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ANALYSIS OF LUNG AIR FROM PATIENTS WITH BRONCHOGENIC CARCINOMA AND CONTROLS USING GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Volatile metabolites present in expired lung air were collected by odor sampling techniques and analyzed by gas chromatography–mass spectrometry. The study population included controls matched for age and smoking history with patients newly diagnosed with lung carcinoma. Significantly greater concentrations of *o*-toluidine were found in the lung air of patients with lung carcinoma than either age-matched or younger controls. Aniline was present in half of the patient population but absent in age-matched controls.

INTRODUCTION

The volatile organic compounds found in expired breath present a potential source of information concerning both systemic and lung physiology. Expired lung air lends itself to easy, non-invasive collection. It contains an array of volatile organic constituents which are likely to be in equilibrium with a number of compartments within the lung and may arise from endogenous or absorbed volatile substances circulating in the blood. In addition, certain substances in lung air may be in equilibrium with alveolar fluid or lining material. Finally, cells

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within the airspaces, including tumor cells per se, mucous glands and cells which are attached to the bronchial epithelium, such as alveolar macrophages, may also contribute to the constituents of lung air.

The chemical identity of many volatile constituents of body fluids has been described in the diagnosis of diabetes, respiratory viral infections and renal insufficiency [1]. In several disease conditions, breath analysis by combined gas chromatography–mass spectrometry (GC–MS) has revealed the presence of simple endogenous alcohols, ketones and amines as well as numerous compounds of endogenous origin [2–7]. Examples include the elevated levels of mercaptans and C_2–C_5 aliphatic acids in the breath samples of patients with cirrhosis of the liver [3–5] and the presence of dimethyl- and trimethylamines in the breath of uremic patients [6]. A previous study of expired air from lung cancer patients suggested that selected volatiles in lung air could differentiate patients and controls [7]. The volatiles that allowed this differentiation were acetone, methyl ethyl ketone and *n*-propanol.

With this background in mind, we examined lung air samples from patients with newly diagnosed lung cancer and compared them to two control groups. End expiratory air was collected, the volatiles in the airspace were concentrated on a polymeric absorbent and the constituents analyzed by combined GC–MS.

EXPERIMENTAL

Subject/patient population

A total of sixteen control subjects and ten patients with bronchogenic carcinoma were studied. They are described separately (see Table I).

Both controls and patients were questioned concerning their food intake 24 h prior to testing. In addition, a medication history was obtained to rule out exogenous materials from prescription and over-the-counter medications.

Control subjects. Two groups of healthy control subjects were employed. One group of eight subjects, 22–41 years of age, was recruited from the Lung Disease Program at the Hospital of the University of Pennsylvania.

The second group had ages similar to the patients (57–66 years). This latter group was recruited from amongst employees of the Monell Center and the Skin Study Center of the University of Pennsylvania's Dermatology Department. These volunteers met the following criteria: (1) freedom from chronic and acute pulmonary disese; (2) no industrial dust exposure; (3) normal chest roentgenogram; and (4) no medications at the time of study. Among the control subjects, nonsmokers were defined as having never smoked or had completely abstained from tobacco products (pipe, cigar or cigarettes) for at least five years. Those controls classified as smokers consumed between a half and two packs of cigarettes per day but did not have chronic bronchitis as defined by the American Thoracic Society.

Patients with suspected bronchogenic carcinoma. Patients were recruited from the Pulmonary Clinic at the Hospital of the University of Pennsylvania who were suspected of having bronchogenic carcinoma. They were evaluated for smoking

TABLE I

PRESENCE OF ANILINE AND *o*-TOLUIDINE IN PATIENT AND CONTROL POPULATION

N.D. = not detected below instrument detection threshold which is approximately 0.1 ng.

