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Review Article

Urinary metabolites for urological cancer detection: a review on the application of volatile organic compounds for cancers

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Abstract: Cancer is one of the most devastating human diseases that causes a great number of mortalities each year worldwide. Thus, finding and treating cancers early is of increasing interest to the public and presents great opportunity for research. It is well known that the metabolism of cancer cells differs from that of normal tissues. Analysis of volatile organic compounds (VOCs), a group of small molecule metabolites, provides an emerging approach for cancer screening and disease monitoring. VOCs are continuously generated in human body and released through breath, blood, skin, urine and fecal samples, which carry information of the physiological and metabolic status. Furthermore, the development of effective analytical methods for VOCs detection is one of the challenging aspects in cancer research. In this review, the analytical methods such as solid-phase mirco-extraction (SPME) and stir bar sorptive extraction (SBSE) coupled with gas chromatography/mass spectrometry (GC-MS), the application of VOCs in urological cancers diagnosis and potential molecules pathways related to VOCs profile for cancer detection are discussed.

Keywords: Urine, metabolomics, cancer, diagnosis, volatile organic compounds (VOCs)

Introduction

Cancer is one of the most devastating human diseases that causes a vast number of mortalities each year worldwide. Since President Richard Nixon declared war on cancer in 1971, the US spends billions of dollars to develop better drugs and therapies that might control cancer cells, but it has yielded insufficient results: the overall cancer mortality rate in the US has fallen by a scant 8 percent since 1975 as heart disease deaths have dropped by nearly 60 percent in that period, by comparison. While the cure-driven approach has dominated the cancer research, finding and treating cancers early continue to present a great research opportunity for science. It is well accepted that cancer metabolism differs from that of normal tissue. An important hypothesis published in the 1950s by Otto Warburg proposed that cancer cells rely on anaerobic metabolism as the source for energy, even under physiological oxygen levels. As a result, cancer central carbon metabolism has been researched extensively. Cancer is known to involve a wide range of metabolic processes, and many more are still to be unveiled. Studying cancer through metabolomics could reveal new biomarkers for cancer that could be useful for its future prognosis, diagnosis and therapy. Using a metabolomics approach, it is possible to detect a range of metabolites in a single assay and therefore metabolomics can be defined as a holistic and data-driven study of the low molecular weight metabolites present in biological systems.

Among the low molecular weight metabolites, volatile organic compounds (VOCs), the majority being organic in nature, are continuously generated in human body and released through breath, blood, skin, urine and fecal samples (**Figure 1**) [1-4]. These VOCs carry information of the physiological and metabolic status of the individual [5]. As VOCs are considered the metabolites of biological activities in human body, they exist in our system as a result of pathological processes and a consequence of

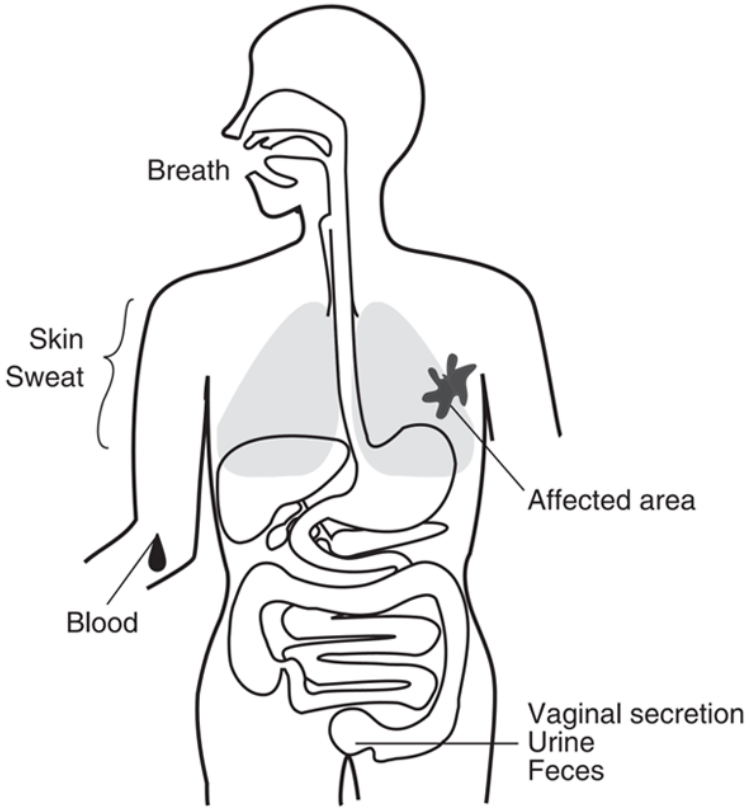

Figure 1. Volatile organic compounds (VOCs) are continuously generated from human body and released through breath, blood, skin, urine and feces. (*Source*: M. Shirasu and K. Touhara, 2011. Copyright © 2011 The Journal of Biochemistry. [10]).

disease. Recent studies have demonstrated that dogs can differentiate cancer patients from control negative by sniffing their biological samples, such as urine [6-9]. The VOCs emitted from human body can be considered as individual 'odor-fingerprints' [10]. Therefore, VOCs could be used as predictive biomarkers for disease detection. In this review, the generation, the analysis and the application of VOCs in urological cancers diagnosis are discussed. Some of the most noteworthy research in the field is highlighted.

