysteine, in cell culture media decreased total lipids and increased TGs in MDA-MB-468 sensitive compared to Met-stress resistant cells. The authors attributed these changes to stress-induced lipid oxidation and the unfolded protein response. Changes in gene expression were also observed, although they were delayed relative to

the changed lipid profile and were better correlated with the unfolded protein response. Additional studies will need to determine the mechanisms by which cancer cells overcome Met dependence and its role in lipid metabolism. Numerous other microenvironmental stressors likely impact the breast cancer cell lipidome including both glucose and L-glutamine which serve as critical carbon sources in many tumor cells. The stress impacted lipidome could be a potential tumor cell vulnerability.

It is well-established that cell culture dimensionality affects cell phenotype [79, 80]. To address this issue, Vidavsky et al. compared the lipid profiles of 2D versus 3D spheroid cultures of a series of MCF10A cells. These included parental MCF10A to mimic "nonmalignant" cells, MCF10DCIS.com to mimic "pre-malignant" cells and MCF10CA1 (HRAS transformed, invasive in vivo) to mimic malignant or "invasive" cells [81]. Storage of lipids upon both conditions was examined using Oil Red O staining. In 2D culture, parental MCF10A cells were void of lipid droplets while MCF10DCIS.com and MCF10CA1 had abundant lipid storage. In 3D culture, parental MCF10A cells showed occasional lipid droplets. Interestingly, the 3D pre-malignant and invasive spheroids exhibited larger lipid droplets (vs 2D) that were concentrated near the spheroid center. To assess global lipid profiles, LC–MS was run on each of the three cell lines in 2D vs 3D cultures. Total phospholipid content differed between 2D vs 3D cells, where 2D cells displayed a higher percentage of PC and PE lipid species. Invasive 3D spheroids also showed increased SM, DG, and acylglycerols compared to pre-cancerous spheroids. Notably, the lysophosphatidylcholines (LPC) pool in MCF10CA1a (invasive cancer) spheroids had shorter chain lengths compared to MCF10DCIS (pre-cancerous) spheroids. These data suggest that growth in 3D profoundly affects lipid production and distribution. We speculate the reduction in total lipid content in 3D spheroids could be indicative of reduced lipid synthesis in a 3D state. Furthermore, the distribution of lipid droplets near the spheroid center could reflect an adaption to meet the energy demands of the surrounding cells through lipid transfer. Tumor centers are typically hypoxic and necrotic. Lipid droplets may serve as a central energy storage since access to nutrients is not evenly distributed in 3D as it is in 2D cell culture. Increased SM levels in invasive 3D spheroids could result from upregulated de novo sphingolipid synthesis. While this has been reported in multiple cancers, it remains unclear whether upregulated sphingolipid biosynthesis is connected to invasiveness or cell survival pathways and requires more study [82]. This study clearly indicates the importance of including 3D culture conditions in the experimental design for future lipid studies in breast cancer. It would also be interesting to determine the effect on including adipocytes in 3D cultures to determine whether the observed changes are intrinsic to the tumor cells, or reflect their growth in 3D in the absence of adipocytes that could serve as a source of lipids for tumor cells.

Breast cancer cell line models that allow for comparison of factors in tightly controlled systems have given a solid indication of how important lipid metabolism is to the disease. However, they must be ultimately confirmed in patient samples. To conduct lipidomics on human tissues, samples are typically flash-frozen at time of tumor-resection or biopsy before sample preparation. Two studies focused specifically on TNBC tumors. Purwaha et al. conducted LC–MS on 70 TNBC tumors looking to identify biomarkers associated with clinical outcome and identify potential therapeutic targets [83]. They found elevated SM and sphingoid bases were correlated with better patient disease-free survival. This is in contrast to the studies in TNBC and MCF10A cells that associated elevated SM with more invasive and metastatic properties and highlights the need for additional in vivo and patient studies to resolve the different observations. above. Notably, Cer levels had no correlation with disease free survival in this study. Hosokawa et al. investigated TNBC patient tumors to define lipid markers correlated with tumor recurrence [84]. Using MALDI-MS on a small set of recurrent (n = 3) and non-recurrent (n = 6) TNBC tumors, PC(32:1) and PC(30:0) were identified as significantly increased in the recurrent tumors. PC(30:0) was identified by two additional studies as associated with TNBC or Grade 3/ER − tumors [85, 86]. Thus, there is some potential for specific lipid species to serve as predictive markers.

