Analysis of VOCs from exhaled breath for the diagnosis of hepatocellular carcinoma

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การวิเคราะห์สารอินทรีย์ระเหยจากลมหายใจเพื่อใช้ในการวินิจฉัยมะเร็งตับ

น.ส.ธนิกานต์ สุขอร่าม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ธนิกานต์ สุขอร่าม : การวิเคราะห์สารอินทรีย์ระเหยจากลมหายใจเพื่อใช้ในการวินิจฉัยมะเร็งตับ. (Analysis of VOCs from exhaled breath for the diagnosis of hepatocellular carcinoma) อ.ที่ปรึกษาหลัก : รศ. ดร.รุ่งฤดี ชัยธีรกิจ, อ.ที่ปรึกษาร่วม : ศ.พิสิฐ ตั้งกิจวานิชย์

ที่มา ความสำคัญ และวัตถุประสงค์: การตรวจสารอินทรีย์ระเหยเป็นวิธีที่สามารถนำมาใช้ในการตรวจวินิจฉัยมะเร็งตับชนิดเฮปาโตเซลลูลาร์คาร์ซิโนมา ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพสารอินทรีย์ระเหยสำหรับการวินิจฉัยผู้ป่วยมะเร็งตับในระยะเริ่มต้นจากกลุ่มผู้ป่วยที่มีความเสี่ยงสูงต่อการเกิดมะเร็งตับ

ระเบียบวิธีการวิจัย: สารอินทรีย์ระเหยจากลมหายใจถูกเก็บจากกลุ่มผู้ป่วยมะเร็งตับจำนวน 90 ราย กลุ่มผู้ป่วยตับแข็งจำนวน 90 ราย กลุ่มผู้ป่วยติดเชื้อไวรัสตับอักเสบบีจำนวน 91 ราย และอาสาสมัครสุขภาพดีจำนวน 95 ราย จากนั้นนำมาวิเคราะห์ด้วยเทคนิคเทอร์มอลดีซอฟชั่น-แก๊สโครมาโตกราฟี-ฟิลด์เอซิมเมตริกไอออนโมบิลิตี้สเปกโตรเมทรี การเปรียบเทียบระดับของสารอินทรีย์ระเหยระหว่าง 4 กลุ่มรวมถึงความสัมพันธ์ระหว่างสารอินทรีย์ระเหยและมะเร็งตับถูกนำมาวิเคราะห์โดยการใช้สถิติการถดถอยโลจิสติก ประเมินประสิทธิภาพการวินิจฉัยผู้ป่วยมะเร็งตับจากสารอินทรีย์ระเหยง่ายโดยใช้พื้นที่ใต้กราฟ ROC และเปรียบเทียบกับแอลฟา-ฟีโตโปรตีน

ผลการศึกษา: จากผลการวิเคราะห์พบปริมาณของ dimethyl sulfide, 1,4-pentadiene, isopropyl alcohol และ acetone มีความแตกต่างระหว่าง 4 กลุ่มอย่างมีนัยสำคัญ เมื่อทำการศึกษาความสัมพันธ์ระหว่างสารอินทรีย์ระเหยและมะเร็งตับโดยคำนึงถึงปัจจัยค่าการทำงานของตับและแอลฟา-ฟีโตโปรตีน พบว่าปริมาณ acetone มีความสัมพันธ์กับมะเร็งตับอย่างมีนัยสำคัญ อีกทั้งยังพบว่า acetone สามารถแยกผู้ป่วยมะเร็งตับจากผู้ป่วยที่ไม่เป็นมะเร็งตับได้ดีกว่าแอลฟา-ฟีโตโปรตีนด้วยความไว (ร้อยละ 88.9 และ 68.2, p=0.017), ความจำเพาะ (ร้อยละ 87.3 และ 63.6, p=0.001) , ความแม่นยำ (ร้อยละ 87.7 และ 65.2, p<0.001) และพื้นที่ใต้กราฟ ROC (0.932 และ 0.725, p=0.001) ตามลำดับ

สรุปผลการศึกษา: สารอินทรีย์ระเหยโดยเฉพาะ acetone มีประสิทธิภาพในการวินิจฉัยแยกผู้ป่วยมะเร็งตับในระยะเริ่มต้นจากผู้ป่วยที่มีความเสี่ยงสูงได้ดีกว่าแอลฟา-ฟีโตโปรตีน อย่างไรก็ตามจำเป็นต้องมีการศึกษาความเที่ยงตรงภายนอกเพิ่มเติมเพื่อตรวจสอบประสิทธิภาพของสารอินทรีย์ระเหยในการเป็นตัวบ่งชี้ทางชีวภาพเพื่อใช้ในการตรวจเฝ้าระวังมะเร็งตับ

สาขาวิชา	วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Thanikarn Suk-aram : Analysis of VOCs from exhaled breath for the diagnosis of hepatocellular carcinoma . Advisor: Assoc. Prof. Dr. ROONGRUEDEE CHAITEERAKIJ, Ph.D. Co-advisor: Prof. PISIT TANGKIJVANICH, M.D.

Background: Volatile organic compounds (VOCs) were shown as promising biomarkers for hepatocellular carcinoma (HCC) diagnosis. We aimed to investigate the performance of VOCs for diagnosing early-stage HCC in patients at-risk for HCC.

Methods: VOCs were identified in exhaled breath samples collected from 90 early-stage HCC patients, 90 cirrhotic patients, 91 HBV-infected patients, and 95 healthy volunteers using thermal desorption-gas chromatography/field-asymmetric ion mobility spectrometry. The VOC levels were compared between the four groups. An association between VOCs and HCC was determined using logistic regression analysis. Diagnostic performance of VOCs was estimated using the AUROC and compared to serum alpha-fetoprotein (AFP).

Results: The levels of dimethyl sulfide, 1,4-pentadiene, isopropyl alcohol, and acetone were significantly different between the four groups. After adjusting for liver function test and AFP, acetone was significantly associated with HCC. Acetone significantly outperformed AFP, with 88.9% vs. 68.2% sensitivity, 87.3% vs. 63.6% specificity, 87.7% vs. 65.2% for accuracy, and AUROC of 0.932 vs. 0.725, p=0.017, 0.001, <0.001, and 0.001, respectively, for differentiating between HCC and non-HCC group.

Conclusion: Acetone has a better performance than AFP for diagnosing early HCC in high-risk patients. Further studies to validate the utility of VOCs as a HCC surveillance tool are needed.

Field of Study:	Medical Sciences	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and one of the leading causes of cancer-related mortality (1, 2). HCC most commonly occurs in patients with underlying cirrhosis, as well as chronic liver diseases, specifically chronic viral hepatitis B (HBV) and C (HCV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease (NAFLD). Early-stage HCC patients are often asymptomatic, and over 50% of cases are diagnosed when effective treatment options are limited. (3). Individuals at risk for HCC are advised to undergo regular surveillance, including abdominal ultrasonography and serum alpha-fetoprotein (AFP) testing, in order to detect early-stage HCC when curative therapeutic interventions are still feasible. Ultrasonography and AFP had sensitivity rates of 84% and 52%, respectively, in detecting HCC at any stage (4, 5). Both surveillance tools demonstrated low sensitivity in detecting early HCC. When used for surveillance in cirrhotic patients, ultrasonography and AFP had sensitivities of only 47% and 44%, respectively, for early HCC detection (6). Therefore, it is crucial to develop a surveillance tool that offers higher sensitivity in detecting early-stage HCC in high-risk populations. This would enable prompt curative treatment and reduce patient mortality associated with HCC.

Volatile organic compounds (VOCs) are endogenous products of intracellular metabolic activity in both physiological and pathological conditions. Accumulating evidence has shown that the analysis of VOCs in exhaled breath is a promising non-invasive diagnostic method for various diseases (7), for example, diabetes mellitus (8), asthma and chronic obstructive pulmonary disease (9), inflammatory bowel disease, and non-alcoholic fatty liver disease (NAFLD) (10) as well as several cancers, e.g. colon, lung and pancreas. (11-13). The initial research conducted a proof-of-concept study that utilized exhaled breath VOCs for the diagnosis of HCC and employed canine scent detection. The results demonstrated a high sensitivity of VOCs in exhaled breath, with a detection rate of 78% (95% CI: 62-90%) (14).

Another three previous studies have found the utility of exhaled VOCs in diagnosing HCC (15-17). 3-hydroxy-2-butanone, styrene, and decane levels were significantly higher in HCC patients than in healthy volunteers (15). Another study reported the higher acetone level but lower isoprene and pentane levels in HCC patients than in cirrhotic patients (16). Further research identified a combination of 18 VOCs that identified patients with HCC from non-HCC groups with 72% accuracy (17). The latest research, which was our previous study (6), had three goals: (1) To develop VOCs as biomarkers for HCC diagnosis in cirrhosis patients, (2) To determine the correlation between VOC levels and HCC stages, and (3) To measure changes in VOC levels after HCC treatment to explore the feasibility of using VOCs for monitoring treatment response. This was achieved using gas chromatography-mass spectrometry and the Support Vector Machine algorithm. The results showed that among the 64 VOCs identified in the study, the combination of 6 VOCs, including acetone, benzene, methylene chloride, 1,4-pentadiene, phenol, and allyl methyl sulfide. The predictive model demonstrated good performance in discriminating HCC from controls in both the training and test sets, achieving an accuracy of 79.6%, sensitivity of 76.5%, and specificity of 82.7% in the training set. In the test set, it provided an accuracy of 73%, sensitivity of 77%, and specificity of 68%. Furthermore, the combination of 6 VOCs is related to HCC stages, and after treatment, the levels of VOCs were significantly altered. The decreased level of acetone predicted response to therapy with satisfactory performance, achieving an accuracy of 79.4%, sensitivity of 77.3%, and specificity of 83.3%. However, all the previous research on VOCs from exhaled breath has shown some limitations. The number of studies remains sparse, and there are problems with the methods of breath sampling, including low stability storage, contamination from unwanted VOCs in the ambient air, and susceptibility to ultraviolet degradation (18). Additionally, techniques for VOCs detection, such as SIFT-MS and GC-MS, require extensive training and expertise for operation, making them costly and time-consuming (19). Furthermore, although the aforementioned findings suggest the potential use

of exhaled VOCs as a non-invasive diagnostic test for HCC, previous studies have included HCC cases at various stages of the disease. Only a small proportion of HCC cases (12.4-33.9%) were in the early stage of the disease (6, 20). In order to apply exhaled VOC testing for HCC surveillance in routine service, it is necessary to study the performance of VOCs using a cohort consisting only of early-stage HCC patients. Some studies included healthy individuals as controls, which may have led to an overestimation of the performance of VOCs. Additionally, most studies did not compare the performance of VOCs to that of AFP, which is the most clinically used HCC biomarker. Furthermore, none of the previous research included chronic HBV-infected patients, who are at a high risk of developing HCC (21).

Considering the limitations of previous research, our aim was to validate the diagnostic performance of exhaled VOCs for early-stage HCC using Thermal Desorption-Gas Chromatography-Field Asymmetric Ion Mobility Spectrometry. We compared the levels of VOCs in exhaled breath between early-stage HCC patients and at-risk groups, which included cirrhotic patients, chronic HBV-infected patients, and healthy volunteers. Additionally, we investigated the association between VOCs and HCC and compared the ability of exhaled VOCs to detect early-stage HCC with that of AFP.

1.2 Research question

- Can VOCs profiles differentiate HCC patients from non-cancer individuals, chronic liver disease patients and cirrhotic patients?
- Are VOC profiles associated with HCC?
- Does the diagnostic performance of VOCs have a better performance than AFP?

1.3 Hypothesis

The change in metabolic pathways in HCC, cirrhotic patients, chronic liver disease patients, and non-cancer individuals results in distinct patterns of VOCs.

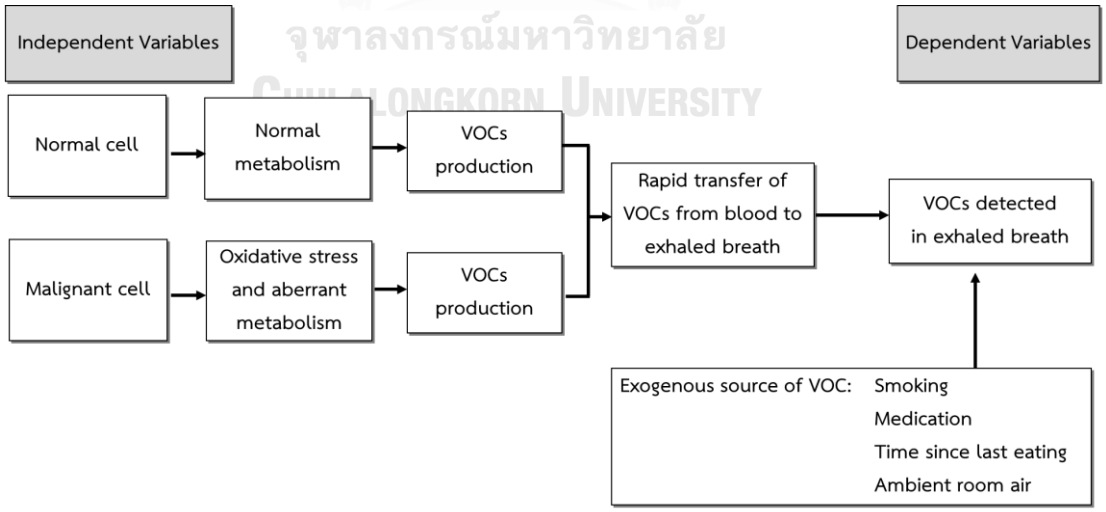
1.4 Research aims

- To identify VOC profile for HCC diagnosis
- To investigate the association of VOC with HCC
- To investigate the diagnostic performance of VOCs in comparison to AFP

1.5 Keywords

Volatile organic compounds (VOCs), Biomarkers, Diagnostic model, Thermal desorption tube (TD tube) and GC-FAIMS technique.

1.6 Conceptual framework



1.7. Research Design

Single center, Analytic study, Cross sectional study

CHAPTER 2 LITERATURE REVIEW

2.1 Hepatocellular carcinoma (HCC)

2.1.1 Epidemiology

HCC is the most common primary liver cancer. In 2020, the world health organization (WHO) recently reported that there were 905,677 new HCC cases worldwide, ranking 6th in global cancer incidence, and HCC is the third cause of cancer-related deaths worldwide (22). In Thailand, HCC is one of the most common cancers and the second cause of cancer-related deaths. In Thailand, 27,394 new HCC cases were diagnosed, with 50% of HCC patients present at an advanced stage (23). The risk of HCC in male patients was three-fold higher than in females (24). Age-standardized incidence rates (ASR) in Thailand were 34.8 per 100,000 males and 11.3 per 100,000 females (24). Risk factors of HCC commonly occur in individuals 80%–90% with underlying cirrhosis and 10%-20% chronic liver diseases, particularly chronic viral hepatitis B and C (HBV and HCV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease (NAFLD).

2.1.2 HCC screening and surveillance method

Ultrasound is the standard screening method for HCC. Although it has a high specificity of 92%, it has a low sensitivity of 47% for detecting early-stage HCC (4, 5). Alpha-fetoprotein (AFP) is the most widely used biomarker for HCC screening. However, AFP has a low sensitivity (52%) and 94% specificity at a 20 ng/mL cutoff value. Moreover, it has 44 % sensitivity and 85 % specificity for early-stage HCC (25). Although serum AFP combined with ultrasonography improved the sensitivity of HCC detection in clinical practice, the performance remained poor (63%). Additionally, radiologic imaging techniques, including CT or MRI, can increase HCC surveillance, given concerns about ultrasound's accuracy and critical response to HCC therapy. However, these techniques are costly and have some drawbacks. Therefore, developing a better screening tool for detecting early HCC and monitoring the treatment response of HCC is needed.

2.1.3 HCC staging system

The Barcelona Clinic Liver Cancer (BCLC) system is a clinical staging system used to guide treatment options and determine the prognosis of HCC patients. This system classifies five stages of HCC patients based on three major factors: tumor burden, liver function, and patient performance status. as follows (26):

1. BCLC stage 0 (Very early stage): Patients with a single nodule less than <2 cm, patients with good performance status (PS 0), patients without vascular invasion or metastases, and patients with well-preserved liver function (Child-Pugh A). Surgical resection, liver transplantation, or local ablation are the curative treatment options.

2. BCLC A (Early stage): Patients with a single nodule diameter of 5 cm or three nodules (≤3 cm) . Patients with PS 0 and Child-Pugh A. Surgical resection, liver transplantation, or local ablation are the curative treatment options.

3. BCLC B (Intermediate stage): Asymptomatic patients with large or multifocal tumors are limited to liver parenchyma. Patients with PS 0 and Child-Pugh A-B.