VOCs emitted from human body

VOCs in blood

Blood directly reflects the internal environment of the body, including nutritional, metabolic and immune status, which highly values the blood samples in disease-specific VOCs studies. The specific VOCs in the blood have been reported to be useful in predicting and diagnosing diseases, such as ovarian cancer, colorectal cancer, lung cancer, and hepatic encephalopathy [11-14]. In the study of Horvath *et al*. [11], the trained dogs could differentiate ovarian cancer patients from the patients with other gynecological cancers and healthy control subjects through sniffing the blood samples from patients. Wang *et al*. [12] carried out a study to identify the blood volatile compounds as biomarkers for colorectal cancer by collecting blood samples from 16 colorectal cancer patients and 20 healthy controls. Four metabolic biomarkers were found at significantly higher or lower level in cancer patients. However, obtaining blood samples is invasive and pre-treatment of blood samples is also time-consuming. These factors have limited the use of VOCs in blood for diagnostic tool development. Further studies are needed to evaluate these results and to apply these findings to clinical diagnoses.

VOCs in breath

Exhaled breath contains VOCs that can be attributed to either exogenous or endogenous volatiles [15, 16]. Endogenous volatiles consist of blood-borne compounds released to the environment via the lungs and/or compounds made from all classes of symbiotic bacteria. Numerous studies were conducted to investigate the potential of VOCs in breath in diseases diagnosis, especially lung cancer. Collecting breath samples is relatively simple, painless and non-invasive as compared to sampling blood. Phillips *et al.* [17] collected breath samples from 108 patients and a combination of 22 VOCs in breath samples distinguished between patients with and without lung cancer. In the study of Peng et al. [18], an array of sensors based on gold nanoparticles were shown to be able to rapidly distinguish the breath of lung cancer patients from the breath of healthy individuals by training and optimizing sensors with the VOCs identified through gas chromatography/mass spectrometry (GC-MS). Exo-

genous volatiles include compounds inhaled from the external environment, such as compounds produced following the oral ingestion of food and compounds derived from smoking cigarettes. It is always challenging to distinguish exogenous compounds of environmental contaminants from endogenously produced VOCs.

VOCs in urine

The VOCs in urine are considered intermediate or end products of metabolic pathways, and may contain a variety of structural motifs, such as ketone, alcohol, furan, pyrrole and sulfide with a particular odor [10]. In some cases, characteristic urine VOCs profile have been directly linked to particular metabolic disorders. Some studies have linked urinary VOCs profiles to infectious diseases [19, 20] and different types of cancers, including prostate cancer (PCa) [21], renal cancer (RCa) [22] and bladder cancer (BCa) [23]. Urinary VOC patterns in cancer patients are often different from the patterns in urine samples from control subjects, although the differences depend on cancer types and even cancer stages. Khalid *et al.* [21] showed that urinary VOCs profile of prostate cancer patients can be discriminated from cancer free controls by using four VOCs, 2,6-dimethyl-7-octen-2-ol, pentanal, 3-octanone, and 2-octanone with accuracy as high as 71%. In an analysis of volatile human urinary metabolome for renal cell carcinoma (RCC), Monteiro *et al.* [22] reported that the volatile urinary metabolome could discriminate between RCC and control patients with 60.33% of the variability in principal component analysis (PCA). And according to Weber *et al*. [23], the best diagnostic performance they obtained through the comparison between healthy volunteers and bladder cancer patients was 70% overall accuracy using a gas sensor array and pattern recognition.

These studies have proved the potential in searching for volatile diagnostic biomarkers in the urine of cancer patients. Due to the complexity of urine components, such as metabolites from ingested foods and drinks, and considerable variation among individuals, caution must be taken when determining the source of candidate VOC biomarkers resulting from disease-related changes in metabolism and advanced computer processing of chromatographic data should be involved in identifying the VOC patterns.

VOCs in other biospecimen

VOCs can also be continuously emitted from skin as sweat. Sweat is one of the less employed bio-fluids for discovery of markers. In the research conducted by Calderón-Santiago *et al.* [24], human sweat was collected and used as clinical sample to develop a screening tool for lung cancer. The five metabolites identified in this study provided 80% specificity and 79% sensitivity to discriminate between patients with lung cancer versus smokers as control individuals. Mi-Jung *et al*. [25] also applied the analysis of sweat volatile organic compounds in forensic science. Although VOCs in sweat could result from internal hormonal or metabolic changes, many VOCs appear to be derived from symbiotic bacteria that live on the skin surface which then metabolize and transform secreted compounds in sweat and sebum. Any alteration in homeostatic balance due to some inherited metabolic disorder or bacterial infection of the diseased area can induce changes in both the quality and quantity of VOCs. For example, some infectious diseases or cancerous wounds develop characteristic and offensive odors [10]. Therefore, the contamination from the environment must also be taken into consideration, including the interference from the ambient air, humidity and cosmetics.

