

Detection of 2-Pentylfuran in the breath of patients with *Aspergillus fumigatus*

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Aspergillus fumigatus produces 2-Pentylfuran (2-PF) when cultured on blood agar, nutrient agar and other media. As 2-PF is not known to be produced by mammalian metabolism we hypothesized that it is detectable in breath of patients colonized or infected with *A. fumigatus*. Breath was tested for 2-PF from normal subjects, those undergoing chemotherapy, and adults at risk of colonization or infection with *A. fumigatus* because of bronchiectasis, cystic fibrosis, or immune suppression. Breath samples were collected in five L tedlar bags and analyzed by Gas Chromatography/Mass Spectroscopy (GC/MS) in MS-MS mode. 2-PF was not detected in breath 14 healthy controls, in one of 10 neutropenic subjects and 16 of 32 patients with lung disease. The sensitivity and specificity of the 2-PF breath tests when compared with recurrent isolation of aspergillus from sputum or from bronchoalveolar lavage over two months was 77% and 78% respectively. As 2-PF is not normally found in human breath its presence may reflect the active metabolism of *A. fumigatus* in the airways and form the basis of a new diagnostic breath test for *Aspergillus* infection.

Keywords *Aspergillus fumigatus*, Breath test, 2-pentylfuran, mass spectroscopy

Introduction

Fungi are important etiologic agents of serious and often fatal infections in individuals who are severely immune compromised [1–3]. The diagnosis of invasive fungal infection remains difficult despite recent advances in such tests as PCR, galactomannan detection and imaging techniques [4–8]. Another possible diagnostic approach is the detection of volatile organic compounds (VOCs) in clinical samples, particularly if they are specific for the organism [9,10].

2-Pentylfuran (2-PF) is a small (MW 138g/mol), volatile (vapour pressure estimated to be 160 PA at

25°C) molecule, that is poorly water soluble (42mg/l at 25°C) with a distinctive mass fragmentation pattern. It is produced *in vitro* by *Aspergillus fumigatus* and other medically important fungi including *Aspergillus flavus*, *Aspergillus niger*, *Scedosporium apiospermum* and *Fusarium* species, but not zygomycetes [9]. 2-PF is generated as part of fungal growth on diverse media including blood agar, nutrient agar and gypsum board [9,11]. The likely metabolic pathway of production is by oxidation of linoleic acid via a lipoxygenase enzyme suggesting that it may be produced in the biological milieu of the human respiratory tract. 2-PF was also found in the headspace of cultures of *Streptococcus pneumoniae* when cultured on blood agar, but not on other culture media, and was not detected from other respiratory tract pathogens [9]. The requisite lipoxygenase pathway is not known to be present in clinically relevant bacteria including *S. pneumoniae*.

2-PF is not known to be produced in normal mammalian metabolism, and has not been found in

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surveys of human breath [9,12]. It is found in the plant kingdom both as a natural product (e.g., coffee and asparagus) and in tainted foodstuffs such as olive oil or canola oil where it is a breakdown product of linoleic acid and it could potentially reach human breath after being ingested [13–17]. As linoleic acid is part of all cell membranes it is possible some is produced *in vivo* by necrotic or inflammatory reactions.

In a preliminary study we found small quantities of 2-PF (pico mol) were detectable in the breath of a small number patients with cystic fibrosis in whom *Aspergillus* was recovered from sputum but not normal controls [9]. These results suggested that 2-PF is possibly produced in sufficient quantities to be detectable in breath samples and could provide a useful marker for the presence of *A. fumigatus* in the respiratory tract. However, it is also possible that lung damage from inflammation or other processes was the major source of 2-PF.

The main hypothesis addressed in this study was that 2-PF is detectable in breath of subjects infected or colonized with *A. fumigatus* but not normal subjects or those undergoing chemotherapy. Because invasive aspergillosis is a rare condition we have studied patients with chronic lung disease who are at risk of colonization and infection with *A. fumigatus* from whom respiratory samples can be readily obtained.