4. BCLC C (Advanced stage): Patients with a mild PS (PS 1 or 2), (Child-Pugh A or B), patients with macrovascular invasion, or patients with extrahepatic spread. This stage is treated with transarterial chemoembolization (TACE) or systemic therapy with sorafenib.

5. BCLC D (Terminal stage): Patients with poor PS (PS 3 or 4) or liver function (Child-Pugh C) indicate a severe tumor or cirrhosis-related disability. Patients in this stage receive the best supportive care.

2.2 Volatile organic compounds (VOCs)

2.2.1 Background

The WHO defined VOCs as carbon compounds whose boiling point is 50 to 260 ^{0}C (27). Compared to other organic compounds, their relatively low boiling point makes VOCs able to evaporate at room temperature and normal atmospheric pressure. VOCs are released from cells and circulate in the blood. After that, VOCs can be released into several bodily fluids, including breath, blood, urine, feces, and skin (1). VOCs can originate from within the body and from an exogenous source such as smoking, medication, time since last eating, and ambient air (28-30).

2.2.2 Previous research on VOCs in cancers

VOCs analysis was a potential method to detect cancers including lung (31), breast (32), oesophageal cancer (33), colon cancer (34), bladder cancer (35), prostate cancer (36), colorectal cancer (37, 38), and irritable bowel syndrome (39) **(Table 1)**. The cancer odor database (COD) reported VOCs as cancer biomarkers consisting of general cancer biomarkers and biomarkers for a specific cancer type (40). The VOCs can be classified into five functional groups of VOCs, including aldehydes (heptanal, hexanal, decanal, nonanal, pentanal, and octanal), ketones (acetone, 3 -heptanone, 2 - butanone, and cyclohexanone), alcohols (2-ethyl hexanol), hydrocarbons (dodecane, 3-methylehexan, 4-methyl octane, and 2,2-dimethyl decane), and aromatic compounds (1,2,4-trimethylbenzene, 1-methyl-4-propan-2 -ylbenzene, and p-xylene) (40). These VOCs are involved in various types of cancer. Moreover, 3 VOCs can be used as general cancer biomarkers, including hexanal, acetone, and ethanol (40) for lung, breast, prostate, and colon cancer. As a result, combining VOCs from general cancer biomarkers and specific biomarkers would be very promising for cancer detection.

Previous studies have shown that tumor cells produce VOCs, including hydrocarbon compounds from oxidative stress, alcohols from hydrocarbon metabolism, ketones from fatty acid oxidation, nitrites, and aromatic compounds from exogenous carcinogens (30). Moreover, cancer cells' metabolism hugely differs from normal cells. This metabolic change is called "the Warburg effect". This effect causes a hugely increased glycolysis rate in cancerous cells, leading to significantly raised metabolites such as lactate and fumarate that could alter the VOC profile (41). Moreover, the increased glycolysis pathway can generate intermediate biosynthetic precursors such as nucleic acid synthesis via the pentose phosphate pathway (PPP), and acetyl-CoA is used to produce lipids. Increased nucleic acid and lipid synthesis promote cancer cell proliferation and growth **(Figure 1)**. Therefore, it can indicate organ dysfunction and be a biomarker in many types of cancer.

Table 1. Previous research on VOCs in cancers.

Sample	**Years**	**Author**	**Diagnosis**	**Sample group**		**Sensitivity**	**Specificity**
				Case	**control**		
Breath	1999	Phillips M. *et al.*	Lung cancer	193	211	100.0%	81.3%
Breath	2006	Phillips M. *et al.*	Breast cancer	51	50	93.8%	84.6%
Breath	2013	Kumar S. *et al.*	Oesophageal cancer	81	129	92.0%	87.0%
Breath	2013	Altomare DF. *et al.*	Colon cancer	37	41	86.0%	83.0%
Urine	2017	HEER H. *et al.*	Bladder cancer	30	30	93.3%	86.7%
Urine	2019	Gao Q. *et al.*	Prostate Cancer	55	53	96.0%	80.0%
Urine	2019	Mozdiak E. *et al.*	Colorectal cancer	113	37	98.0%	82.0%
Feces	2014	de Meij *et al.*	Colorectal cancer	83	50	85.0%	87.0%

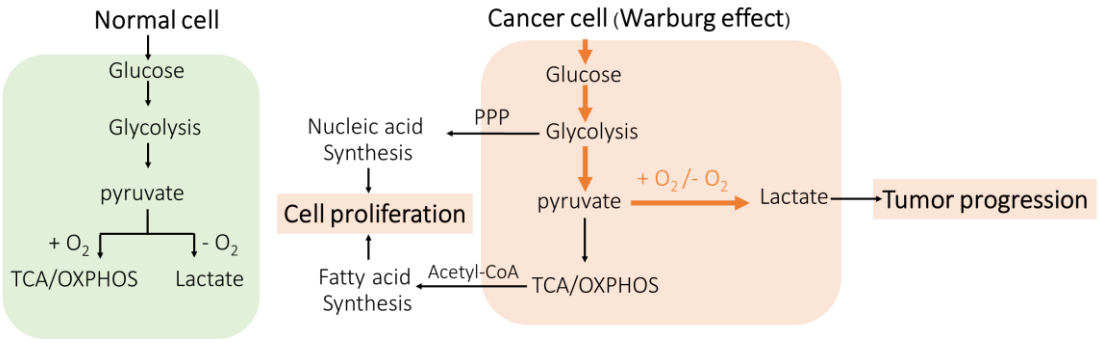


Figure 1. Metabolism of cancer cells modified from reference (42).

2.2.3 Previous research on VOCs in HCC

Only three studies have used breath-based VOCs for the diagnosis of HCC patients. VOCs in 112 HCC patients can be differentially expressed from 30 cirrhosis patients and 54 Healthy volunteers. Then, samples were analyzed by a selective Ion Flow Tube Mass Spectrometer (SIFT-MS). The results showed acetone, acetaldehyde, and dimethyl sulfide were higher in HCC patients than in cirrhotic patients, with 72% accuracy, 73% sensitivity, and 71% specificity (17). In secondary research, VOCs from 3 groups are 30 HCC patients, 27 cirrhosis patients, and 36 healthy volunteers. Breath samples were extracted by solid-phase microextraction (SPME) and analyzed by gas chromatography-mass spectrometry (GC-MS). The result showed that levels of 3-hydroxy-2-butanone, styrene, and decane were significantly increased in HCC patients than in healthy controls and cirrhotic patients, with a sensitivity of 86.7% and specificity of 91.7% (15). In our previous study, we used breath-based VOCs for diagnosis and monitoring of the therapeutic response of HCC by GC-MS. The results from 97 cancer patients and 111 no-cancer patients (78 cirrhotic patients and 33 healthy volunteers) found 6VOCs, including acetone, 1,4-pentadiene, methylene chloride, benzene, phenol, and allyl methyl sulfide that were used as a diagnostic model with high accuracy of 79.6%, with 76.5% sensitivity and 82.7% specificity. Furthermore, acetone level reduction at post-treatment predicted treatment response with 77.3% sensitivity, 83.3% specificity, 79.4% accuracy, and an AUC of 0.784 (43). The difference between our previous

work and this study is patients with chronic hepatitis B virus (HBV) infection as, the most common viral infection in Thailand, were recruited in this study which can increase the performance for HCC screening in Thailand. This study provided a balanced number of each group (HCC, cirrhosis, chronic liver disease, and healthy volunteer) in development cohort and validation cohort for better classification. This method was recruited only very early-stage (BCLC stage 0 tumor size less than 2 cm) HCC that can be used to screen HCC in clinical practice. **(Table 2)**. Moreover, limitations of 3 previous research of VOCs in HCC showed the number of studies remains sparse and the problem with the method of breath sampling (Inert steel bag), which produced biased results because inert steel bags have low stability storage, contaminated unwanted VOCs from the ambient air and suffer from ultraviolet degradation **(Table 3)**.

Table 2. Differences between our previous work and this study.

Parameters	**Our previous work**	**This study**	**Differences**
Chronic liver disease patients	Not studied	Our study recruited patients with chronic HBV infection as the most common viral infection in Thailand	Increase the performance for HCC screening in Thailand
Proportion of sample group	97 HCC 78 Cirrhosis 33 Healthy volunteers	90 HCC 90 Cirrhosis 90 Chronic HBV infected 90 Healthy volunteers	Balanced number of the 4 groups for better classification
Stage of HCC	Our previous work study was recruited from all BCLC stage	Our study recruited only early-stage (0-A) HCC	This method can be used to screen early-stage (0-A) HCC in clinical practice

Table 3. Differences between the previous method and our (TD-GC-FAIMS) method.

Parameters	**Previous method** (6)	**Our method**	**Differences**
Breath collection equipment	Inert steel bag	ReCIVA breath samples system	-Higher sensitivity for trace VOCs (alkene and sulfur compound)
Stability of breath collection equipment	Low (1 day)	High (1 week)	-Higher ability to retain VOCs
Air supply	Room air	Clean air (pure oxygen supply)	-Less affected by ambient room air
Detector	Mass Spectrometry (MS)	Field Asymmetric Ion Mobility Spectrometry (FAIMS)	-Higher sensitivity to detect VOCs Portable -Robust -Inexpensive

2.3 Methods for a breath sample collection

Breath sample collection is one of the most important steps for breath analysis. Researchers should control factors that may interfere with the breath analysis results. The confounding factors include the type and the number of breath collections, the portion of breath used, and the VOCs background from the collection room (18).

2.3.1 Sampling bags

Sampling bags (Tedlar bag) **(Figure 2)** were used as the first approach for breath sample collection. The sampling bags can be connected with Solid-Phase Microextraction (SPME) Fiber to extract and preconcentrate VOCs in breath samples. Polyvinyl fluoride, perfluoroalkoxy polymer, polytetrafluoroethylene, or polyvinylidene chloride (44) was used to make the sampling bags. Sampling bags are covered with aluminum foil that can prevent gas diffusion. Sampling bags can be reused if thoroughly flushed with pure nitrogen gas to eliminate contamination and background VOCs (phenols and N, N-dimethylacetamide) (45). The advantages of sampling bags are inexpensive, easy to use, and chemically stable. However, Sampling bags are low stability storage, contamination unwanted VOCs from the ambient air, and suffer from ultraviolet degradation (18). Sampling bags may provide inaccurate results. Therefore, thermal desorption tubes are more appropriate (46).

Figure 2. Breath sampling bags (Ref: Tedlar® bag, SKC Inc. cat.no. 231-944, 2015)

2.3.2 Sampling bags with Solid-Phase Microextraction (SPME) fiber

SPME fiber is commonly used to extract and analyze volatile analytes from breath, food, and environmental monitoring (46). It is one of the most popular extraction techniques involving a sorbent-coated rod or fiber injected into the sampling bag. The extraction of analytes in a gas sample was absorbed onto a SPME fiber. The fibers consist of a 1–2 cm length of fused silica coated with a thin layer (5–100 nm) of a suitable polymeric adsorbent such as Polydimethylsiloxane (PDMS), Carboxen (CAR), Divinylbenzene (DVB), mixtures of these, and others. The CAR/PDMS stationary phases have shown excellent performance in breath analysis. After sample extraction, SPME fiber was injected into gas chromatography-mass spectrometry (GC-MS) to analyze VOCs in breath samples (46) **(Figure 3)**.

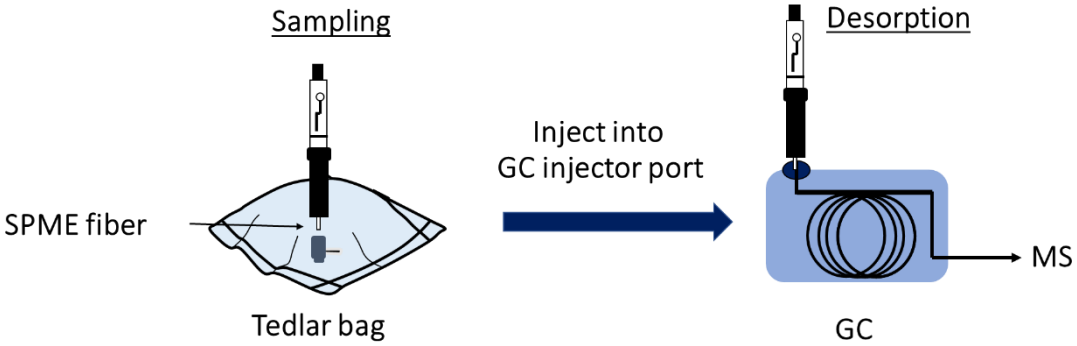


Figure 3. Analysis with SPME- GC-MS.

2.3.3 Thermal desorption tube (TD-tube)

TD-tubes or sorbent tubes are made from the highest specification materials with stainless steel tubes and are suitable for VOCs collection. TD-tube consists of sorbent traps that are adsorption materials contained in a small tube, enabling to retain VOC by capping the sample tubes with special capping tools **(Figure 4)**. TD tubes are one of the most famous for exhaled VOCs analysis because they can collect a wide range of VOCs at low to high concentration (parts per trillion – parts per billion, ppt-ppb), have low cost, high stability to the storage of VOCs, and perform well in pre-concentration and transport of breath samples before analysis. Therefore, TD-tubes are more versatile alternatives to Tedlar bags or other containers for VOC collection (47).

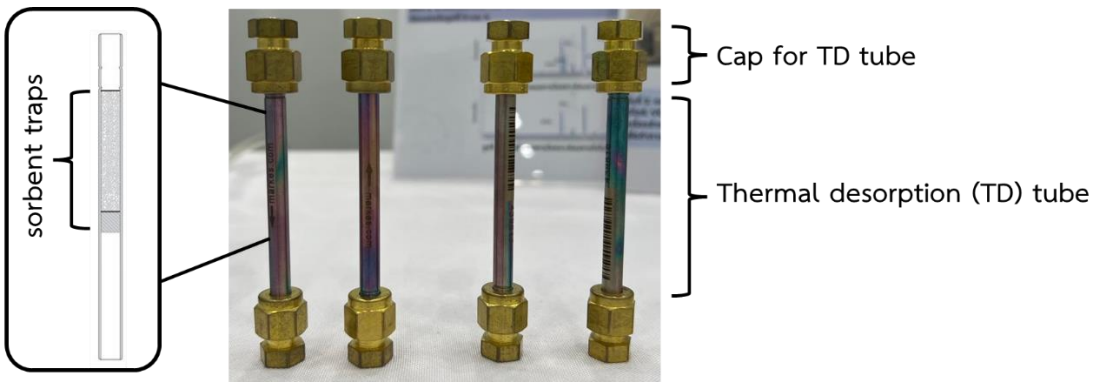


Figure 4. Thermal desorption (TD) tube

2.3.4 The ReCIVA™ breath sample system

The ReCIVA™ breath sample system is a handheld portable device that provide a higher ability to retain VOCs than inert steel bag, higher sensitivity for trace VOCs (alkene and sulfur compound) than inert steel bag and pre-concentrates exhaled VOC onto a TD tube. The direct capture of VOCs from breath samples allows the VOCs enrichment, particularly for those VOCs at low abundance **(Figure 5)**. The ReCIVA™ breath sample system can collect either whole breath sample, i.e., total breath sample or mixed expiratory air, or only the alveolar portions of the exhaled breath by measuring the CO_2 levels **(Figure 6)**. There are 3 phases of breathing for breath collection. Phase 1 is the inspiratory phase or the beginning of expiration, which presents a level of CO_2 of 0. Phase 2 is the exhalation of mixed air, which contains a very rapid increase in CO_2. Phase 3 is the alveolar expiratory phase, consisting of the initial alveolar sampling (Point A) and the final alveolar sampling (Point B). This phase has a constant in CO_2 and starts at the peak at the end of tidal expiration. In this phase, it is close to alveolar carbon dioxide tension. The whole breath is more prone to contamination with exogenous compounds from the oral cavity and may compromise the analysis, whereas the alveolar air is richer in volatile blood-borne compounds (46). The ReCIVA™ breath system is incorporated to a computer's straightforward Breath Biopsy Collect software. The device has a clean air supply system providing the volunteers to limit breath sample contamination from ambient air during breath sampling **(Figure 5)**. After sample collection, the TD tube containing exhaled VOCs from the ReCIVA™ breath system will further analyzed by gas chromatography field asymmetric ion mobility spectrometry (GC-FAIMS) (48).

