A narrative review of urinary phospholipids: from biochemical aspect towards clinical application

Xin Li[1#], Kenji Nakayama[1#], Takayuki Goto[1], Shusuke Akamatsu[1], Takashi Kobayashi[1], Koji Shimizu[2], Osamu Ogawa[1], Takahiro Inoue[3]

[1]Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [2]Clinical Research Center for Medical Equipment Development, Kyoto University Hospital, Kyoto, Japan; [3]Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Tsu, Japan

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[#]These authors contributed equally to this work.

Correspondence to: Professor Takahiro Inoue. Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Tsu, Japan. Email: tinoue28@med.mie-u.ac.jp.

Abstract: As a newly emerged discipline, lipidomic studies have focused on the comprehensive characterization and quantification of lipids in a given biological system, which has remarkably advanced in recent years owing to the rapid development of analytical techniques, especially mass spectrometry. Among diverse lipid classes, phospholipids, which have fundamental roles in the formation of cellular membranes, signaling processes, and bioenergetics have gained momentum in several fields of research. The altered composition, concentration, spatial distribution, and metabolism of phospholipids in cells, tissues, and body fluids have been elucidated in various human diseases such as cancer, inflammation, as well as cardiovascular and metabolic disorders. Among the different kinds of phospholipid sources in the human body, urine has not been extensively investigated in recent years owing to the extremely low concentrations of phospholipids and high levels of salts and other contaminants, which can interfere with precise detection. However, with profound advances and rapid expansion in analytical methods, urinary phospholipids have attracted increasing attention in current biomedical research as urine is an easily available source for the discovery of noninvasive biomarkers. In this review, we provide an overview of urinary phospholipids, including their biochemical aspects and clinical applications, aimed at promoting this field of research.

Keywords: Phospholipid (PL); lysophospholipid (LPL); urine; biomarkers; genitourinary diseases

Introduction

Urine is a unique, easily accessible, and noninvasive sample that is widely used for clinical diagnostics, especially in the field of urology. The analysis of urinary components has often been utilized for assessing metabolic or physiological states of patients or healthy subjects (1). Additionally, urine is an attractive matrix for biomarker discovery, presenting rich metabolite categories and high participant compliance (2).

The concept of lipidomics was introduced by Spener *et al.* (3) and Han and Gross (4) in 2003, which refers to the qualitative and quantitative analysis of global lipids in a given cellular or biological system. In recent years, phospholipids (PLs) have been separately analyzed from other lipids owing to their unique amphipathic properties (hydrophobic fatty acyl tails and hydrophilic head group on the same molecule), diverse bio-functions, and biological

relevance in various diseases (5).

In cells, owing to their amphipathic properties, PLs are the main components of bilayer membranes of the cell and intracellular organelles, providing cellular integrity (5). Additionally, PLs act as key members in cellular signaling or sources of energy for various cellular processes, including homeostasis, cell migration, apoptosis, autophagy, and post-translational modifications (6).

Alterations in the composition of various PLs in cells, tissues, and body fluids, including urine, are associated with numerous urologic and genitourinary diseases and pathophysiological conditions such as genitourinary (prostate, bladder, and kidney) cancers (6), kidney stones (7), inflammatory disorders (8), and sperm quality (9).

Owing to the extremely low concentrations of PLs and high levels of contamination in urine, an effective extraction method, as well as a sensitive detection method for urinary PLs remain crucial for clinical application. Traditional liquid-liquid extraction (LLE) methods, including Folch (10), Bligh-Dyer (11), butanol-methanol (BUME) (12,13), methyl-tert-butyl ether (MTBE) (14), and hexane-isopropanol (HIP) (15), were applied and compared for urinary PLs and lysophospholipids (LPLs) in our previous study (16). Furthermore, diverse analytical methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (17), electrospray ionization mass spectrometry (ESI-MS) (18), liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (19), and nuclear magnetic resonance spectroscopy (NMR) (20) have been utilized for detecting urinary PLs and LPLs in previous reports.

In this review, we focus on the chemical, physical, and biological aspects of urinary PLs and LPLs, as well as their clinical applications in genitourinary diseases. We present the following article in accordance with the Narrative Review reporting checklist (available at http://dx.doi.org/10.21037/tau-20-1263).

Methods

This narrative review designed to collect and review published articles and literatures on the biochemical aspects of PLs and LPLs and the clinical application of urinary PLs and LPLs. A search was carried out on the databases including PubMed, Web of Science and Google Scholar by MeSH-compliant keywords including phospholipid(s), LPL(s), biochemical property, urine, biomarkers, and genitourinary diseases. All related original research studies, letters to the editor, reviews and ebooks written in English and published from January 1950 to August 2020 were screened and checked.

Discussion

Chemical and physical aspects of PLs and LPLs

Chemical structure

PL is composed of a glycerol backbone, two fatty acids, and a phosphate group. The chemical structures of the most common PLs are presented in *Figure 1*. In PLs or LPLs, a glycerol molecule forms the backbone of the whole structure (shown in *Figure 1*). Each carbon in the glycerol molecule is numbered using the "stereospecific numbering" (sn) system. The hydroxyl groups at the sn-1 and sn-2 positions are esterified with two fatty acids (shown in *Figure 1*), and the hydroxyl group at sn-3 is esterified with a phosphate group to form a head group. Variations of head groups (shown in the dashed box in *Figure 1*) extend the subcategories of PLs, resulting in phosphatidic acid (PA, with hydrogen), phosphatidylcholine (PC, with choline), phosphatidylethanolamine (PE, with ethanolamine), phosphatidylserine (PS, with serine), phosphatidylglycerol (PG, with glycerol), and phosphatidylinositol (PI, with inositol). Within each sub-category, the length and saturation of hydrophobic fatty acid chains lead to further characterization of glycerol-PLs. For example, dilauroyl PLs (12 carbon atoms in each fatty acyl chain), dimyristoyl PLs (14 carbon atoms in each fatty acyl chain), or dipalmitoyl PLs (16 carbon atoms in each fatty acyl chain) are referred to based on the length of fatty acyl chains. Dioleoyl PLs (containing one double bond in each fatty acyl chain) and distearoyl PLs (containing two double bonds in each fatty acyl chain) are characterized by the saturation condition of fatty acyl chains. If an acyl group is connected to the glycerol backbone (shown in the left bottom of *Figure 1*), it is termed a LPL, which can be catalyzed by PLs in the cell. Variations of LPL head groups is similar to PLs, resulting in subclasses such as lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), lysophosphatidylglycerol (LPG), and lysophosphatidylinositol (LPI) by connecting the phosphate with hydrogen, choline, ethanolamine, serine, glycerol, and inositol, respectively.
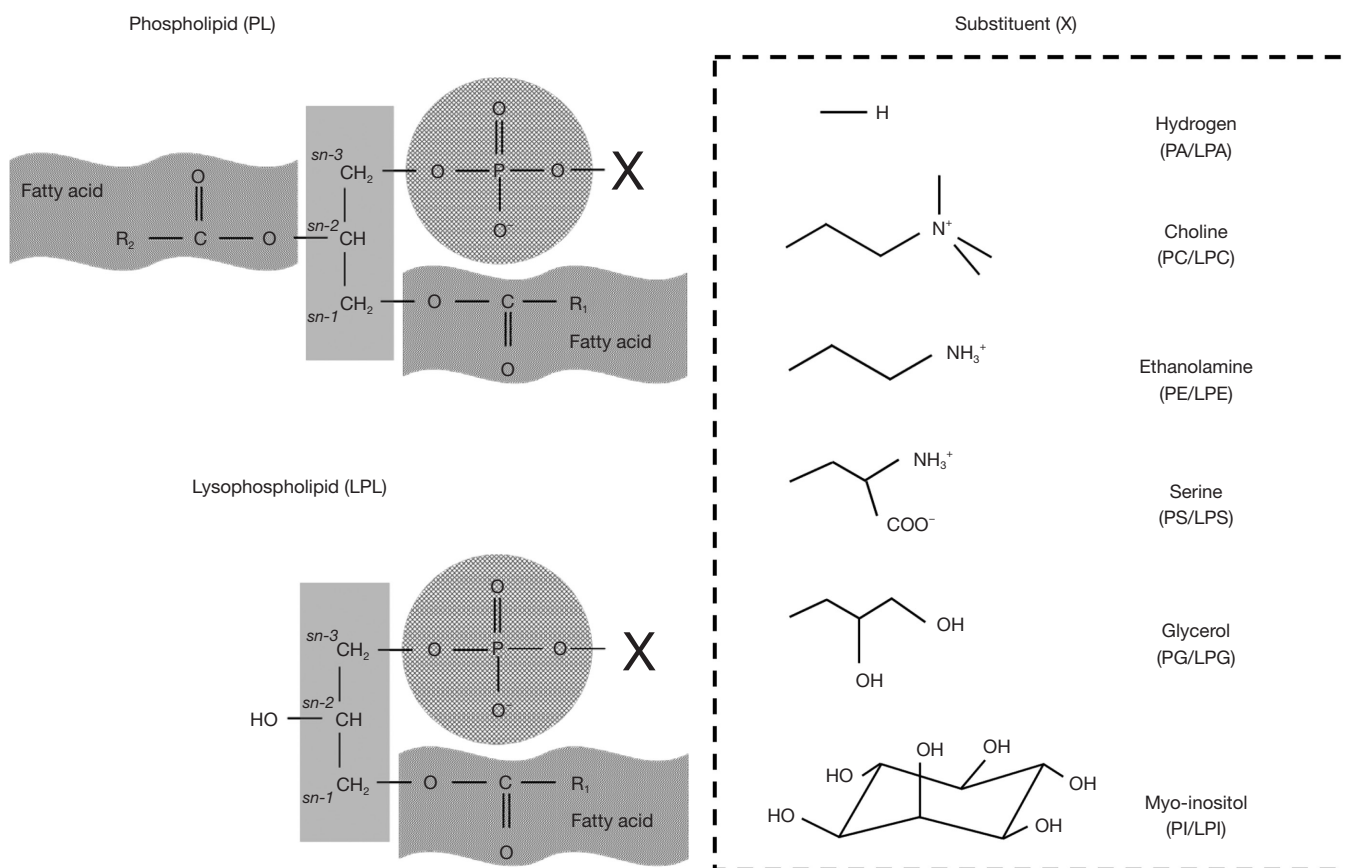
Figure 1 Characteristic structures of the PL subclasses. PLs are composed of three main parts: a three-carbon glycerol backbone (shown in rectangle box); one or two fatty acid chains (shown in wavy shape) esterified to the first and/or second carbons of the glycerol molecule in LPL and PL, respectively; a phosphate head group (shown in circle) that can be modified by substitution X to form different PL subclasses. Different structures of substitution X, which are connected to the phosphate and shown in the dashed box, determine the individual subclass of PL as indicated with abbreviations. (R1 and R2 indicate the fatty acid chains esterified to the sn-1 and sn-2 carbon atoms in the glycerol backbone). PL, phospholipid; LPL, lysophospholipid; PA, phosphatidic acid; LPA, lysophosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; LPS, lysophosphatidylserine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; LPI, lysophosphatidylinositol.