Methods

Subjects

A prospective observational study was conducted by testing for 2-PF in breath samples from subjects at risk of invasion/colonization with *A. fumigatus*, and neutropenic from chemotherapy, as well as healthy controls. Subjects were identified by a research nurse through specialist respiratory services at Christchurch Hospital. A breath sample was obtained and the patient asked to provide a sputum sample. If this was not available at the time, samples were collected at home by the patient and returned to the laboratory for culture. A drug and dietary history over that day was taken in respiratory subjects. Ingestion of the following foods were specifically sought and responses entered onto a standard data sheet: coffee, redbush tea, black tea, black currant juice, asparagus, celery, parsley, soy bean products, beans, chicken, beef, olive oil, canola oil, butter, and margarine. The respiratory diagnoses were confirmed by a specialist respiratory physician (ME) by review of the clinical notes and investigations. Ethical approval for the study was obtained from the Upper South Island A Ethics Committee, and

participants gave their written informed consent to take part in the study. The study was conducted in accordance with the standards for clinical research of the University of Otago.

Subjects at risk of Aspergillus colonization/invasion. All subjects were: (i) under the care of a specialist respiratory physician at Christchurch Hospital, and (ii) had chronic lung disease, primarily bronchiectasis, asthma, COPD or cystic fibrosis, both with and without a history of *Aspergillus* isolation from the sputum, or possible invasive aspergillosis. Prescription of itraconazole or voriconazole was not an exclusion criteria but was noted.

Subjects with neutropenia. All subjects were: (i) under the care of a specialist haematologist or oncologist, (ii) had neutropenia (peripheral blood white cell count of $<1.0 \times 10^9/l$) at the time of sampling and afebrile, (iii) had breath samples taken within 3 days of the development of neutropenia, (iv) had no past history of acute or chronic lung disease or an abnormal chest radiograph, and (v) had no history of known or suspected *Aspergillus* infection.

Healthy subjects. Subjects had: (i) no history of current acute or chronic medical condition including asthma, and (ii) were not taking any over the counter or prescribed medication other than oral contraceptive. Smokers were excluded for these studies.

Definitions

The presence of allergic bronchopulmonary aspergillosis (ABPA) in subjects with chronic lung disease (asthma, COPD, bronchiectasis or cystic fibrosis) was defined in a manner similar to the criteria of de Oliveira and co-workers [18]. *Invasive aspergillosis* was defined according to the criteria of the European Organization of Research and Treatment of Cancer (EORTC) [19]. *Colonization by Aspergillus* was defined as the identification of *A. fumigatus* in at least a single bronchoscopy specimen or at least two sputum samples within a defined time period (two or six months) without evidence of invasive aspergillus infection.

Non-colonization with A. fumigatus was defined as the absence of any positive cultures for *A. fumigatus* within the defined time period (two or six months) with at least two samples having been examined and no clinical suspicion of invasive aspergillosis.

An *indeterminate* case of chronic colonization was defined as a single positive *Aspergillus* culture from a sputum sample within the defined time period. Cases with an inadequate number of sputum samples to be cultured for *Aspergillus* were defined as *not evaluable* and were excluded from the relevant analysis.

Concurrent sputum and breath tests were defined as having been taken within 24 h of each other.

Routine laboratory procedures

All microscopic analyses of sputum samples and their culturing for bacteria and fungi were performed in Canterbury Health Laboratories according to standard diagnostic protocols [20]. Samples were rejected if the quality score was inadequate [21]. Sputa were initially examined in KOH mounts for hyphae and then inoculated onto Columbia Blood Agar, supplemented Chocolate Agar, MacConkey Agar, and Saboraud Dextrose Agar (with chloramphenicol and gentamicin) plates. One of each plate was incubated at 30°C and one at 36°C with CO₂ for seven days unless growth was detected earlier [22]. All plates were obtained from Fort Richard, Auckland, New Zealand. Identification was made by morphology and a flag mount (Lactophenol Cotton Blue) [22].