Human fecal samples represent dietary endproducts resulting from digestive and excretory processes and intestinal bacterial metabolism. The investigation of fecal VOCs may reveal potential health consequences and be the best non-invasive way of diagnosing gastrointestinal diseases. Distinct patterns of VOCs have been associated with fecal samples from patients with some types of bacterial infection, such as Vibrio cholera, Clostridium difficile or Campylobacter jejuni infections [26, 27]. Batty *et al.* [28] reported the use of fecal volatile metabolome in screening for colorectal cancer with 78% specificity and 72% sensitivity. VOCs may also be contained in other types of bio-fluids, such as vaginal secretions, which accurately reflect the stages of menstrual cycles

In summary, VOCs can be emitted from different types of biological fluids of human body and carry “odor fingerprint” of the individuals (**Table 1**). Pathological processes can influence our daily odor fingerprints by producing new VOCs or by changing the ratio of VOCs that are produced normally [10]. These VOCs could poten-

Table 1. VOCs, as "odor fingerprint", could be emitted from different types of biological samples of human body

The origins of odor	Published Paper	Disease Detection	Method	Sample Size	Study Results	
					# of VOCs	How reliable?
Blood	Horvath *et al*. [11]	Ovarian cancer	Trained dogs	N/A	N/A	Tissue tests: 100% sensitivity and 95% specificity. blood tests: 100% sensitivity and 98% specificity
	Wang *et al*. [12]	Colorectal cancer	SPME-GC-MS	16 cancer patients and 20 healthy controls	4	Lower level VOCs (P<0.01): Higher level VOCs (P<0.05):
Breath	Phillips *et al.* [17]	Lung cancer	GC-MS	60 cancer patients and 48 non-cancer controls	22	100% sensitivity and 81.3% specificity
	Peng et al. [18]	Lung cancer	Sensors based on gold nanoparticles	N/A	42	Accuracy >86%
Urine	Khalid *et al.* [21]	Prostate Cancer	SPME-GC-MS	59 cancer patients and 43 non-cancer controls	4	AUC 0.76 Accuracy as high as 74%
	Monteiro *et al.* [22]	Renal cell carcinoma	GC-MS	N/A	N/A	N/A
	Weber *et al*. [23]	bladder cancer	Gas sensor	30 cancer patients and 59 non-cancer controls	N/A	70% overall accuracy; 70% sensitivity and 70% specificity
Sweat	Calderón-Santiago *et al.* [24]	Lung cancer	LC-MS	41 cancer patients and 55 non-cancer controls	16	specificity/sensitivity pair (80 and 79%
Feces	Batty *et al.* [28]	Colorectal cancer	Ion flow tube mass spectrometry (SIFT-MS)	31 high risk patients and 31 low risk or non-cancer controls	N/A	Accuracy 75% with 78% specificity and 72% sensitivity

AUC: Area Under the receiver operating characteristic curve.

tially be the markers for clinical diagnosis and therapeutic monitoring of diseases. However, those VOCs may be affected by various factors, such as age, sex, drug therapy, diet and smoking. Therefore, care must be taken when investigating disease related VOCs in clinical samples.

Extraction and detection of VOCs as potential method for disease diagnosis

VOCs extraction

Since the low concentration of VOCs presents in various biological specimen, the extraction and pre-concentration are crucial for the analysis of VOCs of interest [29] and may affect the reliability and accuracy of the analysis [30].

To increase the reproducibility, selectivity, and extraction capacity of the sample preparation steps, several extraction techniques were developed to facilitate rapid and efficient preparation processes of VOCs [31]. For example, solid-phase micro-extraction (SPME) technique (**Figure 2**) uses a fine bare fused silica fiber or a fine silica fiber coated with a thin layer of a selective coating (either solid or liquid) to extract organic compounds directly from aqueous samples for instrumental analysis by Gas Chromatography (GC) or Gas Chromatography/Mass Spectrometry (GC-MS) [32]. There are two types of extraction based on the different samples: 1) the direct immersion SPME which is immersing the fiber to extract VOCs in liquid samples, and 2) the headspace SPME by suspending fiber in the headspace above the liquid phase. The analytes are firstly adsorbed during extraction on the surface of the fiber materials as a result of chemical bonding, and then absorbed into the coating materials [33]. There are four types of polymers widely used as the coating materials, polydimethylsiloxane (PDMS), divinylbenzene (DVB), polyacrylate (PA), and polyethyleneglycol (PEG). Those materials could also be used through the combination blended with carboxen (CAR) [34]. After preconcentrating, the fiber with analytes trapped on its coating materials is injected to the instruments and release analytes through thermal desorption. Deng *et al*. [13] developed a simple, rapid and sensitive SPME/GC-MS method for the investigation of volatile biomarkers in blood for lung cancer. Poli *et al*. [30] evaluated the potential of aldehydes from exhaled breath in the diagnosis of non-small cell lung cancer by means of SPME/GC-MS with 93% accuracy and precision between 7.2-15.1%. Monteiro *et al.* [22] studied the volatile human urinary metabolome difference of RCC and healthy individuals through the headspace SPME sampling coupled with gas chromatography-ion trap/mass spectrometry (GC-IT/MS). Wang *et al*. [12] analyzed the VOCs in the blood samples from colorectal cancer patients and healthy controls with headspace SPME sampling. Khalid *et al.* [21] also applied SPME in headspace of urine samples to identify the specific urinary VOCs for the detection of prostate cancer.

Similar to the theory of SPME, another novel approach for sample enrichment is referred as stir bar sorptive extraction (SBSE), which was developed by Baltussen *et al*. [35]. SBSE technique uses stir bars coated with the sorbent PDMS. The results of experiments conducted by Baltussen *et al*. indicated that the stir bars present higher efficiency than SPME in the preconcentration of analytes from aqueous samples, with up to a 500-fold increase in sensitivity when stirring between 30 to 60 min. The high efficiency could be contributed to the increased amount of PDMS coated on the stir bars. Furthermore, the volatile compounds can also be easily and conveniently handled due to the absence of drying step. Therefore, SBSE can be applied in the analysis of VOCs in different types of aqueous samples, as well as the biological fluids. Melo *et al*. [36] carried out an analysis of antidepressants in plasma samples using SBSE and liquid chromatography (LC) with high extraction efficiency. Soini *et al.* [37] showed a high reproducibility of using SBSE in quantitative comparisons of the urinary profiles with relative standard deviations (RSD) of 1-5% for a wide range of compounds. In one of our studies, SBSE was successfully applied in identifying the specific urinary volatile organic compounds for the diagnosis of prostate cancer [38]. In addition, the coated stir bar could also be used as headspace sorptive extraction (HSSE) [39, 40] (**Figure 3**).