Lipid signatures of clinical breast cancer specimens has also established potential subtype differences. Hilvo et al. conducted lipidomics on 267 patient tumors, the largest study of this kind thus far [86]. They found PC(14:0/16:0) and PE (18:0/18:1) lipid species were correlated with ER − tumors and PC(16:0/16:0) was associated with decreased patient survival in confirmation of the observations from Hosowaka et al. [84]. A smaller study of 34 tumors reported PC(32:1) and PC(30:0) were increased in TNBC tumors compared to normal adjacent tissue [85]. PC(18:1/16:0) was also consistently present in all breast tumors compared to normal adjacent tissue suggesting PC(34:1) may serve as a general breast cancer biomarker [85]. Notably, HER2 + tumors exhibited elevated levels of short chain PC(16:1) lipids. Collectively, lipidomic analysis on breast cancer cell lines and patient tumors has identified a subset of potential prognostic or predictive lipid markers (Table 1). However, the biological significance of these lipid species and their validation as reliable prognostic markers will require extensive additional study. Additionally, the lipid contribution of stromal or adipose cells were not discussed in many of these publications. This warrants discussion as these cells could play potential roles in lipid trafficking or signaling crosstalk with tumor cells.

Major Players of Lipid Metabolism and their Regulation in Breast Cancer

Deregulated energy metabolism is a hallmark of cancer and is often associated with aberrant glucose metabolism, or the Warburg effect, and glutamine metabolism [13, 87]. An emerging hallmark of cancer metabolism as described by Pavlova et al. is "the use of glycolysis and tricarboxylic acid (TCA) cycle intermediates for biosynthesis", a major component of which is lipid metabolism [87]. FAs are critical components of cell membranes, energy homeostasis, and signaling. The regulation of these processes is only partially understood in breast cancer. Normal breast tissue undergoes extensive metabolic rewiring, largely resulting from transcriptional changes, to prepare for milk production during lactation [reviewed in [44, 88]]. Therefore, breast cancers originate from cells that have the machinery to undergo dynamic lipid remodeling. Whether these processes are retained during tumorigenesis is unclear. However, breast cancer cells show an exceptional ability to utilize anabolic and catabolic lipid metabolism to fulfill survival and proliferative needs. Here we discuss current knowledge on how lipid metabolism is regulated in breast cancer.

Breast cancer cells can obtain lipids through uptake from their microenvironment or through de novo synthesis, therefore the expression of genes involved in lipid transport or fatty acid biosynthesis repress two extreme phenotypes for tumor cells that require lipids, and the ability to toggle between these two states may be critical for metabolic flexibility and tumor survival. Exogenous FA uptake is mediated through specialized transporters that facilitate FA movement across the plasma membrane. FA translocase (FAT/CD36) and six FA transport proteins (FATP1-6/SLC271-6) are the best characterized molecules that mediate uptake and are over expressed in many cancers [89]. High CD36 expression is associated with poor prognosis in breast cancer and reported to enhance therapy resistance in each of the three main breast cancer subtypes [90–92]. Additionally, breast cancer cells can uptake FAs through secondary mechanisms such as endocytosis [93]. Cancer associated fibroblasts are the most abundant cell type in the tumor stroma and have been shown to transfer FAs to breast cancer cells through the extracellular matrix, lipid droplets, and microvessicles [94, 95]. Adipocytes, especially abundant in the breast stroma, can also supply FAs to breast cancer cells [96].

FA de novo synthesis is the anabolic process of building intracellular FAs. Most metabolic processes depend on central pools of acetyl-CoA, a fundamental metabolite building block (Fig. 3). Acetyl-CoA is derived from citrate or acetate – which are either imported or broken down from larger carbohydrates. Acetyl-CoA and malonyl-CoA are the necessary substrates for de novo FA synthesis. Acetyl-CoA is converted to Malonyl-CoA by acetyl-CoA carboxylase (ACC), a rate limiting enzyme in de novo FA synthesis. FA synthase (FASN) is the master enzyme that assembles acetyl-CoA and malonyl-CoA into palmitate (C16), or other FAs. FASN is overexpressed in breast cancer compared to normal, nonlactating tissue, with the highest expression in HER2 + followed by HR + and TNBC [97]. Increased FASN activity has been linked with increased Pentose Phosphate Pathway (PPP) activity in non-Hodgkin lymphoma [98]. The PPP generates NADPH and 5-carbon sugars needed for nucleotide synthesis. FASN consumption of NADPH relieves feedback inhibition of Phosphogluconate Dehydrogenase (PGDH) and resupplies NADP + for PDGH to synthesis ribulose-5-phosphate. These two interdependent biosynthetic pathways are likely essential for lipogenic breast cancer growth and their cooperation warrants further research. In addition, upregulated de novo synthesis may be a metabolic adaptation to breast cancer tumor microenvironment or specific metastatic sites. Ferraro et al. recently showed that FASN activity was required for growth of tumor cells in the brain but not in the mammary gland using their HER2-enriched breast cancer models [99]. This represents the first example of a tissue-specific requirement for FASN and fatty acid biosynthesis and suggests this metabolic change could be required for brain metastasis in this model.