Breath sampling procedure

All breath samples and index sputum samples were collected by a research nurse and sent to the laboratory for analysis in an anonymized fashion. Breath samples were collected in new five L Tedlar bags (Supelco, Bellefonte, USA), which incorporated a Locking Combo® Valve and septum that could be pierced for sampling. Bag lots were tested on a regular basis for the absence of 2-PF in the breath of healthy controls. Sampling was done between 9 a.m. and 5 p.m. Coffee ingestion was permitted for up to 3 h prior to breath collection. All normal controls and subjects with cystic fibrosis and bronchiectasis were studied in both outpatient and inpatient facilities that had a common air conditioning system. Subjects in the bone marrow transplant wards were in a specialized HEPA filtered unit. No other filters or control systems were used. Single breath samples only were collected by forced expiration into the Tedlar bag without a nose clip. Some rest was allowed between breaths but all breath samples were collected within two minutes. The Tedlar bags were taken to the GC/MS laboratory within 30 min of collection, SPME fibres were inserted within 1 h, and bags stored at room temperature for 48 h before analysis. The loss of 2-PF from spiked breath samples

in this system was less than 5% although this is a semi-quantitative technique.

Breath analysis procedures

GC/MS parameters. A Saturn 2200 system (Varian, Palo Alto, USA) was used to perform the GC/MS analysis. A Zebron ZB-Wax 30 m × 0.25 mm × 0.25 µm column (Phenomenex, Auckland, New Zealand) was used. It was connected to a PTV-1079 injector and proved to be superior for the separation of 2-PF from breath matrix.

The temperatures of the injector, ion trap, manifold and transfer line were 250, 200, 60 and 250°C, respectively. The oven program commenced at 60°C for 2 min and was raised to 250°C at a rate of 10°C/min, at which temperature it was maintained for a further 2 min. Helium flow was set at a constant rate of 1.2 ml/min. The split vent was opened to a ratio of 1:50 after 1 min. Identification work and *in vitro* work were performed in the EI-mode as full scan. The retention time for 2P-F under those conditions was 4.5 min.

In order to analyze 2P-F in breath it was necessary to utilize the MS-MS capabilities of the ion trap due to matrix and very low detection level requirements. A reference spectrum and a representative spectrum from breath analysis are shown in Figs. 1 and 2.

GC/MS-MS parameters

The conditioned SPME fibre (StableFlex, DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA) was exposed into the hot injector for 20 min at 250°C, after that a test chromatogram was obtained to check for any remaining impurities and finally the clean fiber was

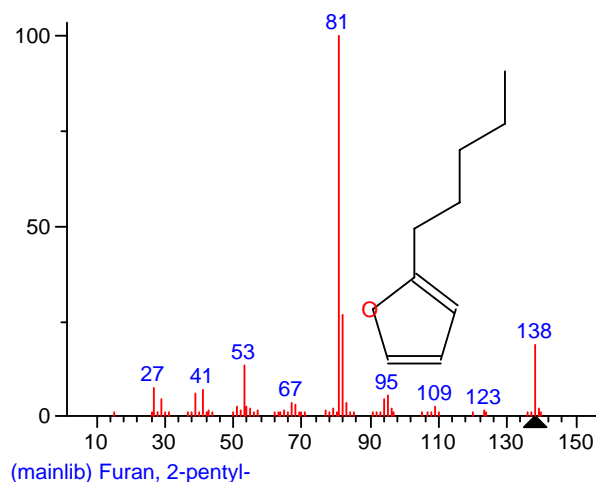


Fig. 1 The structure of 2-Pentylfuran and the NIST library mass spectroscopy spectrum.

Target Compound Report for #1 from patient2_3d 7-23-2007.sms

Sample ID:	Patient2_3d	Operator:	Mona
Instrument ID:	Saturn 2200 GCMSMS	Last Calibration:	2/7/2007 12:49 PM
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	7/23/2007 11:59 AM	Data File:	...t2_3d 7-23-2007.sms
Calculation Date:	7/24/2007 3:56 PM	Method:	..._bag_pf_18h_msms.mth
Sample Type:	Analysis		
Inj. Sample Notes:	None		

Compound Information

Peak Name:	2-Pentylfuran	Compound Number:	1	CAS Number:	3777-69-3	Identified
Result Index:	1					