Other less common extraction techniques, such as purge and trap [41], single drop microextraction [42], were also applied in VOCs extraction. However, their applications in VOCs analysis is relatively limited due to the sensitivity concerns.

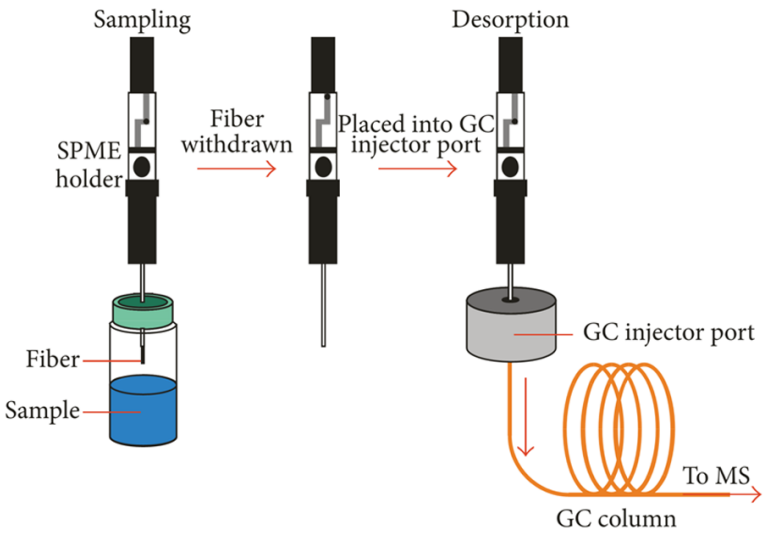

Figure 2. The use of solid phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS) (Source: Kamila Schmidt and Ian Podmore, 2015. Copyright © 2015 The Journal of Biomarkers. [29]).

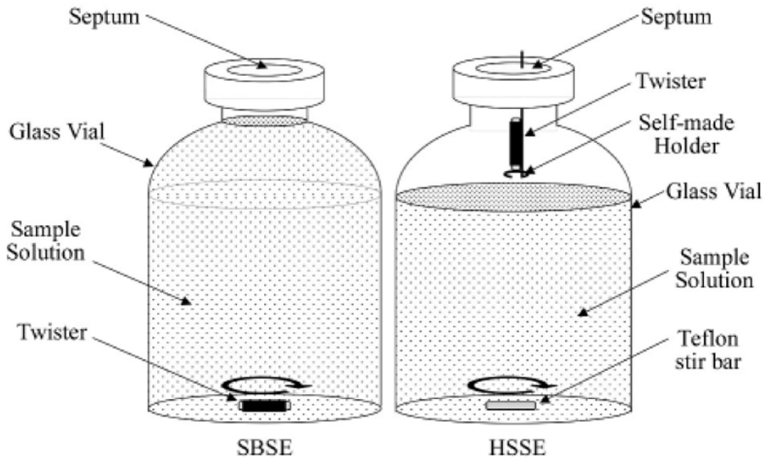

Figure 3. The set-ups of SBSE and HSSE. (Source: Ochiai *et al*., 2001. Copyright © 2001 The Royal Society of Chemistry. [40]).

VOCs detection

VOCs are easy to be detected by using analytic instruments, such as GC-MS, proton transfer reaction-mass spectrometry (PTR-MS), selected ion flow tube-mass spectrometry (SIFTMS), or gas sensors [21, 29, 43-45]. As one of the most commonly used analytical technique, GC-MS is widely used in the investigation of VOC biomarkers because of its sensitivity and reliability in analyte identification [12, 13, 22, 30, 38]. Studies have shown that GC-MS provides an outstanding sensitivity at ppb (parts per billion) and low ppt (parts per trillion) levels in VOC analysis with the pre-concentration steps [46, 47]. Fuchs *et al.* [46] analyzed aldehydes from the breath samples of lung cancer patients using GC-MS. The concentrations in their study ranged from 7 pmol/l (161 pptV) for butanal to 71 nmol/l (1,582 ppbV) for formaldehyde. In another study using GC-MS conducted by Ligor *et al.* [47], the limit of detection was in the range of 0.05 to 15.00 ppb. Meanwhile, it provides the most detailed information of VOCs profiles and identifies analytes with most certainty. However, GC-MS instruments are often expensive. Compared to GC-MS, PTR-MS and SIFT-MS do not require a pre-concentration step and can work in real time, which make these two better instant quantification techniques for VOCs analysis [48, 49]. Wehinger *et al.* [48] identified VOCs in the exhaled breath using PTR-MS to discriminate the primary lung cancer patients and controls. As mentioned in this study, even though the technique is simple and time-saving for larger clinical evaluation, it is not possible for PTR-MS to differentiate between compounds with the same molecular mass.