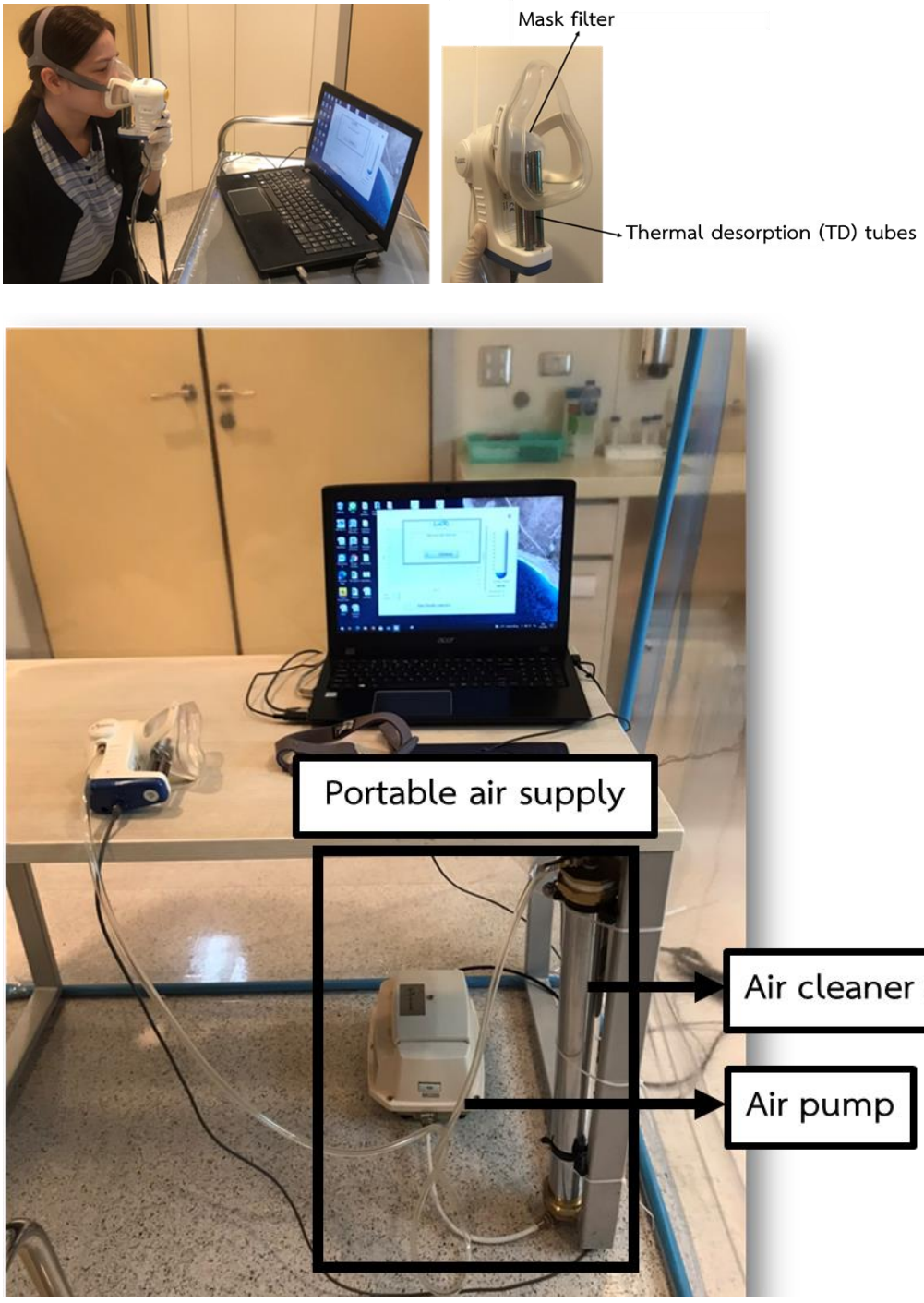


Figure 5. The ReCIVA™ breath sample system

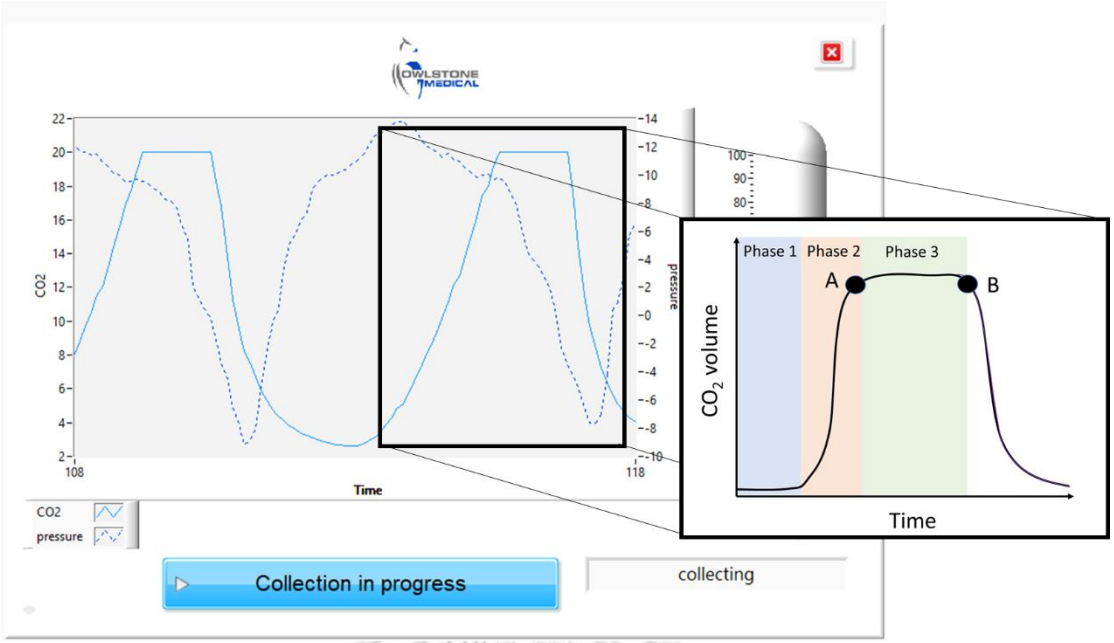


Figure 6. Diagram of the portion of breath in breath sampling process

2.4 VOC detection and measurement techniques

2.4.1 Electronic Nose (E-nose) sensor

In metabolomics, E-nose devices are used for real-time VOC detection in exhaled breath samples (49). The E-nose analyzes the breath sample and provides digital breath patterns that can be recognized by application-specific databases. The breath patterns are shown to be correlated with specific diseases (49). E-nose has the potential to be a non-invasive tool for point-of-care diagnosis of both cancer and non-cancer diseases. The device has been extensively investigated for diagnosis of several lung diseases, including acute respiratory stress syndrome (ARDS), asthma, chronic obstructive pulmonary disease, endocarditis, malignant pleural mesothelioma, pulmonary tuberculosis, upper respiratory tract infections, and ventilator-associated pneumonia (49). The advantages of E-nose over the GC-MS methods are lower cost, easier to use as the operation does not require extensive training, and short operation time. Thus, it provides rapid results, small size, and portable device (50-52). However, E-nose technique is sensitive to water vapor, has a relatively short life of

sensor, and has difficulty in measuring analyte concentrations accurately than GC-MS methods because e-nose suffers from interference caused by background interference and the operation environment (49).

2.4.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is one of the most commonly used techniques for VOCs identification at a molecular level (the MS component). This technique can be used to analyze liquid, gaseous or solid samples. Moreover, GC-MS can be used for qualitative, quantitative analysis, and analysis of the sample in a long linear rang concentration (≥ppb). The GC separates compounds in the sample by GC column before the analyte molecules are eluted into the MS for detection. The results from a GC-MS were shown as a chromatogram and were used mass spectra libraries to identify compounds. Although GC-MS is a commonly used technique for VOCs identification in research, it has several limitations for being applied in clinical settings as it requires: extensive training and expertise for operation. The equipment is costly, and the procedure is time-consuming (19).

2.4.3 Field Asymmetric Ion Mobility Spectrometry (FAIMS)

FAIMS technology is a technique that can be used for both qualitative and quantitative analysis. This method provides a high sensitivity at low concentration (ppt) , selective, and rapid VOCs analysis (53). This method is a gas-phase separation of ions based on their difference mobilities under the effect of an electric field. A FAIMS detector can be combined with GC. In the process of VOCs identification, the VOCs in breath sample passed through the column of GC, where the initial separation of compounds occurs. Subsequently, VOCs are consecutively fed into the ionization chamber of FAIMS, where ionization occurs. VOCs analytes are ionized due to colliding with positively charged proton clusters, forming a positively charged analyte cluster complex. Then passed through the FAIMS drift tube at high and low electric fields depending on their mobility, including size, mass, and volume of VOCs ion. Next, the positively charged VOCs ions are attached to the sensor faraday plate

with opposite polarity and are detected **(Figure 7)**.

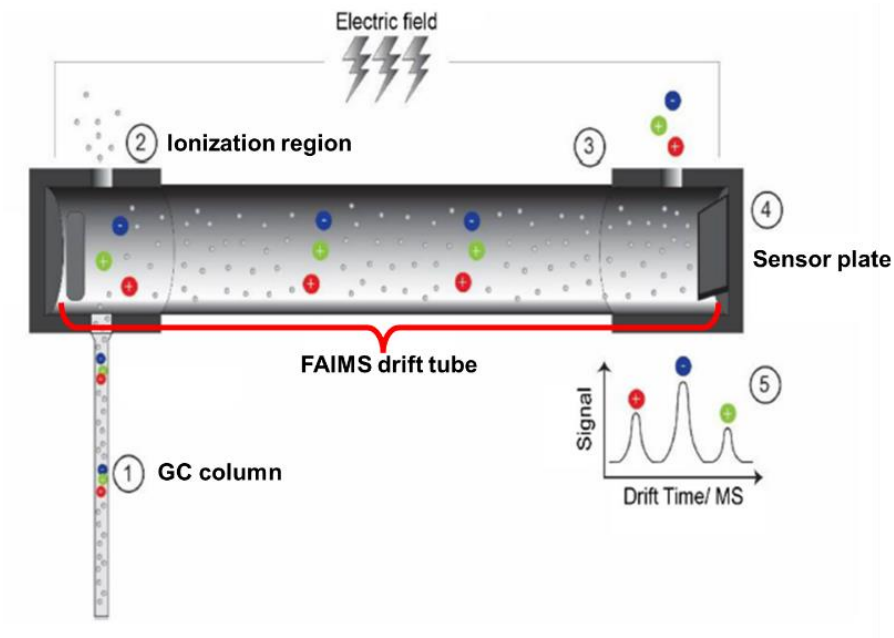


Figure 7. GC– FAIMS technique from reference 34 ; (1) GC column used to separate components; (2,3) FAIMS were ionized components and separated components in drift tube of FAIMS; (4) Sensor plate of FAIMS was detected ion based on ion mobility; (5) Chromatogram from GC-FAIMS technique

CHAPTER 3 METHODOLOGY

3.1 Population

3.1.1 Target population and sample population

Target population

HCC patients

Sample population

Patients in this study were recruited HCC patients, cirrhosis patients, and chronic liver disease from the Chula Excellence Center of Endoscopy, Division of Gastroenterology, Department of Medicine, Chulalongkorn University.

3.1.2 Control population

Healthy volunteers, chronic HBV-infected patients, and cirrhotic patients

3.1.3 Inclusion criteria

The participants were divided into 4 groups as HCC patients, cirrhotic patients, chronic HBV-infected patients, and healthy volunteers. The inclusion criteria of each group are as follows:

3.1.3.1 HCC patients

The HCC patient cohort in the study comprised individuals aged between 18 to 80 years, whose HCC diagnosis was confirmed by either histopathological examination or presence of typical features in computed tomography (CT) and/or magnetic resonance imaging (MRI) (15).

The study specifically focused on HCC patients belonging to the Barcelona clinic liver cancer (BCLC) stage 0 (i.e., very early stage) and stage A (i.e., early stage).

- Patients at BCLC stage 0 presented with a single nodule less than <2 cm, had a good performance status (PS 0), lacked of

vascular invasion or metastases, and demonstrated well-preserved liver function (Child-Pugh A)

- Patients at BCLC stage A presented with a single nodule of ≤ 5 cm or no more than three nodules (≤3 cm), had a PS of 0 and Child-Pugh score of A.

3.1.3.2 Cirrhotic patients

Participants with cirrhosis were aged between 18 to 80 years. The diagnosis of cirrhosis was established based on either histopathological examination or the presence of radiologic features such as a small-sized nodular liver and evidence of portal hypertension (e.g., intraabdominal collateral circulation and/or splenomegaly) in ultrasonography (USG), CT, and MRI.

3.1.3.3 Chronic liver disease patients

Patients with chronic liver disease in this study are those who suffer from a chronic infection with the hepatitis B virus (HBV).

The inclusion criteria for chronic hepatitis B infected patients were those with a positive test result for hepatitis B surface antigen (HBsAg) for more than six months and were on antiviral therapy with virological suppression (21).

3.1.3.4 Healthy volunteers

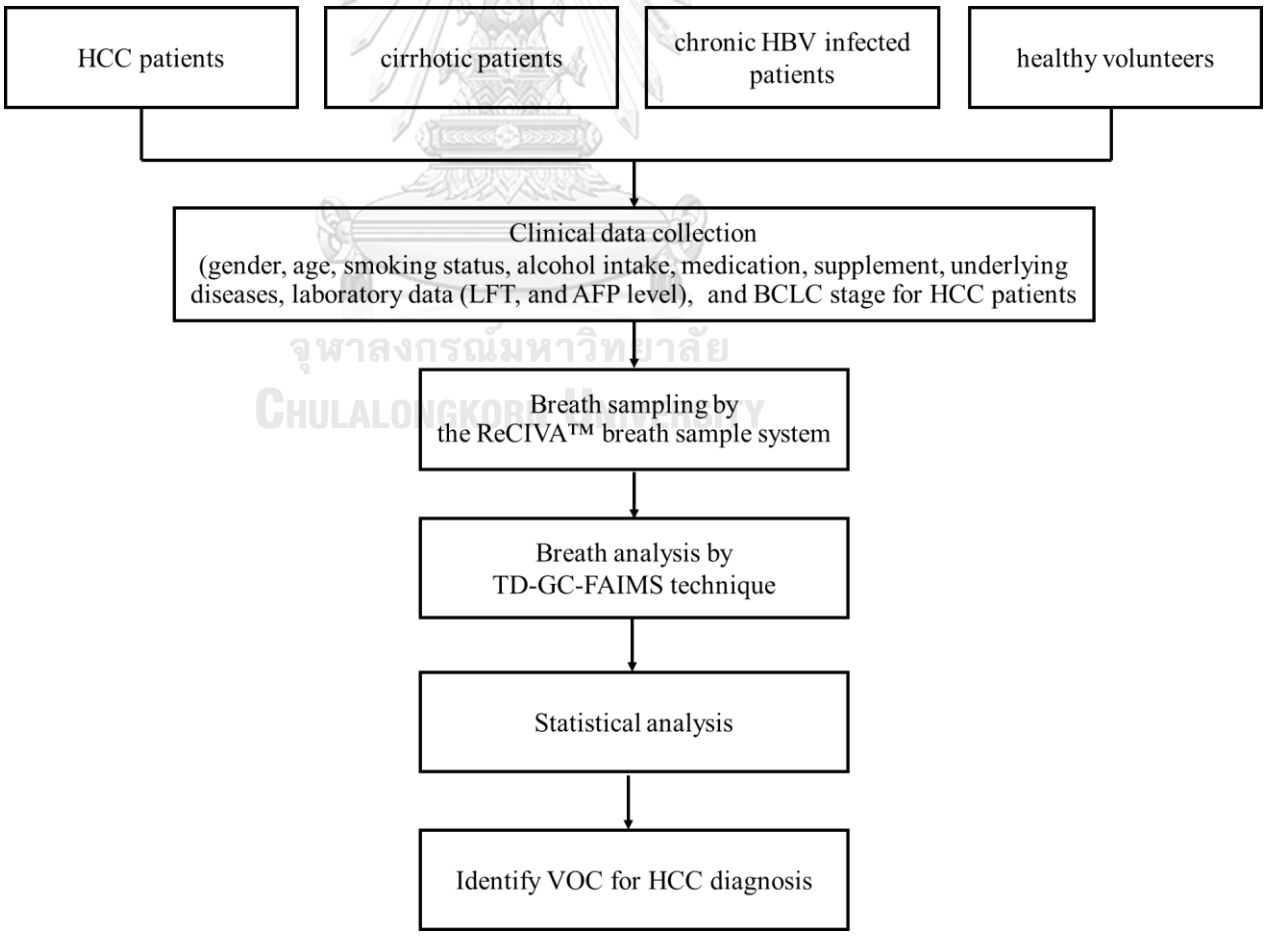
The study recruited healthy volunteers aged between 18 to 80 years, who had no history of chronic liver diseases or malignancies.

3.1.4 Exclusion criteria

-Patients with recent infection within the last three weeks preceding the study were excluded to avoid the potential confounding effects of active infection that can affect the analysis of certain biomarkers, such as volatile organic compounds (VOCs) in breath samples, that may be used for diagnostic purposes.

-Individuals who had used antibiotics or probiotics within the last three weeks were excluded (54).