Physical properties

Solubility

As lipids, PLs and LPLs are poorly soluble in water and other polar solvents but are soluble in organic solvents such as chloroform ($CHCl_3$), methanol (MeOH), hexane, ethanol, isopropanol, or in mixtures these solvents. This property allows PLs and LPLs to be easily separated and extracted from aqueous solution (e.g., urine) by the addition of organic solvents. For example, in our previously optimized extraction method for urinary PLs and LPLs (16), 5 mL of an organic solvent mixture containing $CHCl_3$/MeOH (1:1, v/v) was added to 1 mL of urine, followed by the addition of 1.25 mL of water. The volume ratio of $CHCl_3$, MeOH, and water in the final solution was approximately 1:1:0.9, achieving an optimal condition for transferring PLs from the aqueous to the solvent phase. Accordingly, lipids, including PLs, are isolated and purified from urine.

Electrolysis

Notably, all PLs vary from no net charge (PC/LPC and PE/LPE, also named zwitterionic PLs or LPLs) to anions (PA/LPA, PS/LPS, PG/LPG, and PI/LPI), with none possessing a net positive charge under physiological pH.

Therefore, anionic PLs can provide negative charge densities to the cellular/organelle membranes, necessary for recruitment and interactions with ions and proteins (21). At physiological pH, zwitterionic PLs (PC/LPC and PE/LPE) contain both a negative and positive charge group. A pH change in the aqueous environment causes protonation/deprotonation of the PL head groups, greatly impacting the chemical and physical properties (e.g., hydration, swelling, phase transitions, and the overall polarity) of the PL molecule. The altered PL properties will further influence the interactions with ions and solutes such as peptides and proteins, causing a downstream reaction in the cell/organelle. If the pH of the solution is equal to the isoelectric point of PLs, their physicochemical behavior will resemble that of nonionic lipids (e.g., mono- and digalactosyldiacylglycerols). Furthermore, a change in pH may influence the hydrolytic stability of PLs, which are more rapidly hydrolyzed at high pH than at low pH.

Amphiphilicity

Although poorly water-soluble, as described in the previous section, PLs are readily dispersible in water owing to a feature termed 'amphiphilicity'. The word 'amphi', derived from Greek, means 'both'. In the case of PLs in water, all PL molecules are composed of two parts: a hydrophilic part (the phosphate group) and hydrophobic part (fatty acid chains) (22). The amphiphilic property is essential for the spontaneous formation of the lipid bilayer structure in aqueous environments. The hydrophilic phosphate head group faces water at each surface of the bilayer, whereas the hydrophobic fatty acid tails remain shielded from water in the interior (23). This is the fundamental structure of the cell and organelle membrane, which is further discussed in a later section.

Biological Functions of PLs and LPLs

As described above, physical, and chemical diversities confer PLs versatile bio-functions in cells. Cumulative studies have indicated the three main roles of PLs, including as major components of cellular and organelle membranes, signaling mediators in various cellular processes, and members of energy storage (24). *Table 1* summarizes the main bio-functions of PLs and LPLs described in previous studies.

Membrane

For PLs, the first illustrated biological function is the

formation of cell and organelle membranes, which help provide cellular integrity and separation of subcellular compartments, which are fundamental requirements for all cellular functions. PLs can form an ordered bilayer structure owing to their amphiphilic nature (described above). The hydrophobic fatty acyl tails of PLs line up and face each other, whereas the hydrophilic head group faces both sides of the membrane. This membrane is described as a 'fluid mosaic model' (23), which is semi-fluid and partially permeable, with embedded proteins in the PL matrix.

Among the different subcategories of PLs, PC has demonstrated the highest content in animal membranes (25). It is ideally suited to this role as the width of its polar head group is approximately identical to the sum of its apolar fatty acyl tails (26). Moreover, PC is a zwitterion with a formal positive and negative charge, lacking a net charge. Owing to these two physical properties, PC is deemed relatively stable in membranes, both physically and chemically. PE is the second most prevalent PL in the human body and is particularly rich in nervous tissues. As another zwitterionic PL, the primary role of PE is as a structural lipid in the cell membrane (25). For PE, the head group is small and poorly hydrated, imparting a pyramidal shape, with a wide base (fatty acyl chains) and a narrow head. Owing to its amine-containing head group, PEs are also chemically active and can form hydrogen bonds with other neighboring polar groups, thus impacting the membrane structure (27).

To considerably lower levels when compared with PC and PE, other anionic PLs (i.e., PA, PS, PG, PI, and those LPLs) also play important roles, with additional functions in the cell membranes. For example, the net charge of PA at physiological pH is only 1.5, and such a strong anion contributes negative charge density to the membrane, which is necessary for membrane vesicle fission, as well as the activities of some membrane proteins (30). Like the anionic role of PA, PS, PI, and PG additionally act as important anions at physiological pH and thus contribute to the negative charge density of lipid bilayer membranes. Indeed, they possess additional functions in cell signaling and regulation in addition to structural lipids in the cells; this will be discussed in the next section.

