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Cancer sniffer dogs: how can we translate this peculiarity in laboratory medicine? Results of a pilot study on gastrointestinal cancers

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Abstract

Background: Identification of cancer biomarkers to allow early diagnosis is an urgent need for many types of tumors, whose prognosis strongly depends on the stage of the disease. Canine olfactory testing for detecting cancer is an emerging field of investigation. As an alternative, here we propose to use GC-Olfactometry (GC/O), which enables the speeding up of targeted biomarker identification and analysis. A pilot study was conducted in order to determine odor-active compounds in urine that discriminate patients with gastrointestinal cancers from control samples (healthy people).

Methods: Headspace solid phase microextraction (HS-SPME)-GC/MS and GC-olfactometry (GC/O) analysis were performed on urine samples obtained from gastrointestinal cancer patients and healthy controls.

Results: In total, 91 key odor-active compounds were found in the urine samples. Although no odor-active biomarkers present were found in cancer carrier's urine, significant differences were discovered in the odor activities of 11 compounds in the urine of healthy and diseased people. Seven of above mentioned compounds were identified: thiophene, 2-methoxythiophene, dimethyl disulphide, 3-methyl-2-pentanone, 4-(or 5-)methyl-3-hexanone, 4-ethyl guaiacol and phenylacetic acid. The other four compounds remained unknown.

Conclusions: GC/O has a big potential to identify compounds not detectable using untargeted GC/MS approach. This paves the way for further research aimed at improving and validating the performance of this technique so that the identified cancer-associated compounds may be introduced as biomarkers in clinical practice to support early cancer diagnosis.

Keywords: cancer sniffer dogs; gastrointestinal cancer; VOC.

Introduction

Gastrointestinal (GI) cancers encompass malignancies affecting different organs and tracts of the digestive apparatus, which together account for around 40% of cancer-related deaths worldwide [1–3]. Among the GI cancers, colorectal cancer is the most frequent, with a prognosis highly dependent on the stage at diagnosis [2]. Also common is gastric cancer, which is the third most deadly tumor [1, 4], partly due to late diagnosis [5]. Indeed, early-stage disease is often curable, but usually it remains undiagnosed until late stage [1]. The GI cancer with the worst prognosis, however, is the pancreatic cancer, the fourth most lethal cancer worldwide, with mortality approaching incidence [6, 7]. This cancer owes its poor clinical outcome to late-stage detection, biological aggressiveness of the disease, and resistance to conventional therapies [6, 8]. It is therefore evident that for GI tumors early detection is essential to give patients the best chance of treatment effectiveness. Regrettably, early diagnosis is often difficult

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to achieve, since some screening tests may be expensive, ineffective in finding small tumors, or may even introduce additional health risks (e.g. exposure to radiations, biopsies and other invasive exams) [9]. Diagnosis of GI cancers is usually based on invasive endoscopic procedures [10, 11]. For these reasons, new and non-invasive detection methods endowed with sensitivity and specificity are needed. In recent decades great efforts have been devoted to discovering candidate biomarkers which could allow a non- invasive, cost-effective identification of the disease.

Recently, the analysis of volatile organic compounds (VOCs) as a diagnostic tool has attracted much attention [10], supported by the evidence that a specific profile of odorous compounds discriminating healthy and neoplastic people can be successfully sensed by the canine olfactory system [12–14]. Lippi and Cervellin [12] reviewed recent studies reporting that appropriately trained dogs (the so called "sniffer-dogs") exhibit an extraordinary ability to detect various types of cancers by recognizing a distinctive "odor signature" in urine, sweat, breath and blood.