	Age (years)	Sex	Smoking*	Diagnosis**	Concentration*** (ng/20 l lung air)	
					Aniline	*o*-Toluidine
Patients						
1	66	M	SS8; 30 pk yr	Squamous cell	2.01	6.26
2	59	F	S; 40 pk yr	Squamous cell	17.56	9.55
3	68	M	SS3; 40 pk yr	Squamous cell	13.87	19.45
4	68	M	SS5; 40 pk yr	Squamous cell	N.D.	5.26
5	77	M	SS10; 50 pk yr	Squamous cell	24.08	9.00
6	70	M	S; 50 pk yr	Squamous cell	N.D.	N.D.
7	62	F	SS1; 40 pk yr	Undifferentiated large cell	N.D.	1.53
8	76	M	SS15; 30 pk yr	Undifferentiated large cell	N.D.	2.24
9	54	F	S; 40 pk yr	Adenocarcinoma	7.44	6.20
10	63	M	S; 45 pk yr	Adenocarcinoma	N.D.	8.77
Age-matched controls (57-66 years)						
AC1	66	M	S; 45 pk yr		N.D.	0.181
AC2	64	M	S; 40 pk yr		N.D.	2.94
AC3	57	F	S; 25 pk yr		N.D.	4.30
AC4	55	M	S; 30 pk yr		N.D.	2.82
AC5	54	F	NS		N.D.	4.92
AC6	58	F	SS1; 40 pk yr		N.D.	2.94
AC7	63	F	NS		N.D.	N.D.
AC8	59	M	NS		N.D.	N.D.
Young controls (22-41 years)						
YC1	27	F	S; 5 pk yr	N.D.	N.D.	
YC2	24	M	NS	N.D.	N.D.	
YC3	25	F	S; 8 pk yr	N.D.	N.D.	
YC4	27	F	NS	N.D.	2.80	
YC5	22	F	NS	8.6	N.D.	
YC6	41	F	SS6; 15 pk yr	N.D.	N.D.	
YC7	34	F	S; 16 pk yr	5.89	11.05	
YC8	22	F	NS	N.D.	0.88	

*S = smoker; NS = non-smoker; SS*X* = stopped smoking *x* years prior to study; *y* pk yr = *y* pack years.

**Diagnosis by cell types which were either squamous cell carcinoma, undifferentiated large cell carcinoma or adenocarcinoma.

***Quantitation is based on the amount recovered from 20-l bags. The efficiency of transfer of aniline and *o*-toluidine from the bags to the GC–MS system is approximately 4.34 ± 0.56 and $8.60 \pm 0.75\%$, respectively.

history and with respect to industrial and occupational exposures to suspected carcinogens.

This patient population included seven males (ages 66–77 years) and three females (ages 54–62 years, see Table I). All patients were or had been heavy smokers, although five of the male patients had stopped smoking for over three years before the study. Each patient provided a sample of lung air on that patient's initial visit to the clinic and prior to diagnosis and treatment. Subsequent chest X-rays, bronchoscopy and biopsy confirmed the diagnosis of either squamous cell carcinoma (six patients), undifferentiated large cell carcinoma (two patients) or adenocarcinoma (two patients) (see Table I).

Lung air collection

All patients were asked to exhale end-expiratory air into a tube connected to a 20-l Tedlar bag (Cole-Parmer). The bags were returned immediately to the laboratory and the contents transfered to a Tenax adsorbent tube (300 mg Tenax GC, 60–80 mesh, Alltech) by use of a vacuum pump. The tubes were sealed and frozen at −60°C until analyzed (with one week of collection). The volatiles are thermally desorbed from the tubes onto the capillary column of the GC–MS system.

Volatile transfer and analysis

For desorption and analysis, tubes were placed in a semi-automated tube desorber (Envirochem) and the volatiles desorbed over a 3-min period with rapid heating to 250°C in a helium stream. The volatiles were condensed onto the first 15–20 cm of the capillary column which was cooled with nitrogen passed through a copper coil immersed in liquid nitrogen.

Analyses of the volatile substances were done using a Finnigan 4510 GC–MS–data system equipped with a split/splitless injector, a fused-silica capillary column and with the capability of operating in both electron-impact and chemical-ionization modes. The column employed for chromatography was a CP Wax-57 CB (25 m × 0.32 mm I.D.) with a 1.2-μm coating (Chrompack, Bridgewater, NJ, U.S.A.). The gas chromatograph was programmed from 60°C (8 min hold) to 220°C at 3°C/min. The spectrometer was interfaced with a Nova 3 computer which utilizes the Incos software for data acquisition and analysis. The mass range of m/z 40–350 was scanned once each second and a typical run included 4000 scans. The data system included the NBS library of 31 000 compounds. Identifications were based on a comparison of the unknown spectra with the NBS library and manual interpretation of the resulting comparison with mass spectra generated from commercially available standard compounds. In addition, the relative chromatographic retention times of unknowns and known standards were compared. A mixture of fatty acid ethyl esters was used to determine relative retention times (FAEE Index). In the case of the anilines, authentic samples were used for comparison of retention times (scan numbers), mass spectra and quantitation.