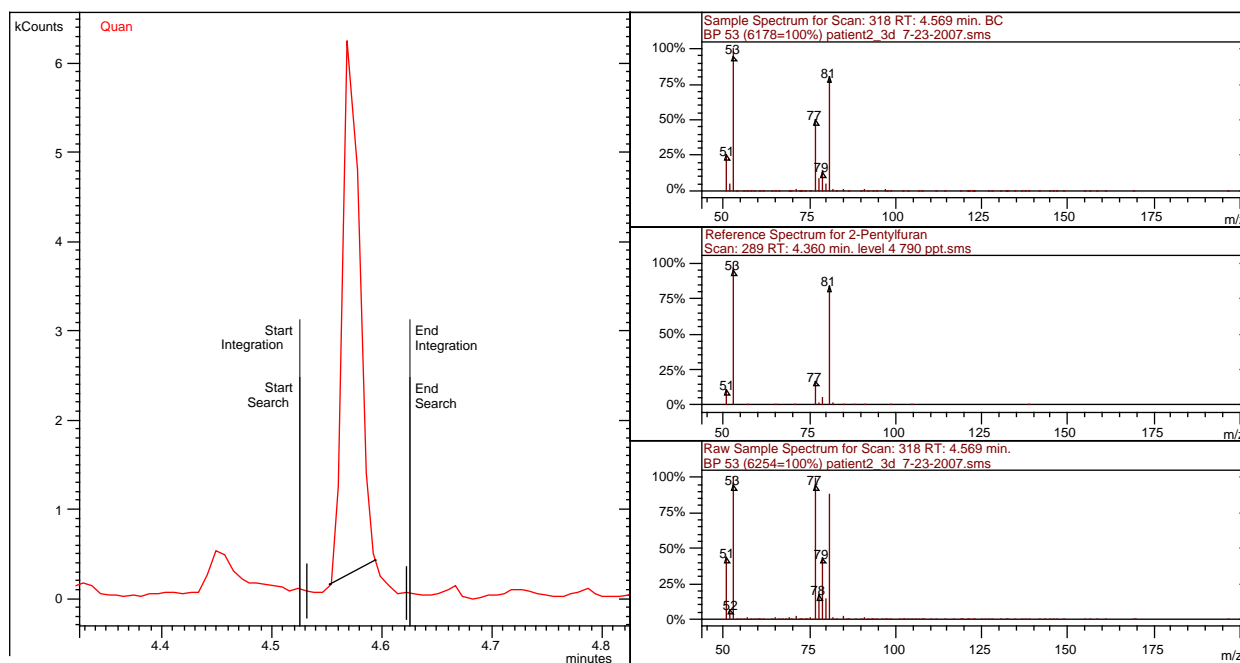
Identification

Parameter	Specification	Actual	Status
Search Type	Spectrum		
Retention Time	4.576 +/- 0.050	4.571 min.	Pass
Match Result	N-R >= 500	939	Pass
Qual. Ion Ratio (3 ions)	m/z 80.8:83.7%	63.7% - 103.7%	82.4%
	m/z 76.8:23.2%	3.2% - 43.2%	43.1%
	m/z 51.0:12.7%	0.1% - 32.7%	18.9%

Integration and Quantitation

Parameter	Specification	Actual	Status
Quan Ions	53.0		
Calibration Equation	Linear, Include, None	y = +146.0377x +671.7178	
Area	>=10	6122	Pass
Height		5974	
Amount	>= 0.000 ppt	37.320 ppt	Pass

Match Types: N-R : Normal-Reverse



Seg 2, 2-PF, Time: 4.00- 5.00, EI-Auto-MRM, First Precursor: 81.0

Fig. 2 Target report for 2-PF in breath sample.

placed into the collection bags for 48 h. The fiber was then desorbed directly into the injection port for the entire run (15 min).

Ion preparation for MS-MS analysis was EI mode; the selected parent ion was m/z 81 with an isolation window of m/z 3; non-resonant, excitation storage level was 35; excitation amplitude was 35; the resulting

MS-MS spectra featured two main peaks at m/z 53 (100) and m/z 81 (82).

Statistical analysis

The Chi squared test was used to compare non-paired data. The comparator standards for the sensitivity and

Table 1 Characteristics of subjects in the study.