Despite its potential, canine olfactory detection of cancers in the clinical practice has some limitations: dogs have to be suitably trained and this is expensive and time-consuming [15]; the performance may be influenced by polymorphisms in the olfactory receptor gene and thus by the breed of dogs [13, 16]. Moreover, it is still unclear what dogs recognize in cancer samples [12], whether a single or rather a mix of odor-active compounds [14]. To address all these issues, the olfactory cancer detection by dogs should be substantiated with laboratory tests [12]. A well-established technique for detecting VOCs in biological fluids, which can be used in laboratory medicine, is represented by gas chromatography/mass spectrometry (GC/MS) [17]. An appealing alternative approach to this kind of study, which could help to shed light on the scientific bases of canine cancer detection, is represented by gas chromatography-olfactometry (GC/O), which couples the traditional GC separation with human olfactory detection in order to identify odor-active compounds within a mixture [18]. Each odorous compound eluted by GC is directed through an odor port to trained human assessors which can detect, measure and describe the quality of the odor [18, 19]. This technique allows traces of molecules with extremely low odor threshold (not visible by GC/MS, but with strong odor and therefore detectable by GC/O). To date, it has mainly been applied in the analysis of food and drink aromatic compounds and in the field of fragrances and perfumery, and its application in the medical field is only recently emerging as a promising tool to help diagnoses [18].

Comparing different biological samples, the urine has proven to contain a greater variety of VOCs [20, 21], reflecting the products of the overall body metabolism and its alterations [21]. Moreover, relatively large volumes of sample can be easily obtained [22]. VOC levels extracted from the headspace of urine have been investigated and found to discriminate healthy subjects from patients suffering from lung [21, 23], prostate [24], biliary [25] and renal cancer [26]. As for the GI cancers, VOCs have been determined in the urine of colorectal cancer patients [27] and in a pilot study on the urine of patients with biliary malignancy [25]. To the best of our knowledge, no previous analysis of VOCs in the urine of gastric and pancreatic cancer patients has been performed. Further, no application of GC/O in the medical field for the detection of potential cancer biomarkers has been reported. For this purpose, we performed for the first time a pilot study on urine samples from GI cancer-affected people (above all gastric and pancreatic ones) using Headspace solid phase microextraction (HS-SPME)-GC/MS and GC-Olfactometry (GC/O) analysis in order to discover one or more odor-active compounds differentiating cancer and cancer-free subjects that could be eligible as potential GI cancer biomarkers.

A deeper exploration of this area would be desirable since the identification of one or more chemical markers of disease would be of great impact in the development of diagnostic tests.

Materials and methods

Patient's selection and sample's preparation

Twenty-three patients with a confirmed diagnosis of gastrointestinal cancer, referred to our Gastroenterology Department of the "Casa Sollievo della Sofferenza" Hospital of San Giovanni Rotondo, Italy, and fifteen healthy donors (controls) were enrolled in the study. All patients signed an informed consent approved by the Local Ethics Committee. The consent included clinical-pathological patient's features. The latter are reported in the Table 1. Urine from both patients and controls were collected and stored at –20 °C until the analysis. For GC/O and GC/MS analysis, 2 mL of urine (defrosted to room temperature) was measured into 20 mL glass headspace vial with glass-covered magnetic stir-bar, 0.8 g of sodium chloride was added and pH was adjusted to 1–2 using hydrochloric acid (HCl:H_2O 1:1) as suggested by Silva et al. [28]. Vials were capped with PTFE-silicon septa and placed in an autosampler tray at room temperature just before analysis. Samples were brought one-by-one into magnetic stirring chamber for volatile extraction. Pre-incubating time was 5 min and adsorption time was 45 min (250 rpm). Pre-incubation and adsorption temperatures were both 40 °C. For SPME, 30/50 µm DVB/Car/PDMS Stableflex 2 cm long fiber from Supelco (Bellefonte, PA) was

Table 1: Demographic and clinical features of patients and controls.

	Cancer patients	Healthy controls
Number	23	15
Mean age (±SD)	69.5 ± 10.8	58.2 ± 8.9
Sex (male/female)	12/11	6/9
Cancer type	12 gastric, 8 pancreatic, 2 colon, 1 bile duct	–
Current smoker	2	5
Ex-smoker	6	–

used. After extraction the fiber was injected to GC/MS-TOF, GC/qMS or GC/O for desorption (10 min).

Chemicals and consumables

Sodium chloride, hydrochloric acid and alkane mix (C8-C22) were purchased from Sigma-Aldrich (Germany).

