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Breath biomarkers of liver cirrhosis

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ABSTRACT

The diagnosis of asymptomatic cirrhosis in patients with liver disease is of importance to start screening for complications in due time. Liver biopsy is neither sensitive nor practical enough to be used as a frequent follow-up test in patients with chronic liver disease. The volatile organic compounds present in exhaled breath offer the possibility of exploring internal physiologic and pathologic process in a non invasive way. This study examined whether a specific pattern of biomarkers can be found in breath samples of patients with cirrhosis. To this aim samples of alveolar breath from patients with cirrhosis and healthy volunteers were analyzed using gas chromatography–mass spectrometry. When linear discriminant analysis was used to search for a model(s)/pattern of compounds characteristic for liver cirrhosis, 24 models of 8 independent compounds could distinguish between the groups. The sensitivity and specificity (between 82% and 88%, and 96% and 100%, respectively) of the models suggest that a specific pattern of breath biomarkers can be found in patients with cirrhosis, which may allow detecting this complication of chronic liver disease in an early stage.

1. Introduction

Once a patient with chronic liver disease develops cirrhosis, his prognosis is impaired due to the development of complications. Besides the risk for hepatocellular carcinoma (HCC), the most important lethal complications are portal hypertension, predominantly due to the development of fibrosis into the liver and characterized by ascites and gastro esophageal varices; and finally the loss of function due to the disappearance of functional liver mass and portosystemic shunting characterized by jaundice and/or hepatic encephalopathy [1].

Liver biopsy, the 'gold standard' to diagnose cirrhosis, is a highly invasive test, presents problem of sample error and it does not assess the functional capacity of the liver. Therefore, biopsy is neither sensitive nor practical enough to be used as a frequent follow-up test. To overcome these inconveniences several attempts have recently been made to develop non-invasive tests to assess the degree of fibrosis [2–4]. But again these tests do not assess the degree of liver function failure. Currently, the best test to assess the liver function is based on some biochemical tests. This model for end-stage liver disease (MELD score) only becomes abnormal in the more advanced stage of cirrhosis [5]. Early detection of cirrhosis is of value in order to start screening to esophageal varices or HCC in due time [6,7].

Analysis of volatile organic compounds (VOCs) in human breath has been reported to provide information for several clinical conditions including, between others, lung cancer, diabetes mellitus and oxidative stress [8]. Early studies identified methyl mercaptan (MM) and dimethyl sulphide (DMS) as present in the breath of cirrhotic patients in hepatic coma [9]. Subsequently, the research groups of Kaji [10,11] and Tangerman [12,13] used improved gas chromatographic methods to demonstrate that the levels of all of these sulphur containing molecules were elevated in the breath of patients with cirrhosis even outside liver coma. More recently, in a preliminary study of breath biomarkers in liver diseases, Sehnert and coworkers reported that isoprene, carbonyl sulphide and carbonyl disulphide concentrations were significant different from the values in normal subjects [14]. In a previous report of our group [15] breath analysis by means of thermal desorption coupled with gas chromatography–mass spectrometry (GC–MS) made it possible

Abbreviations: AUC, area under the curve; DEHP, di (2-ethylhexyl) phthalate; DMS, dimethyl sulphide; GC–MS, gas chromatography–mass spectrometry; HCC, hepatocellular carcinoma; H_2S, hydrogen sulphide; MELD-score, model for end-stage liver disease; MM, methyl mercaptan; NPV, negative predicted value; PPV, positive predicted value; VOCs, volatile organic compounds.

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Table 1
Characteristics of the study population.

Group	*N*	Female/male	Smokers	Alcoholic etiology	Child-Pugh (mean ± sd)	MELD (mean ± sd)
Healthy controls	49	29/20	8	n.a.	n.a.	n.a.
Cirrhosis	35	14/21	9	26	8 ± 2	16 ± 4
Cirrhosis (validation group)	12	4/8	5	11	7 ± 1	14 ± 4

n.a.: not applicable.

to discriminate patients with breath malodour related to hepatic pathologies. Fetor hepaticus in case of hepatic encephalopathy was mainly caused by DMS and in a lower extent by the ketones acetone, 2-butanone and 2-pentanone.