3.2 Research framework



3.3 Sample size calculation

A value of the mean and SD of acetone as biomarker from exhaled breath for HCC diagnosis were based on a study by Sukaram T., *et al.* (2022) (43). The sample size of this study was calculated using 1-way ANOVA Pairwise as follows:

$$n = 2\left(\sigma \frac{Z_{1-\alpha/(2\tau)} + Z_{1-\beta}}{\mu_A - \mu_B}\right)^2$$

n = sample size

σ = the standard deviation of acetone in HCC = 69.3[43]

τ = the number of comparisons = 4

μ = mean of acetone in HCC and non-HCC group

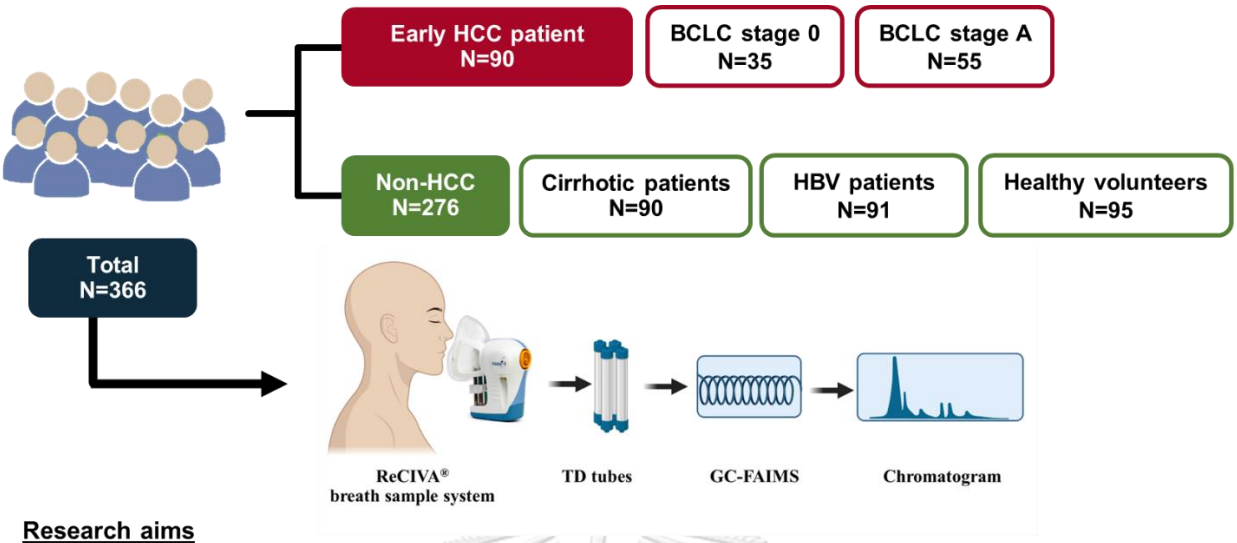
(94.42 and 40.90 respectively)[43]

α = Type I error = 0.05

$1-\beta$ = power =0.80

The calculated sample size was 360 (90 HCC patients, 90 cirrhotic patients, 90 chronic liver disease patients, and 90 healthy volunteers).

3.4 Study design



Research aims

- **To identify VOC profile for HCC diagnosis**
- **To investigate the association of VOC with HCC**
- **To investigate the diagnostic performance of VOCs in comparison to AFP**

3.5 Clinical data collection

The study collected several patient characteristics including gender, age, smoking history, alcohol consumption, current medication. and supplement use, underlying medical conditions (chronic hepatitis B and C infection, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, and diabetes mellitus), and laboratory data such as liver function tests, and AFP level. In addition, the diagnosis of HCC patients was determined based on histopathology and/or imaging results from CT and MRI scans. The staging of HCC was categorized based on the BCLC staging system, which considers three primary factors: tumor burden, liver function, and patient performance status.

3.6 Breath sample collection

As a preventive measure in the COVID-19 situation, all participants were presented with a negative result from an antigen test kit, and their breath samples were collected using a plastic particle barrier shown in **Figure 8**. All participants were fasted, stopped current medications, smoking, and alcohol consumption at least 6 hours before breath sampling to minimize the risk of oral contamination or food intake as a confounder (33). A total of 100 ml of exhaled breath was collected from each participant at a flow rate of 50 ml/min using the ReCIVA™ breath sample system (Owlstone Medical, Cambridge, UK), as shown in **Figure 9**. This system comprises a handheld unit connected to a laptop for real-time monitoring. The ReCIVA™ breath sample software is shown in **Figure 10.** This system has a disposable mask with a filter and 4 Thermal desorption (TD) tubes **(Figure 9),** and a constant supply of clean air is supplied to the patient's mask. Exhaled breath samples were trapped onto TD tube. Finally, TD tubes were analyzed by TD-GC-FAIMS technique.

Figure 8. Plastic particle barrier

Figure 9. *ReCIVA™ breath sample system*

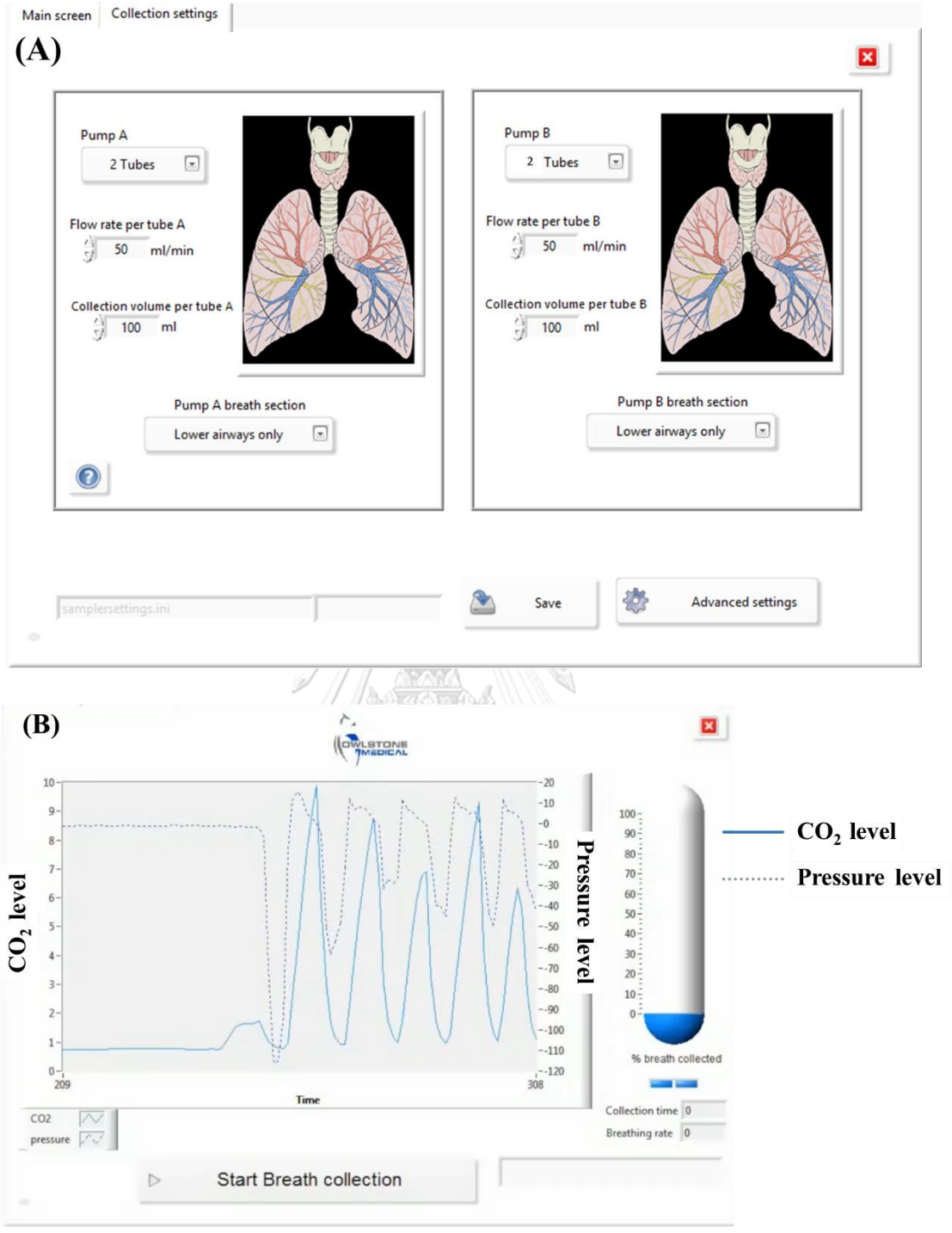


Figure 10. ReCIVA™ breath sample software; (A) collection settings, (B) Main screen

3.6 VOCs extraction and measurement

Breath samples underwent analysis using Thermal Desorption-Gas Chromatography-Field Asymmetric Ion Mobility Spectrometry (TD-GC-FAIMS) as shown in **Figure 11A**. The breath samples were extracted using a TD tube (Unity™ -xr, Markes International Ltd, Llantrisant, UK) in a two-stage desorption method with a constant flow of helium at 50ml/min. The extraction process consisted of two stages:

- The first stage involved dry-purging the TD tube for 1 min and heating it at 280 °C for 5 min.

- In the second stage, the cold trap (U-T12ME-2S, Markes International Ltd, Llantrisant, UK) was rapidly heated to from 10 °C to 290 °C to complete the extraction process. The VOCs in the sample were then transferred to GC-FAIMS **(Figure 11B)** through a capillary line that was heated to 130 °C.

Gas chromatography - Field Asymmetric Ion Mobility Spectrometry (GC-FAIMS)

GC-FAIMS analysis was performed using GC (Thermo Scientific, TRACE 1310) with FAIMS system (Owlstone Medical Lonestar VOC Analyze). GC system consists of an HP-PLOT U GC column (30m x 0.32mm ID x 10µm df; Agilent Technologies, USA) with helium as the carrier gas at a flow rate of 1.0 ml/min. The temperature program of GC column was heated at 40 °C for 2 min and ramped to 130 °C with flow rate of 10 °C /min. The FAIMS transfer line temperature was 130 °C with a drift tube length of 7.5 cm and drift voltage of 5 kV. The FAIMS system was operated at 40 °C and the ambient pressure at 10 mbar with the purified air as the drift gas at a flow rate of 150 mL/min.

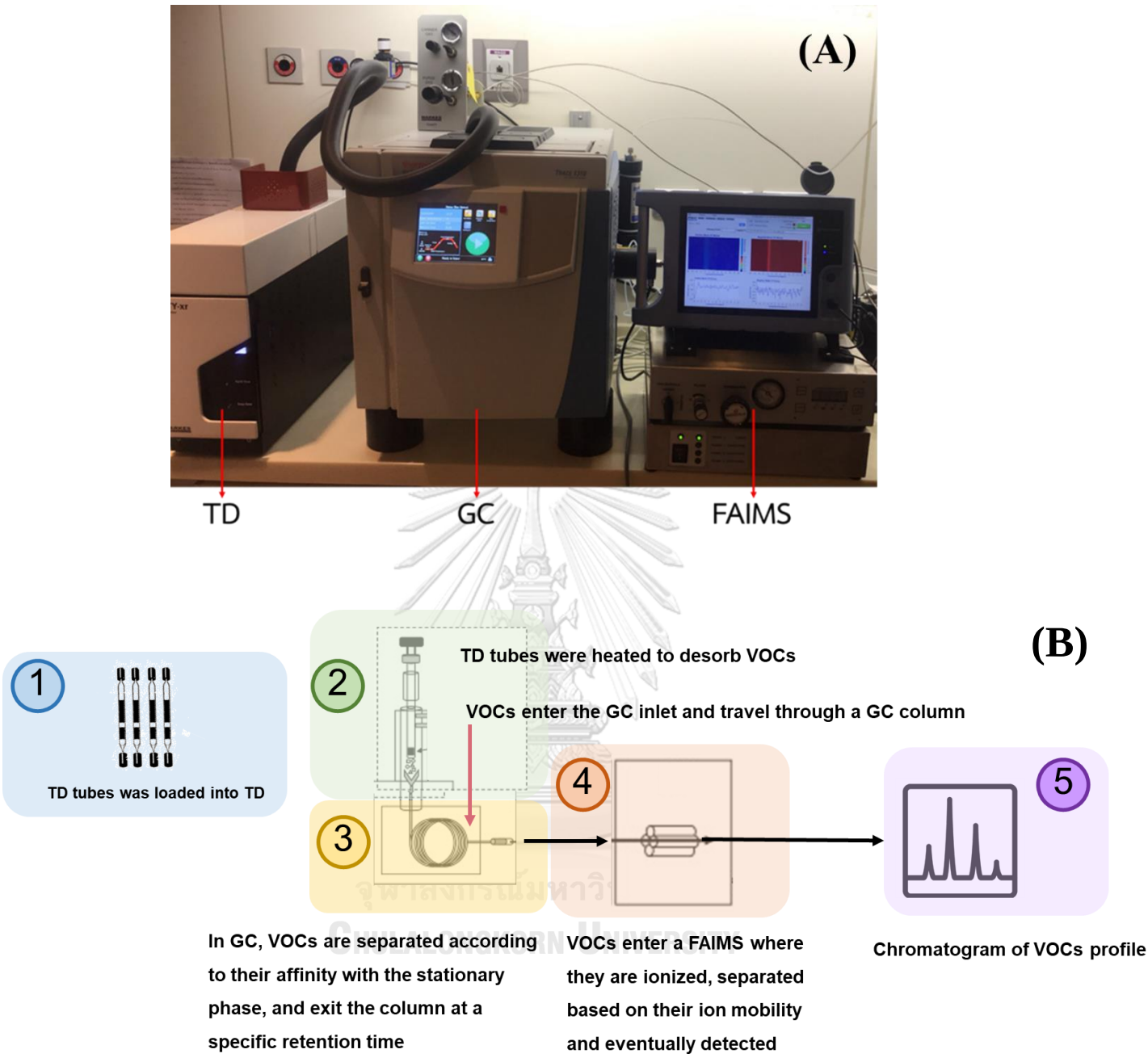


Figure 11. (A) TD-GC-FAIMS machines, (B) Schematic diagram of TD-GC-FAIMS

3.7 Analytical procedure for VOCs identification

The VOCs were profiled by targeted metabolomics and performed a semiquantitative analysis approach on TD-GC-FAIMS. The standard gas of 1,000 parts per billion (ppb) of ethanol, acetone, 1,4-pentadiene, dimethyl sulfide, benzene, isopropyl alcohol, acetonitrile, and toluene was prepared by diluting the standard solution with nitrogen gas in a Tedlar bag and heated at 80°C for 5 minutes. Then, 1.0 mL of the standard gas was injected into TD tubes **(Figure 12)** and analyzed using the TD-GC-FAIMS technique. The identification of the VOCs was based on comparing the retention times with those of the standard solutions. The acceptance criteria for compound identification were a maximum retention time difference of 5% relative standard deviation (RSD) between the sample and the standard solution values, in accordance with the guidelines of the Association of Official Analytical Chemists (AOAC) (55).

Figure 12. The method for sampling the standard gas from the Tedlar bag into the TD tube

3.8 Data analysis

In this study, patient characteristics were analyzed by summarizing continuous variables as mean and standard deviation (SD), or median (range), as appropriate.

-The Mann-Whitney U and Kruskal-Wallis tests were used for comparing continuous variables, while the $\chi 2$ test was used for categorical variables as count (percentage).

-A logistic regression analysis was used to identify VOCs that were independently associated with HCC. VOCs significantly associated with early HCC in the univariable model were included in the multivariable logistic regression analysis, controlled for age, gender, liver function tests and serum AFP.

- Receiver-operating characteristic (ROC) analysis was used to determine the performance at optimal cutoffs of VOC and AFP for early-stage HCC diagnosis. The entire patient cohort was divided into two independent sets: a training set (n = 293) and a test set (n = 73). The training set consisted of 72 early HCC patients, 72 cirrhosis patients, 73 chronic HBV infected patients, and 76 healthy controls. This set was used to determine the performance of VOC at the optimal cutoff. A leave-one-out cross-validation was performed within the training set. The same optimal cutoff for VOC was selected and evaluated for its performance using the test set, which included 18 early HCC patients, 18 cirrhosis patients, 18 chronic HBV infected patients, and 19 healthy controls. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy of VOCs and AFP were compared using McNemar test. Area Under the ROC Curves (AUCs) were compared using concordance statistics (c-statistics).

-To reduce the chances of type I errors, statistically significant differences in VOC levels between groups were considered at the p-value corrected using the Bonferroni correction method for a multiple pairwise comparison, which included the number of VOCs tested and the number of study groups. A p-value of <0.05 and a corrected p-value of <0.0056 were

considered statistically significant. SPSS version 22.0.0 (SPSS Inc, Chicago, IL, USA) was used to perform all statistical tests.