GC/O and GC/MS apparatus parameters

GC/O was equipped with odor detection port ODP3 (Gerstel, Germany). GC/MS-TOF (GCT Premier CAB021, Waters, UK) and GC/qMS (5975 Agilent Technologies, USA) were used to identify the compounds. The GC column for GC/MS-TOF and GC/O was DB5-MS, 30 m × 0.25 mm × 1.0 µm (Phenomenex, USA). For GC/qMS HP-5MS 30 m × 0.25 mm × 0.25 µm (Agilent Technologies) and ZB-Wax plus 30 m × 0.25 mm × 0.25 µm (Phenomenex, USA) was used. Both GC/qMS and GC/O inlets were Merlin Microseal (Agilent, USA) and GC/MS tof had PTV (CIS-4, Gerstel, Germany) inlet. All were run in splitless mode. A 0.75 mm i.d. liner at 250 °C was used in all injectors. The carrier gas was He (5.0, AGA Estonia, Estonia) with a flow 1.0 mL/min in both GC/MS and 2.0 mL/min in GC/O. For the GC/MS identification ChromaLynx program (Waters, UK), Chemstation (Agilent Technologies, USA), NIST05 library, retention indices and odor qualities (Flavornet.org, Pherobase.com and Thegoodscentcompany.com) were used. Different oven programs were used for GC/MS-TOF, GC/qMS and GC/O analysis. For the GC/MS-TOF and GC/qMS HP-5MS column, the following program was used: starting at 45 °C, 5 °C/min to 280 °C, holding time 3 min. Total run time was 50 min. For the GC/qMS ZB-Wax column, the program was 35 °C, 10 °C/min to 250 °C (total run 21.5 min). The GC/O oven program was shorter to avoid assessor fatigue, starting at 35 °C, 17 °C/min to 280 °C, holding time 4 min (total run time 17.41 min).

Data analysis

In GC/O analysis three trained assessors (two female, one male) were used to detect the odors and assess the intensities in a 5-point scale (1 – fairly detectable, 5 – extremely strong) and describe odor quality in one parallel [29]. GC/O data were processed using modified frequency formula:

$$MF(\%) = \sqrt{F(\%) * I(\%)}$$

where F(%) is the detection frequency expressed as percentage of maximum detection and I(%) is a sum of odor intensities of different assessors expressed as percentage of maximum sum of intensity. MF(%) shows the importance of the compound in a sample. Key odor-active compounds were identified as compounds with MF(%) value of at least 11.2%. Compounds with low MF(%) were not screened as compounds which may come from the environment (e.g. diet-derived) and are not specific to cancer metabolism.

Statistical analysis

Results are expressed as mean ± SD. Comparisons were made using a Student's t-test. Differences were considered as statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**).

Results

Comparative GC/O analysis of VOCs in urine samples of GI cancer patients and healthy controls

In total, 91 key odor-active compounds were found in the urine samples, whose retention indices, odor qualities and MF(%) are reported in Table 2.

According to GC/O data, no odor-active biomarkers present only in a cancer carrier's urine were found. Despite this, significant differences were discovered in the odor activities of 11 compounds in the urine of healthy and diseased people (Table 3). Seven of above mentioned compounds were identified, and other four remained unknown. Among the identified compounds, the sulfur compounds were as follows: thiophene, 2-methoxythiophene and dimethyl disulphide; ketones: 3-methyl-2-pentanone, 4-(or 5-) methyl -3-hexanone; and aromatic compounds: 4-ethyl guaiacol and phenylacetic acid.

Average modified frequency values were calculated for the seven identified compounds that differentiate the urine of healthy and diseased people (Figure 1). Dimethyl disulphide, 2-methoxythiophene and phenylacetic acid odor activities were elevated in cancer carrier's urine and thiophene, 3-methyl-2-pentanone, 4-(or 5-) methyl-3-hexanone, 4-ethyl guaiacol odor intesities were higher in the urine of healthy people.

GC/O method has a large variability due to the human factor. The results depend on the assessor's physiological state: tiredness, breathing rhythm, genetic background etc. Thus, only significant differences in the odor activities of compounds were taken into account. Difference was defined as significant when odor activities between

Table 2: Odor quality and odor activities of key odor compounds in healthy individuals and cancer carrier urine samples among three GC/O assessors.