Quantitation of aniline and o-toluidine from lung air

The Incos software (suplied by Finnigan/MAT) was used to quantitate the desired compounds by using the Targeted Compound Analysis (TCA) package. This software package locates and quantitates individual compounds by employing retention times and the intensities of key ions designated by the user. For each of the compounds, we used quantitation based on the molecular ion obtained in the mass spectrum. The standard curves for aniline and *o*-toluidine consisted of the absoute computer-generated intensities of the molecular ions for each compound at different concentration versus the ratio of that intensity divided by the intensity of the molecular ion of d_3-anisole generated by 100 ng of that compound. The amounts of aniline and *o*-toluidine used for each standard curve were 0.5, 1, 5 and 10 ng.

RESULTS

Table II lists all the major compounds found in the combined study of the patient and control populations. The compounds in the table are divided into those thought to be of metabolic origin, those thought to be from exogenous sources (including those from food and environmental exposure), in addition to aniline and toluidine. The major components in the lung air from patients with lung carcinoma and control subjects (regardless of age) were qualitatively similar. However, differences in several minor components were discernible after careful examination of each peak in the reconstructed ion chromatograms generated by the collected lung air constituents. Aniline and a methylaniline were initially

TABLE II

COMPOUNDS IDENTIFIED IN LUNG AIR

Peak*	Metabolic origin	Peak*	Exogenous origin
A	Acetone	B	Toluene
C	Dimethyldisulfide	D	Limonene
E	Pyridine	F	Styrene
H	Acetoin	G	C_3 Alkylbenzene
L	Benzaldehyde	I	Dichlorobenzene
M	Octanol	J	N,N-Dimethylacetamide
P	Acetophenone	K	2-Ethyl-1-hexanol
R	Cumene alcohol	S	Butylatedhydroxytoluene
U	Dodecanol	T	Benzothiazole
V	Phenol	N	Menthol
W	Cresol	O	Propoxybenzene
Y	Indole	Q	Naphthalene
Z	Diphenylamine	X	Diethylphthalate
			Other**
			Aniline
			o-Toluidine

*These letters correspond to the labeled peaks in the chromatograms shown in Fig. 1.
**See Fig. 1 for the location of aniline and *o*-toluidine.