Some other detection techniques are also used in the analysis VOCs emitted from human, such as ion mobility spectrometry (IMS) [50]. Compared with GC-MS, IMS gives a tenfold higher detection rate of VOCs (500 seconds for IMS vs. 1 h for GC-MS per sample). In one study of detecting VOCs in exhaled breath of patients with lung cancer, the IMS was used by Westhoff *et al.* [50] and a combination of 23 peak regions were identified to discriminate the cancer patients and controls without error.

In addition, several types of electronic noses have been used in the studies of VOCs in cancer [18, 23, 51]. Natale *et al.* [51] investigated

the possibility of using electronic nose to identify the lung cancer patients from controls. The results in their study indicated a 100% of classification of lung cancer affected patients and 94% of controls. These sensors used in this study showed a good sensitivity towards the compounds identified previously as potential lung cancer markers. However, electronic noses are designed to recognize the VOCs found in established studies but not to identify any unknown VOC patterns. Compared to the mass spectrometry based techniques, the electronic nose is less time consuming and enables the potential of cheap, rapid, simple, and miniature detection devices [52, 53]. However, electronic noses are sensitive to moisture, less sensitive, and with poor reproducibility [54, 55]. Additionally, electronic noses can only allow the semi-quantitative detection of VOCs [56].

VOCs and urological cancers

Cancer is a leading cause of death and disability globally, impacting more than 14 million people each year [57]. Urological cancers, such as prostate cancer (PCa), renal cancer (RCa), and bladder cancer (BCa), are a major cause of morbidity and mortality worldwide [58]. In 2018, about 164,690 new cases of PCa, 65,340 of RCa, 81,190 of BCa and about 29,430 deaths in PCa, 14,970 in RCa, 17,240 in BCa are estimated in United States according to the American Cancer Society [59]. In the United States, PCa is the most common cancer and the third leading cause of death in men [59]. RCa and BCa also account for more than 2% and 4% of cancer mortality in the United States [59].

Diagnosis and treatment for these urological cancers are associated with different but overlapping clinical challenges [58]. High-throughput genomic screening, proteomic profiling, and metabolomics analysis of related functional protein molecules provide a large amount of informational data and overview of clinical changes of cancer development and progression. The cells, proteins, and metabolites in urine originated from kidney, prostate, and bladder could provide information for biomarkers searching, such as genomics, proteomics, and metabolomics [60-62]. Urine, as a source of excretion from the urological system, is an ideal body fluid for the investigation and detection of biomarkers for those urological cancers. Moreover, urine collection is an easy and non-invasive procedure which increases the feasibility of point-of-care clinical application.

As early diagnosis and treatment of those urological cancers will improve the quality of care and reduce mortality, there is a high demand of reliable, quick and patient-friendly diagnostic method for cancer screening. As aforementioned, several studies have demonstrated that sniffer dogs can differentiate cancer patients from controls by sniffing their urine [6-9]. Cornu *et al.* [7] reported the trained dog detected PCa by smelling urine with 91% of both sensitivity and specificity. The study from Willis *et al.* [9] also provided further evidence that volatile compounds found in urine can be identified by trained dogs with 73% sensitivity and 92% specificity. Additionally, VOCs are easy to be detected by using analytic instruments, like gas chromatography-mass spectrometry (GC-MS), or further developed gas sensors [21, 43-45]. All of those proved that VOCs, particularly in urine, could be desirable disease markers for their non-invasiveness, easy detection, high sensitivity and high specificity. As one of the most promising metabolomics approaches in cancer detection, the analysis of VOCs can potentially serve as a safe, non-invasive, and specific test for the early detection of those urological cancers.

VOCs in prostate cancer

Currently, PCa are screened by the prostate-specific antigen (PSA) blood test and/or the digital rectal exam (DRE). If the PCa is suspected based on the results of screening tests or other symptoms, further tests, such as prostate biopsy will be required to confirm the diagnosis [63]. Furthermore, techniques used in advanced stages, such as bone scans, computed tomography (CT) scan, and magnetic resonance imaging (MRI), may involve X-rays, magnetic fields, sound waves and radioactive substances which can lead to the second injury of cancer patients [63]. Diagnostic methods which can reduce stress and be more patient-friendly are needed.

Adding to the fact that PSA is not cancer specific, there is no reliable PSA threshold that can accurately distinguish men with or without cancer [64] resulting in over-diagnose of the dis-

Table 2. Comparison in sensitivity, specificity, and AUC from various biomarkers in prostate cancer diagnosis

	VOCs based biomarkers [21, 38]	Other potential biomarkers					
		Iso-PSA [71]	PCA3 [72]	TMPRSS2: ERG [73]	4K scor [66]	PHI [69]	ConfirmMDx [70]
Sensitivity	0.74-0.96	0.90	0.68	0.24			0.68
specificity	0.53-0.80	0.48	0.58	0.93			0.64
AUC	0.71-0.92	0.79	0.68	0.59	0.82	0.68	

ease. As high as 80% of men were found PCa negative based on their biopsy results [65]. Therefore, there is a significant interest in finding a more accurate PCa-specific biomarker. Khalid *et al.* [21] showed the discrimination power of urinary VOCs profile in differentiating PCa patients from controls with 71% accuracy based on only 4 VOCs. In one of our studies [38], the performance of VOCs has been tested and validated with AUC 0.92 (96% sensitivity and 80% specificity). The VOCs based prostate cancer diagnosis tool would be promising in future clinical use.