Cell signaling

Beyond their role as a fundamental structure of lipid bilayers, PLs participate in cellular processes such as signaling. Owing to their transmembrane location and

Table 1 Summarized biological functions of PLs and LPLs

Lipids	Subclass	Main biological functions	Refs
PLs	PC	Major components of cell and organelle membranes	(25,26)
	PE	Cell and organelle membrane fusions; precursors of PC and PS synthesis; formation of autophagosome	(25,27-29)
	PA	Negative charge density to the membrane; intermediate for the biosynthesis of other PLs; recruits effector proteins to the membrane	(30-45)
	PS	Platelet activation; "eat me signal" on surface of apoptotic cells	(28,46,47)
	PG	Anionic lipids in membranes; RNA synthesis; required in PKC signaling	(48,49)
	PI	Phosphorylated PI play central role in multiple cell signaling and regulation	(27,30,50-58)
LPLs	LPC	Induce oxidative stress and inflammatory responses	(59-62)
	LPE	Neuronal differentiation via activation of MAPK; increase intracellular Ca^{2+} and stimulate migration and invasion of cancer cells	(63-65)
	LPA	Lipid mediator regulating multiple pathways	(66-68)
	LPS	Lipid mediator involved in platelet activation, inflammation, atherosclerosis	(69-71)
	LPG	Increase intracellular Ca^{2+} and stimulate ERK activity in cancer cells; inhibit the chemokine-induced migration and IL-1β production in phagocytes	(72,73)
	LPI	Insulin release; mitogenic factor; inducing fat deposition and wound healing	(74-79)

PLs, phospholipids; LPLs, lysophospholipids; PA, phosphatidic acid; LPA lysophosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; LPS, lysophosphatidylserine; PG, phosphatidylglycerol; LPG lysophosphatidylglycerol PI, phosphatidylinositol; LPI, lysophosphatidylinositol; PKC, protein kinase C; MAPK, mitogen-activated protein kinase, Ca^{2+}, calcium ion; ERK, extracellular signal regulated protein kinase.

diversity in structural modification, PLs play crucial roles in various cellular functions by directly regulating enzymes or acting as precursors of signaling molecules. Herein, we briefly introduce several widely investigated PLs demonstrating important biological functions in cell signaling.

Phosphoinositides

Phosphoinositides, the phosphorylated derivatives of PI, play a central role in cell signaling and regulation (50,51). Frequently, PI is phosphorylated at various positions on the inositol chain. The D-3, D-4, and/or D-5 positions of the hydroxyl groups of the inositol ring result in the formation of seven distinct phosphoinositide species, including phosphatidylinositol 3-phosphate (PI3P), PI4P, PI5P, phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], PI(3,5)P2, PI(4,5)P2, and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3]. Phosphoinositides are localized on inner plasma membranes, including various organelle (endosomal, Golgi, and nuclear) membranes (mainly monophosphates) and inner plasma membranes (mainly di- and triphosphates), tightly regulated by a network of kinases and phosphatases.

Phosphoinositides regulate cell processes through direct interaction and modeling of membrane proteins (such as ion channels) or recruitment of cytosolic proteins to membranes, and activating downstream pathways [such as protein kinase B, also known as Akt, can be recruited and activated by PI(3,4)P2 or PI(3,4,5)P3], thus regulating cellular processes such as proliferation, apoptosis, and migration. Phosphoinositide signaling affects various cellular processes, including proliferation (52), survival (53), migration (54), cytoskeletal rearrangement (55), vesicle trafficking (56), membrane dynamics (57), and apoptosis (58).

Phosphatidic acid

As the simplest PL with relatively low abundance (covering only 1–4% of the total PLs), PA is reportedly known as an intermediate for the biosynthesis of other PLs following dephosphorylation to diacylglycerol (DAG). In recent years, PA has been recognized as a signaling messenger (31). In cells, PA levels are dynamic and controlled (i.e., synthesis and catabolism) by several enzymes, including phospholipases (e.g., phospholipase A and D), lipid kinases

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(e.g., PA kinase, DAG kinase), and phosphatases (e.g., lipid phosphate phosphatase). The cellular level of PA is highly increased under stress conditions such as injury, freezing, and infection (31). Although yet to be thoroughly elucidated, PA reportedly interacts with effector proteins in the "electrostatic/hydrophobic switch mechanism" (32) and "electrostatic/hydrogen-bond switch mechanism" (33), performing its role as a cellular messenger. PA recruits effector proteins to the membrane and regulates the activities of proteins in cellular pathways. Some effector proteins known to interact with PA are Son of sevenless (Sos) (34), kinase Raf-1 (35), mammalian target of rapamycin (mTOR) (36), Gardner-Rasheed feline sarcoma viral (FGR) (37), and sphingosine kinase (38), which are involved in multiple signaling pathways such as Akt (39), Hippo (40), and vascular endothelial growth factor (VEGF) (41). These PA-protein interactions are involved in a wide range of cellular processes, including cell proliferation, apoptosis, vesicular trafficking and secretion, and cytoskeletal organization (42), as well as pathophysiological conditions like inflammation (43), oncogenesis, and metastasis (44). Importantly, PA is known to play an important role in spermatogenesis by facilitating the generation of piwi-interacting RNA (piRNA), a critical event in germline development (45).

Phosphatidylserine (PS)
Reportedly, PS is involved in cell-cell recognition and communication processes during apoptosis (46). In contrast to other PLs, PS is located almost exclusively in the membrane inner leaflet, which is transported and maintained by ATP-dependent amino-PL translocases (also known as flippases) (46). Extracellularly, PS is involved in platelet activation (46) and also serves as an "eat me" signal for the clearance of apoptotic cells (47). Intracellularly, several pathways depend on PS. For example, the PS-specific binding of Raf kinases is involved in the activation of mitogen-activated protein kinase (MAPK) signaling (28).

Phosphatidylethanolamine (PE)
One important and attractive function of PE in cells is its involvement in autophagosome biogenesis during autophagy. In the autophagic process of mammalian cells, PE attaches to microtubule-associated protein light chain 3 (LC3), which functions as an anchor to autophagosomal membranes for LC3 (29). Reportedly, increased intracellular PE levels significantly increased autophagic flux (80). Furthermore, PEs possess other diverse cellular functions,

including serving as a precursor for PC and PS synthesis, a determinant of membrane protein topology, and promoting the membrane fusion of cells and organelles by accumulation on the external leaflet (29).

Phosphatidylglycerol (PG)
PG is the second most abundant lipid present in the lung surfactant (LS) of mammals, demonstrating a fluidizing effect on LS. In fetuses, PG levels in the LS are used as indicators of lung maturity, and a lack of PG causes respiratory distress syndrome (RDS) (81). However, the role of PG in human cell signaling remains limited. Previous *in vitro* studies have indicated that PG is involved in the activation of RNA synthesis (48). In cells, the lack of PG leads to defective cell wall synthesis, and PG is required in protein kinase C (PKC) signaling (49).

LPLs as lipid mediators
LPLs, particularly LPAs, are important bioactive lipids in the human body and can act as hormones in the blood by combining with a group of specific G protein-coupled receptors (GPCRs) expressed on the surfaces of target cells (69). As the most investigated LPL, LPA is an extracellular mediator catabolized from substrate LPC and PA. Reportedly, six GPCRs were determined for LPA ligand recognition. On binding to these receptors, LPA activates or inhibits downstream secondary messengers, including MAPK, phosphoinositol 3-kinase (PI3K), Rho, Ras, DAG, adenylyl cyclase (AC) inositol 1,4,5-trisphosphate (IP3), and Ca^{2+}, in various pathways (66). LPA signaling plays key roles in tumor migration and invasion, smooth muscle contraction, vascular development, inflammation, endothelial integrity, neurogenesis, implantation of fertilized eggs, and lymphocyte homing (67-69). Although not as popular as LPA, LPC, which is the most abundant LPL in blood, has attracted increasing attention as a key lipid factor in various pathophysiological conditions such as inflammation, fatty acid oxidation, endothelial activation, and atherogenesis (59-62). Currently, three LPS GPCRs have been identified, and their roles have attracted attention in the immunomodulation (69). In previously reported studies, LPS signaling was involved in platelet activation, inflammation, and the pathogenesis of atherosclerotic diseases (69-71). LPE was found to play important roles in neuronal differentiation via the activation of MAPK (63). In addition, LPE can increase intracellular calcium, thus facilitating the migration and invasion of ovarian and breast cancer (BC) cells (64,65). Like LPE, LPG has

been found to stimulate calcium signaling and increase intracellular calcium in ovarian cancer cells (72). Moreover, this ability of LPG to increase intracellular calcium can inhibit chemokine-induced migration and interleukin-1β production in human phagocytes (73). The first reported biological role of LPI was the stimulatory effect of insulin release by pancreatic islets (74,75). Since then, a growing number of LPI bio-functions/roles have been revealed, including as a potent mitogenic factor, and in fat deposition and wound healing (76-79).

Although current studies mainly used serum or plasma as sources of LPLs biomarkers, a previous review indicated that urine could be utilized as another promising sample for LPL assays as urine requires no burden on the subject. Furthermore, LPLs in the urine are relatively more stable than in blood, which comprises enzymes that introduce undesired and artificial effects, thus facilitating the *in vitro* manipulation of LPLs (69).