The aim of the present study was to examine whether other specific biomarkers of liver cirrhosis could be found making use of the entire breath composition.

2. Materials and methods

2.1. Patients

The study population included 35 patients with established cirrhosis proven by histology and 49 healthy volunteers. An independent group of 12 patients with cirrhosis was used for further validation. Patients with end-stage liver disease were excluded defined as: MELD > 20, a Child-Pugh C and hepatic encephalopathy gr 3–4. The baseline characteristics of the study population are shown in Table 1.

All subjects have given their consent and the research was approved by the Clinical Trials Committee of the University Hospital Leuven.

2.2. Sample collection and VOC analysis

Sample collection and VOCs analysis have been described in detail in previous reports [16]. Briefly, breath sample collection was done using a commercial device (Bio-VOC® sampler, Markes International Limited, Rhondda Cynon Taff, UK). Alveolar air was transferred immediately from the sampler to a sorbent tube containing 200 mg TenaxTA and 200 mg Unicarb (carbonized molecular sieve) (Markes International Limited) to capture all VOCs present in a sample. Analysis of samples was performed by combining thermal desorption (Unity®, Markes International Limited) with gas chromatography (capillary column, HP5MS, 30 m × 0.25 mm × 0.25 μm film thicknesses, HP6890N Agilent Technologies, Diegem, Belgium). Identification of VOC's occurred in a mass spectrometer (HP5973, Agilent Technologies).

2.3. Data management

Data from each chromatographic peak comprising retention time, library identification (NIST 98 library and a self-created database) and peak area (AUC, area under curve, response) were downloaded into a spreadsheet. VOCs were quantified by their ratio to the internal standard. Background VOCs were subtracted from VOCs in alveolar breath. In this way, the gradient of each VOC was calculated as previously suggested by Phillips [17]:

$$\text{Gradient} = \frac{\text{AUC}_{\text{VOC in breath}}}{\text{AUC}_{\text{internal standard}}} - \frac{\text{AUC}_{\text{VOC in room air}}}{\text{AUC}_{\text{internal standard}}}$$

A positive gradient indicates that the compound was formed endogenously, while a negative gradient indicates that the compound was derived from the environment.

2.4. Statistical analysis

Data analyses were performed using R for Windows, version 2.11. Differences between groups were assessed using a Mann Whitney *U*-test with Bonferroni correction to minimize the effect of multiple testing. Linear discriminant analysis was applied for the model construction.

3. Results

A total of 891 compounds were detected at least once in the breath of the volunteers. After exclusion of the compounds possibly introduced by the technique (i.e. several siloxanes, silicone, methyl alcohol and benzene); 881 compounds where considered for analysis. Siloxanes were discarded from the analysis since they are generally considered in the literature as main background interferences, apparently stemming from the capillary column stationary phase [18].

Benzene was excluded from the analysis based on its presence in the 50% of the runs of empty sorbent tubes (blank). No other compound was detected in the empty sorbent tubes.

In a first step only those compounds present in at least half of the members of any of the groups under study (healthy volunteers or patients with cirrhosis) showing a positive mean (endogenous production) in at least one of the groups, were selected. This resulted in 68 compounds. After manual revision of the list one of the compounds (4-heptanone) has been excluded of the analysis. 4-heptanone is a major metabolite of di (2-ethylhexyl) phthalate (DEHP), a plasticizer used in polyvinyl chloride products, not associated with insulin resistance or liver metabolism [19]. It is incorporated in many medical devices like tubing, infusion sets, and storage bags for blood, parenteral nutrition and dialysis fluids. The frequency of detection of the 67 remaining compounds is listed in Table 2. The 67 compounds selected were then tested for differences between the groups (Mann Whitney *U*-test with Bonferroni correction). As a result, 28 compounds showed a significant difference in alveolar air from healthy volunteers and patients with cirrhosis (Table 3) and were further selected to be used to perform the linear discriminant analysis. A complete flowchart of the compounds' selection and models' construction is presented in Fig. 1.