	Normal subjects	Neutropenic subjects	Subjects with Respiratory Disease
Number (2-PF positive ^a)	14 (0)	10 (1)	32 (17)
Age years (mean, range)	38.8 (25–57)	45.5 (21–72)	60.5 (18–85)
Male (%)	11 (79)	6 (60%)	16 (50%)
Itraconazole/Voriconazole ^b	0	0	6
Diagnoses ^c (number)		Acute myeloid leukaemia (3) Breast cancer (3) Aplastic anaemia (2) Burkitt's lymphoma (1) Non-Hodgkin's Lymphoma (1) Multiple myeloma (1)	Bronchiectasis (19) Asthma (11) ABPA (1), Possible ABPA ^d (8) Cystic fibrosis (6) Chronic obstructive pulmonary disease (3) Lung transplant (1) Kidney transplant (1) Lung infiltrates (1)

^aAs measured by GC/MS in breath.

^bTreatment at time of study entry.

^cSome patients had multiple diagnoses.

^dABPA – Allergic Bronchopulmonary Aspergillosis.

specificity estimations of 2-PF in the breath were: (i) the isolation of *A. fumigatus* from the index respiratory specimen and lack of recovery of *A. fumigatus* from these samples; (ii) isolation of *A. fumigatus* from at least two sputum samples or one bronchoalveolar lavage (BAL) within the last two months and inability to obtain the fungus from at least two sputum samples or one BAL tested within the last two months; and (iii) isolation of *A. fumigatus* from at least two sputum samples or one BAL within the last six months and lack of recoverable *A. fumigatus* with at least two sputum samples or one BAL tested. McNemar's test was used to compare different testing methods.

Results

Fifty six subjects, including 14 normal individuals, 10 neutropenic patients not thought to be at risk for *Aspergillus* infection and 32 with chronic respiratory disease were studied and their clinical characteristics are shown in Table 1. 2-PF was not detected in breath samples of any of the 14 controls but was detected in one of 10 neutropenic patients and in 17 of 32 subjects with respiratory disease (Table 1).

2-PF and isolation of *A. fumigatus* in respiratory disease

Twenty-two patients with respiratory disease had concurrent breath and respiratory samples, the latter were cultured for *A. fumigatus*. The sensitivity and specificity on this subgroup were 67% (95% CI 30–93%) and 69% (95% CI 39–91%) respectively (Table 2).

In a separate subset of 22 subjects, *A. fumigatus* had been isolated in 13 subjects from at least two

respiratory samples or one BAL within the last two months, but could not be isolated in nine subjects over that time. The sensitivity and specificity of the 2-PF breath tests was 77% (95% CI, 46–95) and 78% (95% CI, 40–97) respectively, in this group (Table 2). A further subset of 14 subjects had at least two samples which were culture positive for *A. fumigatus* within the last six months and 12 subjects were culture negative over the same period. The sensitivity and specificity of the 2-PF breath tests compared with culture results were 79% (95% CI 49–95) and 75% (95% CI 42–95), respectively. *A. fumigatus* was isolated from the washings of four patients who had undergone BAL, three of whom had a positive 2-PF breath test. If these were removed from the analyses there was minimal effect on the sensitivity and specificity analyses.

The performance of 2-PF testing was similar to recovery of *Aspergillus* in culture from index sputum, two months results and six month results ($P = 0.39$, 1.0, and 1.0, respectively; Table 2).

Clinical details of subjects at risk of Aspergillus colonization or infection. The clinical details of the subjects are shown in Table 1. The neutropenic subject who had a positive 2-PF breath test had prolonged fever that diminished when placed on amphotericin B therapy. A CT scan showed an area of unsuspected bronchiectasis but no radiological features suggestive of fungal infection and no cause of fever was identified. The first had undergone a lung transplantation and had probable invasive aspergillosis according to EORTC criteria. *A. fumigatus* was isolated from two sputum specimens and from BAL five days prior to breath testing and was

Table 2 Sensitivity and specificity testing of 2-PF in breath compared with sputum and bronchoalveolar lavage cultures for *Aspergillus fumigatus*.