Several biomarkers have been developed to improve upon the limitations of serum PSA including Iso-PSA, prostate cancer antigen 3 (PCA3), 4Kscore, Prostate Health Index (PHI), TMPRSS2:ERG and ConfirmMDx [66-71]. Among those markers, IsoPSA, PHI and 4Kscore are all PSA-based assay for PCa risk assessment [66, 69, 71]. PCA3 is a noncoding RNA that is prostate specific and highly overexpressed in prostate cancer [72]. TMPRSS2-ERG gene fusions are reported to be the predominant molecular subtype of prostate cancer [73]. ConfimMDx is an epigenetic test for PCa diagnosis before prostate biopsy [70]. Again, VOCs based prostate cancer diagnosis tool has shown a more satisfactory screening capability for PCa then these methods (**Table 2**).

VOCs in renal cancer

The most common type of kidney cancer is renal cell carcinoma (RCC) consisting about 90% of kidney cancer cases. RCC is a heterogeneous malignancy, both morphologically and genetically, which is classified into different histologic subtypes, including clear cell RCC (most common one), papillary RCC, chromophobe RCC and other less common subtypes [74-76]. The outcome of RCC is usually unpredictable even after a long period of asymptomatically development and progression [77]. Therefore, its diagnosis is often incidental through the use of medical imagology and is frequently detected at an advanced stage and metastatic when detected clinically [78]. Additionally, RCC is particularly challenging to treat because of its relative insensitivity to radiotherapy and conventional chemotherapy drugs [79]. The early screening of RCC could improve the outcome of diagnosis. However, no early screening method is recommended to screen for kidney cancer clinically in people at average risk or increasing risk.

The potential of urinary VOCs used in RCC diagnose has been highlighted in previous studies [22, 80, 81]. The purpose of most previous studies were focused on the searching of specific VOCs in RCC patients without further validation [22, 80]. In the study reported by Marica Monteiro in 2017 [81], the selected VOCs was validated in different patients group besides the searching of specific VOCs, but the performance of VOCs in differentiating RCC patients and controls was not determined. Besides, two urinary exosomal proteins, AQP-1 and PLIN2 have shown promise as the biomarkers in RCC diagnosis [82]. It should be noted that AQP-1 and PLIN2 can be found in clear cell and papillary RCC but not in the chromophobe subtype of RCC. However, VOCs based screening has great potential to be developed as a more universal screening tool of almost all types of RCC or even specific screening tool for each type of RCC because of the metabolic distinction shown with each selected VOC between cancer patients and controls, Unlike the ELISA detection methods of AQP-1 and PLIN2, the VOCs based diagnostic model could be developed as a high throughput and fast screening method in clinic enabled by high performance GC/MS and statistic assistance.

VOCs in bladder cancer

Bladder cancer (BCa) is the second most common genitourinary malignant disease in United States [83]. And it is also a heterogeneous malignancy, with different histologic subtypes, including transitional cell carcinomas (90%),

squamous cell carcinomas (5%), and adenocarcinomas (less than 2%) [84]. The most common symptom of BCa (in 80%-90% of the patients) [85] is hematuria, or blood in the urine, and others including complaints of dysuria (painful urination), increased frequency or urgency of urination, failed attempts to urinate, a mass in the bladder or a ureteral obstruction [86, 87]. Intravenous pyelography, cystoscopy, transurethral biopsy, and imaging techniques, such as magnetic resonance imaging and computerized tomography scan, are always involved in the clinical diagnosis procedures of potential BCa [87, 88]. Like renal cancer, no early screening method for bladder cancer is recommended in United States [89].

Many studies are attempting to identify genetic and chemical markers in order to complement the use of clinical features and better assess the risk level of BCa [90, 91]. The overexpression of the *p53* gene, cells containing multiple aneuploid cell lines, and the expression of the Lewis-x blood group antigen were found to be the markers of high risk BCa [90]. Furthermore, nuclear matrix protein 22 (NMP22) and bladder tumour antigen (BTAstat) are more sensitive (50-85% and 50-70%), but less specific (60-70%), than urine cytology, which have been approved by FDA as protein markers of bladder cancer [23, 92].

Recently, VOCs are also suggested in different studies that have potential in differentiating patients of BCa from controls. And according to Weber *et al*. [23], the best diagnostic performance they obtained through the comparison between healthy volunteers and bladder cancer patients was 70% overall accuracy (70% sensitivity and 70% specificity) using a gas sensor array and pattern recognition. The results of another study using gas sensors, reported from Khalid *et al.* [93], also showed potential of VOCs for the diagnosis of bladder cancer (the best performance: 100% sensitivity and 94.6% specificity). All those studies have revealed the potential of VOCs used in bladder cancer diagnosis.

Potential molecules pathways related to VOCs profile for cancer direction

Androgen signaling and one-carbon metabolism

The androgen receptor (AR), is a nuclear receptor that is activated by binding either of the androgenic hormones, testosterone, or dihydrotestosterone in the cytoplasm and then translocating into the nucleus [94, 95]. It plays an essential and important role in PCa initiation, progression, and metabolic adaptation that takes place during PCa progression. As a transcription factor, the AR directly affects essential catabolic and biosynthetic pathways through modulating the expression of related effectors and regulators. On the other hand, the AR, as a modulator of the one-carbon metabolism, can also affect epigenetic processes, DNA metabolism, and redox balance indirectly, which are all important factors in tumorigenesis [96].