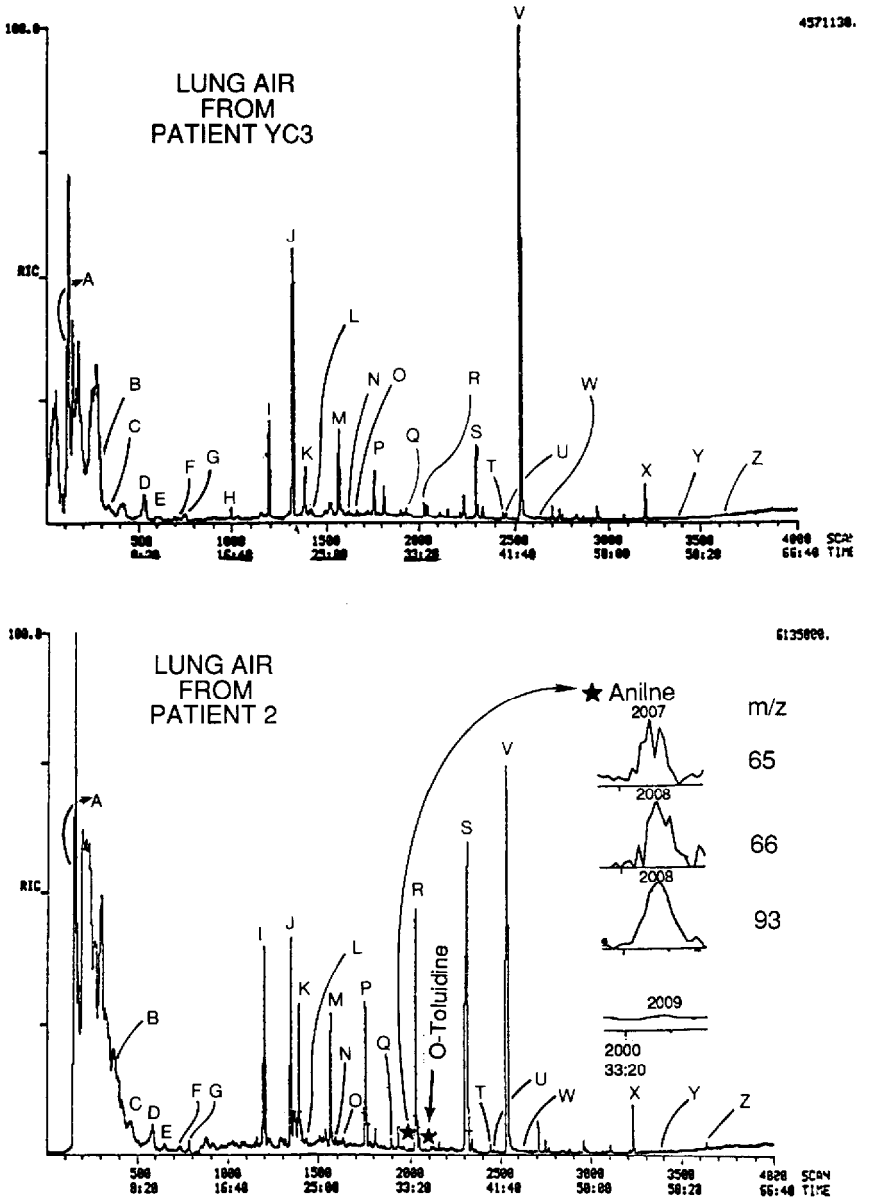

Fig. 1. Chromatograms of volatile substances from the lung air of control YC3 (Table I) and patient 2 (Table I). The places where aniline and *o*-toluidine elute are shown for patient 2. The insert for aniline shows the mass chromatogram maxima at scans 2007–2008 for the ions *m/z* 93 (molecular ion), *m/z* 66 and *m/z* 65 which are indicative of aniline. Other compounds shown to elute in these chromatograms are as follows: A = acetone; B = toluene; C = dimethyldisulfide; D = limonene; E = pyridine; F = styrene; G = C_3 alkylbenzene; H = acetoin; I = dichlorobenzene; J = N,N-dimethylacetamide; K = 2-ethyl-1-hexanol; L = benzaldehyde; M = octanol; N = menthol; O = propoxybenzene; P = acetophenone; Q = naphthalene; R = cumene alcohol; S = butylated hydroxytoluene; T = benzothiazole; U = dodecanol; V = phenol; W = *p*-cresol; X = diethylphthalate; Y = indole; Z = diphenylamine.