	Number sputums cultured/subject Mean (range)	2-PF positive	2-PF negative	Sensitivity (95% CI)	Specificity (95% CI)
Breath test within 1 day of sputum					
<i>Aspergillus</i> in index sputum (n = 9)	1	6	3	67% (30–93)	69% (39–91)
<i>Aspergillus</i> not in index sputum (n = 13)	1	4	9		
Sampling within 2 months of breath sample ^a					
<i>Aspergillus</i> present >2 sputa or 1 BAL (n = 13)	2.3 (1–4)	10	3	77% (46–95)	78% (40–97)
<i>Aspergillus</i> absent – at least 2 samples tested (n = 9)	2.8 (2–4)	2	7		
Sampling with 6 months of breath sample ^b					
<i>Aspergillus</i> present >2 sputa or 1 BAL (n = 14)	4.2 (2–8)	11	3	79% (49–95)	75% (42–95)
<i>Aspergillus</i> absent – at least 2 samples tested (n = 12)	4.1 (2–8)	3	9		

^a10 excluded: 7 as 1 sample tested within 2 months, 3 indeterminant.

^b6 excluded: 3 one sample tested in time frame, 3 indeterminant.

BAL = bronchoalveolar lavage.

place on voriconazole for three days. The day the breath test was performed, the sputum sample provided to be negative for *Aspergillus* but the breath test was positive for 2-PF.

2-Pentylfuran and other organisms isolated from sputum samples at the time of breath test. Bacteria were identified from the index sputum samples of 28/32 subjects (median 2, range 0–5). Among the 17 patients who were 2-PF positive, the organisms recovered were identified for 15 of them (number 26, median 1, range 0–4). These were *Pseudomonas aeruginosa* seven, *Staphylococcus aureus* six, *Stenotrophomonas maltophilia* four, *Candida albicans* three, viridans streptococci three, *Haemophilus influenzae* two, *Mycobacterium avium intracellulare* one. Among the 15 subjects who were 2-PF negative on breath testing, the microorganisms recovered were identified for 13 of them (number 30, median 2, range 0–5). These were *Ps. aeruginosa* eight, *Staphylococcus aureus* four, *Stenotrophomonas maltophilia* three, *Candida albicans* two, viridans streptococci five, *H. influenzae* three, *Escherichia coli* two, *Achromobacter sp.* one, *Mycobacterium fortuitum* one. *S. pneumoniae* was identified in the sputum of one subject.

All sputum samples cultured in the preceding six months were reviewed. *S. pneumoniae* was identified in a single culture in one subject whose sputum sampled did not yield *A. fumigatus*.

2-Pentylfuran and food

Food and fluid records were collected from 14 of the 17 subjects with 2-PF detected in the breath and of these 10 had ingested 14 foods of interest (Table 3). Food records were obtained in all 15 with negative 2-PF breath tests and of these 10 had ingested 13 food items of interest. The nature of the food and drinks and the times ingested, were very similar in the two groups and do not account for the differences in 2-PF observed.

Discussion

In this proof of concept study we have demonstrated that 2-PF was more commonly present in the breath of subjects with *Aspergillus* present in respiratory specimens, whereas 2-PF was not associated with normal controls and only one of the subjects with neutropenia had 2-PF detected in a breath sample. These results support the hypothesis that 2-PF in the breath is related to the presence of *Aspergillus* in the respiratory tract and does not normally come from the lungs.

In the absence of a recognized, readily applicable gold standard to determine infection/colonization by *Aspergillus*, concurrent testing and evidence of repeated isolation of *A. fumigatus* over a prolonged period was used as the comparator. The results demonstrated a higher sensitivity and specificity for 2-PF as compared to the more stringent criteria for colonization/infection

Table 3 Food records from subjects with respiratory disease.

	2-PF positive (<i>n</i> = 17)	Time ingested prior to breath test (hours)	2-PF negative (<i>n</i> = 15)	Time ingested prior to breath test (hours)
Food/drink record obtained	14		15	
No food/drink ingested	10		10	
Black tea	1	2.0	0	
Coffee	2	3.3, 3.5	1	
Butter	3	2, 2.5, 3.3, 5.0	6	1.25, 2.0, 3.0, 3.0, 3.5, 6.0
Margarine	4	1.5, 2.0, 2.5, 5.3,	4	2.0, 3.0, 4.0, 5.15.
Beef	2	0.75, 1.25	2	2.0, 5.0.
Oil	1	1.5	0	
Parsley	1	1.25	0	

with *A. fumigatus* in the respiratory tree. In contrast, culture of *A. fumigatus* from a single sputum sample is of limited significance even in severely immune compromised patients and the sensitivity and specificity of 2-PF in the breath was lower in this group [23–25]. These results suggest that 2-PF in the breath was associated with the presence of *A. fumigatus* in the respiratory tract.