One-carbon metabolism involves a complex network with two central cycles: (1) the folate cycle and (2) the methionine cycle. One-carbon metabolism also regulates essential processes including DNA synthesis and repair, epigenetic methylation reactions, redox homeostasis, and protein synthesis. The balanced flux through these four pathways, e.g. folate cycle, methionine cycle, transsulfuration pathway, and polyamine synthesis, is essential for cellular homeostasis. (**Figure 4A**) [96], and disruptions of the balanced flux could contribute to the pathogenesis of many diseases, including cancer [97]. Cancer creates a demand and dependency on one-carbon metabolism. For example, methyl group availability for methyltransferases that modulate gene expression via epigenetic mechanisms is influenced by flux within the folate cycle and methionine cycles [98, 99]. Alterations in one-carbon metabolism may contribute to tumorigenesis through fueling DNA synthesis, changing the DNA and histone methylomes, promoting protein translation, driving cell cycle progression, and modulating redox balance. These changes can in turn promote sustained proliferation, induce tumorigenic gene expression changes, contribute to genomic instability, and promote survival-all important processes in tumorigenesis and cancer progression [96].

The progression and metastasis of tumors were associated with metabolite increases in glutathione and cysteine/methionine metabolism pathways. For example, clear cell RCC is characterized by broad shifts in central carbon metabolism, one-carbon metabolism, and antioxidant response, reported by Hakimi *et al.* [100]. Bridging the gap between the Cancer Genome Atlas (TCGA) transcriptomic profiling

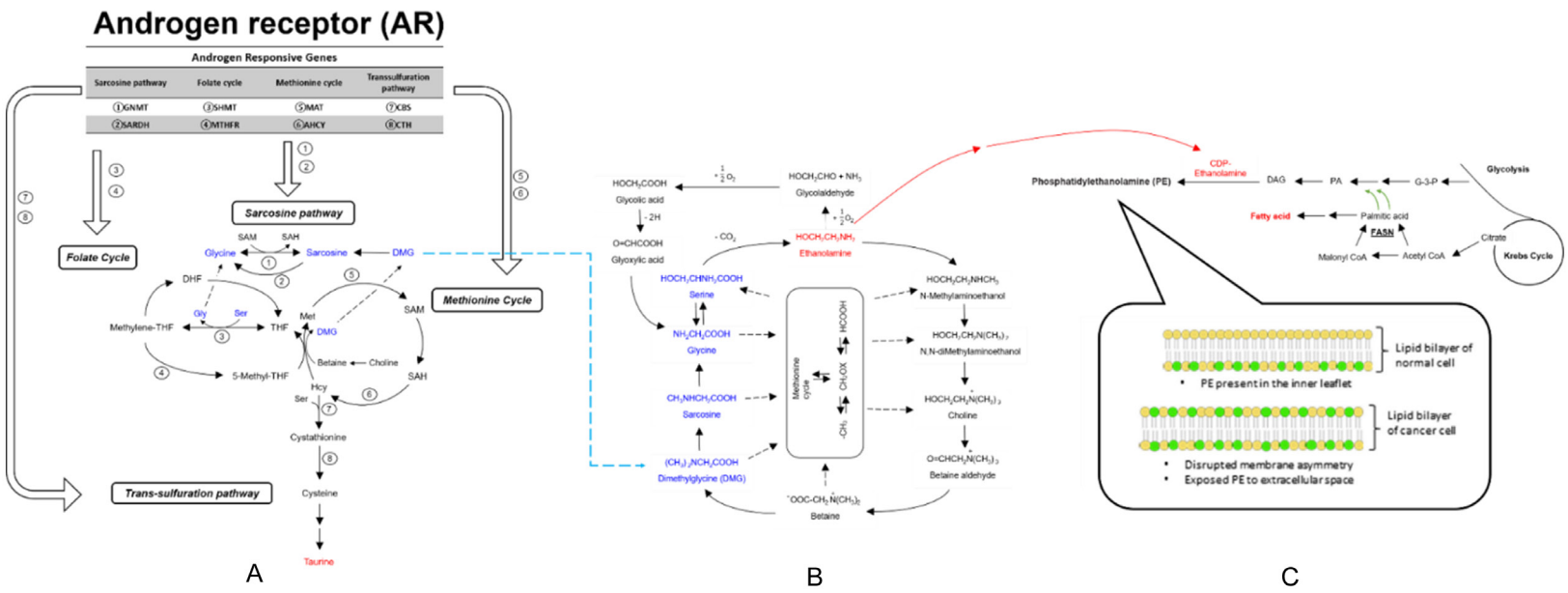

Figure 4. A comprehensive illustration of Androgen signaling, one-carbon metabolism, and metabolic phenotype. (A) One-carbon metabolism involves a complex network with four pathways: (1) folate cycle; (2) methionine cycle; (3) transsulfuration pathway; (4) sarcosine pathway. In the prostate, androgens and the AR regulate the activity/expression of several enzymes involved in the one-carbon metabolism pathway. Enzyme abbreviations are as follows: SARDH: Sarcosine Dehydrogenase; SHMT: Serine hydroxymethyltransferase; GNMT: Glycine-N-methyltransferase; MTHFR: Methylene tetrahydrofolate reductase; MAT: Methionine adenosyltransferase; AHCY: S-adenosylhomocysteine hydrolase; CBS: Cystathionine beta-synthase; CTH: cystathione gamma-lyase or gamma-cystathionase. (B) Hypothetical cycle of metabolism involving glycine, serine, ethanolamine, choline, and betaine. [101] (C) Enhanced lipogenesis, arising from increased activities of fatty acid biosynthetic enzymes (including ACC1, FASN, and stearoyl CoA desaturase (SCD1)), is a metabolic hallmark of many cancer cells. [106-108] In addition, the plasma membrane of normal cells is characterized by an asymmetric distribution of various phospholipids over two membrane leaflet. PE resides in the inner leaflet facing the cytosol. The disrupted membrane asymmetry of cancer cell exposes PE to extracellular space. Furthermore, PE is also highly exposed on endothelium cells in tumor vasculature. PA, phosphatidic acid; PC, phosphatidylcholine; DAG, diacylglycerol; CDP-ethanolamine, Cytidine diphosphate ethanolamine.

and the metabolomic data in their studies, the authors were able to integrate the pathway-level metabolic atlas and to demonstrate discordance between transcriptome and metabolome.