Upstream regulation of de novo synthesis occurs largely through sterol regulatory element-binding proteins (SREBPs). There are two SREBP genes in mammals that encode three isoforms (SREBP1a, SREBP1c, and SREBP2) [100]. SREBPs reside in the endoplasmic reticulum or golgi depending on high or low cholesterol levels, respectively [101, 102], and must be cleaved in order to translocate the nucleus where they activate transcription of lipogenic genes including FASN, ACC, and ATP citrate lyase (ACLY) [103, 104]. PPARγ, NR1H2/3, and CEBPs are additional transcription factors that regulate lipid enzyme transcription. SREBP regulation of lipid biosynthetic pathways was largely defined in the lactating mammary gland where SREBP1c plays a critical role in initiating milk-globule production [105]. HER2 is also reported to induce FASN expression, however it is unclear if this is in an SREBP-dependent manner [70]. In HR + breast cancers, both estrogen and progesterone are reported to increase lipogenic gene expression [106]. Thus, HR + and HER2 + breast cancers have highly activated lipid biosynthetic pathways, and we speculate that this may be retained from cells that originally required these processes for lactation, that will require further study.

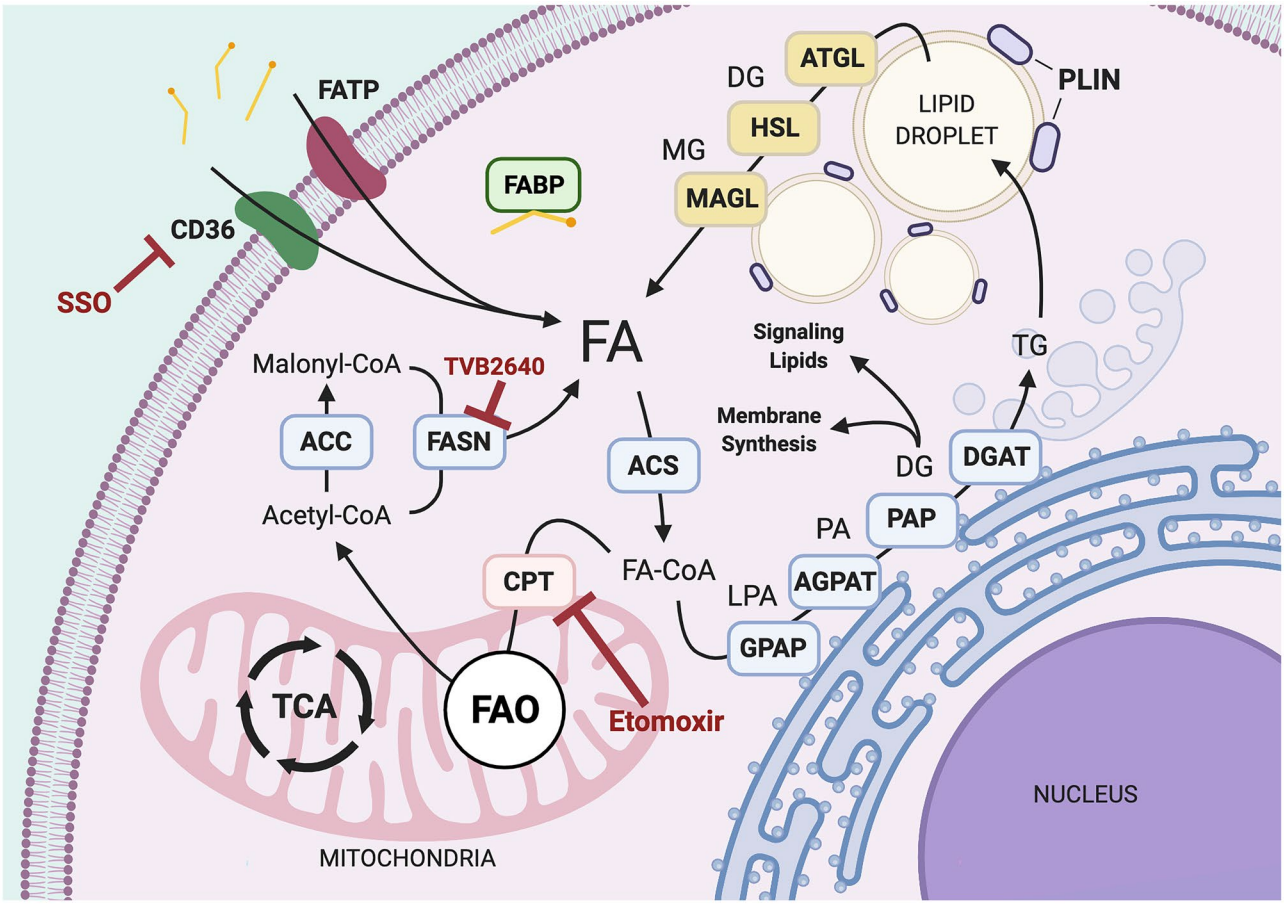

Fig. 3 Overview of Lipid Metabolism. General anabolic and catabolic pathways for intracellular fatty acids. Acetyl-CoA carboxylase (ACC), acetyl-CoA synthase (ACS), 1-acyl glycerol-3-phosphate acyltransferase (AGPAT), adipose triglyceride ligase (ATGL), cluster of differentiation 36 (CD36), carnitine palmitoyl transferase 1/2 (CPT1/2), diacylglycerol (DA), diacylglycerol acyltransferase (DGAT), fatty acids (FA), fatty acid binding protein (FABP), fatty acid oxidation (FAO), fatty acid synthase (FASN), fatty acid transport protein (FATP), glycerol-3-phosphate acyltransferase (GPAT), hormone sensitive lipase (HSL), lysophosphatidic acid (LPA), monoacylglycerol (MG), monoacylglycerol lipase (MGL), phosphatidic acid (PA), phosphatidic acid phosphatase (PAP), perilipin (PLIN), triacylglycerol (TG), tricarboxylic acid cycle (TCA)

Palmitate, the most abundant and fundamental saturated FA, can be further processed into glycerolipids by enzymes in the golgi (GPAT, LPAT, PAP) and shuttled into lipid storage, membrane synthesis, or signaling lipids. Palmitate itself serves as important protein lipid modification in cancer cells. The Wnt signaling pathway is an important driver of several cancers and is frequently activated in breast cancer cells. Wnt ligands undergo palmitoylation and depalmitoylation for trafficking between the plasma membrane and cytosol. Palmitoylation occurs