Cellular bioenergetics

Moreover, PLs regulate the architecture, biogenesis, and degradation of lipid droplets (LDs), which play critical roles in the energy homeostasis of cells. LDs consist of a core of neutral lipids wrapped by a PL monolayer with integral/peripheral proteins. This lipid-rich cellular organelle is believed to play a central role in the storage and supply of energy in nearly all cell types (82). LDs are highly dynamic organelles that alternate between periods of formation and consumption (83). The LD monolayer not only provides a barrier between LD and the cytoplasm, but is also involved in the communication between LDs and other cellular organelles (e.g., endoplasmic reticulum, mitochondria, and lysosomes) (84). This communication determines the formation and mobilization of LDs in conditions of surplus nutrients or starvation. Furthermore, a recent study indicated that PLs also determine the positions of LDs; PLs with large positive molecular curvature (such as LPLs) facilitate the emergence of LDs from the endoplasmic reticulum (ER), while negative molecular curvature PLs (such as PE) favor the embedding of LDs in the ER (85).

Methods for measuring urinary PL and LPLs

Owing to their extremely low concentration and high diversity in structures, comprehensive analysis and quantification of PLs and LPLs present a considerable challenge. For urinary PL and LPL analysis, the general

workflow mainly includes lipid extraction from the urine, as well as qualitative and quantitative analysis by diverse approaches. A wide range of technologies such as MS, chromatography including high-performance liquid chromatography (HPLC) (86), thin-layer chromatography (TLC) (87), and NMR spectroscopy (88) are commonly utilized for the detection of PLs. Among these, the MS-based method is considered the primary choice for the identification and quantification of PLs and LPLs in the urine. Thus, we mainly focused on the application of MS for the analysis of urinary PLs and LPLs. A common workflow for the MS-based urinary PL and LPL study is shown in *Figure 2*, which includes three main steps of sample preparation (i.e., collection of urine samples and extraction of urinary lipids), data acquisition by MS analysis, and MS data processing by bioinformatics for the screening potential biomarkers. We summarized urinary PLs and LPLs detected in our laboratory using MALDI-TOF/MS in both positive and negative modes.

Extraction

The extraction of lipids from biological samples such as urine is the first step in any lipid analysis method. Prior to analysis, enrichment and purification of PLs from urine are necessary owing to the considerably low lipid concentration when compared with the high abundance of salts and other charged compounds such as amino acids (17).

LLE using a mixture of organic solvents is the most commonly used method to solubilize and separate lipids from the matrix (e.g., tissues and fluids), which are largely insoluble in these organic solvents. Furthermore, solvent extraction can help to minimize the oxidation of unsaturated fatty acyl chains in PLs (89). The most popular choice of organic solvents is a mixture of $CHCl_3$ and MeOH, applied in two methods that were developed by Folch *et al.* (10) in 1951 and Bligh and Dyer (11) in 1959. Although introduced more than 60 years ago, these two methods remain widely utilized and play a fundamental role in several other modified approaches. The Folch method uses $CHCl_3$ and MeOH in a 2:1 ratio, which is suitable for lipid extraction from tissues, whereas the Bligh and Dyer (B&D) method uses $CHCl_3$ and MeOH in a 1:1 ratio and is commonly designed for lipid extraction from fluids with a large amount of endogenous water. These $CHCl_3$/MeOH based methods have been widely applied in urinary lipid studies. For instance, Min *et al.* used the Folch method to extract urinary PLs and LPLs from lyophilized urine powder (90);
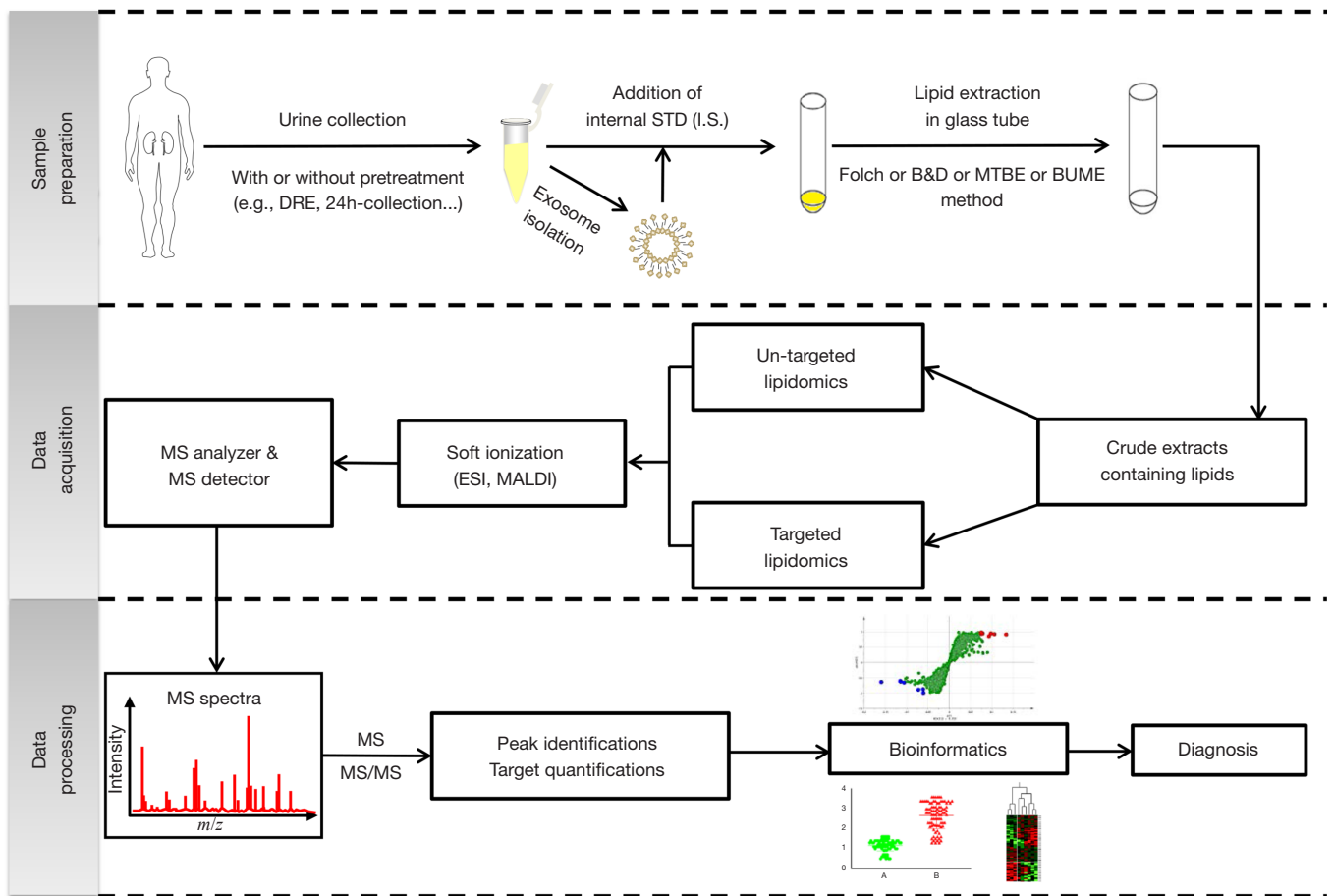
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Figure 2 A typical workflow for urinary PL and LPL study using MS. There are three main steps including sample preparation, MS-based data acquisition, and data processing in the urinary PL and LPL study using MS. Unfractionated urine samples or isolated urinary exosomes spiked with IS can be extracted using organic solvents. Two main approaches, untargeted or targeted lipidomics, can be selected for the analysis of urinary lipid extracts using ESI or MALDI platform. The untargeted lipidomics method is a direct ionization/analysis of the crude extracts without prior separation to obtain the full scanning of the lipidome; in targeted lipidomics, lipid extracts pass through a chromatography instrument to separate lipids based on different classes or molecular species before MS analysis. More detailed information regarding the MS processes is presented in section "Mass spectrometry-based phospholipidomics". The data processing step mainly focuses on the identification and quantification of lipid peaks obtained in the MS analysis. Identification of individual PLs can be achieved by detecting its characteristic molecular structure in tandem MS. Quantification of the identified PLs is based on the spiked IS with a known concentration in the urine sample. By comparing the relative intensities of the target and IS peaks, concentrations of the target can be calculated. Quantitative information regarding targets can be further evaluated by various bioinformatic methods/software to unravel potential biomarker candidates. Abbreviations: DRE, digital rectal examination; IS, internal standard; B&D, Bligh and Dyer lipid extraction method; MTEB, methyl tertiary butyl ether/methanol lipid extraction method; BUME, butanol/methanol lipid extraction method; MS, mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization.