Studies in PCa cell lines demonstrate AR-regulation of one-carbon metabolism enzymes, and altered cellular methylation potential in response to androgens [101-104]. For example, sarcosine, a methylated metabolite of the one-carbon pathway, was found be accumulated in PCa clinical samples [102]. In the prostate, androgens and the AR regulate the activity and/or expression of several enzymes involved in the one-carbon metabolism pathways, specifically enzymes involved in S-adenosyl-methionine (SAM) homeostasis and the entry into the transsulfuration and polyamine synthesis pathways (**Figure 4A**). Studies directed to identify AR transcriptional networks in different models of PCa have demonstrated an involvement of the AR in global metabolism by directly targeting enzymes involved in several metabolic processes [105-108]. These findings illustrate the role of the AR in PCa tumorigenesis by controlling metabolism, and the value of integrating metabolomic profiling and gene expression analysis for the identification of new biomarkers and therapeutic targets. Also, these observations emphasize the link between the AR and one-carbon metabolism, and the potential effects that changes in AR signaling, that can occur with disease progression, may have on essential cellular processes.

The effect of metabolic phenotype on fatty acid and phospholipid synthesis

The zinc accumulating and citrate synthesizing phenotype is the hallmark of the healthy prostate epithelial cell [109, 110]. However, PCa cells reverse this phenotype and adopt a zinc wasting, citrate oxidizing phenotype, thereby representing a major shift in energy metabolism [111]. This shift allows these cells to utilize the Krebs cycle and subsequent oxidative phosphorylation (**Figure 4B** and **4C**). It has long been identified that PCa cells do not conform to the standard Warburg effect seen in most cancers, which described in the early to mid-1900s by Otto Warburg [112]. Malignant cells shift their dominant ATP producing pathway away from oxidative phosphorylation to aerobic glycolysis [112]. Unlike most cancer cells that resort to aerobic glycolysis, prostate cancer cells exhibit a higher level of citric acid cycle activity compared to benign cells [110]. The increased activity of citric acid cycle, essential for the progression of malignancy, was induced by the inability of malignant prostate cells to accumulate high zinc levels, which inhibits citrate oxidation [113].

Another metabolic hallmark of many cancer cells is the enhanced lipogenesis, arising from increased activities of fatty acid biosynthetic enzymes [114-116]. Clear cell RCC (ccRCC) is histologically defined by its lipid and glycogen-rich cytoplasmic deposits [117, 118]. In the study of Du *et al.* [118], the lipid deposition of ccRCC was investigated with focus on the carnitine palmitoyltransferase 1A (*CPT1A*), as a direct HIF target gene. Prostate cancer cells often utilize lipids derived from androgens through the expression of the AR [119]. However, these cells can also utilize *de novo* lipid synthesis to produce fatty acids in order to obtain energy. This shift to a lipid-producing phenotype is a key turning point in the progression of prostate cancer. The *de novo* lipid producers have ability to produce the key energetic molecules for growth without the regulation of androgens (**Figure 4C**) [120]. Clinically, this is problematic as it represents a disease that is unresponsive to androgen deprivation therapy, known as castration-resistant prostate cancer [121]. These producers include fatty acid synthase (FASN), sterol regulatory element binding protein 1 (SREBP1), and steroyl CoA desaturase. Among them, the enzyme FASN functions to help synthesize long-chain fatty acids. It is believed that unregulated FASN activity within prostate tissue is the beginning of malignant phenotype, and has been argued to be necessary for PCa growth maintenance [122]. The use of lipid by the PCa cells illustrates that these cells bypass potential degenerative pathways, and rather utilize the anabolic pathways in order to maintain energy and growth [123]. A variety of fatty acid moiety were detected in our preliminary study and that supports the importance of specific VOCs in PCa.

Additionally, phospholipids, also as the downstream products of enhanced lipogenesis, in the cancer cell membrane have been found to be abnormal compared with normal cells. The plasma membrane of normal cells is characterized by an asymmetric distribution of various

phospholipids over two membrane leaflet. Phosphatidylethanolamine (PE) resides in the inner leaflet facing the cytosol (**Figure 4C**). The disrupted membrane asymmetry of cancer cell exposes PE to extracellular space, which can serve as a molecular target for anticancer therapy [124]. The increasing need of PE in cancer cell may correlate the excessive consumption of ethanolamine and enhanced lipogenesis.

In conclusion, the use of urinary VOCs has demonstrated a potential application in cancer diagnosis as biomarkers for the assessment or detection of disease. Although the pathways affecting VOCs production are yet to be fully understood, the VOCs identified to be related to cancers may provide valuable information to study the pathways of VOC production in the context of cancer.

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Disclosure of conflict of interest

None.

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