The following strategy was used to identify the compounds that discriminate best between healthy volunteers and patients with cirrhosis. First, volunteers and patients were randomly assigned to one of two groups: a *training set* in order to create a model for liver disease (25 healthy volunteers, 18 patients with cirrhosis) and a *prediction set* (24 healthy volunteers, 17 patients with cirrhosis) to test the model. The *validation set* consists in an independent group of 12 patients with cirrhosis. Then, all possible combinations (376,740) of 6 compounds out of the 28 compounds selected as described in the paragraph above were assessed. From these 376,740, two hundred and sixty two (262) combinations of six compounds each were able to distinguish between the two groups of subjects (healthy volunteers and patient with cirrhosis) with four or less misclassification (<5% of the total set). In order to reduce the number of possibilities, the occurrence of the compounds in these 262 models was assessed and the most frequent ones ($n = 20$) have been selected for further investigation. Again, all the possible combinations, this time including 8 compounds out of the set of 20 were assessed. Eight was chosen because it offers the best compromise between feasibility and prediction power of the analysis. Five

Table 2
Compounds detected in at least half of the breath of healthy volunteers (healthy) and/or patients with cirrhosis (cirrhosis).

Compound name	Frequency of appearance (%)		Compound name	Frequency of appearance (%)	
	Healthy	Cirrhosis		Healthy	Cirrhosis
beta-Pinene	100	88	Unknown derivative 3	63	16
Acetone	100	100	2-Propenal, 2-methyl-	60	39
Isoprene	97	100	Benzaldehyde	60	78
2-Methyl-1-propene	94	59	Butane, 2-methyl	60	53
Acetic acid, methyl ester	94	96	Styrene	60	22
Caryophyllene	94	92	Tetradecane	60	16
Dimethyl sulfide	94	76	Thiophene, 2-methyl	60	90
Furan, 2-methyl	94	96	2-Butanone, 3-hydroxy	57	51
Propane, 1-(methylthio)	94	94	BCA (27.00)	57	0
Octane	91	69	Butane	57	57
gamma-Terpinene	89	76	Hexane	54	41
alpha-Pinene	86	90	Phenol	54	98
2-Pentanone	86	94	1,4-Pentadien-3-one	51	39
Unknown derivative 1	86	0	1-Propanamine, N,N-dipropyl	51	0
D-Limonene	86	84	3-Buten-2-one	49	59
Alkylbenzene (34.64)	83	0	Pentane, 3-methyl	49	53
1-Propene, 1-methylthio-(E)	80	65	1-Hexene, 4-methyl	46	51
alpha-Terpinolene	80	39	Hexane, 3-methyl	46	69
Furan, 2-pentyl	80	76	Methane, dimethoxy	46	80
2-Butanone	77	61	Carbonic acid, dimethyl ester	43	94
Benzoic acid, methyl ester	77	86	Cyclopentane, methyl	43	49
Unknown derivative 2	77	0	Camphene	40	65
Heptane	77	76	Cyclohexane, methyl	40	61
Nonane	77	39	Unknown (22.25)	40	65
Tridecane	77	35	2-Propenoic acid, methyl ester	31	84
alpha-Terpinene	74	65	Hexane, 2-methyl	17	61
1-Propene, 1-methylthio-(Z)	71	82	Sulfide allyl methyl	17	55
Cyclohexane	71	49	Indole	14	80
Pentadecane	71	59	AlkylBbenzene (34.46)	9	61
Acetophenone	69	59	Dimethylselene	9	67
BCA (48.31)	69	20	Unknown (32.64)	9	76
Undecane	69	47	BCA (48.51)	6	55
Pentane, 2-methyl	66	59	Acetic acid	0	51
Eucalyptol	63	61			

BCA: branched chain alkane.