3.9 Ethical Consideration

The study was performed after the approval by the Ethics and Research Committee of the Faculty of Medicine, Chulalongkorn University. Written consent forms were obtained from all the participants before enrollment. The study protocol has been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 058/64)**.**

3.10 Expected benefits and applications

Successfully identifying VOCs could potentially be a non-invasive test and effective measurement for early detection of HCC and improve patients' outcomes. Additionally, VOCs could potentially be useful as an adjunctive tool to improve the performance of ultrasound for HCC detection, and the VOC platform and analysis method in this study can be further applied to identify potential VOC markers for diagnosis of other cancers.

3.11 Challenges

The use of VOCs as a diagnostic tool for early HCC care surveillance also poses some challenges. One major challenge is the complexity and variability of VOC profiles, which can be affected by various factors such as time since last eating, smoking status, alcohol status, and medication, making it difficult to identify specific VOC markers that are truly indicative of HCC. These are some steps that can be taken to minimize the impact of confounding factors:

- Standardize the collection procedure: Establish a standardized protocol for breath sample collection to ensure consistency in the collection process. This includes ensuring that subjects are in a standardized condition (such as fasting for a specific duration) before breath

collection, using standardized collection tubes, and ensuring that collection procedures are consistent across all subjects.

- Control for environmental factors: Avoid exposure to external VOCs by collecting breath samples in a controlled environment, such as a well-ventilated room. Minimize exposure to smoking, alcohol, and other potential VOC confounders in the sample collection environment.

- Account for lifestyle factors: Record lifestyle factors, including smoking status, alcohol consumption, and medication use, for each subject in the study. These factors can be used as potential confounders in data analysis.

- Match subjects: To minimize the impact of confounding factors, match subjects based on demographic characteristics and lifestyle factors, such as smoking status and alcohol consumption. This can be done through careful selection of subjects or by randomization.

By following these steps, the potential influence of confounding factors can be minimized, ensuring more accurate and reliable VOC profiles for early detection of HCC.

CHAPTER 4 RESULTS

4.1 Baseline patient characteristics

Of the 366 enrolled patients, there were 90 HCC patients, 90 cirrhotic patients, 91 HBV-infected patients, and 95 healthy volunteers. The cohort's mean age was 60.9 ± 10.7 years, and 181 (49.5%) were males. **Table 4** shows the baseline characteristics of the four groups. The mean age and proportion of males in the four groups, as well as proportions of smoking status, alcohol consumption, patients with cirrhosis, cirrhosis severity, chronic HBV and HCV infection, NAFLD, and diabetes, were not significantly different. The severity of liver impairment was comparable between the HCC and cirrhosis groups, with 79 (90.8%) vs. 82 (91.1%) for Child-Pugh class A patients and 8 (9.2%) vs. 8 (8.9%) for Child-Pugh class B patients **(Figure 13)**.

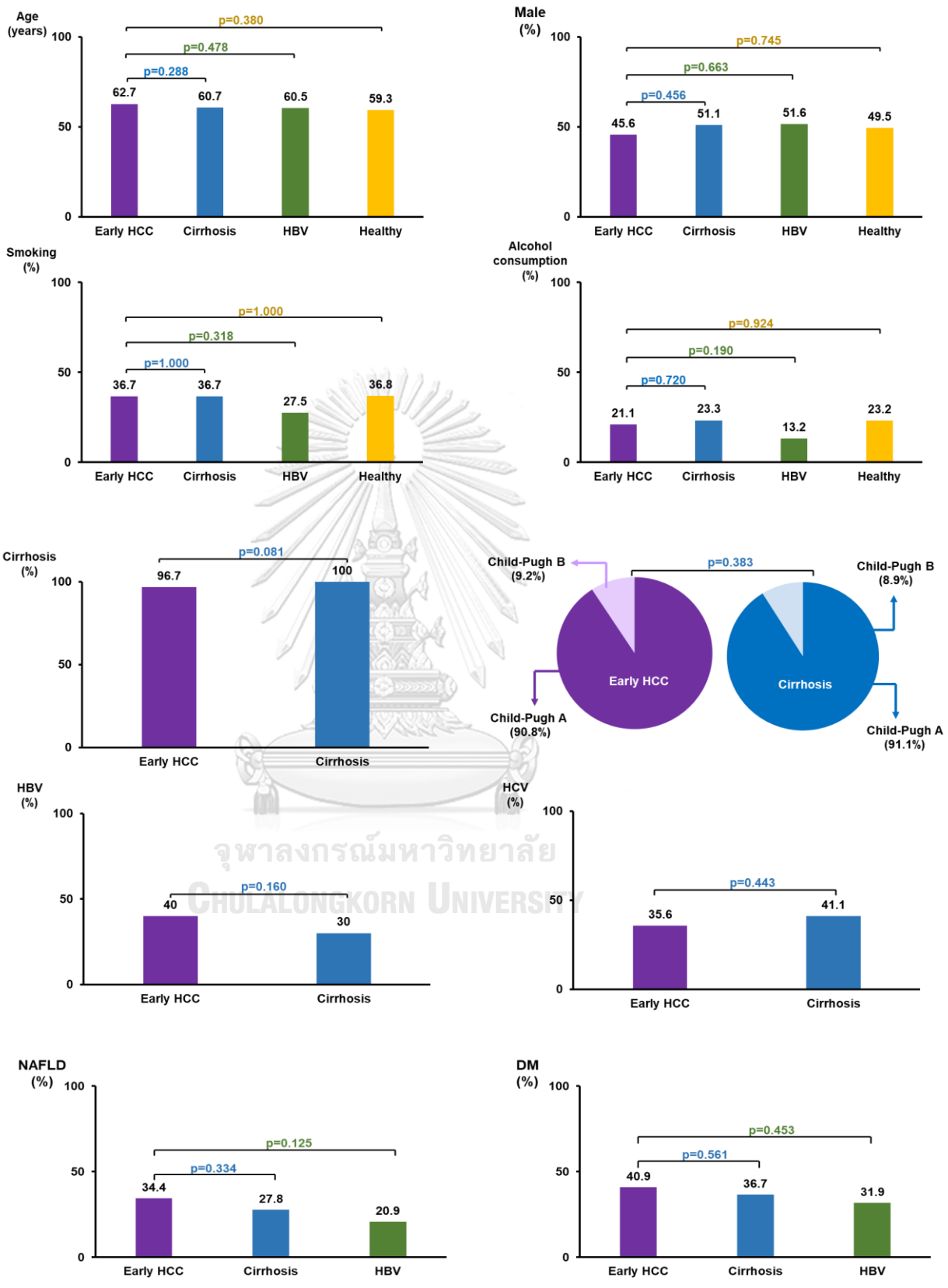


Figure 13. Baseline patient characteristics

The median levels of total bilirubin (TB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and alkaline phosphatase (ALP) did not show significant differences between early HCC and those with cirrhosis. However, the HCC group had significantly higher levels compared to the cirrhosis, HBV, and healthy volunteer groups **(Figure 14)**.

The median AFP levels in the HCC, cirrhosis, HBV, and healthy volunteer groups were 4.8 (range: 1-643) vs. 3.0 (1-18) vs. 1.9 (1-8) vs. 3.1 (1-11), respectively, $p<0.001$. In the HCC groups, there were 35 (38.9%), 55 (61.1%) patients with BCLC stage 0 and A, respectively. The mean tumor size of the entire cohort was 2.7±2.6 cm, with 1.5±0.4 cm for BCLC stage 0, and 3.5±3.0 cm for BCLC stage A, respectively. Among BCLC stage A patients, 35 (63.6%) had a single lesion and 19 (36.5%) had two or three lesions**.**

Table 4. Baseline characteristics of early HCC, cirrhosis, HBV, and healthy volunteers

Variables	Early HCC (n=90)	Cirrhosis (n=90)	HBV (n=91)	Healthy (n=95)	p^a	p^b	p^c
Age (years), mean ± SD	62.7 ± 10.71	60.7 ± 9.3	60.5 ± 10.9	59.3 ± 9.1	0.288	0.478	0.380
Male, n (%)	41 (45.6%)	46 (51.1%)	47 (51.6%)	47 (49.5%)	0.456	0.663	0.745
Smoking, n (%)	33 (36.7%)	33 (36.7%)	25 (27.5%)	35 (36.8%)	1.000	0.318	1.000
Alcohol consumption, n (%)	19 (21.1%)	21 (23.3%)	12 (13.2%)	22 (23.2%)	0.720	0.190	0.924
Cirrhosis, n (%)	87 (96.7%)	90 (100.0%)	0 (0.0%)	0 (0.0%)	0.081		
Child-Pugh class, n (%)					0.383		
A	79 (90.8%)	82 (91.1%)	0 (0%)	0 (0.0%)			
B	8 (9.2%)	8 (8.9%)	0 (0%)	0 (0.0%)			
Alcoholic liver disease	21 (23.3%)	12 (13.3%)	0 (0.0%)	0 (0.0%)	0.083		
HBV, n (%)	36 (40.0%)	27 (30.0%)	91 (100%)	0 (0.0%)	0.160		
HCV, n (%)	32 (35.6%)	37 (41.1%)	0 (0.0%)	0 (0.0%)	0.443		
NAFLD, n (%)	31 (34.4%)	25 (27.8%)	19 (20.9%)	0 (0.0%)	0.334	0.125	
Diabetes mellitus, n (%)	36 (40.9%)	33 (36.7%)	29 (31.9%)	0 (0.0%)	0.561	0.453	
TB (mg/dL)*	0.9 (0.3-5.2)	0.9 (0.1-7.8)	0.8 (0.2-2.0)	0.7 (0.4-1.6)	0.826	0.073	0.101
Albumin (g/dL)*	4.0 (2.7-4.9)	4.1 (2.7-4.8)	4.3 (4.0-4.8)	4.4 (4.0-4.6)	0.114	<0.001	<0.001
AST (U/L)*	33.5 (13.0-466.0)	32.0 (14.0-241.0)	21.0 (16.0-89.0)	21.0 (16.0-37.0)	0.187	<0.001	<0.001
ALT (U/L)*	31.50 (7.0-489.0)	27.0 (13.0-109.0)	19.0 (7.0-184.0)	21.5 (14.0-40.0)	0.110	<0.001	0.001
ALP (U/L)*	92.5 (45.0-398.0)	83.0 (28.0-261.0)	68.0 (40.0-184.0)	64.5 (44.0-140.0)	0.144	<0.001	0.002
AFP (ng/mL)*	4.8 (1-643)	3.0 (1-18)	1.9 (1-8)	3.1 (1-11)	<0.001	<0.001	0.001

* Shown as median (range),

p^a for HCC vs. cirrhosis, p^b for HCC vs. cirrhosis vs. HBV, p^c for HCC vs. healthy

Abbreviations: AFP – alpha fetoprotein, ALP – alkaline phosphatase, ALT – alanine aminotransferase, AST – aspartate aminotransferase, HBV – hepatitis B virus, HCC – hepatocellular carcinoma, HCV – hepatitis C virus, NAFLD – non-alcoholic fatty liver disease, TB – total bilirubin

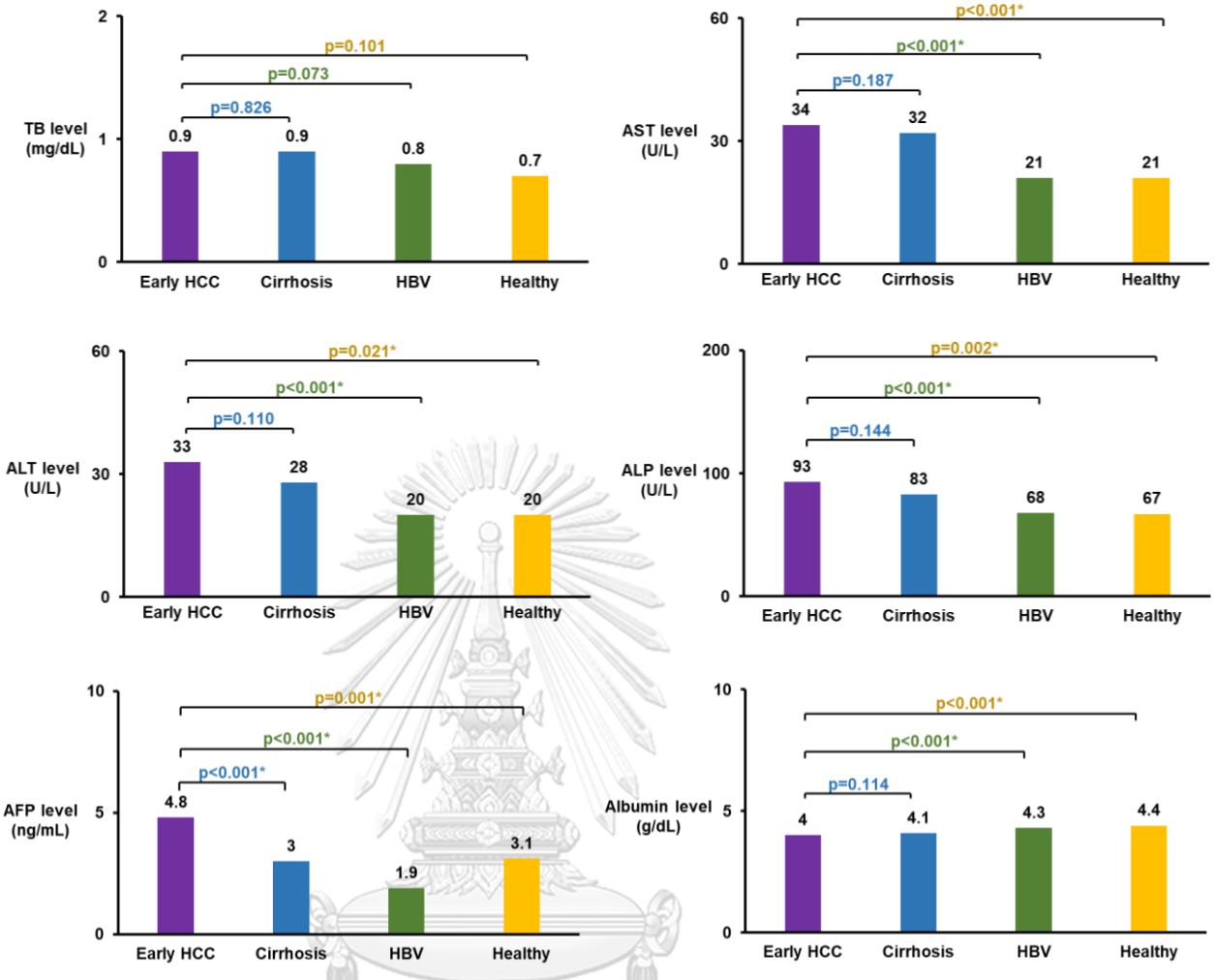


Figure 14. Liver function test (LFT)

4.2 Identification of VOCs

The VOCs were profiled using a targeted metabolomics approach on TD-GC-FAIMS. The VOCs were identified by comparing their retention times with those of the standard solution, as shown in **Figure 15.** The acetone compound exhibited two peaks, namely the acetone monomer and acetone dimer peaks. The FAIMS technique consists of beta radiation as a light source that emits electrons, which can ionize air and moisture, resulting in the formation of hydronium ions and hydrated oxygen ions. These ions can react with compounds that have high proton or electron affinity, such as acetone, replacing one or two water molecules in the hydronium ion to form monomer or dimer ions, respectively. Therefore, a chromatogram combining the monomer and dimer peaks at their maximum peak heights was generated for the acetone level. Furthermore, we also identified another VOC (ethanol, 1,4-pentadiene, dimethyl sulfide, benzene, isopropyl alcohol, acetonitrile, and toluene) as shown in **Figure 16.**

The results showed that the % Relative Standard Deviation (%RSD) of the retention time in the standard solution was close to the mean of the observed levels in the breath sample. The %RSD falls within the range of 1.0-3.2% **(Table 5)**. Therefore, eight VOC compounds (ethanol, 1,4-pentadiene, dimethyl sulfide, benzene, isopropyl alcohol, acetone, acetonitrile, and toluene) identified in this study are presented in **Figure 17.**