LRI (GC/O)	Odor quality	Cancer			Control		
		A1	A2	A3	A1	A2	A3
–	Rotten cabbage	76.4	90.9	79.2	72.5	82.0	74.4
–	Burnt	0.0	18.4	7.8	0.0	0.0	8.6
504	Sour, medicinal	0.0	0.0	17.3	0.0	0.0	24.3
520	Rubber, burnt, sulphur	0.0	0.0	11.6	0.0	0.0	7.0
534	Burnt	7.0	22.1	0.0	13.7	69.6	0.0
554	Urine, sulphur	0.0	0.0	7.1	0.0	0.0	22.8
574	Butter, sour	2.0	0.0	0.0	12.5	0.0	0.0
599	Butter, sour	10.0	65.6	0.0	12.5	64.4	7.0
604	Acidic, vinegar	9.5	17.8	3.2	0.0	62.9	0.0
623	Sour, acidic, vinegar	0.0	0.0	3.9	0.0	0.0	14.1
631	Acidic, sharp	0.0	0.0	0.0	11.2	0.0	7.0
660	Coffee, sweet	28.2	0.0	0.0	5.6	0.0	0.0
678	Sulphur, garlic	26.9	39.3	32.4	57.0	78.9	69.9
695	Butter	2.5	18.8	3.2	0.0	14.1	0.0
703	Sweat, urine, roast	8.1	25.8	10.3	6.9	0.0	7.0
760	Fruit, solvent	21.1	6.7	0.0	49.1	41.6	0.0
761	Sulphur, garlic, cabbage	10.4	55.0	36.3	0.0	19.9	50.9
762	Bread, cheese, berries	17.8	71.9	3.9	4.0	60.3	8.6
782	Rubber,solvent	50.7	48.5	35.4	62.3	65.9	47.1
802	Green, apple	60.5	73.1	45.8	65.2	80.3	57.3
794	Fruity, sweet	10.9	25.6	45.2	5.6	58.0	22.8
815	Boiled potato	50.1	75.4	0.0	68.0	78.3	0.0
825	Cheese	12.9	0.0	79.5	18.5	0.0	85.6
826	Garlic, sulphur	49.5	76.0	31.0	68.0	88.2	7.7
836	Garlic	19.6	33.2	0.0	43.3	67.4	0.0
838	Chemical, sour	24.7	91.8	75.8	15.1	88.9	76.7
854	Fruity, sweet with fermented, bready nuances	22.0	34.6	43.8	40.5	65.0	57.3
861	Coffee, roast	0.0	0.0	3.2	0.0	0.0	15.7
879	Pear, barberry, apple, solvent, chemical	57.5	20.9	65.1	59.7	47.1	81.7
875	Roast	0.0	70.8	75.6	7.9	86.9	73.0
887	Onion, rubber	5.0	66.4	47.1	0.0	29.8	0.0
900	Boiled potato	13.6	4.3	0.0	0.0	7.0	0.0
907	Dried fish, green, fatty	40.0	65.7	14.1	28.5	64.4	24.3
918	Green, boiled potato	63.3	89.4	0.0	58.2	85.6	7.0
928	Sulfurous, burnt, urine, sweat	38.6	74.5	84.3	75.8	100.0	86.3
961	Sweat, urine	2.9	0.0	0.0	14.8	0.0	0.0
972	Sweat, urine, sulphur	14.7	3.0	3.9	0.0	7.0	7.0
982	Mushroom	42.4	70.6	73.3	30.6	81.7	74.5
988	Metal, dry	26.6	58.3	38.4	23.7	43.0	38.5
998	Roast, garlic	77.8	98.6	89.4	81.4	97.8	90.7
1011	Rubber, chemical	51.4	80.3	87.8	69.4	83.0	93.1
1036	Dirty, sweat, musty	0.0	54.1	60.3	0.0	8.6	78.2
1037	Sweat, rubber, plastic, dust	30.2	45.8	54.1	29.1	41.6	14.1
1046	Coffee, chemical	20.5	54.1	22.6	30.6	18.6	28.6
1054	Floral, sweet, fresh, spicy	12.9	36.9	0.0	0.0	37.2	0.0
1061	Sweet, honey, vegetative	2.9	52.6	23.2	11.2	65.7	34.4
1056	Caramel, coffee, sweet	44.0	35.0	22.6	53.9	18.6	28.7
1067	Urine, sour	81.7	11.7	45.0	64.2	0.0	40.0
1077	Fecal	76.2	54.0	40.0	84.3	51.6	46.9
1097	Sour, chemical, urine	22.3	29.5	21.9	7.9	28.5	24.3
1099	Plasticine, green	48.7	52.2	49.6	79.7	39.8	74.4
1104	Sweet,phenolic, spicy, woody, green	65.6	85.7	87.0	79.0	98.9	97.8
1127	Medicinal, green, rubber, coffee	36.1	13.5	79.4	33.5	8.6	82.3
1140	Sweat, dirty	0.0	6.7	21.9	0.0	0.0	22.8
1134	Vegetative, sweat	0.0	3.0	11.6	0.0	7.0	0.0
1158	Green, fresh, floral, fruity	28.9	75.6	59.1	69.4	34.4	69.6