when a protein forms an enzyme-mediated thioester bond with a palmitoyl group and this process is responsible for tethering a number of proteins to the plasma membrane such as Ras, and CD36. Palmitoylation and myristoylation are just a few of the key protein lipid modifications prevalent in cancer which are being explored as therapeutic targets [107]. Glycerolipids destined for storage are processed into MG, DG, and TGs. The PLIN family of proteins package these species into lipid droplets with cholesterol. Lipid droplets visible by microscope are associated with a lipogenic cellular phenotype and commonly reported in HR + and HER2 + breast cancer [108]. Lipid droplets mainly serve as energy storage but may have other consequential effects. For example, lipid droplets in breast cancer cells have been shown to provide cytotoxic protection by sequestering chemotherapeutic agents [109]. Under times of nutrient deficiency, lipases associated with lipid droplets (ATGL, HSL, MGL) can release FAs from their glycerol backbone through hydrolysis reactions. Intracellular FAs can recycle to other anabolic synthetic pathways or be shuttled for oxidation.

FAO is a catabolic process that breaks down FAs into acetyl-CoA. This process begins in the mitochondria with

transport proteins bound to the mitochondrial membrane that participate in the carnitine shuttle. Mitochondria consist of two membranes, the inner and outer membranes. Carnitine palmitoyltransferase I (CPT1) is incorporated in the outer mitochondrial membrane and facilitates transfer of FAs across this membrane, while CPT2 coordinates FA transport across the inner membrane. Through a series of reactions, FAs are broken down to yield acetyl-CoA, NADH, and FADH2. This mechanism serves as an alternative to drive TCA cycle movement during insufficient glucose or glutamine availability. Breakdown of branched and very long chain FAs require α- and β-oxidation by peroxisomes [110]. Once these FAs are converted to shorter chain FAs, they can be imported into the mitochondria via the CPT-mediated carnitine shuttle to complete further oxidation steps. FAO is emerging as an important metabolic process that contributes to deregulated breast cancer metabolism, especially in TNBC [111]. The MYC oncogene is frequently amplified in TNBC and has been shown to drive FAO in addition to glycolysis [112, 113]. TNBCs with MYC overexpression have been shown to upregulate PGC1α, CPT1B, and CDCP1 while downregulating FASN and ACC [113]. In addition, FAO gene signatures have been associated with poor clinical outcome in MYC-expressing TNBCs, suggesting that this process is a contributing factor to TNBC pathogenesis [113].