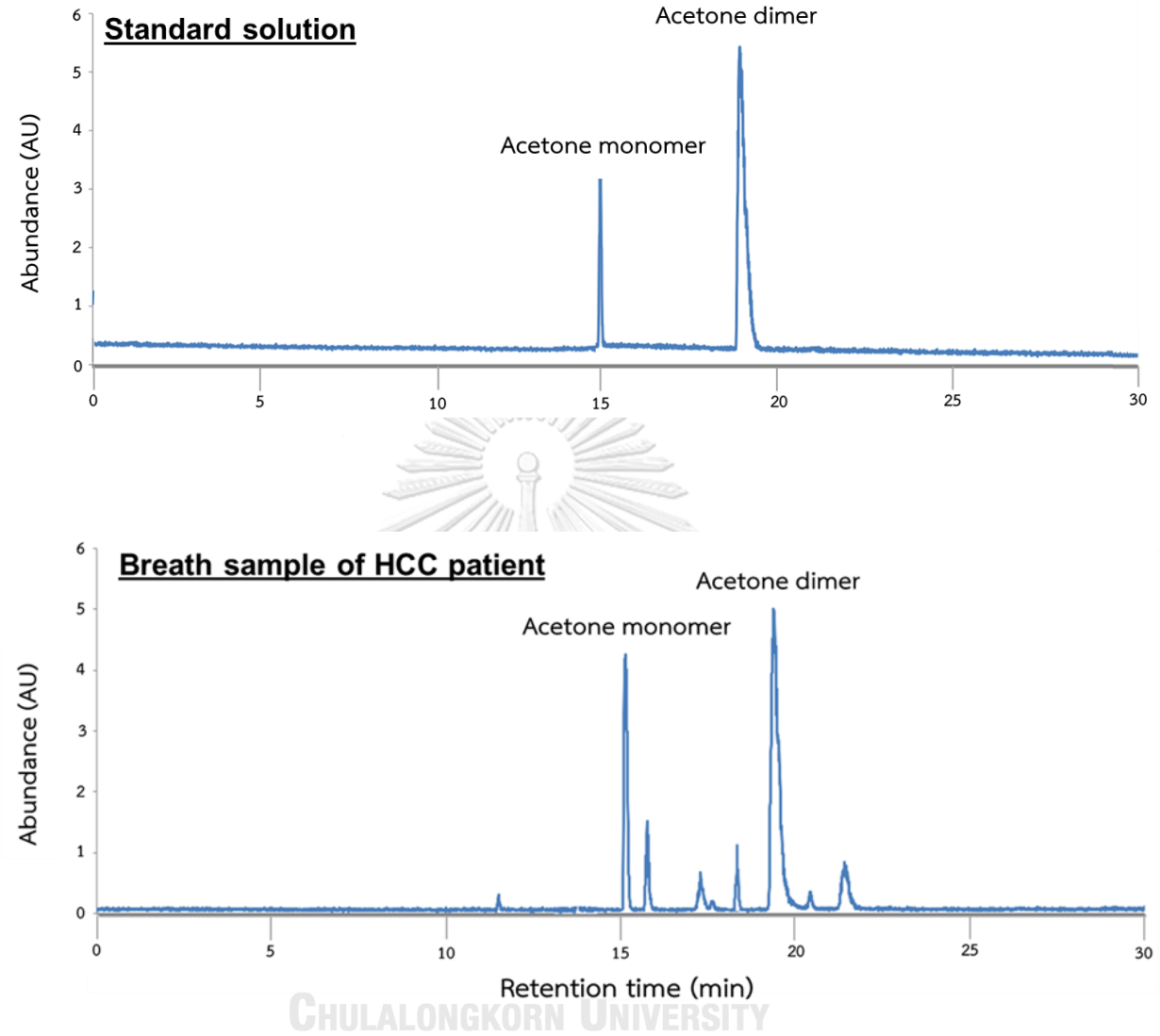


Figure 15. The retention times of acetone in the standard solution and breath sample of the HCC patient

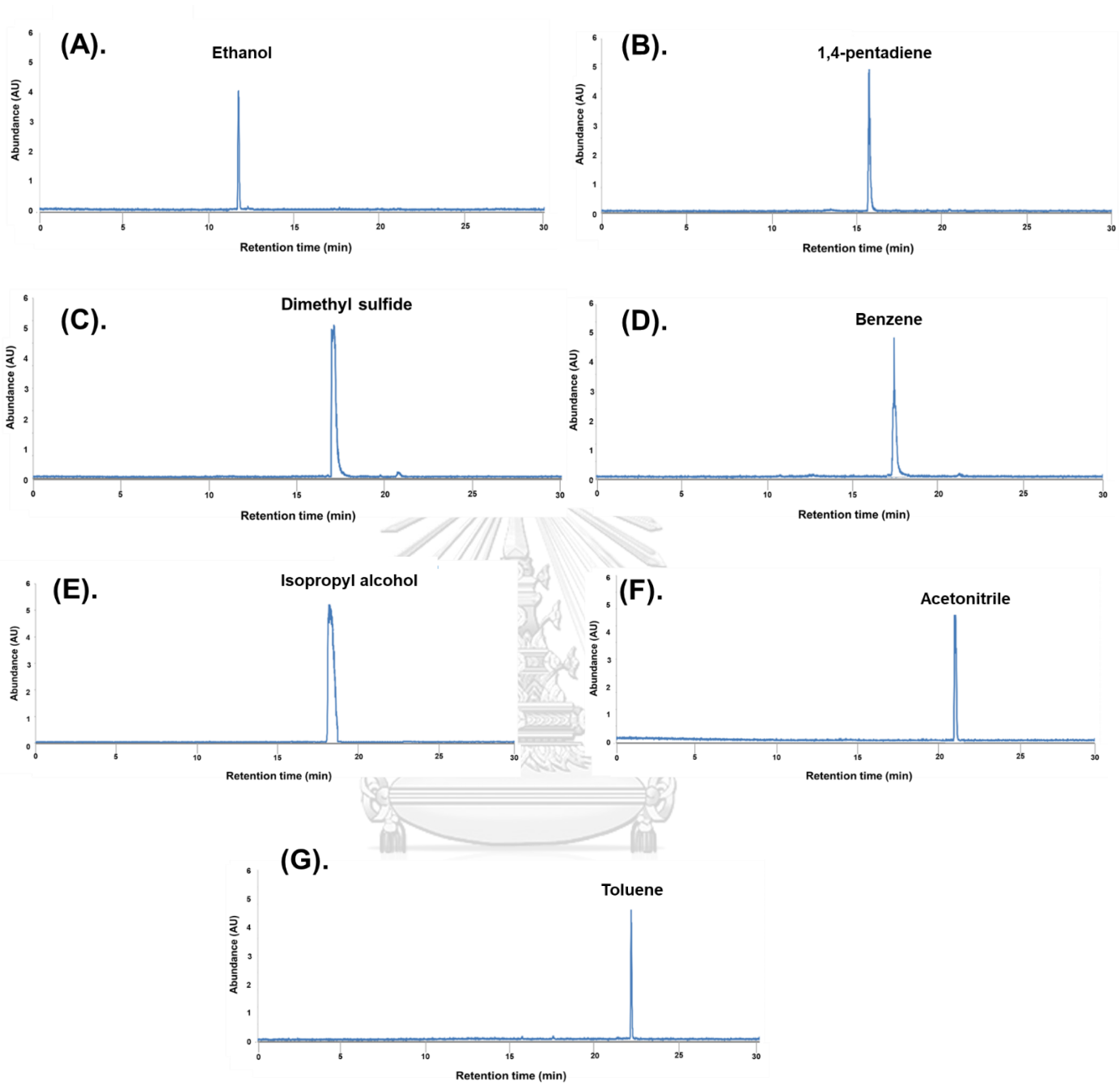


Figure 16. The retention times of the standard solution; (A) ethanol, (B) 1,4-pentadiene, (C) dimethyl sulfide, (D) benzene, (E) isopropyl alcohol, (F) acetonitrile, and (G) toluene

Table 5. Retention time of VOC compounds in this study

Compound	Chemical Abstracts Service (CAS)*	Retention time, R_t (min)	% RSD **
Ethanol	64-17-5	11.30	1.1
Acetone monomer	67-64-1	14.79	1.2
1,4-pentadiene	591-93-5	15.67	2.4
Dimethyl sulfide	75-18-3	16.96	3.2
Benzene	71-43-2	17.83	2.1
Isopropyl alcohol	67-63-0	18.26	1.9
Acetone dimer	26776-70-5	18.69	1.0
Acetonitrile	75-05-8	20.00	2.9
Toluene	108-88-3	22.11	2.7

***** Chemical Abstracts Service (CAS) registry number is a unique identifier assigned to every chemical substance described in scientific literature

*** *** %RSD= (SD/mean) x100

%RSD <5% as Expected precision according to Association of Official Analytical Chemists (AOAC)

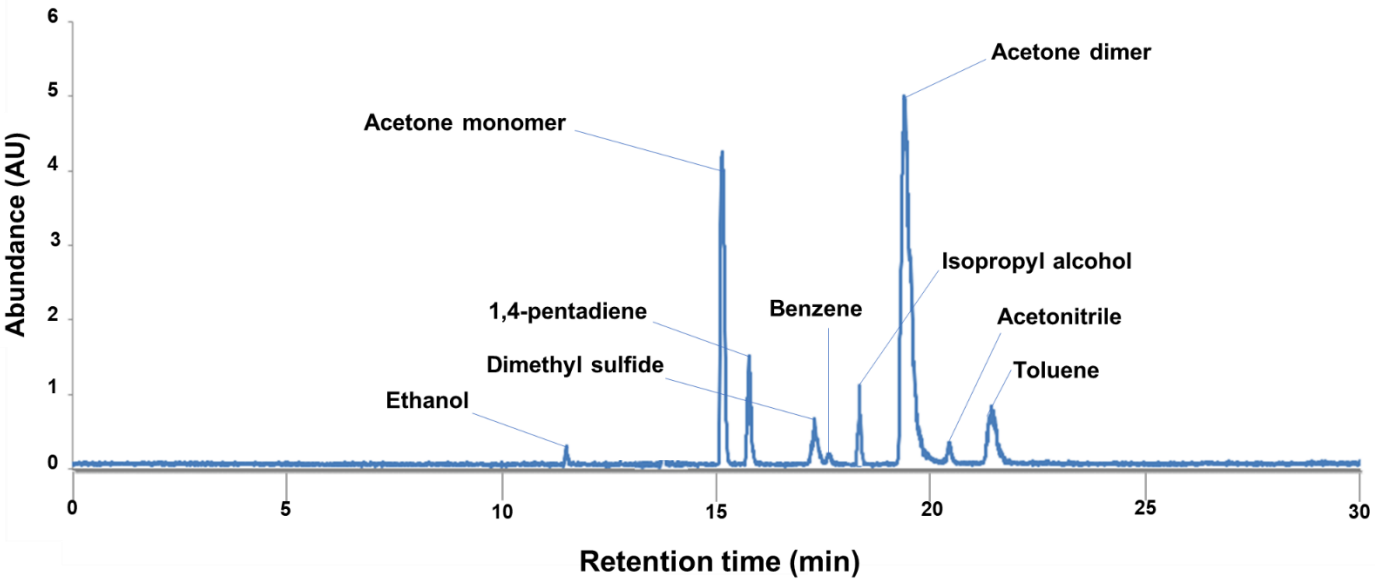


Figure 17. Chromatogram of the VOC profile in the breath sample of the HCC patient

4.3 Levels of VOCs in the study cohort

Table 6 shows the levels of 8 exhaled VOCs in this study. Four VOCs (acetone, 1,4-pentadiene, isopropyl alcohol, and dimethyl sulfide) were found in significantly different levels in the four groups. The levels of the 4 VOCs of HCC, cirrhosis, HBV groups, and healthy were as follows: for acetone, the levels were 9.05 vs. 8.08 vs. 7.90 vs. 7.81 AU , respectively, obtained by combining the monomer and dimer peaks at their maximum heights; for 1,4-pentadiene, the levels were 0.76, 0.97, 1.04, and 1.10 AU; for isopropyl alcohol, the levels were 0.32, 0.28, 0.17, and 0.19 AU; and for dimethyl sulfide, the levels were 0.63, 0.44, 0.45, and 0.35 AU, $p<0.001$, $p<0.001$, $p=0.002$, and $p=0.001$, respectively.

When comparing HCC and cirrhotic patients, significantly different levels of three VOCs were observed. Early HCC patients exhibited significantly higher levels of acetone ($p<0.001$) and dimethyl sulfide ($p=0.004$), but lower levels of 1,4-pentadiene ($p<0.001$) compared to cirrhotic patients **(Table 6)**. In comparison to HBV and healthy groups, HCC patients also showed significant differences in the levels of these four VOCs. Acetone, isopropyl alcohol, and dimethyl sulfide levels were significantly higher, whereas the level of 1,4-pentadiene was lower **(Table 6)**.

When comparing early HCC with BCLC stage 0 and A, significant differences were observed in the levels of two VOCs. Early HCC with BCLC stage 0 exhibited lower levels of isopropyl alcohol and dimethyl sulfide compared to early HCC with BCLC stage A, with a mean ± standard deviation (SD) level of (0.20 ± 0.18 vs. 0.40 ± 0.44, $p=0.008$) for isopropyl alcohol, and (0.55 ± 0.79 vs. 0.68 ± 0.65, $p=0.035$) for dimethyl sulfide **(Table 7)**. Acetone and 1,4-pentadiene levels in BCLC stage 0 were also lower than in BCLC stage A but the difference did not reach statistical significance (9.14 ± 0.77 vs. 8.99 ± 1.10 AU, $p = 0.369$ for acetone, and 0.80 ± 0.34 vs. 0.71 ± 0.33 AU, $p = 0.201$ for 1,4-pentadiene). However, the small sample size made it difficult to statistically identify even a moderately large difference. These findings provide a rationale for replication with a larger sample of patients.

When comparing early HCC (BCLC stage 0, A) with lesions ≤ 2cm to the non-HCC group, early HCC showed significantly higher levels of acetone than the non-HCC group (9.12 ± 0.77 vs. 7.93 ± 0.95, p<0.001), respectively **(Table 8)**.

Table 6. VOCs levels of early HCC, cirrhosis, HBV, and healthy groups*

VOCs	Early HCC (n=90)	Cirrhosis (n=90)	HBV (n=91)	Healthy (n=95)	p^a	p^b	p^c	p^d	p^e
Acetone	9.05 ± 0.99	8.08 ± 0.73	7.90 ± 0.98	7.81 ± 1.09	<0.001	<0.001	<0.001	<0.001	<0.001
1,4-pentadiene	0.76 ± 0.33	0.97 ± 0.46	1.04 ± 0.37	1.10 ± 0.32	<0.001	<0.001	<0.001	<0.001	<0.001
Isopropyl alcohol	0.32 ± 0.37	0.28 ± 0.31	0.17 ± 0.15	0.19 ± 0.17	0.111	<0.001	0.003	0.005	0.002
Dimethyl sulfide	0.63 ± 0.71	0.44 ± 0.36	0.45 ± 0.30	0.35 ± 0.23	0.004	0.004	<0.001	0.004	0.001
Ethanol	0.25 ± 0.05	0.24 ± 0.06	0.26 ± 0.07	0.26 ± 0.06	0.070	0.111	0.111	0.130	0.082
Benzene	0.22 ± 0.15	0.28 ± 0.21	0.22 ± 0.13	0.23 ± 0.15	0.016	0.111	0.111	0.059	0.094
Acetonitrile	0.19 ± 0.10	0.18 ± 0.09	0.19 ± 0.17	0.20 ± 0.12	0.111	0.111	0.111	0.668	0.668
Toluene	0.51 ± 0.36	0.55 ± 0.46	0.49 ± 0.24	0.49 ± 0.45	0.111	0.111	0.111	0.574	0.355

*VOCs levels are expressed as mean ± standard deviation in arbitrary unit.

p^a for HCC vs. cirrhosis, p^b for HCC vs. HBV, p^c for HCC vs. healthy,

p^d for HCC vs. cirrhosis vs. HBV, p^e for HCC vs. cirrhosis vs. HBV vs. healthy

A p value of <0.0056 was considered statistically significant.

Abbreviations: HBV – hepatitis B virus, HCC – hepatocellular carcinoma, VOC – Volatile organic compound

Table 7. VOCs levels of early HCC patients with BCLC stage 0 and A

VOCs	HCC BCLC 0 (n=35)	HCC BCLC A (n=55)	*p*
Acetone	9.14 ± 0.77	8.99 ± 1.10	0.369
1,4-pentadiene	0.80 ± 0.34	0.71 ± 0.33	0.201
Isopropyl alcohol	0.20 ± 0.18	0.40 ± 0.44	0.008
Dimethyl sulfide	0.55 ± 0.79	0.68 ±0.65	0.035
Ethanol	0.25 ± 0.06	0.25 ± 0.05	0.963
Benzene	0.21 ± 0.14	0.22 ± 0.15	0.548
Acetonitrile	0.18 ± 0.10	0.20 ± 0.10	0.146
Toluene	0.52 ± 0.33	0.50 ± 0.38	0.446

Table 8. VOCs levels of early HCC patients with lesion ≤ 2cm and non-HCC group*

VOCs	HCC with lesion ≤ 2cm (n=52)	non-HCC* (n=276)	*p*
Acetone	9.12 ± 0.77	7.93 ± 0.95	<0.001
1,4-pentadiene	0.81 ± 0.37	0.90 ± 0.42	0.185
Isopropyl alcohol	0.28 ± 0.34	0.20 ± 0.21	0.470
Dimethyl sulfide	0.55 ± 0.66	0.42 ± 0.31	0.556
Ethanol	0.25 ± 0.06	0.25 ± 0.06	0.918
Benzene	0.23 ± 0.16	0.25 ± 0.17	0.465
Acetonitrile	0.17 ± 0.09	0.19 ± 0.13	0.372
Toluene	0.50 ± 0.32	0.49 ± 0.40	0.442

* non-HCC group included patients with cirrhosis, chronic viral hepatitis B infection, and healthy volunteer.