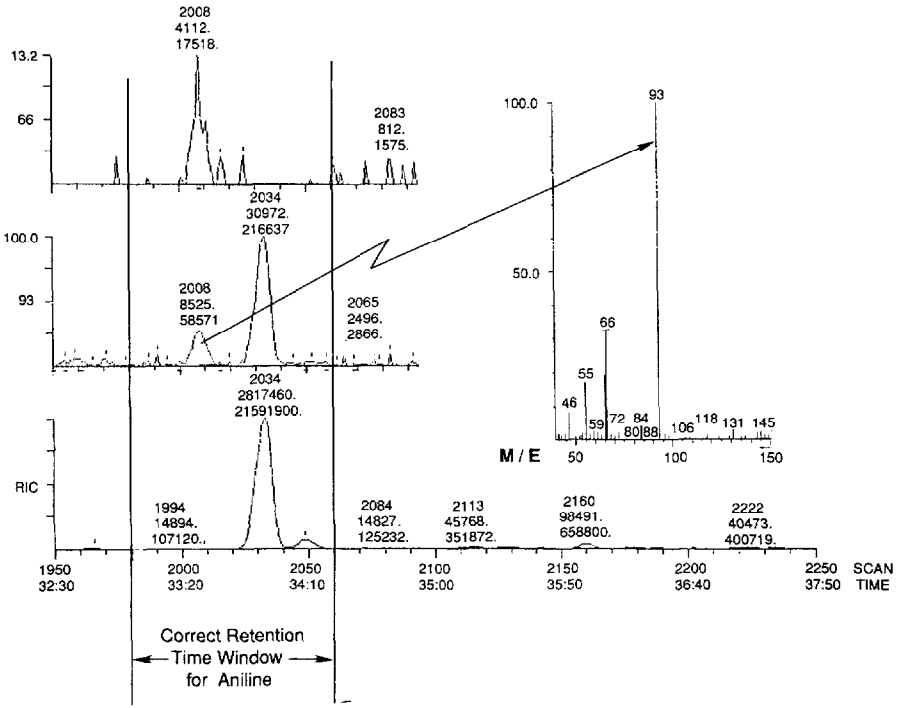

Fig. 2. Retention time window where aniline elutes is shown for patient 2. The peak centered at scan 2008 in the reconstructed ion chromatogram yielded the correct mass spectrum for aniline shown in the insert. The molecular ion (M^+) is seen at m/z 93 and the characteristic ions at m/z 66 and 65.

found in the expired lung air of one carcinoma patient (see Fig. 1). The *o*-methylaniline (*o*-toluidine) was determined to be present from comparison of the retention times of standards to the unknown. Subsequently, the corresponding retention time window of each chromatogram generated from all participants was searched for these compounds according to the key ions in their mass spectrum (m/z 93 and 66 for aniline; m/z 106 and 107 for *o*-toluidine) (see Figs. 2 and 3). Aniline was found in five of the ten cancer patients, none of the age-matched controls (55–66 years) and two of the eight younger controls (22–41 years). *o*-Toluidine was found in nine of the ten cancer patients, three of the eight younger controls and six of the eight age-matched controls. Table I summarizes the results of the quantitative analysis for the presence of aniline and *o*-toluidine in each of the patients and controls.

The level of *o*-toluidine found in the cancer patients appeared to be higher than the level found in either control group. The differences in the levels of *o*-toluidine in patients versus aged-matched controls was tested using the Mann–Whitney *U*-test. The result of this is shown in Fig. 4 which depicts the median and interquartile range for each group ($U = 17$, $p < 0.05$). Thus, more *o*-toluidine was present in patients with bronchogenic carcinoma than either control group.

The greatest levels of aniline were found in four of the six patients with squamous cell carcinoma suggesting a possible relationship between this cell type and