Table 2 (continued)

LRI (GC/O)	Odor quality	Cancer			Control		
		A1	A2	A3	A1	A2	A3
1166	Grass, cucumber	8.6	26.4	12.0	6.9	25.8	17.2
1168	Gouache, green, rubber	81.6	82.5	74.4	94.9	94.3	81.0
1184	Leather, green, metal, herb	18.7	17.8	31.0	22.4	44.4	59.5
1188	Citrus, peppermint	0.0	0.0	20.0	0.0	0.0	0.0
1199	Boiled potato	20.3	63.4	24.5	9.7	77.0	28.6
1201	Coffe, roast, sweet	0.0	0.0	0.0	0.0	0.0	35.8
1205	Green, floral	40.4	64.3	76.8	23.7	67.0	28.5
1214	Mushroom	0.0	7.4	0.0	0.0	34.4	17.2
1217	Burnt plastic, rubber, medicinal	19.3	10.4	70.5	14.7	0.0	61.7
1227	Honey, sweet, waxy, green	75.7	90.4	70.1	96.2	41.0	83.0
1244	Honey, floral with fecal and urine nuances	45.7	75.3	64.4	32.1	47.1	78.9
1255	Chalk, musty	0.0	0.0	7.1	0.0	0.0	24.3
1267	Waxy, dirty, sweet, honey	4.1	14.3	29.7	34.3	18.6	32.7
1260	Dill, peppermint	0.0	8.0	7.8	0.0	8.6	14.1
1289	Spicy, clove-like, floral	6.1	3.7	16.1	0.0	45.8	64.4
1304	Mushroom	18.0	83.6	87.2	57.3	89.4	83.0
1312	Woody, metal, thyme	5.4	14.8	34.6	15.8	27.2	34.4
1329	Sweet, woody, floral, clove, cinnamon, honey	57.6	83.0	73.5	62.8	78.3	77.5
1335	Rubber, medicinal	0.0	0.0	12.3	0.0	0.0	0.0
1352	Natural vanilla, phenolic, woody	48.1	65.7	85.5	62.3	78.9	91.3
1362	Fatty, dirty, fresh, chalk	39.7	20.2	83.4	19.4	8.6	72.4
1369	Mould	0.0	77.7	0.0	5.6	55.8	0.0
1381	Coconut, dill, woody	11.0	31.9	6.7	9.7	31.4	17.2
1388	Cherry, sweet, plum, rose	23.6	53.9	66.1	33.1	74.5	76.0
1406	Cherry, sweet	30.8	88.3	68.4	24.7	88.2	79.6
1425	Cream, vanilla	37.4	54.1	58.3	55.3	60.3	64.4
1428	Urine, fecal	8.6	26.4	0.0	0.0	17.2	0.0
1435	Dill, coconut, cream	37.4	0.0	43.1	34.5	8.6	55.8
1456	Raspberry, sweet, berries, floral, cream	66.5	0.0	27.1	68.6	0.0	25.8
1474	Sulphur, burnt	2.0	7.4	3.2	0.0	22.8	22.8
1479	Coconut, cream, peach	8.6	78.7	44.3	5.6	74.5	45.5
1495	Dill, coconut, cream, sweet	66.2	78.7	76.8	68.9	74.5	62.9
1557	Soap	15.1	62.3	66.0	17.7	71.7	67.4
1579	Fecal, urine	36.9	68.6	0.0	36.4	76.0	0.0
1685	Fresh, dirty, waxy with citrus notes	54.3	88.9	63.2	53.9	86.9	65.7

Compounds with two or more times different odor activities of at least two assessors are highlighted.