Oncogenes play a critical role in regulating lipid metabolism. *PIK3CA* is one of the most commonly mutated genes in carcinomas with up to 40% of breast cancers exhibiting gain-of-function mutations [114, 115]. The protein generated by *PIK3CA*, phosphoinositide 3-kinase (PI3K), participates in the PI3K/AKT/mTOR signaling axis which regulates cell growth and proliferation as well as sensing availability of nutrients, hormones, and growth factor stimulation [116]. Loss of the tumor suppressor PTEN also occurs frequently in breast cancer; PTEN acts as a lipid phosphatase, converting phosphatidylinositol (3,4,5)-trisphosphate (PIP3), to phosphatidylinositol (4,5)-bisphosphate (PIP2), thereby depressing PI3K and AKT activation. 80% of HER2 + breast cancer tumors display increased phosphorylation AKT, an indicator of active PI3K signaling [117]. The connection between active PI3K signaling and a lipogenic phenotype in HER2 + breast cancer is not fully understood but is suggested to be a consequence of AKT downstream targets [118, 119]. As discussed, MYC is associated with activating glycolysis and lipogenesis/FAO in TNBC [113, 120]. MYC is also frequently mutated or amplified in breast cancer and likely contributes to breast cancer aggressiveness through its regulation of multiple branches of metabolism [115]. Thus, the various genetic changes present in the different breast cancer subtypes can drive changes in lipid metabolism. There are two excellent recent revies on breast cancer oncogenes and metabolism [121, 122].

Serum Lipidomics: Clinical Diagnostic Potential?

Preclinical and clinical studies to date support that lipid metabolism is aberrantly altered in breast cancer compared to benign breast tissue. A key question is whether breast tumor lipid metabolites in patient serum have diagnostic potential. Mammograms and magnetic resonance imaging (MRI) are the current standard for breast cancer screening, yet mammograms alone display a rate of over-diagnosis between 0–30% [123]. Follow up MRIs and tissue sampling can be costly and inconclusive. In addition, some subtypes such as TNBC can be difficult to detect by mammogram until tumors are of a size that negatively impacts treatment and outcome [124]. Thus, there remains a need for cost-effective breast cancer screening alternatives. Serum tumor markers provide an alternative, noninvasive and less costly methods for breast cancer diagnostic screening. For example, advances in capturing circulating tumor cells and circulating tumor DNA provides prognostic and disease-state information [125]. Current advancements in mass spectrometry detection of lipids may offer an additional serum screening option.

Several recent studies conducted lipidomic analysis of serum from breast cancer patients and non-cancer controls to determine if tumor-associated lipids could be detected. Three independent studies identified increased levels of PC(32:1) in serum from women with breast cancer compared to non-affected women [126–128]. PC(32:1) was also an increased lipid analyte detected in studies of TNBC tumors described previously [84, 85]. In addition, total TGs were increased in breast cancer patient serum compared to control [129]. Total serum TGs also distinguished menopausal and HR status in breast cancer patients as well as pathological complete response rate to neoadjuvant chemotherapy [126, 127]. Notably, TGs containing mainly oleic acid (C18:1) were associated with decreased disease-free survival in breast cancer patients [129]. Additionally, serum LPC and cholesterol esters were elevated in breast cancer patients compared to healthy control [129].

One study investigated specific lipid signatures as a diagnostic test. Using 166 plasma samples, Eghlimi et al. established a 19-lipid biomarker panel capable of distinguishing early stage TNBC from controls (AUROC = 0.93, sensitivity = 0.89, specificity = 0.76), as well as a 5-lipid biomarker panel differentiating ES-TNBC from non-ES-TNBC serum samples (AUROC = 0.95, sensitivity = 0.95, specificity = 0.87) [128]. Of the 19-lipid panel, stearic acid and Cer(43:1) are elevated (fold change (FC) > 1.3) in TNBC while DGs and LPCs were generally decreased compared to serum from non-affected women (FC < 0.7). The smaller 5-lipid panel was sufficient to detect DG(34:2)

as significantly decreased (FC > 0.2) in TNBC. In this study, a statically tested lipid biomarker panel was established, however, clinical use of this panel still requires further validation with larger patient serum cohorts. Despite these challenges there is sufficient promise in the utility of serum-based biomarkers for breast cancer detection that merit further study.