Table 3
Compounds with significant difference between healthy volunteers and patients with cirrhosis.

Compound name	Healthy			Cirrhosis		
	Median	LQ	UQ	Median	LQ	UQ
beta-Pinene	0.50	0.14	1.09	2.39	1.43	4.77
Acetone	22.89	14.88	36.06	109.12	62.91	222.99
Isoprene	19.01	14.50	29.40	43.73	28.47	59.64
2-Methyl-1-propene	0.08	0.0	0.17	0.26	0.15	0.47
Caryophyllene	0.34	0.15	0.59	0.77	0.37	1.54
Dimethyl sulfide	0.54	0.16	1.18	1.60	0.94	2.97
Propane, 1-(methylthio)	0.27	0.12	0.53	0.82	0.41	1.63
Octane	0.03	−0.01	0.07	0.21	0.12	0.37
gamma-Terpinene	0.26	0.08	0.43	1.21	0.41	2.31
alpha-Pinene	0.85	0.37	2.15	2.32	0.55	4.21
2-Pentanone	0.14	0.08	0.32	0.70	0.20	1.44
Unknownderivative 1	0.0	0.0	0.0	1.64	0.61	2.91
D-limonene	2.28	0.62	4.05	33.32	2.99	109.23
Alkylbenzene (34.64)	0.0	0.0	0.0	1.27	0.28	2.02
Furan, 2-pentyl	0.33	0.03	0.71	1.55	0.63	3.82
2-Butanone	0.05	−0.03	0.19	0.44	0.0	1.31
Unknownderivative 2	0.0	0.0	0.0	0.69	0.0	1.42
Nonane	−0.08	−0.32	0.07	0.14	0.02	0.30
Tridecane	−0.08	−0.27	0.06	0.10	−0.06	0.30
BCA (48.31)	0.0	0.0	0.0	0.12	0.0	0.18
Unknownderivative 3	0.0	0.0	0.0	0.33	0.0	1.01
Styrene	−0.18	−0.33	−0.02	0.07	−0.03	0.30
Tetradecane	−0.31	−0.52	−0.07	0.07	−0.26	0.38
BCA (27.00)	0.0	0.0	0.0	0.01	0.0	0.06
Phenol[a]	1.4	0.85	1.97	0.23	0.0	0.53
Hexane, 2-methyl	0.02	−0.02	0.11	−0.12	−0.35	0.0
Indole[a]	0.33	0.13	0.75	0.0	0.0	0.0
Dimethylselene[a]	0.14	0.0	0.24	0.0	0.0	0.0

BCA: branched chain alkane; LQ: lower quartile; UQ: upper quartile.
[a] Compounds significantly lower in patients with cirrhosis.