Rockwell *et al.* used the B&D method for liquid urine samples (91); Skotland *et al.* used a modified Folch method for extracting lipids from urinary exosomes (92). Although demonstrating high PL recovery rates, the high toxicity of $CHCl_3$ has promoted the exploration of alternative

organic solvents/methods for achieving the goal of a "green extraction process". Matyash *et al.* developed a mixture of methyl tertiary butyl ether (MTBE)/MeOH for replacing $CHCl_3$ lipids and revealed similar or even better recoveries for species of most major lipid classes when compared with

the Folch or B&D methods in *E. coli*, mouse brain tissue, human plasma, and C. elegans embryos (14). Additionally, this method has been applied in lipid extractions from urine (93) and urinary exosome (94) in later studies. LLE using other solvents such as 1-butanol/MeOH (BUME) (13) has been performed for the evaluation of urinary PLs and LPLs (95). To optimize the process of the urinary PL and LPL workflow and select one suitable lipid extraction for MALDI based urinary PL evaluation, our group tested six different lipid extraction methods, including the Folch, B&D, acidified B&D, MTBE, BUME, and hexane/isopropanol (HIP). We observed that using MALDI-TOF/MS as a detection system with 9-aminoacridine as the matrix, the acidified B&D method showed excellent recovery rates for both positively and negatively charged PLs in urine (16). This method may offer a rapid and comprehensive screening for PLs and LPLs in urine samples.

Diverse analytical technologies

After the extraction of urinary lipids, the crude extracts are analyzed to exploit qualitative and quantitative information regarding the lipid class or individual molecular species using diverse techniques. Commonly used methods involve NMR, which is extremely suitable for PL identification but requires a relatively high sample content and expensive instrumentation, basic chromatography that simply separates lipid classes but cannot isolate individual lipid species, and MS-based lipidomics with high sensitivity, resolution, and throughput, which has become the primary choice for identification and quantification of urinary PLs and LPLs.

Chromatography-based phospholipidomics

Chromatography is a technique generally used for separating complex mixtures into simpler groups. There are three main basic methods, including TLC, HPLC, and gas chromatography (GC), commonly used for lipid studies. Among them, TLC (87) or HPLC (86) are more commonly utilized than GC for the detection of PLs as GC is best suited for volatile lipids that are thermally stable and have a molecular weight less than 500 Da (87).

TLC separates a sample mixture by migrating the organic solvents on a silica-coated glass plate. Samples deposited on a solid surface are separated based on affinity to the solvent, and molecules that are more preferred to the solvent (e.g., lipids) can move further up on the plate than those that prefer the solvent. The separated lipids can be visualized or further analyzed by MS. TLC can further resolve lipids by class, or by the length or saturation of acyl chains. The advantages of TLC include a simple and fast experimental process and inexpensive instrumentation, rendering it easy to implement in laboratories. However, the main disadvantage of TLC is the limited resolution that fails to separate individual lipid species, and thus demonstrating limited application in advanced lipid separation. Using TLC, Boonla *et al.* separated and identified major lipid classes in total lipid extracts obtained from urine and stones collected from patients with nephrolithiasis (96). In lipid research, a more important application of TLC is to combine TLC separation with MS analysis. The identification of PL species can be performed by coupling MS on the TLC plate (97).

The HPLC system separates each component in a mixture by pumping a liquid solvent containing the sample mixture (mobile phase) that flows through a solid column (stationary phase). All components within the sample mixture possess different molecular weights and slightly different affinities to the stationary and mobile phases, thus demonstrating different flow speeds, and finally leading to their separation while flowing through the column. HPLC is a versatile chromatographic technique that enables the separation of most lipids and PL classes, using a combination of numerous columns and solvents. Importantly, the coupling of HPLC with MS has dramatically increased the utility of HPLC (will be discussed later). Several previous studies have used the HPLC-MS technique for the sensitive detection of urinary PLs and LPLs (94,98,99).

NMR spectroscopy-based phospholipidomics

In addition to chromatography, different spectroscopic techniques have been used for lipid analysis. Among these, NMR spectroscopy is capable of providing the most detailed information regarding the lipid composition of a mixture. One convenient method in PL research is 31P NMR—when PL amounts are in the 100 μg range (20). By using a suitable solvent system, highly resolved 31P NMR spectra can be obtained in a few minutes, even from complex crude mixtures. These spectra allow the differentiation of all major PL classes and clear differentiation of the fatty acyl residues in PLs (100) (i.e., saturated, moderately unsaturated, and highly unsaturated lipids can be differentiated). Additionally, a positional analysis of the fatty acyl residues is possible using this technique (101). For instance, isomers

of LPC (sn1- and sn2-LPC) can be differentiated utilizing their different chemical shifts. However, NMR requires larger amounts of PLs than chromatography or MS (20). Another disadvantage is the high price of 31P NMR, limiting its application. Schiller and coworkers developed protocols for combining 31P NMR with MALDI-TOF/MS, revealing both excellent sensitivity detection, as well as precise identification and quantification of PLs (102).

Mass spectrometry-based phospholipidomics

As mentioned above, MS plays a central role in the analysis of PLs, either combined with chromatography or NMR. Typically, a mass spectrometer consists of three main parts: an ion source that generates ionized/vaporized analytes, a mass analyzer system that separates the ions according to their mass to charge (m/z) ratio, and a detector that records the signal intensities of different ionized molecules with different m/z values. By plotting the m/z value (x-axis) and signal intensity (y-axis), a mass spectrum can be displayed. Mass spectrometers can be classified into different types based on different types of ion sources and mass analyzers. Within the scope of PL studies, soft ionization methods involving ESI and MALDI are used to avoid molecular fragmentation. Mass analyzers used for PL analysis mainly include linear ion trap (quadrupole), triple quadrupole, time-of-flight (TOF), Orbitrap, and a hybrid of these techniques (103). The analytical chemistry background of these techniques is beyond the scope of this review. Thus, we focused on the introduction of established MS approaches for urinary PL studies—targeted and untargeted lipidomics using ESI-MS or MALDI MS platform.

Typically, in the ESI platform, according to whether prior chromatographic separation (usually HPLC) of the lipid mixture has been performed before the MS analysis, the ESI lipidomic approach for urinary PL evaluation can be divided into two strategies: untargeted lipidomics (also termed shotgun lipidomics) and targeted lipidomics (LC-ESI-MS/MS). Direct-infusion ESI untargeted lipidomics was developed by Han and Gross approximately 20 years ago and is currently one of the most widely used methods in PL and LPL studies (104). This was initially performed using direct-infusion ESI coupled with a triple quadrupole (Q) analyzer; however, presently, several newly developed technologies, including chip-based nanoLC-ESI, Q-TOF, Fourier transform–ion cyclotron resonance (FT-ICR), and orbitrap analyzers, have dramatically enriched the choice of available methods that enhance mass resolution and detection limits under various conditions (105). As the

goal of untargeted lipidomics is to identify and quantify the maximum number of lipid molecular species in a single run, the ability to measure all detectable lipids and quantify targets with just one non-endogenous internal standard for each lipid class remain the main advantages of this method. These advantages establish untargeted lipidomics as a promising strategy to screen PL biomarkers in complex biological samples such as urine. However, untargeted lipidomics is prone to the ion suppression effect observed with minor lipid molecules with poor ionization properties mediated by major lipid molecules with strong ionization properties. Another disadvantage is the failure or difficulty in separating isobaric and isomeric lipid molecules when analyzing complex samples.

Compared with the untargeted lipidomics strategy, targeted lipidomics utilizes a pre-separation before MS analysis using HPLC instruments. As mentioned above, this combined technology overcomes some drawbacks presented by untargeted lipidomics, permits effective separation of PL isomers and isobars, and reduces the ion suppression effects. There are three types of HPLC columns used for PL investigations: reversed-phase LC (RPLC), which separates PLs according to the length of fatty acid chains and the number of double bonds; normal-phase LC (NPLC), which separates PLs mainly based on chemical structures of the head groups; hydrophilic interaction chromatography (HILIC), a variant of NPLC that enables the effective separation of small polar compounds in polar stationary phases (24). The main disadvantages of targeted lipidomics include the complexity in choosing a suitable column for different target molecules and the need for several internal standards per lipid class as the species may spread across a wide retention time window.