Therapeutic Targets in Tumor Lipid Biology and Metabolism

As discussed in this review, potential lipid metabolism targets have been identified in each breast cancer subtype. Cancer metabolism therapies have largely been unsuccessful in clinical trials, with the exception of isocitrate dehydrogenase 1 inhibitors. However, our understanding of cancer metabolism continues to improve. Here, we give a brief overview of several lipid metabolic and transport inhibitors at preclinical or clinical trial stages for breast cancer.

FASN is perhaps the most widely targeted lipogenic enzyme in breast cancer due to its consistent overexpression. Genetic or pharmacological inhibition of FASN in preclinical studies has shown efficacy in decreasing cell proliferation in vitro and tumor growth in vivo in all subtypes of breast cancer, and there are excellent reviews on the topic [130]. Unfortunately, clinical translation has not been successful. Existing selective inhibitors of FASN have limited solubility and adverse side effects that have prohibited their clinical use. Cerulenin, C75, and C93 are inhibitors that target the β-ketoacyl synthase domain of FASN induce anorexia and weight loss in murine models [131, 132]. EGCG, G28UCM, GSK2194069, and GSK837149A also target the β-ketoacyl synthase domain but are ineffective in vivo due to low solubility [133–135]. To date, TVB-2640 is the only FASN inhibitor that is has reached a phase II clinical trial for breast cancer. Trial NCT03179904 is testing the efficacy of TVB-2640 in combination with paclitaxel and Trastuzumab in breast cancer patients with metastatic HER2 + disease. While the trial is still ongoing, additional inhibitors, such as Fasnall, that block FASN co-factor binding, are under preclinical study [136]. Furthermore, FASN inhibitors have shown promise against some forms of endocrine resistant HR + breast cancers in vivo [137]. Some preclinical studies have also tested inhibiting alternative de novo synthesis targets such as ACC [138].

Elevated cholesterol has long been associated with increased risk of breast cancer and specific metabolites, such as 27-hydroxycholeesterol, have been shown to facilitate metastasis and evasion of immune cells in breast cancer [139]. Farnesyltransferase or HMG-CoA reductase inhibitors (statins) that target the mevalonate pathway and are commonly used to treat hypercholesterolemia and meta-analyses have shown statin users have reduced in breast cancer specific mortality [140]. Statin drugs are an appealing therapeutic target especially in advanced HER2 + and TNBC that exhibit enhanced cholesterol dependency. Preclinical studies have shown efficacy of statins in therapy-resistant HER2 + breast cancer models [141]. Simvastatin has reached phase II clinical trial (NCT03324425) in combination with targeted HER2 therapies for advanced HER2 + breast cancer. For TNBC, Atorvastatin is being evaluated in conjunction with the bisphosphonate zoledronate and adjuvant chemotherapy in phase II clinical trial NCT03358017. Some studies show adverse effects of statins such as increased expression of cholesterol synthesis enzymes through heightened feedback regulation from SREBP2 [142]. To circumvent this resistance mechanism, alternative targets of cholesterol synthesis are being investigated. For example, RORγ was recently identified as new upstream target for mevalonate pathway inhibition in TNBC [143, 144].

There is also interest in targeting lipid transporters, which has mostly centered on the best described FA transporters CD36 and FATP. However, there are likely many other promiscuous FA transporters of the SLC family. In low nutrient conditions, breast cancer cells can bypass dependency on de novo synthesis by increasing FA uptake. For example, studies show that drug resistant HER2 + breast cancer cells compensate for FASN inhibition by increasing extracellular FA uptake [91, 145]. Feng et al. showed that lapatinib-resistant breast cancer cells upregulate CD36 and regain drug sensitivity under CD36 inhibition [91]. Unfortunately, there are few available CD36 inhibitors, likely due in part to incomplete understanding of CD36 mechanisms and functions. Sulfo-N-succinimidyl esters of long-chain FAs, such as Sulfosuccinimidyl Oleate (SSO), efficiently inhibit CD36 and have been used in multiple in vitro studies. Large chemical screens have identified additional potential inhibitors; however, further development is needed for these compounds to advance into preclinical studies [146]. It may be important to use lipid transport inhibitors in conjunction with inhibitors of fatty acid synthesis to prevent resistance to the latter by increased lipid transport.

FAO or beta-oxidation is the mitochondrial break down of FAs to provide metabolic fuel. FAO has emerged as an attractive target in breast cancer. TNBC cells in particular have been reported to utilize FAO [92]. Since FAO occurs within the mitochondria, the primary target for this pathway is the outer mitochondrial membrane transporter and rate-limiting enzyme CPT1. The best known CPT1 inhibitor is Etomoxir which continues to be widely used in preclinical studies. Etomoxir failed in clinical trials due to cardiotoxicity. There is a continued effort to develop tolerable anti-CPT1 analogs to target FAO-dependent breast cancers.