Table 3: Odor activity of the compounds differentiating the urine samples of healthy and deseased people.

RI (GC/0)[a]	Compound	Odor quality	Cancer			Control		
			A1[b]	A2	A3	A1	A2	A3
631	Unknown	Acidic, sharp	0.0	0.0	0.0	11.2	0.0	7.0
678	Thiophene	Sulfur, garlic	26.9	39.3	32.4	57.0	78.9	69.9
760	3-methyl-2-pentanone	Fruit, solvent	21.1	6.7	0.0	49.1	41.6	0.0
761	Dimethyl disulphide	Sulphur, garlic, cabbage	10.4	55.0	36.3	0.0	19.9	50.9
794	4-(or 5-)methyl-3-hexanone	Fruity, sweet	10.9	25.6	45.2	5.6	58.0	22.8
836	Unknown	Garlic	19.6	33.2	0.0	43.3	67.4	0.0
887	2-methoxythiophene	Onion, rubber	5.0	66.4	47.1	0.0	29.8	0.0
1214	Unknown	Mushroom	0.0	7.4	0.0	0.0	34.4	17.2
1244	Phenylacetic acid	Honey, floral	45.7	75.3	64.4	32.1	47.1	78.9
1289	4-ethyl guaiacol	Spicy, clove-like, floral	6.1	3.7	16.1	0.0	45.8	64.4
1474	Unknown	Sulphur, burnt	2.0	7.4	3.2	0.0	22.8	22.8

[a]Retention indexes, calculated based on GQ'O retention times of compounds. Calculated Rl-s are equal to the Rl-s found in the scientific literature in the range of ±20. [b]A1-3: modified frquencies of three GC/O assessors.

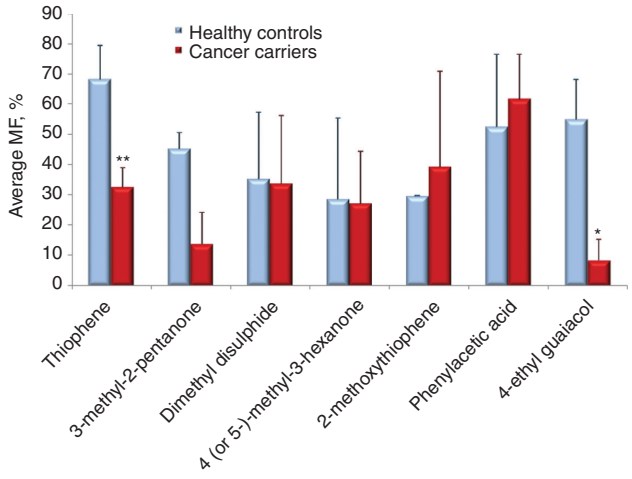

Figure 1: Odor activity of identified compounds differentiating the urine of healthy controls and cancer carriers.
Differences were considered as statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**). The average of modified frequency (MF [%]) is calculated among the determinations of all three assessors on all healthy and diseased people urine samples.

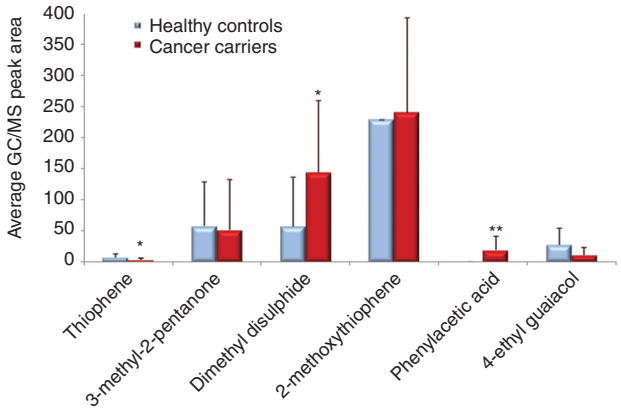

Figure 2: The average GC/MS peak areas of the compounds differentiating the urine of healthy controls and cancer carriers.
Differences were considered as statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**). The average is calculated among all healthy and diseased people urine samples independently of detection of compound in a precise sample during GC/O analysis. Peak area of 2-methoxythiophene is referred to the only one control sample where it was detected.