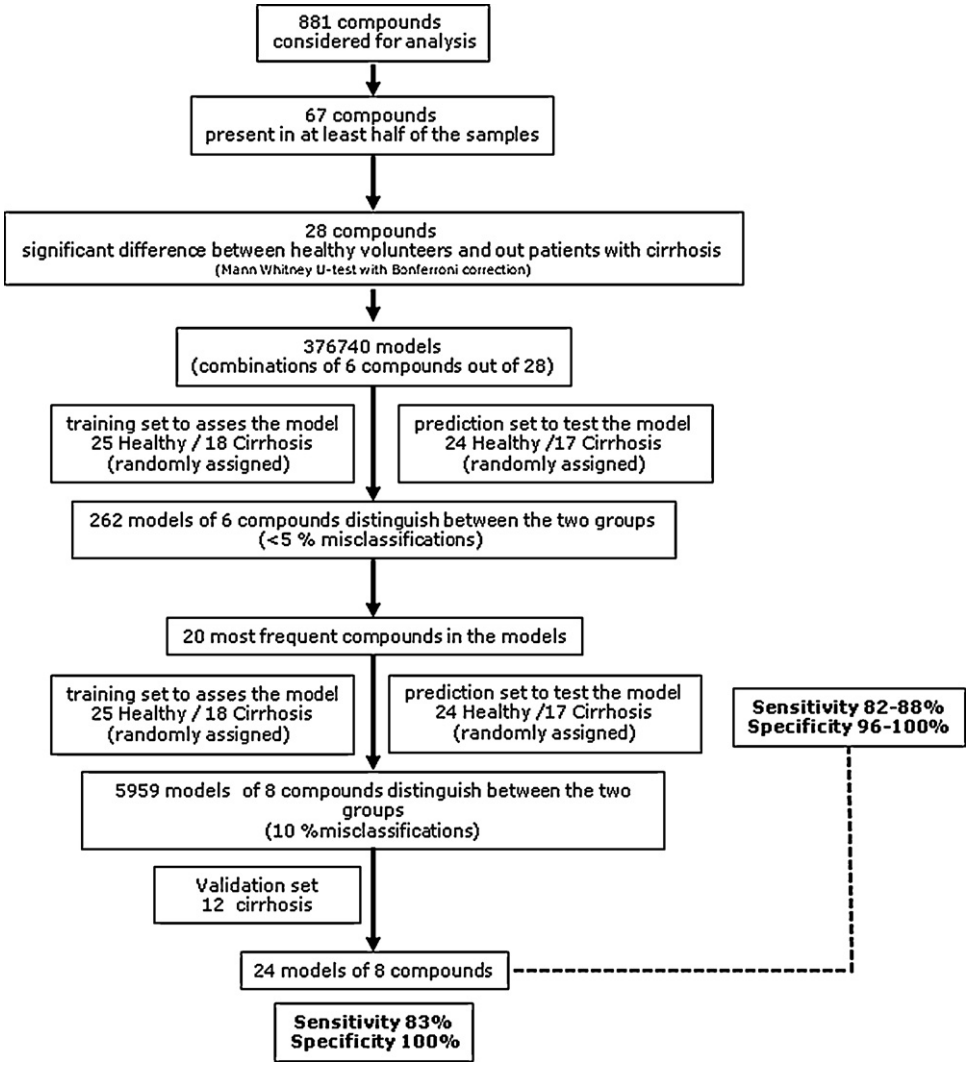

Fig. 1. Flowchart of compounds and model selection.

thousands nine hundred and fifty nine models (5959) with 5 or less misclassification (<10% of the total set) were found. The low number of bad classifications in both data sets supports the hypothesis of good *adequacy* and *generality* for these combinations. The models were then applied to the *validation set*. In this case 24 models were able to distinguish well between the patients' groups. The frequency of appearance of the compounds in the models is presented in Table 4.

In resume, using linear discriminant analysis the 881 compounds under analysis were restricted to 20 compounds which were able to, in models of 8 independent compounds; discriminate well between the two groups. When these models were applied to the *validation set* 24 of them showed a sensitivity and specificity of 83.3% and 100%, respectively.

All compounds with exception of *indole, phenol* and *dimethyl selenide* (Table 3) were significantly higher in the breath of patients with cirrhosis than in the healthy population. The proposed reasons for the observed differences are summarized in Table 5 and discussed more in extend below.

When considering the initial data set (healthy volunteers and patients with cirrhosis), the sensitivity and specificity of the models varied between 82% and 88%, and 96% and 100%, respectively. The positive and negative predicted values (PPV, NPV) varied between 93% and 100%, and 88% and 92%, respectively.

Table 4
Frequency of appearance of the compounds included in the 24 models of 8 compounds that differentiate the best between healthy volunteers and patients with cirrhosis.

Compound name	Frequency of appearance (%)
Acetone	100.0
Styrene	95.8
Branched chain alkane (48.31)	91.7
Dimethyl sulfide	87.5
Dimethylselene[a]	79.2
Phenol[a]	62.5
Tetradecane	62.5
Branched chain alkane (27.00)	45.8
Indole[a]	37.5
Unknown derivative 2	33.3
Octane	29.2
Isoprene	25.0
Nonane	16.7
gamma-Terpinene	12.5
2-Methyl-1-propene	4.2
2-Butanone	4.2
beta-Pinene	4.2
Caryophyllene	4.2
Unknown derivative 3	4.2
Unknown derivative 1	0.0

[a] Compounds significantly lower in patients with cirrhosis.