Diabetic drugs may have efficacy in breast cancer treatment. Metformin, the most common drug taken for diabetes,

mediates hepatic glucose production and insulin sensitivity through inhibition of mitochondrial complex 1 and AMPK pathway activation [147]. Women with Type 2 diabetes taking Metformin have decreased risk of post-menopausal breast cancer [148]. Metformin is currently involved in 18 clinical trials for breast cancer, several of which are investigating its efficacy as a neoadjuvant treatment (NCT04387630, NCT04170465, NCT03238495). While the effects of Metformin on breast cancer lipid metabolism remain unclear, current studies suggest Metformin may be a promising therapeutic for FAO-dependent breast cancers [149].

In addition to enzymes and transporters, lipids themselves are believed to hold therapeutic potential. Omega3 or PUFA supplementation is currently under investigation for use in neoadjuvant breast cancer therapy (NCT02831582). Joint pain or discomfort is a common side effect of endocrine therapies and PUFAs have been shown to reduce joint inflammation by competing with pro-inflammatory prostaglandin signals [150]. Three clinical trials are investigating the benefits of PUFA supplementation with standard endocrine and chemotherapies. PUFAs could potentially be advantageous for breast cancers exhibiting enhanced lipid uptake and warrants further study.

Another novel therapeutic approach is to target tumor cell lipidomes. There is increased interest in lipidome-based therapies as we learn more about tumor specific lipid dependencies [reviewed in [151]]. For example, Toric et al. successfully screened a library of layer-by-layer nanoparticles to determine the surface layer that best interacts with STAT3 expressing TNBC cell membranes [152]. The NP coating identified from the screen allowed selective cisplatin NP delivery to STAT3-active TNBC cells, avoiding non-STAT3 activated cells. This study demonstrates the potential of exploiting distinct lipidomes for targeted drug delivery. Other novel methods of lipidome targeting are certain to emerge.

Summary and Future Perspectives

Advancements in mass spectrometry-based lipidomics have increased our understanding of breast cancer lipidomes, yet there remain several obstacles to accelerating the field. The first obstacle resides within the technique itself (see Fig. 2). Numerous forms of mass spectrometry have been utilized (i.e., shotgun MS, LC–MS, LC–MS/MS, GC–MS), each using various methods of sample separation, detection, and identification, which is also dependent on whether the desired approach is global or targeted. The lack of a technical "gold standard" makes data sharing and comparison difficult. However, the two main data repositories, Metabolomics Workbench (https://www.metabolomicsworkbench.org/) and Metabolites (https://www.ebi.ac.uk/metabolights/), accept lipidomics data in different formats but require thorough methodological detail to aid in interpretation of shared data. In addition to methodological discrepancies, global lipidomics conducted on any biological sample can detect over 1800 validated lipid species which adds to the difficulty of connecting lipidome alterations to biological consequence or changes in cellular phenotype [153, 154]. Despite these attempts to achieve methodological transparency, it remains difficult to compare different datasets unless similar MS methods and data handling were used.

The second obstacle is the types of breast cancer models used in previous published work. The majority of lipidomic studies discussed in this review used either breast cancer cell lines [69, 71, 73, 76, 78, 81] or primary tumor samples [83–86]. As highlighted in Table 1, there is a clear disparity between lipid analytes detected in 2D cell line monoculture versus patient tumor samples, with only several key overlapping lipid species. While primary tumor samples may be the most directly relevant, they have several variables that can complicate interpretations including tumor heterogeneity and inclusion of multiple cell types in the microenvironment. In addition, factors such as diet, body mass index, and tumor stage/burden may impact results. While breast cancer cell lines eliminate many of these variables, whether they reflect the more complex in vivo situation is uncertain. As discovered by Vidavsky et al. in comparing lipidomics in MCF10A 2D monoculture vs 3D spheroids, spatial orientation within a 3D cell structure significantly impacts lipid content [81]. In addition, the lipid content of cell culture medium directly impacts cellular morphology and behavior [155]. The FA content of fetal bovine serum can vary between commercial source and lot number. Many companies do not provide detailed FA information since it is difficult to measure and not a concern for all consumers. Charcoal stripped serum is frequently used to remove hormones, particularly estrogens, and likely also removes a subset of lipids. To our knowledge, additional lipidome studies on breast cancer 3D cultures, tumor xenografts, or syngeneic mammary tumor models have not been reported. There is therefore a need for future pre-clinical lipidomic studies to utilize 3D cultures, organoids or cell line or patient-derived xenograft models to better incorporate spatial influences.