Although the application of MALDI for lipid analysis is not as extensive as that of the ESI platform, interest in MALDI has continued to increase owing to the progress in instrumentation, as well as its obvious advantages of fast and highly sensitive data acquisition. A previous opinion regarding the disadvantage of MALDI MS analysis is the weak quantitative ability owing to uneven molecular distribution in the solid analyte-matrix crystal on the MALDI plate. However, this might apply to polar biomolecules (such as proteins), but not PLs (106). This is because both PLs and the matrix are readily solved in organic solvents, and by selecting proper solvents for lipid and matrix, homogeneity of matrix/analyte crystallization can be achieved, offering reliable quantification even in a short analysis (107-109). The other main drawbacks of

Table 2 Identified urinary LPLs and PLs in the positive ionization mode

No.	Assignment	Detected *m/z*	Adduct ion
1	LPC (16:0)	496.3/518.3	[M+H]$^+$/[M+Na]$^+$
2	LPC (18:2)	520.3/542.3	[M+H]$^+$/[M+Na]$^+$
3	LPC (18:1)	522.3/544.3	[M+H]$^+$/[M+Na]$^+$
4	LPC (18:0)	524.3/546.3	[M+H]$^+$/[M+Na]$^+$
5	LPC (20:0)	552.4/574.4	[M+H]$^+$/[M+Na]$^+$
6	PC (26:1)	648.5/670.5	[M+H]$^+$/[M+Na]$^+$
7	PC (26:0)	650.5/672.5	[M+H]$^+$/[M+Na]$^+$
8	PC (30:1)	704.5/726.5	[M+H]$^+$/[M+Na]$^+$
9	PC (32:5)	724.5/746.5	[M+H]$^+$/[M+Na]$^+$
10	PC (32:4)	726.5/748.5	[M+H]$^+$/[M+Na]$^+$
11	PC (32:1)	732.6/754.6	[M+H]$^+$/[M+Na]$^+$
12	PC (32:0)	734.6/756.6	[M+H]$^+$/[M+Na]$^+$
13	PC (34:5)	752.4/774.4	[M+H]$^+$/[M+Na]$^+$
14	PC (34:3)	756.6/778.6	[M+H]$^+$/[M+Na]$^+$
15	PC (34:2)	758.6/780.6	[M+H]$^+$/[M+Na]$^+$
16	PC (34:1)	760.6/782.6	[M+H]$^+$/[M+Na]$^+$
17	PC (34:0)	762.6/784.6	[M+H]$^+$/[M+Na]$^+$
18	PC (36:7)	776.1/798.1	[M+H]$^+$/[M+Na]$^+$
19	PC (36:6)	778.9/800.9	[M+H]$^+$/[M+Na]$^+$
20	PC (36:5)	780.4/802.4	[M+H]$^+$/[M+Na]$^+$
21	PC (36:4)	782.5/804.5	[M+H]$^+$/[M+Na]$^+$
22	PC (36:3)	784.5/806.5	[M+H]$^+$/[M+Na]$^+$
23	PC (36:2)	786.5/808.5	[M+H]$^+$/[M+Na]$^+$
24	PC (36:1)	788.5/810.5	[M+H]$^+$/[M+Na]$^+$
25	PC (38:4)	810.7/832.7	[M+H]$^+$/[M+Na]$^+$
26	PC (38:3)	812.4/834.4	[M+H]$^+$/[M+Na]$^+$
27	PC (38:2)	814.4/836.4	[M+H]$^+$/[M+Na]$^+$
28	SM (d18:1/16:0)	703.6/725.6	[M+H]$^+$/[M+Na]$^+$
29	SM (d18:1/20:0)	759.6/781.6	[M+H]$^+$/[M+Na]$^+$
30	SM (d18:1/22:0)	787.6/809.6	[M+H]$^+$/[M+Na]$^+$
31	SM (d18:1/24:1)	813.7/835.7	[M+H]$^+$/[M+Na]$^+$
32	SM (d18:1/24:0)	815.7/837.7	[M+H]$^+$/[M+Na]$^+$
33	PE (O-34:2)	702.5/724.5	[M+H]$^+$/[M+Na]$^+$
34	PE (34:2)	716.5/738.5	[M+H]$^+$/[M+Na]$^+$

Table 2 (*continued*)

Table 2 (*continued*)

No.	Assignment	Detected *m/z*	Adduct ion
35	PE (34:1)	718.5/740.5	[M+H]$^+$/[M+Na]$^+$
36	PE (34:0)	720.5/742.5	[M+H]$^+$/[M+Na]$^+$
37	PE (O-36:5)	724.5/746.5	[M+H]$^+$/[M+Na]$^+$
38	PE (36:4)	740.4/762.4	[M+H]$^+$/[M+Na]$^+$
39	PE (36:3)	742.4/764.4	[M+H]$^+$/[M+Na]$^+$
40	PE (36:2)	744.5/766.5	[M+H]$^+$/[M+Na]$^+$
41	PE (36:1)	746.6/768.6	[M+H]$^+$/[M+Na]$^+$
42	PE (O-38:5)	752.6/774.6	[M+H]$^+$/[M+Na]$^+$
43	PE (38:4)	768.5/790.5	[M+H]$^+$/[M+Na]$^+$

MALDI lipid analysis, such as matrix overlap of the analytes, could be resolved by the progress in matrix science (110), which has conferred MALDI a promising method in PL investigation, including urinary PLs and LPLs biomarker screening.

Similar to the ESI platform, MALDI can be placed in two strategies based on whether a chromatographic separation is performed prior to MS analysis. As MALDI is mainly recognized as a fast tool for sensitive molecule detection, the major application of this technique is the direct analysis of crude lipid extraction. Matrices applied in lipid research include 2,5-dihydroxybenzoic acid (DHB), α-cyanocinnamic acid (CHCA), and 9-aminoacridine (9-AA), or a mixture of selected options at an appropriate concentration ratio (110).

In our group, we achieved the sensitive detection of urinary PLs and LPLs in the femtomole grades using 9-AA as the matrix (16). We detected urinary PC, LPC, and SM in the positive ionization mode (summarized in *Table 2*), while analyzing PA, PI, PG, PE, and PS in the negative ionization mode (summarized in *Table 3*). The relative quantification of the urinary PLs and LPLs has been achieved by the addition of internal standards before lipid extraction. Only two internal/ionization standard species (i.e., non-endogenous commercial PC for the positive ionization mode and non-endogenous commercial PG for the negative ionization mode) can offer a reliable quantification of the bio-PLs by careful optimization of the matrix, solvent systems, and ionization modes (16). Herein, we defined and named this quantification method as a quantitative shotgun MALDI (qShot MALDI) lipidomics, which relies on non-

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Table 3 Identified urinary LPLs and PLs in the negative ionization mode

No.	Assignment	Detected m/z	Adduct ion
1	LPA (16:0)	409.2	[M-H]⁻
2	LPA (P-18:0)	421.3	[M-H]⁻
3	LPA (18:1)	435.3	[M-H]⁻
4	LPA (18:0)	437.3	[M-H]⁻
5	LPA (20:4)	457.3	[M-H]⁻
6	LPA (20:2)	461.3	[M-H]⁻
7	LPA (20:1)	463.3	[M-H]⁻
8	LPA (22:6)	481.2	[M-H]⁻
9	LPA (22:4)	485.3	[M-H]⁻
10	LPA (22:2)	489.3	[M-H]⁻
11	LPE (16:1)	450.3	[M-H]⁻
12	LPE (P-18:0)	464.3	[M-H]⁻
13	LPE (18:2)	476.3	[M-H]⁻
14	LPE (18:1)	478.3	[M-H]⁻
15	LPE (18:0)	480.3	[M-H]⁻
16	LPE (20:5)	498.3	[M-H]⁻
17	LPE (20:4)	500.3	[M-H]⁻
18	LPE (20:3)	502.3	[M-H]⁻
19	LPE (20:2)	504.3	[M-H]⁻
20	LPE (20:1)	506.3	[M-H]⁻
21	LPE (22:2)	532.3	[M-H]⁻
22	LPE (22:1)	534.4	[M-H]⁻
23	LPS (18:2)	520.3	[M-H]⁻
24	LPS (18:1)	522.3	[M-H]⁻
25	LPS (22:5)	570.4	[M-H]⁻
26	LPG (22:6)	555.3	[M-H]⁻
27	LPI (16:0)	571.3	[M-H]⁻
28	LPI (18:1)	597.3	[M-H]⁻
29	LPI (18:0)	599.3	[M-H]⁻
30	LPI (20:4)	619.3	[M-H]⁻
31	PA (23:2)	517.3	[M-H]⁻
32	PA (35:1)	687.5	[M-H]⁻
33	PA (36:2)	699.5	[M-H]⁻
34	PA (36:1)	701.5	[M-H]⁻
35	PA (38:6)	719.5	[M-H]⁻