healthy and diseased people were differentiating two or more times in the same way, and that in case of at least two assessors. Although phenylacetic acid does not have drastic changes in MF(%) value, it was included in the list because assessor A2 detected it almost in all cancer samples with greater intensity than in controls. As for thiophene and 4-ethyl guaiacol, differences between healthy controls and cancer carriers proved statistically significant (Figure 1).

Confirmation of GC/O results by GC/MS

To ensure that the differences in odor activities are caused by actual differences in the content of certain compounds and not by occasional variability in MF(%) values, average peak areas on GC/MS chromatograms of above mentioned compounds were calculated (Figure 2). Peak areas indirectly represent the concentration of compounds in the sample. The average peak area is not shown for 4-(or 5-)methyl-3-hexanone as it was present in samples at trace levels.

Comparing data in Figures 1 and 2 it is clearly seen that the differences in odor activities of compounds are caused by roughly similar differencesin their contents in the samples based on GC/MS data. Statistically significant differences between the two groups were obtained for thiophene, dimethyl disulphide and phenyl-acetic acid (Figure 2). Although the peak area for 2-methoxythiophene appear to be the same between control samples and cancer' patients in Figure 2, it is noteworthy that GC/MS detected this compound in only one of healthy controls while it was detected in about half of cancer patients.

Discussion

Fiction and action movies often show escaped prisoners chased by junkyard dogs, reminding us of the dog's ability to recognize chemical mixtures and track them to their sources [30]. Crime-related chemical residues, such us narcotics and explosives, can be easily detected by well-trained sniffer dogs [31, 32]. The dogs' sense of smell is highly developed; their olfaction allows them to perceive odorous compounds present in as little as parts per trillion [33], due to anatomical, physiological and genetic factors. Dogs perceive odours by sniffing, during which short air inhalations enter the nose and reach the olfactory receptors on neurons of the nasal olfactory epithelium. The axons of all these cells form the olfactory nerve bundles, which converge to the olfactory bulb in the brain. The perception of scent occurs in the frontal cortex [34]. In recent years, a dog's ability to recognize, by sniffing, biological samples from cancer patients has emerged, so that the involvement of dogs in the clinical setting has been suggested since urinary volatile organic compounds (VOCs) have been proposed as useful biomarkers for assessing tumor presence [12–14]. Diagnosing cancer at its earliest stages is crucial to give patients the best chance of survival and treatment options [35]. Nowadays, scientists are struggling to discover novel biomarkers that may help in

early and non-invasive diagnosis of malignancies. To date several clinical markers (mostly detected in the blood serum) have been proposed for GI cancers: the most frequently used both for pancreatic and for gastric cancer are the carbohydrate antigen 19-9 (CA 19-9) and the carcinoembryonic antigen (CEA) [1, 5, 36]. However, the sensitivity and specificity of these markers are inconsistent as they are unfrequently elevated in the early stage of the cancerogenesis [37], and may be over-expressed in various inflammatory conditions [38, 39]. Interestingly, the use of these and other proposed markers (such as a CEACAM1, CA-125 for pancreatic cancer) [40] has been assessed as a prognostic and surveillance rather than a diagnostic tool [1, 41].

Great effort has been devoted to studying VOCs [10], which may be differentially recognized by dogs in biological samples from healthy and diseased people [12–14]. Nevertheless canine olfactory detection in the clinical setting to help diagnosis has some limitations: i) dogs need to be well trained to recognize a particular odor signature, and this may be expensive and time-consuming [15]; ii) genetic factors such as a dog's breed or polymorphisms in the olfactory receptors may influence the sensory ability of the animal [13, 16], so that performances from different dogs may be variable. Moreover, another disadvantage of using dogs to detect cancer is that it is still not known whether they recognize a single compound or a mixture of compounds [14, 42], while identifying individually the molecules that distinguish diseased from healthy samples could open the way to the discovery of new therapeutic targets.