Both presumed false positive and negative results of 2-PF were found compared with recurrent isolation of *A. fumigatus* from sputum samples or the recovery of *A. fumigatus* from BAL fluids. It is possible that false positive tests resulted from ingestion of 2-PF as it has been identified in food such as boiled beef, coffee, asparagus and as a breakdown product of vegetable oils including olive and canola oil [13–18]. To minimize this possibility we took breath samples three hours after the last coffee ingestion but did not place any restriction on timing of eating for any of the subjects. There were no apparent differences in food ingestion on the day of testing between the 2-PF positive and 2-PF negative groups, nor did we find 2-PF in the breath of healthy controls or 9/10 neutropenic subjects, making ingestion of 2-PF an unlikely explanation. However it is difficult to exclude an unsuspected food source as a potential cause of false positive tests.

It is also difficult to exclude the possibility that 2-PF might be produced by in some circumstances by tissue inflammation or breakdown in bronchiectatic lungs giving a false positive breath test, although there were many subjects with bronchiectasis who had negative breath tests suggesting this was uncommon. 2-PF can be produced from oxidation of linoleic acid by both lipoygenase and oxygen radicals. Lipoygenases are present in human white blood cells but the preferred substrate is arachidonic acid rather than linoleic acid

making this less likely [26]. Oxygen radicals are formed during inflammation but formation of 2-PF by this pathway has not been documented in humans [27,28]. Thus, it is conceivable that detectable amounts could be produced in subjects with very severe and extensive lung inflammation. An equally plausible explanation is that 2-PF was produced from undetected fungal infection/colonization of the lung.

It is unlikely that 2-PF was produced by bacteria within the lungs. Lipoygenases required for the production of 2-PF are normally present in fungi and plants, but not bacteria and we were unable to demonstrate 2-PF in headspace of cultures of the respiratory pathogens tested, other than for cultures of *S. pneumoniae* on a single culture medium [29]. *S. pneumoniae* was present in the sputum of only one subject, who did not have 2-PF detected in the breath sample. Although adjunctive testing for *S. pneumoniae* such as antigen detection in the urine were not performed, it is doubtful that undetected infection or colonization with this organism has confounded the study.

It is unclear why there would be false negative tests. Possible explanations include some 2-PF interaction with the tedlar bag, uneven pulmonary ventilation, especially in the presence of mucus plugging, limiting 2-PF transmission into exhaled breath, low level of colonization with an associated low 2-PF production, and intermittent production of 2-PF by aspergillus, perhaps related to growth cycle.

This study demonstrates that 2-PF in breath probably comes from the metabolism of *A. fumigatus*, but does not exclude a contribution from the respiratory tract in some circumstances. Furthermore it establishes that detection for diagnostic purposes is feasible and that identification of a VOC specific for a particular

organism is a useful strategy to pursue. Further studies are needed to establish the sensitivity and specificity of 2-PF breath testing compared to other diagnostic tests in both severely immune compromised patients at risk from invasive aspergillosis, and intermediate risk groups such as those with HIV infection, solid organ transplantation and corticosteroid use. The procedure is attractive because it is non-invasive, extremely patient friendly and repeatable and may be best used as a screening tool to identify patients in whom more invasive investigations should be undertaken. At present detection requires a high degree of expertise and sophisticated technologies such as GC/MS-MS but advances in technology are very likely to make detection much more accessible.

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Conflicts of interest

Stephen T. Chambers holds an interest in the patent for the detection of 2-Pentylfuran as a breath test for aspergillus. Mona Syhre holds an interest in the patent for the detection of 2-Pentylfuran as a breath test for aspergillus infection. David R. Murdoch – none. Fiona McCarten – none. Michael Epton – none.

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