Table 3 (*continued*)

Table 3 (*continued*)

No.	Assignment	Detected m/z	Adduct ion
36	PA (38:4)	723.5	[M-H]⁻
37	PE (28:0)	634.6	[M-H]⁻
38	PE (32:1)	688.6	[M-H]⁻
39	PE (34:1)	702.5	[M-H]⁻
40	PE (P-36:4)	722.5	[M-H]⁻
41	PE (36:2)	742.5	[M-H]⁻
42	PE (36:1)	744.6	[M-H]⁻
43	PE (38:4)	766.5	[M-H]⁻
44	PE (O-40:3)	778.6	[M-H]⁻
45	PE (40:4)	794.6	[M-H]⁻
46	PG (34:1)	747.5	[M-H]⁻
47	PG (34:0)	749.5	[M-H]⁻
48	PG (36:3)	771.5	[M-H]⁻
49	PG (36:2)	773.5	[M-H]⁻
50	PS (34:2)	758.5	[M-H]⁻
51	PS (34:1)	760.5	[M-H]⁻
52	PS (34:0)	762.5	[M-H]⁻
53	PS (36:4)	782.5	[M-H]⁻
54	PS (36:2)	786.5	[M-H]⁻
55	PS (36:1)	788.5	[M-H]⁻
56	PS (38:4)	810.5	[M-H]⁻
57	PS (38:1)	816.5	[M-H]⁻
58	PS (40:6)	834.5	[M-H]⁻
59	PS (40:4)	838.6	[M-H]⁻
60	PS (40:2)	842.6	[M-H]⁻
61	PS (40:1)	844.6	[M-H]⁻
62	PS (42:8)	850.6	[M-H]⁻
63	PS (44:12)	878.5	[M-H]⁻
64	PS (44:6)	890.6	[M-H]⁻
65	PI (32:0)	809.5	[M-H]⁻
66	PI (34:1)	835.6	[M-H]⁻
67	PI (36:4)	857.7	[M-H]⁻
68	PI (36:1)	863.6	[M-H]⁻
69	PI (38:6)	881.6	[M-H]⁻
70	PI (38:4)	885.5	[M-H]⁻
71	PI (40:6)	909.6	[M-H]⁻
72	PI (42:6)	937.6	[M-H]⁻

endogenous internal/ionization standard species to rapidly evaluate the relative PL concentration in crude extracts.

Furthermore, the pre-separation of lipid classes or species has been achieved by the ingenious combination of MALDI with chromatography in previous studies (111-113). In contrast to ESI, MALDI is usually linked with TLC plates but not LC separation because MALDI is mostly performed under vacuum and is not suitable for combination with LC. Utilizing TLC separation, PLs can be separated and subsequently analyzed by MALDI-TOF/MS, and PLs even less than 1% of the total extract can be sensitively and undoubtedly detected without overlap with other molecules, and matrix peaks can be obtained (111). Additionally, this method has been validated as a reliable system as the relative composition of PLs in the mixture remained constant and only marginal changes were observed in the fatty acyl composition during TLC separation (112).

Clinical application of urinary PLs and LPLs

Owing to the ease of sample collection and rapid development of analytical techniques such as MS, urine has become an attractive source of biomarkers for clinical diagnostics and/ or prognostics in various diseases, including genitourinary cancers and renal diseases. The clinical applications of urinary PL and LPLs as potential diagnostic markers for cancerous and benign diseases are summarized in *Table 4*.

PLs under normal physiological

Urine contains trace amounts of PLs under normal circumstances. Although present at markedly low concentrations, urinary species are quite variable. The Human Urine Metabolome Database (https:// urinemetabolome.ca/) has collected information regarding ~3,100 small molecule metabolites found in human urine, which includes approximately 600 PLs and LPLs. Furthermore, several previous studies reported interesting information about urinary PLs and LPLs under normal conditions. Using the nanoflow liquid chromatography-electrospray ionization tandem MS (nanoLC-ESI-MS/ MS) approach, Kim *et al.* performed a comprehensive analysis of human urinary PLs and identified 75 PL species including 22 PCs, 14 PEs, 15 PIs, 13 PSs, 7 PAs, and 4 PGs in healthy individuals (19). In another study that focused on normal urinary PL secretion (123), lipidomic profiles were quantified in urine samples collected from 16 healthy individuals (including 8 men and 8 women) at 5 fractions

over 24 h. Using the shotgun lipidomic approach, six subclasses of PLs (PC, PE, PE-O, PI, PG, and PS) were detected, and a sexual dimorphism between men and women was observed mainly for PC and PE species. In both men and women, PLs were highly correlated with the excretion of urinary creatinine and albumin.

PLs as versatile cancer biomarkers

Altered PL profiles in urine samples have been used to identify specific biomarkers and/or evaluate anticancer treatment in various cancer types, including genitourinary (prostate, bladder, and kidney) and BC.

Genitourinary cancers

As the most concerning disease in urology, urologic malignancies have attracted major investigations within the scope of urinary PL and LPL biomarker screening. Several researchers have selected urinary PLs and LPLs as sources of cancer biomarker candidates because of their noninvasive availability. Min *et al.* performed qualitative and quantitative profiling of urinary PLs and LPLs using nanoLC-ESI-MS/MS in urine samples collected from 9 prostate cancer (PCa) patients, compared with 10 healthy control individuals. In total, 70 PLs, including 21 PCs, 11 PEs, 17 PSs, 11 PIs, 7 PAs, and 3 PGs were identified (90). Among them, two PS species [PS (18:0/18:1) and PS (16:0/22:6)] showed significantly higher concentrations, while the other two PS [PS (18:1/18:0) and PS (18:0/20:5)] and two PI species [PI (18:0/18:1) and PI (16:1/20:2)] presented significantly lower concentrations in the PCa patient group when compared with the healthy control group. Yang *et al.* investigated the size distributions, as well as the lipid compositions of urinary exosomes isolated from pooled urine samples from four PCa patients and healthy controls each. Notably, the PCa group demonstrated an increased exosome size. Moreover, 286 lipids, including 118 PL species from urinary exosomes, were identified and quantified. The lipidomic data revealed a general increase in PL amounts in smaller-sized exosomes isolated from PCa patient samples. Among them, PG (22:6/22:6) was approximately 7.5 times higher in the cancer group (94). Additionally, using urinary exosomes, another group of researchers (92) performed high-throughput MS quantitative lipidomic analysis and identified 107 lipid species including 36 PLs. On comparing 15 PCa patients and 13 healthy controls, urinary exosome-derived PC (16:0/18:2) was significantly higher in the PCa group,

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Table 4 List of PLs and LPLs identified as potential diagnostic markers in urine

Diseases		Sample type	Analytical platform	Discriminatory PL/LPL species	Refs
Cancers	Prostate	Urine	nanoLC-ESI-MS/MS[†]	↑PS(18:0/18:1), ↑PS(16:0/22:6), ↓PS(18:1/18:0), ↓PS(18:0/20:5), ↓PI(18:0/18:1), ↓PI(16:1/20:2)	(90)
	Prostate	Urinary exosome	nanoLC-ESI-MS/MS[‡]	↑PG (22:6/22:6)	(94)
	Prostate	Urinary exosome	LC-ESI-MS/MS[§]	↑PC(16:0/18:2), ↓PS(16:0/18:1), ↓PS(18:1/18:1), ↓PS(18:0/18:1)	(92)
	Prostate	Urinary exosome	LC-ESI-MS[¶]	↓PC(18:2/20:4), ↓PC(14:0/20:4)	(98)
	Kidney	Urinary exosome	LC-ESI-MS[§]	↑PA(28:0), ↑PE(32:2), ↑PG(35:4), ↑PC(46:5)	(114)
	Breast	Urine	nanoLC-ESI-MS/MS[†]	↑PC(16:1/16:0), ↑PC(16:0/18:2), ↑PC(18:1/16:1), ↑PC(16:0/16:0), ↑PE(16:0/20:4), ↑PE(16:0/18:2), ↑PE(20:0/18:4)	(115)
	Breast	Urine	nanoLC-ESI-MS/MS[†]	↑PS(18:1/18:1), ↑PS(18:2/18:0), ↓PI(18:0/20:4)	(116)
	Breast	Urine	LC-ESI-MS[§]	↓LPE(18:2), ↓LPC(14:1), ↓LPE(20:4), ↑PG(O-36:1), ↑PA(O-16:0), ↑PC(34:1), ↑PA(37:6), ↑PA(33:3)	(117)
Benign Diseases	DN	Urine	LC-ESI-MS/MS[¶]	↑LPA(16:0), ↑LPA(20:4), ↑LPC(16:0), ↑LPC(18:2), ↑LPC(18:1), ↑LPC(18:0)	(118)
	FSGS	Urine	LC-ESI-MS[§]	↑LPC(14:0), ↑LPC(18:1), ↓PC(38:4)	(119)
	CKD	Urine	LC-ESI-MS/MS[¶]	↑PC(16:0/16:0), ↑PC(16:0/22:3), ↑PC(16:0/18:1), ↑PC(16:0/18:2)	(120)
	UUO	Urine (Rat mode)	LC-ESI-TOF/MS	↑PE(34:1), ↑PE(34:2), ↑PE(32:1), ↑PE(36:2), ↑PG(34:1), ↑PG(32:1), ↑PG(34:2), ↑PG(36:2)	(121)
	PD (with LRRK2 mutation)	Urine	LC-ESI-MS/MS[§]	↑di-(18:1)-BMP, ↑di-(22:6)-BMP	(122)