4.4 Association between VOCs and HCC

When comparing early HCC to the non-HCC group, acetone, 1,4-pentadiene, and isopropyl alcohol were found to be significantly associated with HCC **(Table 9)** in the univariate analysis. Additionally, in the multivariate analysis adjusted for age, gender, liver function tests, and AFP level, the association of acetone, 1,4-pentadiene, and isopropyl alcohol with HCC remained significant, with an adjusted odds ratio (OR) of 3.46 (1.73-6.90), p<0.001 for acetone, 0.06 (0.01-0.38) 0.003, p=0.003 for 1,4-pentadiene, and 11.49 (1.30-101.69), p= 0.028 for isopropyl alcohol.

Similarly, when comparing early HCC to HBV patients **(Table 9)**, acetone, 1,4-pentadiene, and isopropyl alcohol were found to be significantly associated with HCC, with adjusted ORs of 7.00 (3.43-14.29), p<0.001 for acetone, 0.04 (0.01-0.21), p<0.001 for 1,4-pentadiene, and 29.01 (2.21-381.21), p=0.010 for isopropyl alcohol.

When comparing early HCC to cirrhosis patients, acetone and 1,4-pentadiene were found to be significantly associated with HCC, with adjusted ORs of 3.56 (2.28-5.57), p<0.001 for acetone and 0.19 (0.06-0.57), p=0.003 for 1,4-pentadiene.

Furthermore, when comparing early HCC to healthy volunteers, only acetone was found to be significantly associated with HCC, with an adjusted OR of 0.25 (0.14-0.47), p<0.001. Therefore, acetone was the VOC most significantly associated with early HCC. Based on these findings, acetone was chosen as a VOC candidate for early HCC detection.

Table 9. Associations between VOCs and HCC

Comparison	**OR (95%CI)**	***p***	**Adjusted OR (95%CI)**	***p***
HCC vs. non-HCC*				
Acetone	5.04 (3.34-7.61)	<0.001	3.46 (1.73-6.90)	<0.001
1,4-pentadiene	0.16 (0.07-0.35)	<0.001	0.06 (0.01-0.38)	0.003
Isopropyl alcohol	3.67 (1.23-10.94)	0.020	11.49 (1.30-101.69)	0.028
Dimethyl sulfide	2.05 (1.19-3.54)	0.009	2.47 (0.64-9.57)	0.190
HCC vs. HBV				
Acetone	4.20 (2.57-6.85)	<0.001	7.00 (3.43-14.29)	<0.001
1,4-pentadiene	0.11 (0.04-0.28)	<0.001	0.04 (0.01-0.21)	<0.001
Isopropyl alcohol	19.42 (1.94-194.66)	0.012	29.01 (2.21-381.21)	0.010
Dimethyl sulfide	1.93 (1.01-3.68)	0.045	1.26 (0.45-3.52)	0.662
HCC vs. cirrhosis				
Acetone	3.57 (2.34-5.44)	<0.001	3.56 (2.28-5.57)	<0.001
1,4-pentadiene	0.23 (0.09-0.56)	0.001	0.19 (0.06-0.57)	0.003
Isopropyl alcohol	1.34 (0.41-4.36)	0.623		
Dimethyl sulfide	1.95 (1.03-3.70)	0.040	1.70 (0.72-4.03)	0.228
HCC vs. healthy				
Acetone	0.24 (0.15-0.39)	<0.001	0.25 (0.14-0.47)	<0.001
1,4-pentadiene	0.48 (0.19-1.17)	0.106		
Isopropyl alcohol	0.12 (0.02-0.81)	0.029	0.96 (0.01-1.05)	0.055
Dimethyl sulfide	0.23 (0.08-0.67)	0.007	0.37 (0.09-1.43)	0.149

Adjusted for: age, gender, TB, Alb, AST, ALT, ALP, and AFP

* non-HCC group included patients with cirrhosis, chronic viral hepatitis B infection, and healthy volunteer.

Abbreviations: AFP – alpha fetoprotein, ALP – alkaline phosphatase, ALT – alanine

aminotransferase, AST – aspartate aminotransferase, HCC – hepatocellular carcinoma, OR– odds ratio, TB – total bilirubin

4.5 Performance of exhaled VOC for diagnosis of early HCC

Table 10 presents the performance of acetone and AFP at their respective optimal cutoffs. For acetone, with a cutoff of 8.68 AU, the training set demonstrated an accuracy of 87.7%, sensitivity of 88.9%, specificity of 87.3%, positive predictive value (PPV) of 69.6%, negative predictive value (NPV) of 96.0%, and an area under the receiver operating characteristic curve (AUROC) of 0.932. In the test set, acetone achieved an accuracy of 79.5%, sensitivity of 83.3%, specificity of 78.2%, PPV of 55.6%, NPV of 93.5%, and an AUROC of 0.870.

Regarding AFP as a biomarker for HCC diagnosis, it was found that at a cutoff of 2.99 ng/mL, AFP levels showed a sensitivity of 88.9% compared to 68.2% for distinguishing HCC patients from non-HCC patients. The specificity was 87.3% versus 63.6%, and the accuracy was 87.7% versus 65.2%, with p-values of 0.017, 0.0001, and <0.001, respectively. Acetone exhibited a significantly higher AUROC than AFP, with values of 0.932 vs. 0.725 (p=0.001) **(Figure 18)**.

Table 10. Performance of acetone dimer and AFP for early HCC diagnosis

Early HCC vs. non-HCC*	Acetone (AU)	AFP (ng/mL)	*p* [a]
Sensitivity	88.9	68.2	0.017
Specificity	87.3	63.6	0.001
PPV	69.6	50.4	0.017
NPV	96.0	78.6	0.001
Accuracy	87.7	65.2	<0.001
AUROC	0.932	0.725	0.001

* non-HCC group included patients with cirrhosis, chronic viral hepatitis B infection, and healthy volunteer.

p [a] for acetone at the optimal cutoffs (8.68 AU) vs. AFP at the optimal cutoffs (2.99 ng/mL)

Abbreviations: AFP – alpha fetoprotein, AU – arbitrary unit, AUROC – area under the ROC curve, HCC – hepatocellular carcinoma, NPV – negative predictive value, PPV – positive predictive value

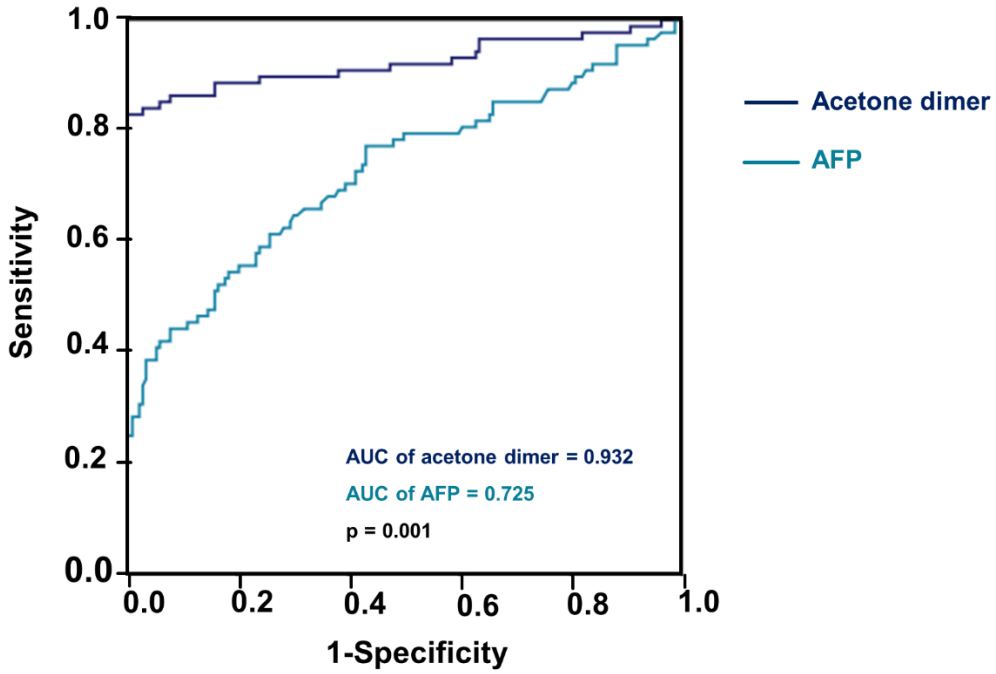


Figure 18. AUROC curves of the acetone and AFP for differentiating early HCC vs. non-HCC patients

CHAPTER 5 DISCUSSION

In Thailand, hepatocellular carcinoma (HCC) is highly prevalent and considered one of the most common cancers in the country. Unfortunately, the majority of HCC cases in Thailand are diagnosed at an advanced stage, rendering curative treatments unfeasible. Consequently, HCC ranks as the 3rd leading cause of cancer-related deaths worldwide and the 2nd leading cause in Thailand (56). However, the common HCC screening and surveillance methods have limitations and show low sensitivity in detecting early-stage HCC. Previous studies on volatile organic compounds (VOCs) in cancers have suggested that the alteration in metabolic pathways in HCC patients leads it to distinct patterns of VOCs of HCC patients for non-cancer individuals.

In this study, volatile organic compounds (VOCs) were utilized to evaluate the diagnostic performance of VOCs in identifying early-stage HCC in patients at risk for HCC. The levels of VOCs in exhaled breath were compared between 90 early-stage HCC patients and at-risk groups, including 90 cirrhotic patients and 91 chronic HBV-infected patients, as well as 95 healthy volunteers. Additionally, the ability of exhaled VOCs to detect early HCC was compared to that of AFP.

The baseline characteristics of the four groups, including the mean age and proportions in each group, as well as the proportions of gender, smoking status, alcohol consumption, patients with cirrhosis, cirrhosis severity, chronic HBV and HCV infection, NAFLD, and diabetes, were not significantly different among these characteristics across the four groups

The liver function test (LFT) and cirrhosis severity were not different between patients with early HCC and those with cirrhosis. Therefore, the association between VOCs and HCC was unlikely to be attributed to impaired liver function. However, the HCC group exhibited significantly higher levels of total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and alpha-fetoprotein (AFP), but lower albumin levels compared to the control group. These results were expected because HCC

patients often exhibit abnormalities in liver function tests (LFT).

The levels of the levels of 4 exhaled VOCs in this study (acetone, 1,4-pentadiene, isopropyl alcohol, and dimethyl sulfide) were found in significantly different levels in the four groups. Overall, the results supported our exploratory research (6, 14). Notably, acetone outperformed serum AFP at the optimal cutoff and AFP at the positive cutoff for HCC surveillance. Exhaled VOC analysis could be useful as a noninvasive test for early HCC detection due to the significant discriminatory performance of VOCs, particularly acetone. The VOCs identified in this study were consistent with those previously reported in other cancers including acetone, 1,4-pentadiene, isopropyl alcohol, and dimethyl sulfide.

Acetone was the VOC most strongly associated with HCC. Acetone levels in HCC patients were significantly higher than in cirrhotic, HBV-infected patients, and healthy volunteers. Acetone has been identified as the primary source of energy production in cancer cells, reflecting the dysregulation of glucose metabolism (21). It is one of the ketone bodies produced in hepatocytes through the decarboxylation of excess acetyl-CoA (22). Ketone bodies act as chemo-attractants for cancer cells, promoting tumor growth and metastasis, supporting the concept of the "reverse Warburg effect." This metabolic interplay between cancer cells and cancer-associated fibroblasts (CAFs) is characterized by enhanced glycolysis and catabolism of glucose into ketone bodies within the cytoplasm of CAFs **(Figure 19)**. Consequently, HCC patients exhibit elevated levels of acetone due to these metabolic dysregulations (6). Another source of acetone was the oxidation-reduction reaction catalyzed by the alcohol dehydrogenase, which converted isopropyl alcohol to acetone (23). These likely explain the observation that HCC patients had increased acetone and isopropyl alcohol levels than non-HCC patients.

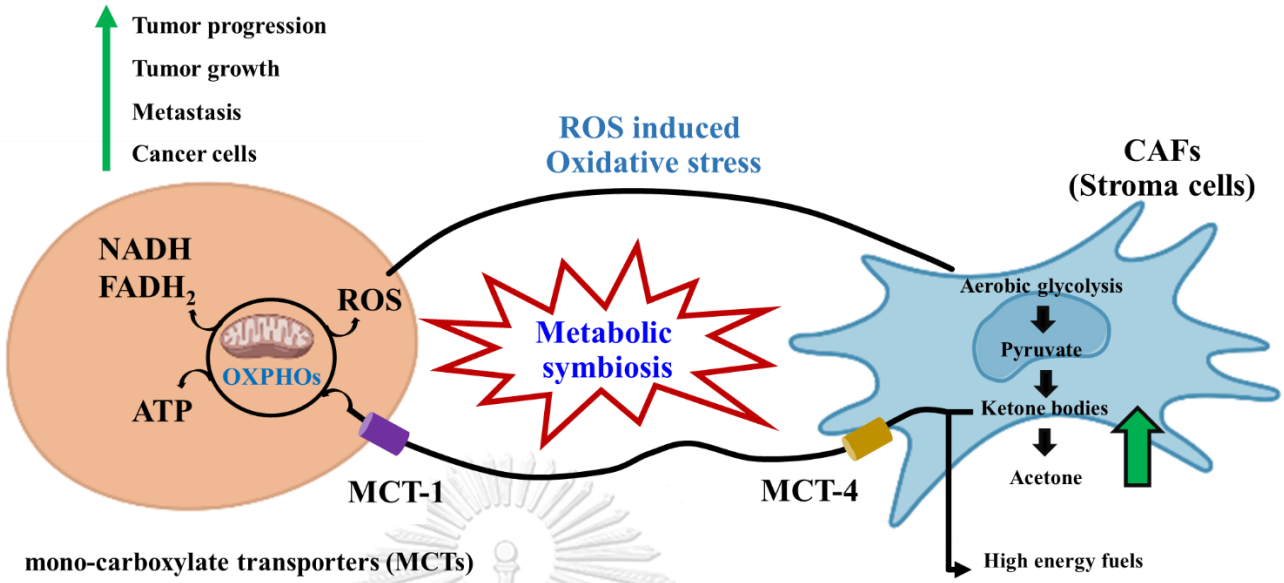


Figure 19. The metabolic interplay between cancer cells and cancer-associated fibroblasts (CAFs)

1,4-pentadiene (hydrocarbon compounds) are products of oxidative stress, which are produced by the induction of cytochrome P450 (CYP450) activity (57). CYP450 is known to be overexpressed in cancer cells and promotes tumor growth by converting endogenous compounds into metabolites that increases angiogenesis (40). Altered levels of hydrocarbon compounds have been observed in a number of cancers. In this study, we found that HCC patients had lower levels of exhaled 1,4-pentadiene than controls. This finding was consistent with the previous reports showing that the level of this compound was decreased in urine of rats with breast cancer and in exhaled breath of patients with lung cancer(58, 59).

Dimethyl sulfide (sulfur compounds) is produced from the process of cysteine and methionine degradation. Cysteine is an essential element for the formation of glutathione (GSH), a well-known antioxidant that scavenges ROS. The reduction of GSH appears to play a role in cancer development. We found the sulfur compound was significantly different in HCC patients to non-HCC patients, consistent with findings previously observed in studies of gastric and colon cancers (60-62). Sulfur compounds was shown to have a chemo preventive effect and to suppress the cancer cell proliferation through the induction of apoptosis (61).

Sulfur compounds also modulated the activity of several metabolizing enzymes that detoxify carcinogens and inhibit the formation of DNA adducts (61).

Due to the limited diagnostic ability of serum AFP, several attempts have been made to discover novel biomarkers. The majority of biomarkers investigated remained in phase II of cancer biomarker studies **(Figure 20)** in which HCC cases and non-HCC controls were enrolled, similar to the present study (24). The sensitivities of these biomarkers studied ranged from 34 to 79% for early HCC detection **(Table 11)** (24). This study, however, found that acetone had a sensitivity of 88.9% for identifying HCC patients among at-risk groups, outperforming AFP, which had a sensitivity of 68.2%. This finding raises the possibility of using exhaled VOC analysis for HCC surveillance in at-risk population.