A third variable lies in technological limitations in studying specific lipid analytes. Unlike genetic or pharmacological manipulation of an individual gene product, lipid metabolites are not as easily modified. Studying the relevance of individual lipid species or classes requires an understanding of the proteins that regulate their synthesis, uptake, degradation, an intracellular location. However, there are tools that are routinely used. These include fluorescent tagged lipid species (i.e. BODIPY) for tracking cell uptake and location and the long standing method of stable isotope tracing which allows us to depict FA usage in cells over time [156]. Stable

isotope lipids containing C14 within its structure are routinely used in lipidomics and aid in absolute quantification of analytes in interest [157].

Analyzing lipids in patient serum samples has diagnostic potential. The studies discussed herein defined preliminary panels of tumor-associated lipids in patient serum that could aid in breast cancer diagnoses [126–129]. This method could be particularly useful for early detection of breast cancer subtypes that are difficult to detect in mammograms [158]. Patient-specific factors such a dietary lipids, lifestyle, and menopausal status may complicate the efficacy of these panels. For example, overweight or individuals with obesity have increased levels of total serum lipids and lipoproteins compared to normal individuals [159]. Diets high in palmitic acid (palmitate) have also been shown to increase an individual's serum cholesterols levels [160]. Once protocols are established to account for these factors, serum lipid panels may serve as an alternative or complementary diagnostic test to the mammogram. Serum lipidomics could also be used to predict patient response to specific therapies. For example, Hilvo et al. identified lipid analytes associated with positive response to chemotherapy [129]. A lipid panel could prospectively be developed for endocrine therapies. In time, we predict serum lipidomics will indicate useful clinical information such as tumor burden, therapeutic response, and development of metastases.

In summary, the breast is a dynamic organ which responds to hormonal and environmental cues to undergo drastic remodeling and lipid production. Breast cancer has well documented reliance on lipid metabolism – however a link between processes in normal and malignant breast tissues has been difficult to define – as have consistent targetable lipid dependencies. This is underscored by a general paucity of understanding of the lipidome in breast cancer cells and the complication of different breast cancer subtypes and significant intra- and inter- tumoral heterogeneity. The studies highlighted have made significant progress in understanding the global lipidome and its impact on breast cancer cell phenotype. However, there remain multiple gaps in our knowledge, including how lipids are impacted by spatial location of the cell withing the tumor, tumor microenvironment, metastasis, and resistance to drug treatment etc. With emerging models such as tumor-derived organoids and patient-derived xenografts, coupled with continuous improvements to lipidomics and analysis tools, these gaps will be hopefully become filled and lipids a regular measurement of breast cancer cell state and therapeutic vulnerability.

Abbreviations ACC: Acetyl-CoA carboxylase; APCI: Atmospheric pressure chemical ionization; APPI: Atmospheric pressure polarization ionization; BmP: Bis(monoacylglycero)phosphate; BUME: Butanol/methanol; Cer: Ceramide; ChEBI: Chemical entities of biological interest; CL: Cardiolipin; CPT1: Carnitine palmitoyltransferase I; DESI: Desorption electrospray ionization; DG: Diacylglycerol; ER: Estrogen receptor; ESI: Electron spray ionization; FA: Fatty acid; FAO: Fatty acid oxidation; FASN: Fatty acid synthase; GC-MS: Gas chromatography mass spectrometry; GlcCer: Glucosylceramide; HER2+: Human epidermal growth factor receptor 2-positive; HexCer: Hexosylceramide; HMBD: Human metabolome data base; HR: Hormone receptor; LacCer: Lactosylceramide; LION: Lipid Ontology Enrichment Analysis; LIPEA: Lipid Pathway Enrichment Analysis; LPC: Lysophosphatidylcholine; MALDI: Matrix assisted laser desorption/ionization; Met: Methionine; MRI: Magnetic resonance imaging; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; MTBE: Methyl tert-butyl ether; MUFA: Monounsaturated fatty acid; NMR: Nuclear magnetic resonance; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol (4,5)-trisphosphate; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; PKC: Protein kinase C; PLIN: Perilipin; PR: Progesterone receptor; PS: Phosphatidylserine; PUFA: Poly unsaturated fatty acid; SCD: Stearoyl-CoA desaturase; SIMS: Secondary ion mass spectrometry; SM: Sphingomyelin; SREBP: Sterol regulatory element-binding protein; SSO: Sulfosuccinimidyl oleate; TCA: Tricarboxylic acid; TG: Triacylglycerol; TNBC: Triple negative breast cancer

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