(↑) indicates increased PL or LPL level in the patient sample, (↓) indicates decreased level in the patient sample. LC, liquid chromatography; ESI, electrospray ionization; TOF, time of flight; DN, diabetic nephropathy; FSGS, focal segmental glomerulosclerosis; CKD, chronic kidney disease; UOO, unilateral ureteral obstruction; PD, Parkinson's disease; PL, phospholipid; LPL, lysophospholipid; PA, phosphatidic acid; LPA lysophosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; LRRK2, leucine-rich repeat kinase 2; BMP bis(monoacylglycerol) phosphate. MS analyzer techniques: [†]Ion trap; [‡]ion trap-orbitrap; [§]quadrupole TOF; [¶]triple quadrupole.

whereas 3 PS species [PS (18:0/18:1), PS (16:0/18:1) and PS (18:1/18:1)] revealed significantly lower concentrations in the same group. The combination of several PL molecules showed a clear separation between cancer patients and healthy controls, which might be used as a diagnostic index. Clos-Garcia *et al.* (98) performed a targeted metabolomics analysis of urinary extracellular vesicles (EVs) and compared results between 31 PCa patients and 14 benign prostate hyperplasia (BPH) controls. These results showed decreased levels of PCs [e.g., PC (18:2/20:4), PC (14:0/20:4)] in the PCa group when compared with the BPH group.

In contrast to PCa, urinary biomarker studies of bladder or kidney cancer are limited, although urine is considered a good source for screening specific PL biomarkers in these two diseases. A previous study (114), which utilized a microLC-Q-TOF-MS platform for lipidomic profiling of urinary exosome, was performed using urine samples from 8 renal cell carcinoma (RCC) patients and compared with 8 healthy controls. The results showed that some PL species such as PA (28:0), PE (32:2), PG (35:4), and PC (46:5) were only detected in the RCC group, and could be applied as potential biomarkers for RCC.

Other cancer types

To the best of our knowledge, except in genitourinary cancers, other applications of urinary PLs and LPLs as

specific biomarker candidates are mainly in BC. Kim and coworkers (115) qualitatively and quantitatively analyzed urinary PCs and PEs extracted from 5 patients with BC (both before and after surgery) and 5 non-cancer controls. In total, 21 PCs and 12 PEs were identified, 4 urinary PC species [PC (16:1/16:0), PC (16:0/18:2), PC (18:1/16:1), and PC (16:0/16:0)] increased by over two-fold in patients with BC, and these PC species decreased to normal or undetectable levels after surgery. Similarly, in PE, some species, including PE (16:0/20:4), PE (16:0/18:2), and PE (20:0/18:4), were prominently decreased after surgery.

In another study, the same group (116) focused on four other types of PLs (PA, PS, PG, and PI) for screening BC biomarker candidates. Overall, 34 urinary PLs (including 6 PAs, 12 PSs, 4 PGs, and 12 PIs) were quantitatively examined in urine samples collected from 5 patients with BC (both before and after surgery) and 5 non-cancer controls. Among them, two PS species [PS (18:1/18:1) and PS (18:2/18:0)] showed significantly higher concentrations in the BC patient group than in the control group, and their concentrations were found to be reduced to normal levels after surgery. Moreover, one PI molecule [PI (18:0/20:4)] showed a significantly lower concentration in the BC group, decreasing even further after surgery. Cala *et al*. (117) evaluated urinary lipid alterations in Hispanic Colombian women diagnosed with BC and observed that some LPLs, including LPE (18:2), LPC (14:1), and LPE (20:4), were decreased, whereas PLs such as PG (O-36:1), PA (O-16:0), PC (34:1), PA (37:6), and PA (33:3) were increased in women with BC than in healthy controls.

PLs in benign diseases

Some urinary LPA and LPC species, which are known to promote renal inflammation, were evaluated in urine samples obtained from 41 type II diabetic patients with nephropathy symptoms and 41 type II diabetic controls without nephropathy symptoms (118). The results indicated that the concentrations of six urinary LPA and LPC species [LPA (16:0), LPA (20:4), LPC (16:0), LPC (18:2), LPC (18:1), and LPC (18:0)] were significantly higher in diabetic nephropathy cases than in the non-nephropathy controls (118). In another study investigating renal disease (119), urinary lipid profiles were determined in patients with focal segmental glomerulosclerosis (FSGS) and healthy controls. The results showed an increased concentration of LPC (14:0) and LPC (18:1)

and decreased PC (38:4) in patients with FSGS. These species could be used as biomarkers for the diagnosis of FSGS and help understand the mechanism of tubular and podocyte damage in FSGS (119). Yang and coworkers evaluated the concentration of urinary PLs extracted from 26 patients with chronic kidney disease (CKD) (including glomerulonephritis cases and tubulointerstitial injury cases), as well as 13 healthy controls (120). The results showed that some PC species [PC (16:0/16:0), PC (16:0/22:3), PC (16:0/18:1), and PC (16:0/18:2)] were significantly higher in the patient group than in the healthy control group.

Using the unilateral ureteral obstruction (UUO) rat model, Yoshioka and coworkers compared the urinary PL profiles before and after UUO for two weeks. They reported that PE and PG with monoenoic or dienoic fatty acyl chains [PE (34:1), PE (34:2), PE (32:1), PE (36:2), PG (34:1), PG(32:1), PG (34:2), and PG (36:2)] were significantly higher in the UOO group than in the control cases, and thus could be used as potential biomarkers for kidney injury caused by UOO (121). Regarding neurological disorders, a previous study (122) focused on urinary bis (monoacylglycerol) phosphate (BMP, a special LPA species) levels in the leucine-rich repeat kinase 2 mutation carrier (LRRK2 mutation, the most common genetic cause of Parkinson's disease), revealing that four urinary BMP isoforms were significantly higher in LRRK2 mutation carriers than in non-carriers, correlating with worse cognitive status.

Future aspects

Despite dramatic advancements in techniques (especially MS) for the identification and quantification of urinary PLs and LPLs, allowing the detection of numerous urinary PL and LPL species, there remain several challenges for the reliable detection of PLs at markedly low concentrations, which have great potential as disease-specific biomarkers. This relies on continued improvements in sample preparation methods (especially for urinary exosome study), as well as detection methods. Furthermore, the poor inter- and intra-laboratory reproducibility of urinary PL and LPL quantification and biomarker validation should be closely considered owing to diverse methodologies and limited cohort sizes in previous studies. Furthermore, bioinformatics or analytical strategies for combining urinary lipidomic profiling with other omics (e.g., proteomics) are required to thoroughly understand the metabolic pathways involved in disease development.

Conclusions

PLs have diverse biological functions in cells and play important roles in disease development, thus presenting immense potential for use as biomarker candidates. Urine is a versatile clinical sample allowing convenient and noninvasive collection, and is considered an ideal source of novel PL biomarkers. Although present at extremely low concentrations, the detection of urinary PLs and LPLs is now feasible owing to the rapid development of analytical techniques, revealing attractive clinical prospects in the screening of biomarkers. In previous studies, several urinary PLs or urinary exosome PLs have been determined as biomarker candidates. With further method development for analyte detection and data processing, urinary PL applications will provide a promising future for disease biomarker screening in clinical practice.

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Footnote

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