Table 5
Possible reasons for significant difference between healthy volunteers and patients with cirrhosis.

Compound class	Model compound	Possible reason
Alkanes	Octane Nonane Tetradecane Branched chain alkane	Oxidative stress
Alkenes	Isoprene 2-Methyl-1-propene	Oxidative stress Impairment of liver metabolism
Aromatic	Styrene	Impairment of liver metabolism
	Phenol Indole	Low serum albumin Low binding capacity of albumin
Ketones	Acetone 2-Butanone	Insulin resistance
Sulfur compounds	DMS	Impairment of liver metabolism
Terpenes and terpenoids	gamma-Terpinene beta-Pinene Caryophyllene	Impairment of liver metabolism
Others	Dimethylselene	Selenium deficiency

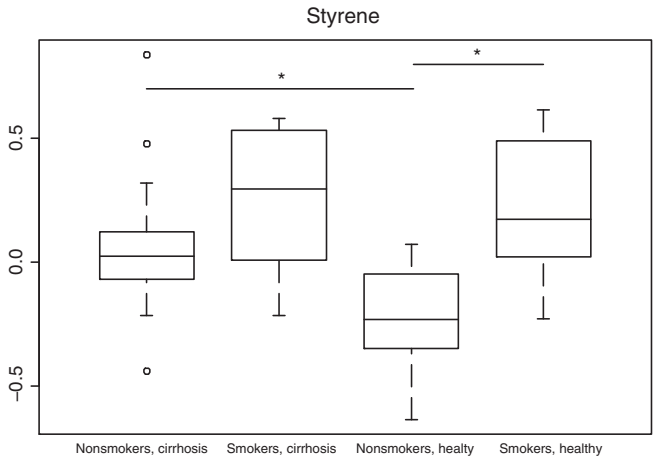

Fig. 2. Box plots of styrene in smokers and non smokers of healthy volunteers and patients with cirrhosis.

4. Discussion

Blood-borne, the VOCs present in exhaled breath offer the possibility of exploring internal physiologic and pathologic process in a non invasive way. In this study, it was examined whether analysis of alveolar breath air, using a previously described method [15,16], has also the potential to detect a specific pattern of compounds for patients with cirrhosis in order to use in the future as a non-invasive test to detect cirrhosis in a clinical asymptomatic stage.

Recently, Netzer and colleagues developed a new ensemble-based feature selection strategy to analyze data generated by ion molecule reaction mass spectrometry (IMR-MS) from patients with liver pathology [20]. In our case, linear discriminant analysis was chosen in this study because it is easy to perform by standard statistical software. Even when this technique may not be the most powerful one, the results obtained support the efficacy of the approach. Moreover, even though we did not restrict manually the compounds to those metabolically related with liver, and thus we might have somewhere worsened the results, the sensitivity and specificity of the models varied between 82% and 88%, and 96% and 100% respectively.

In general the increased breath level of most of the compounds presents in the model can be explained by the disturbance in their liver metabolism. If the function of the liver fails, the concentration of several metabolites will increase in the systemic circulation and they will appear in higher levels in exhaled breath.

Sulfur containing compounds are generated by incomplete metabolism of sulfur containing amino acids in the transamination pathway. Increased levels of these compounds have been reported, already many years ago, during liver function impairment [9–11]. DMS has been implicated as the primary cause of fetor hepaticus, the typical smell of the breath present in some liver patients [21,22]. In contrast with other sulfur compounds, DMS is a neutral molecule that is stable in blood from where it can be transported into the alveolar air and expired [23].

A common complication in patients with cirrhosis is the hepatic insulin resistance. Insulin resistance leads to an increase of triglycerides and free fatty acids and ketones like acetone and 2-butanone are formed during lipolysis. Moreover, early research in rats has shown that the inhibition of CYP2E1 causes a remarkable increase of both compounds in breath [24].