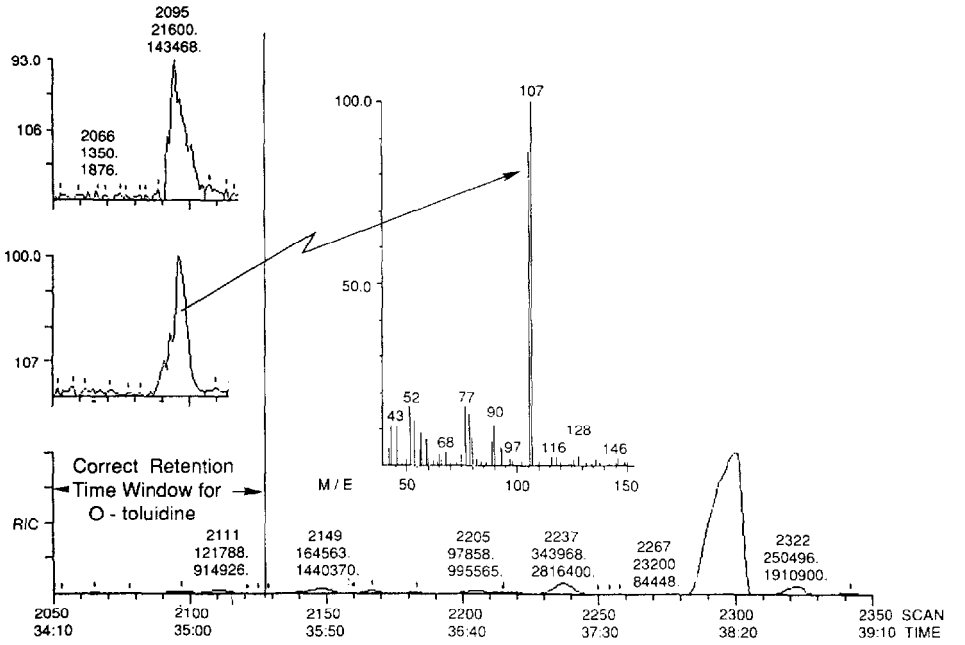

Fig. 3. Retention time window where *o*-toluidine elutes is shown for patient 3. The peak centered at scan 2096 in the reconstructed ion chromatogram yielded the correct mass spectrum for *o*-toluidine shown in the insert. The molecular ion (M^+) is found at *m/z* 107; $M^+ - 1$ is seen at *m/z* 106.

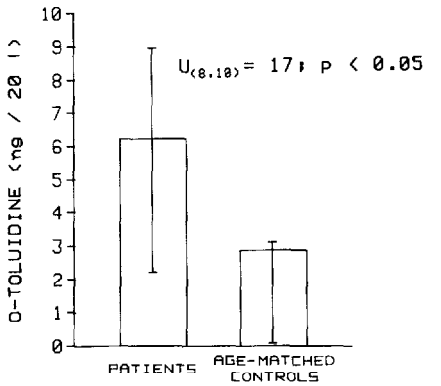

Fig. 4. Comparison of the concentration of *o*-toluidine in cancer patients and age-matched controls. The histograms show the median value and the interquartile range for this compound in each subject group. The Mann-Whitney *U*-test performed on these data reveals a significant difference between the two groups.

production of the aniline. The elevated levels of aniline and/or *o*-toluidine seen in two of the young controls could not be explained since the trend in these subjects was to have little or none of the aromatic nitrogen compounds.

In order to determine how much aniline and *o*-toluidine were being transferred from the 20-l bags to the GC–MS system, 10 ng of each compound (10 μl of 1 ng/μl solutions) and 100 ng of internal standard (5 μl of 20 ng/μl solutions) were

added to the bags as methanol solutions. The bag was then filled with prepurified nitrogen and the 20-l volume desorbed onto a Tenax-filled tube and subsequently analyzed on the GC–MS system. Five repetitions of this procedure showed that the efficiency of transfer of aniline and *o*-toluidine from the Tedlar bags to the mass spectrometer was 4.34 ± 0.56 and $8.60 \pm 0.75\%$, respectively. In contrast, when the same concentrations of these compounds were placed on the Tenax tubes (via injection) and transferred to the mass spectrometer, 95% of the original concentrations were seen. Consequently, although the precision of the entire procedure is good, the efficiency of transfer from bags to tubes is low.

DISCUSSION

In this study, the majority of compounds identified (Table II) have been previously reported as being volatile constituents of either respiratory air or other body specimens [1,2,8,9]. These include many of the volatile, nitrogen-containing compounds such as benzothiazole, indole, pyridine, methylpyridine and diphenylamine. Diphenylamine has been reported in salivary volatile compounds [10]. Pyridine and alkyl pyridines have been detected in the headspace above human saliva and are believed to arise from degradation of collagen [10,11]. Menthol, although detected in the air samples obtained from several of the cancer patients, is a pervasive compound. It is present in cigarettes as well as personal health and toiletry preparations, and, consequently, was thought to be exogenous rather than tumor-related.

Previous attempts to correlate odor with cancer development have been related to the presence of bacteria. Odors have been observed to originate directly from anaerobic bacterial colonization of tumors and were reduced by the use of antibacterial agents [12]. Increased breath methane levels were proposed as indicators of colon cancer although later studies indicated that the correlation was not significant [13]. In addition, the profile of volatile urinary amines has been reported to be indicative of breast cancer [14].