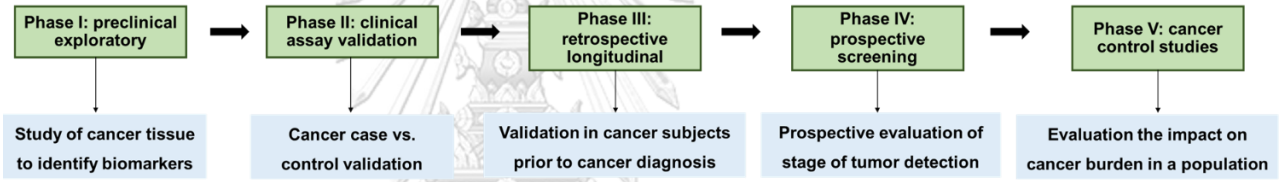


Figure 20. Phase of cancer biomarker studies

Table 11. The sensitivities of biomarkers studied for early HCC detection

Biomarkers	**Sensitivity (%)**	**Phase of development**
AFP in this study cohort	63.6	2
AFP	39-64	2/3
DCP	34-40	2/3
Osteopontin	49	2
MDK	79	2/3
DKK1	41-74	2
GPC-3	55	2
AFU	56	2
GP-73	62-79	2

The study's strength lies in its design. Unlike most cancer biomarker studies, which frequently include a control group of healthy individuals and a case group of mixed early- and advanced-stage cancer patients, making it possible to overestimate the performance of the studied biomarkers, this study's cohort included only early-stage HCC patients as cases and HBV-infected patients and cirrhotic patients as controls. The current study population was representative of the target population for the future clinical use of this biomarker for early-stage HCC detection. The baseline characteristics, specifically liver function tests and cirrhosis severity, were not different between patients with early HCC and those with cirrhosis. Accordingly, the association between VOCs and HCC was unlikely to be due to impaired liver function. Consistent with the results of our previous work (14), this proof-of-concept study validated the performance of exhaled VOCs in diagnosing HCC. This study has some limitations. Exhaled VOCs were not compared to abdominal ultrasonography, which is the standard HCC surveillance tool. However, ultrasound performance reported in several previous studies (4, 25, 26) showed lower sensitivities for early HCC diagnosis than acetone, 21.4-47% vs. 88.9%, respectively. Despite the encouraging findings, a phase III study in a longitudinal cohort of at-risk patients is required to determine the VOC's performance in

detecting HCC at the preclinical stage of disease. It is also worthwhile to investigate the use of VOCs as an adjunctive tool for improving the detection rate of HCC by ultrasound. Exogenous VOCs from environment and occupational exposure, as well as underlying comorbidities, could possibly alter the VOCs profiles when used in real-world practice. The external validity of the diagnostic performance of VOCs remains unknown as the present study and the previous study were conducted in the same center. The generalizability of the VOC analysis must be further investigated in other independent cohorts before applying the VOC analysis in clinical practice. Although smaller, less expensive, and less cumbersome than the gas chromatography-mass spectrometry, the instrument used in this study remains too large to be used as a screening or surveillance tool at the point-of-care setting. Developing a portable analyzer, such as targeted sensors, would improve the clinical utility of exhaled VOC analysis in detecting early HCC.

Conclusion

Exhaled VOCs differ significantly between patients with early-stage HCC and those at high risk of developing HCC, and they could be used as biomarkers to improve the performance of HCC surveillance tool. Prospective longitudinal cohort studies are needed to validate the VOC performance in detecting HCC at the preclinical stage.

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REFERENCES

1. Kulik L, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. Gastroenterology. 2019;156(2):477-91 e1.
2. Llovet JM, Pena CE, Lathia CD, Shan M, Meinhardt G, Bruix J, et al. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. Clin Cancer Res. 2012;18(8):2290-300.
3. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362(9399):1907-17.
4. Tzartzeva K, Obi J, Rich NE, Parikh ND, Marrero JA, Yopp A, et al. Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients With Cirrhosis: A Meta-analysis. Gastroenterology. 2018;154(6):1706-18 e1.
5. Chaiteerakij R, Addissie BD, Roberts LR. Update on biomarkers of hepatocellular carcinoma. Clin Gastroenterol Hepatol. 2015;13(2):237-45.
6. Sukaram T, Tansawat R, Apiparakoon T, Tiyarattanachai T, Marukatat S, Rerknimitr R, et al. Exhaled volatile organic compounds for diagnosis of hepatocellular carcinoma. Sci Rep. 2022;12(1):5326.
7. Sethi S, Nanda R, Chakraborty T. Clinical application of volatile organic compound analysis for detecting infectious diseases. Clin Microbiol Rev. 2013;26(3):462-75.
8. Phillips M, Cataneo RN, Cheema T, Greenberg J. Increased breath biomarkers of oxidative stress in diabetes mellitus. Clin Chim Acta. 2004;344(1-2):189-94.
9. Boots AW, van Berkel JJ, Dallinga JW, Smolinska A, Wouters EF, van Schooten FJ. The versatile use of exhaled volatile organic compounds in human health and disease. J Breath Res. 2012;6(2):027108.
10. Probert CS, Ahmed I, Khalid T, Johnson E, Smith S, Ratcliffe N. Volatile organic compounds as diagnostic biomarkers in gastrointestinal and liver diseases. J Gastrointestin Liver Dis. 2009;18(3):337-43.
11. Di Lena M, Porcelli F, Altomare DF. Volatile organic compounds as new biomarkers for colorectal cancer: a review. Colorectal Dis. 2016;18(7):654-63.
12. Oguma T, Nagaoka T, Kurahashi M, Kobayashi N, Yamamori S, Tsuji C, et al. Clinical contributions of exhaled volatile organic compounds in the diagnosis of lung cancer. PLoS One. 2017;12(4):e0174802.
13. Princivalle A, Monasta L, Butturini G, Bassi C, Perbellini L. Pancreatic ductal adenocarcinoma can be detected by analysis of volatile organic compounds (VOCs) in alveolar air. BMC Cancer. 2018;18(1):529.
14. Kitiyakara T, Redmond S, Unwanatham N, Rattanasiri S, Thakkinstian A, Tangtawee P, et al. The detection of hepatocellular carcinoma (HCC) from patients' breath using canine scent detection: a proof-of-concept study. J Breath Res. 2017;11(4):046002.
15. Qin T, Liu H, Song Q, Song G, Wang HZ, Pan YY, et al. The screening of volatile markers for hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev. 2010;19(9):2247-53.
16. Acevedo-Moreno L SK, Firl D, McVey J, Berber E, Miller C, et al. . Investigating the breath metabolome as a diagnostic tool for hepatocellular carcinoma in cirrhotic patients. HPB. 2018;20:S82-S3.

17. Miller-Atkins G, Acevedo-Moreno LA, Grove D, Dweik RA, Tonelli AR, Brown JM, et al. Breath Metabolomics Provides an Accurate and Noninvasive Approach for Screening Cirrhosis, Primary, and Secondary Liver Tumors. Hepatol Commun. 2020;4(7):1041-55.
18. Sun X, Shao K, Wang T. Detection of volatile organic compounds (VOCs) from exhaled breath as noninvasive methods for cancer diagnosis. Anal Bioanal Chem. 2016;408(11):2759-80.
19. Md. Musfiqur Rahman AMAE-A, Jeong-Heui Choi,Ho-Chul Shin,Sung Chul Shin,Jae-Han Shim. Chapter 3 Basic Overview on Gas Chromatography Columns. Analytical Separation Science.823-35.
20. Sukaram T, Apiparakoon T, Tiyarattanachai T, Ariyaskul D, Kulkraisri K, Marukatat S, et al. VOCs from Exhaled Breath for the Diagnosis of Hepatocellular Carcinoma. Diagnostics (Basel). 2023;13(2).
21. Sachar Y, Brahmania M, Dhanasekaran R, Congly SE. Screening for Hepatocellular Carcinoma in Patients with Hepatitis B. Viruses. 2021;13(7):1318-15.
22. McGlynn KA, Petrick JL, El-Serag HB. Epidemiology of Hepatocellular Carcinoma. Hepatology. 2021;73 Suppl 1:4-13.
23. Ferlay J EM, Lam F, Colombet M, Mery L, Pi.eros M. Global Cancer Observatory: Cancer Today. . International Agency for Research on Cancer. 2020 [cited 2021 Nov 29]. Available from: https://gco.iarc.fr/today.
24. Wong MC, Jiang JY, Goggins WB, Liang M, Fang Y, Fung FD, et al. International incidence and mortality trends of liver cancer: a global profile. Sci Rep. 2017;7:45846-54.
25. Lu Q, Li J, Cao H, Lv C, Wang X, Cao S. Comparison of diagnostic accuracy of Midkine and AFP for detecting hepatocellular carcinoma: a systematic review and meta-analysis. Biosci Rep. 2020;40(3):1-11.
26. Richani M, Kolly P, Knoepfli M, Herrmann E, Zweifel M, von Tengg-Kobligk H, et al. Treatment allocation in hepatocellular carcinoma: Assessment of the BCLC algorithm. Ann Hepatol. 2016;15(1):82-90.
27. World Health O. Air quality guidelines for Europe. WHO Reg Publ Eur Ser. 2000(91):V-X, 1-273.
28. Curran AM, Rabin SI, Prada PA, Furton KG. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. J Chem Ecol. 2005;31(7):1607-19.
29. Gallagher M, Wysocki CJ, Leyden JJ, Spielman AI, Sun X, Preti G. Analyses of volatile organic compounds from human skin. Br J Dermatol. 2008;159(4):780-91.
30. Schmidt K, Podmore I. Current Challenges in Volatile Organic Compounds Analysis as Potential Biomarkers of Cancer. J Biomark. 2015;2015:981458-73.
31. Phillips M, Altorki N, Austin JH, Cameron RB, Cataneo RN, Greenberg J, et al. Prediction of lung cancer using volatile biomarkers in breath. Cancer Biomark. 2007;3(2):95-109.
32. Phillips M, Cataneo RN, Ditkoff BA, Fisher P, Greenberg J, Gunawardena R, et al. Prediction of breast cancer using volatile biomarkers in the breath. Breast Cancer Res Treat. 2006;99(1):19-21.
33. Kumar S, Huang J, Abbassi-Ghadi N, Mackenzie HA, Veselkov KA, Hoare JM, et al. Mass Spectrometric Analysis of Exhaled Breath for the Identification of Volatile Organic Compound Biomarkers in Esophageal and Gastric Adenocarcinoma. Ann Surg.

2015;262(6):981-90.
34. Altomare DF, Di Lena M, Porcelli F, Trizio L, Travaglio E, Tutino M, et al. Exhaled volatile organic compounds identify patients with colorectal cancer. Br J Surg. 2013;100(1):144-50.
35. Heers H, Gut JM, Hegele A, Hofmann R, Boeselt T, Hattesohl A, et al. Non-invasive Detection of Bladder Tumors Through Volatile Organic Compounds: A Pilot Study with an Electronic Nose. Anticancer Res. 2018;38(2):833-7.
36. Gao Q, Su X, Annabi MH, Schreiter BR, Prince T, Ackerman A, et al. Application of Urinary Volatile Organic Compounds (VOCs) for the Diagnosis of Prostate Cancer. Clin Genitourin Cancer. 2019;17(3):183-90.
37. Mozdiak E, Wicaksono AN, Covington JA, Arasaradnam RP. Colorectal cancer and adenoma screening using urinary volatile organic compound (VOC) detection: early results from a single-centre bowel screening population (UK BCSP). Tech Coloproctol. 2019;23(4):343-51.
38. de Meij TG, Larbi IB, van der Schee MP, Lentferink YE, Paff T, Terhaar Sive Droste JS, et al. Electronic nose can discriminate colorectal carcinoma and advanced adenomas by fecal volatile biomarker analysis: proof of principle study. Int J Cancer. 2014;134(5):1132-8.
39. Rossi M, Aggio R, Staudacher HM, Lomer MC, Lindsay JO, Irving P, et al. Volatile Organic Compounds in Feces Associate With Response to Dietary Intervention in Patients With Irritable Bowel Syndrome. Clin Gastroenterol Hepatol. 2018;16(3):385-91 e1.
40. Janfaza S, Khorsand B, Nikkhah M, Zahiri J. Digging deeper into volatile organic compounds associated with cancer. Biol Methods Protoc. 2019;4(1):1-11.
41. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016;41(3):211-8.
42. Jozwiak P, Forma E, Brys M, Krzeslak A. O-GlcNAcylation and Metabolic Reprograming in Cancer. Front Endocrinol (Lausanne). 2014;5:145-57.
43. Sukaram T, Tansawat R, Apiparakoon T, Tiyarattanachai T, Marukatat S, Rerknimitr R, et al. Exhaled volatile organic compounds for diagnosis of hepatocellular carcinoma. Sci Rep. 2022;12(1):5326-34.
44. Beauchamp J, Herbig J, Gutmann R, Hansel A. On the use of Tedlar(R) bags for breath-gas sampling and analysis. J Breath Res. 2008;2(4):046001.
45. Trabue SL, Anhalt JC, Zahn JA. Bias of Tedlar bags in the measurement of agricultural odorants. J Environ Qual. 2006;35(5):1668-77.
46. Pereira J, Porto-Figueira P, Cavaco C, Taunk K, Rapole S, Dhakne R, et al. Breath analysis as a potential and non-invasive frontier in disease diagnosis: an overview. Metabolites. 2015;5(1):3-55.
47. Woolfenden E. Monitoring VOCs in Air Using Sorbent Tubes Followed by Thermal Desorption-Capillary GC Analysis: Summary of Data and Practical Guidelines. Journal of the Air & Waste Management Association. 1997;47(1):20-36.
48. Karl A. Holden WI, Dahlia Salman, Rebecca Cordell, Teresa McNally, Bharti Patel, Rachael Phillips, Caroline Beardsmore, Michael Wilde, Luke Bryant, Amisha Singapuri, Paul Monks, Chris Brightling, Neil Greening, Paul Thomas, Salman Siddiqui, Erol A. Gaillard. Use of the ReCIVA device in breath sampling of patients with acute breathlessness: a feasibility study. ERJ. 2020;6(4):00119-2020.
49. Wilson AD. Advances in electronic-nose technologies for the detection of

volatile biomarker metabolites in the human breath. Metabolites. 2015;5(1):140-63.
50. Wilson AD, Baietto M. Applications and advances in electronic-nose technologies. Sensors (Basel). 2009;9(7):5099-148.
51. Wilson AD. Diverse applications of electronic-nose technologies in agriculture and forestry. Sensors (Basel). 2013;13(2):2295-348.
52. Haick H, Broza YY, Mochalski P, Ruzsanyi V, Amann A. Assessment, origin, and implementation of breath volatile cancer markers. Chem Soc Rev. 2014;43(5):1423-49.
53. Covington JA, van der Schee MP, Edge AS, Boyle B, Savage RS, Arasaradnam RP. The application of FAIMS gas analysis in medical diagnostics. Analyst. 2015;140(20):6775-81.
54. Smolinska A, Tedjo DI, Blanchet L, Bodelier A, Pierik MJ, Masclee AAM, et al. Volatile metabolites in breath strongly correlate with gut microbiome in CD patients. Anal Chim Acta. 2018;1025:1-11.
55. Mochalski P, Wiesenhofer H, Allers M, Zimmermann S, Guntner AT, Pineau NJ, et al. Monitoring of selected skin- and breath-borne volatile organic compounds emitted from the human body using gas chromatography ion mobility spectrometry (GC-IMS). J Chromatogr B Analyt Technol Biomed Life Sci. 2018;1076:29-34.
56. Devarbhavi H, Asrani SK, Arab JP, Nartey YA, Pose E, Kamath PS. Global burden of liver disease: 2023 update. J Hepatol. 2023.
57. Phillips M, Cataneo RN, Saunders C, Hope P, Schmitt P, Wai J. Volatile biomarkers in the breath of women with breast cancer. J Breath Res. 2010;4(2):026003.
58. Rudnicka J, Kowalkowski T, Buszewski B. Searching for selected VOCs in human breath samples as potential markers of lung cancer. Lung Cancer. 2019;135:123-9.
59. Woollam M, Teli M, Angarita-Rivera P, Liu S, Siegel AP, Yokota H, et al. Detection of Volatile Organic Compounds (VOCs) in Urine via Gas Chromatography-Mass Spectrometry QTOF to Differentiate Between Localized and Metastatic Models of Breast Cancer. Sci Rep. 2019;9(1):2526.
60. Mochalski P, Leja M, Gasenko E, Skapars R, Santare D, Sivins A, et al. Ex vivo emission of volatile organic compounds from gastric cancer and non-cancerous tissue. J Breath Res. 2018;12(4):046005.
61. Melino S, Sabelli R, Paci M. Allyl sulfur compounds and cellular detoxification system: effects and perspectives in cancer therapy. Amino Acids. 2011;41(1):103-12.
62. Porto-Figueira P, Pereira JAM, Camara JS. Exploring the potential of needle trap microextraction combined with chromatographic and statistical data to discriminate different types of cancer based on urinary volatomic biosignature. Anal Chim Acta. 2018;1023:53-63.

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