For all these reasons, it would be desirable to set up alternative approaches to sniffer dogs, based also on automated systems which allow separation and identification of single compounds.

In order to shed light on what dogs actually detect in cancer samples and whether these compound(s) can be used as biomarker(s), we used GC/O as a diverse approach aimed at identifying a single or a panel of odor active compounds which could be measured in urine to discriminate between GI cancer carriers and cancer-free subjects, as it was already performed for other kinds of tumors using different techniques [13, 21, 23–27]. A well-established technique for VOC analysis in biological samples is represented by GC/MS. It should be considered, however, that not all VOCs are odor-active [43] and that is why traditional techniques based only on chemical detection of metabolites would not help understand the molecular bases of dogs' cancer detection. Moreover, GC/O has the potential to detect compounds present at trace levels [19], which escape GC/MS detection but are sensed by GC/O due to their odorous properties. In our study, for example, this was the case of 4-(or 5-)methyl-3-hexanone, which was barely detectable by GC/MS but had a considerable odor activity. Moreover, the literature reports examples of urine compounds undetected with traditional techniques which were identified for the first time by GC/O [44]. Certainly human olfaction is much less sensitive than the canine olfaction. From the anatomic point of view, the olfactory epithelium of dogs is about 20-fold larger than in humans and can thus accommodate a greater number of neuronal cells and olfactory receptors [45]. Moreover, it is estimated that dogs possess an approximately 30% higher repertoire of genes encoding olfactory receptors when compared to humans, which in turn have a larger number of inactive pseudogenes [45]. Furthermore, it is believed that the way dogs sense the odours by sniffing makes perception more efficient than with normal breathing: briefly inspiring through the nostrils while the mouth is closed would reduce the distance between inhaled air and receptors in the nose, thus improving the olfactory acuity [31].

Although less sensitive than canine olfaction, however, it is documented that human olfaction is superior to any other chemical detector of odorous compounds [19], such as an electronic nose. For this reason, we considered GC/O combined with GC/MS a valid approach to pursue our aim in order to overcome the dog's weaknesses mentioned above.

The data obtained in the current study indicated that dimethyldisulphide, 2-methoxythiophene and phenylacetic acid odor activities were elevated in cancer carrier's urine and thiophene, 3-methyl-2-pentanone, 4-(or 5-)methyl-3-hexanone, 4-ethyl guaiacol odor intensities were higher in the urine of healthy people.

An increase in the content of 2-methoxythiophene has previously been observed in breast cancer cells [46] and an increase of dimethyl disulphide in melanoma cells [47] which parallel our results. However, in other kinds of cancers such as breast cancer [48], colorectal cancer, leukemia and Hodgkin lymphoma [28], dimethyl disulphide was found down-regulated compared to controls. Furthermore, while in our analysis 5-methyl-3-hexanone was found decreased in cancer patients compared to healthy controls, in a previous study on fecal samples it was found to be a marker of gastrointestinal disease [49, 50]. The reasons for some inconsistencies with previous studies might be due to individual differences in the patients, different biology of the tumors and/or differences in the method of VOC extraction and detection. As concerns the other four compounds we found altered, differences in the content of thiophene, 3-methyl-2-pentanone,

phenylacetic acid and 4-ethyl guaiacol in metabolites of cancer patients have not been reported previously.

Possible causes for failure of finding, among odor-active compounds, a unique biomarker only present in cancer patients and not in controls, may be due to several factors: i) low concentration of analytes detected with very low MF(%); ii) genetic specificity of assessors; iii) lacking of a specific receptor for the compound in humans or compound concentration smaller than the odor treshold barrier for humans. Nevertheless, in the vast majority of literature reports and in our study, no unique biomarker was identified incancer patients' samples compared to controls, but rather profiles of differentially represented compounds were found between the two groups [23, 25–27, 48].

Although further work is needed and larger cohorts of patients and controls should be analyzed, our preliminary results are encouraging and suggest that GC/O is a feasible and reliable method for the differential detection of VOCs in the urine of healthy and diseased people. Should the identified cancer-associated compounds be confirmed in a larger study, this would offer the potential for future development of screening tests which could be used in the clinical practice to support diagnosis.

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