A previous study of respiratory air by GC–MS has shown the presence of simple endogenous alcohols, ketones, amines and numerous compounds of exogenous origin [2]. This study also found that acetone, isoprene and acetonitrile comprised 51% of the volatile substances. These researchers have also recently investigated the expired air from patients with bronchogenic carcinoma [7]. In this study, exhaled air from twelve lung cancer patients and seventeen control subjects were analyzed by GC–MS. When the data were analyzed using general computerized statistical procedures, the results showed that selected volatile substances could almost completely differentiate between patients and controls; the selected volatile substances allowing this differentiation were acetone, methyl ethyl ketone and *n*-propanol. In our analysis of expired lung air, acetone has been consistently seen in both patients and controls. However, due to the large number of unresolved, low-molecular-weight compounds that elute within the first 6–8 min of our chromatogram, reliable quantitation of acetone was not attempted. In addition, since methyl ethyl ketone and *n*-propanol elute in this same area, their identification was difficult, particularly since the ions characteristics of these

compounds are common to many low-molecular-weight molecules. However, our data suggest that a higher molecular weight and more easily analyzed compound (i.e., *o*-toluidine) appears in greater concentrations in patients with lung carcinoma than in control populations.

Aniline, methylanilines, N-ethyl- and N,N-dimethylanilines have been reported in cigarette smoke. Specifically, levels of aniline and *o*-toluidine have been reported as 364 ng and 162 ng, respectively, in main-stream cigarette smoke and are considerably higher in side-stream cigarette smoke [15,16]. Nevertheless, although aniline was detected in this study in the lung air samples of five cancer patients, only two were currently smokers. Consequently, we did not feel these compounds were attributable to cigarettes because aniline was also detected in three patients who had not smoked for 3–10 years as well as one control who never smoked (see Table I). Similarly, *o*-toluidine was detected in all patients who have not smoked for 3–15 years. Aniline, diphenylamine and benzothiazole have been reported to be present in various industrial emissions [17]. Diphenylamine and benzothiazole were found in all patients and controls whereas aniline, as noted above, was only present in five patients and two younger controls. There was no evidence in the histories of these patients or controls to suggest an unusual environmental exposure.

A recent study suggests that aniline and *o*-toluidine may be present in human body fluids whether or not there has been any exposure to cigarettes [18]. El-Boyoumy et al. [18] have found approximately equal concentrations of aniline and *o*-toluidine in the urine of smokers and non-smokers. In addition, Stillwell et al. [19] examined the hemoglobin adducts of several aromatic amines in both cigarette smokers and non-smokers. These amines included *o*-, *m*- and *p*-toluidine and aniline. The concentration of the adducts of *o*- and *p*-toluidine were greater in smokers than non-smokers; however, the levels of aniline adduct were similar in both groups. While these studies suggest sources other than cigarettes for these compounds in humans, it does not readily explain why the cancer patients we studied had higher levels of aniline and *o*-toluidine than controls.

Our data as well as the studies by El-Boyoumy et al. [18] and Stillwell et al. [19] suggest that pathways other than cigarette smoking may give rise to the aniline and *o*-toluidine. Dietary pathways have also been suggested [18] since these compounds are known to occur in vegetables such as celery, cauliflower and carrots. In addition, these authors also suggest broiled foods might yield these compounds via pyrolysis of amino acids. In addition, El-Boyoumy et al. [18] have suggested that anilines may be formed from aromatic amino acid metabolism. However, no metabolic relationship has been proposed by any of these researchers concerning the relationship between aniline and *o*-toluidine.

None of the foods discussed above were reported to have been eaten by our patient population. Further, patients 2 and 3 (Table II) who had two of the highest levels of aniline and *o*-toluidine had not eaten for more than 14 h prior to testing. One of our controls (Table II, YC8) ate celery the day prior to testing and another had tea (Table II, YC5), though not specifically black tea, which is also reported to contain aniline in its aroma [18]. Consequently, the presence of aniline or *o*-toluidine in these two controls may be linked to these dietary factors.

Our initial effort to analyze expired air volatiles attempted to determine whether or not certain lung air constituents might differentiate, in a non-invasive manner, patients with bronchogenic carcinoma from controls. Our results suggest that there are some differences in the response of volatile compounds. The anilines do not appear to be of exogenous origin and may be linked to some metabolic processes in the subjects who displayed them. The presence of *o*-toluidine in expired lung air at the concentration seen in the cancer patients, particularly in conjunction with the levels of aniline reported here, may be suggestive of abnormal metabolic processes.

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