Under normal conditions, saturated hydrocarbons are metabolized by hepatic cytochrome P450 enzymes to alcohols [25]. Impairment of liver function will render an increase of hydrocarbons in blood and subsequently in breath. Moreover, lipid peroxidation is a free-radical-mediated degradative process which involves polyunsaturated fatty acids and release VOCs that are excreted in the breath. In particular, increased levels of ethane and pentane have been related to oxidative stress in several critical conditions [26], including chronic liver disease [27]. Methylated alkanes have also been proposed as markers of oxidative stress [28].

Isobutene or 2 methyl-1-propene has been reported as normal component of human breath [29]. The source of this compound is speculated to be terpenes or ubiquinones. Animal models have proven that it is metabolized by cytochrome P450 enzymes to epoxides [30,31]. Terpenes and terpenoids are the primary constituents of the essential oils of many plants. They are widely used as natural flavor additives for food, cosmetics, household products and tooth pastes. Terpenes are metabolized by cytochrome P450-enzymes in the liver and are mainly excreted in urine and a smaller fraction in breath [32]. The disturbed liver metabolism could be an explanation for the increased concentrations.

Isoprene is the basic unit of the terpenes. It is the most common hydrocarbon in breath, which is formed along the mevalonic pathway of cholesterol synthesis. It is metabolized by liver monooxygenase to the corresponding mono-epoxides [33]. Isoprene is speculated to be a biomarker for oxidative stress and elevated isoprene exhalation has already been associated with various conditions [34,35].

Styrene derives from exogenous sources like industrial materials (e.g. plastic), cigarette smoke, exhaust gases, food. It is oxidized by citochrome P450 (CYP) to styrene-7,8 oxide. CYP2E1 is the main isoform responsible for the styrene metabolism in humans [36,37] and has been reported to be decreased in cirrhotic liver samples [38]. In our population styrene gradient was higher in smokers than in non smokers for both groups of patients. These differences were significant in the group of healthy volunteers ($p < 0.0001$) but not in the group of liver patients ($p = 0.070$). The differences between smokers of both groups were no significant ($p = 0.96257$), while the opposite was seen between the non smokers ($p < 0.0001$) (Fig. 2). These results support the hypothesis that, independently of the source, the compound is not metabolized completely by the liver of patients with cirrhosis.

Phenol and indole are derived from the catabolism of tyrosine and tryptophan, respectively. In patients with liver function

impairment there is evidence that the degradation of these aromatic amino acids by the liver is impaired leading to higher levels of free tyrosine and tryptophan [39]. As a consequence the metabolites of these compounds like indole and phenol are also increased in plasma of these patients and some of them like oxindole are even suggested as potential mediators for the development of hepatic encephalopathy [40]. The reason for the decreased breath concentration of indole and phenol is not clear. Both indole and phenol bind to albumin in blood. The lack of albumin and/or its lower binding capacity for endogenous and exogenous compounds in liver patients are potential reasons for the low breath levels [41].

Dimethyl selenide is an excretion product of the essential micronutrient selenium [42]. Selenium levels are decreased in patients with chronic liver disease [43,44]. This may explain the low levels of dimethyl selenide in breath of these patients.

Even though the results here presented are very promising some limitations of the study should be acknowledged. We have compared VOCs' profiles in alveolar breath only in two groups of volunteers (healthy and with cirrhosis); therefore it should be kept in mind that some of the compounds included in the models might also be presents in other pathologies reducing their discriminant power. Unequivocal identification of the compounds was not possible for all detected VOC. Moreover, the analytical characteristics of the used sorbent material and columns might have limited the recovery of some polar compounds.

In conclusion, within the limits of these settings we have identified 24 models of 8 independent compounds that discriminate well between healthy volunteers and patients with cirrhosis. Prospective validation studies to assess whether this non-invasive test can be used for the early diagnosis of cirrhosis are in progress.

Conflict of interest

None.

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