CLINICAL PROTOCOL

Full Title	Non-invasive testing for the diagnosis and assessment of colorectal disease
Short Title	Colorectal breath analysis (COBRA)
Sponsor	Imperial College London Ruth Nicholson Room 501B, 5th Floor Lab Block, Charing Cross Hospital Fulham Palace Road London W6 8RF
Chief Investigator	Professor George Hanna Head of Division of Surgery Imperial College Healthcare NHS Trust
Principal Investigator	Professor George Hanna
Management Group	Dr Georgia Woodfield, Mr Geng-Ping Lin, Mr Piers Boshier, Mr Andrea Romano
NCT number	NCT03699163

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1. SIGNATURE PAGE

Chief Investigator Agreement

The clinical study as detailed within this research protocol **()**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Chief Investigator Name:

Chief Investigator Site:

Signature and Date:

Principal Investigator Agreement

The clinical study as detailed within this research protocol **()**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Principal Investigator Name:

Principal Investigator Site:

Signature and Date:

2. SUMMARY

Short Title	Colorectal cancer breath analysis (COBRA)
Methodology	Cohort Observational & Laboratory study
Research Site	St Mary's Hospital Charing Cross Hospital St Mark's Hospital St George's Hospital Homerton University Hospital Chelsea and Westminster Hospital West Middlesex Hospital Royal Marsden Hospital
Aims	Determine the diagnostic accuracy of the exhaled breath and urine test for the prediction of colorectal disease. To assess the acceptability of using breath and urine testing
Main Inclusion Criteria	Any patient who: 1. Patients aged 18 - 90 years 2. Patients seen in secondary care with symptoms of colorectal disease or referred for a lower gastrointestinal endoscopy as part of their routine clinical investigation programme 3. Patients able to understand and retain the information provided, thereby being able to give informed consent for inclusion in this study
Number of Participants	Target 2000
Endpoints	*Primary endpoint* • Once samples have been collected from 2000 patients.
Study Duration	April 2017 to April 2022

3. INTRODUCTION

3.1 Background

Colorectal cancer (CRC) is the 2nd commonest cause of cancer death in the UK. Survival rates are among the lowest in Europe (1) and 26% of patients present as an emergency, frequently having visited their GP before diagnosis (2-4). If diagnosed early, CRC survival rates exceed 90% (5). Whilst, reducing delay in diagnosis is therefore considered key to improving outcomes [6], initiatives to reduce delay have thus far been largely unsuccessful (6, 7). In order to improve the diagnosis of CRC, efforts should be made to determine how the risk of CRC can be assessed using sensitive primary care tests that are: valid; easy to perform, and; well tolerated by patients.

CRC screening programmes rely on detection of early-stage cancer using the faecal occult blood test (FOBT) with colonoscopy in those patients with a positive test. Trials have shown that this screening method reduced CRC mortality but not incidence rates. Even if UK CRC incidence rates remain constant, as they have for the past 30 years, with an aging population the number of cases and treatment cost are set to double in the next 50 years. The Bowel Scope programme is also being rolled out across the UK currently, aiming to perform flexible sigmoidoscopy on every person at the age of 55. Invasive screening reduces mortality but is not without risk to patients and pressurises an already over burdened healthcare service.

Risk of CRC in symptomatic patients can be assessed by different methods:

(I) *Flexible sigmoidoscopy and colonoscopy*: The NHS currently has insufficient trained endoscopists to perform the number of procedures that would be required if flexible sigmoidoscopy were used as a first-line test for symptoms with predictive values below 3%.

(II) *Guaiac FOBT* has good sensitivity (87-98% in CRC detection), but highly variable and often unsatisfactory specificity (13-79%), requiring the repetition of the test on multiple stool samples. To date, the FOBT is neither recommended nor available for use as an intermediate test (8-11).

(III) *Faecal immunochemical testing (FIT)* requires sampling only a single stool. It has been extensively tested; and is now used by many CRC screening programmes with very low prevalence rates of CRC (12). Four FIT systems are fully automated, and provide a quantitative measure of haemoglobin, allowing selection of a threshold of positivity to fit specific circumstances. As a result the research data available on sensitivity and specificity for CRC is based on small numbers of cancers. The few studies that have included adequate numbers of cancers have used older qualitative tests with a fixed threshold for positivity and were not automated. Nevertheless, the data suggest that, depending on the selected threshold for positivity, sensitivity for CRC varies between 35% and 86% with specificity between 85% and 95% (8, 9). However, there are no data on the sensitivity of the newer quantitative test for early-stage cancers.

(IV) Multi-target stool DNA testing: When compared with the FIT, in a large multicentre study, the test showed better specificity (92 *vs.* 73%), but lower sensitivity (90 *vs.* 96%) (13).

At Imperial College London, we have developed a breath test for the detection of oesophagogastric adenocarcinoma from biomarker discovery to clinical trials. The assessment of volatile organic compound (VOCs) within exhaled by selected ion flow tube mass spectrometry (SIFT-MS) (14) was followed by the construction and validation of a diagnostic model using a panel of 9 VOCs in 225 patients with an area under the ROC curve (±SE) of 0.92±0.01 and 0.87±0.03 for the model and validation subsets respectively (15). We also performed cross-platform chemical validation using GC-MS. A NIHR-funded multicentre blind validation study on 396 patients (16) showed a sensitivity of 86% and specificity of

80%. In parallel with this work we have also investigated the exhalation kinetics and molecular drivers of VOCs in oesophagogastric adenocarcinoma (17-19).

In colorectal cancer we performed a prospective pilot study to identify discriminative VOCs (n=150); a diagnostic validation study (n=110); a clinical prognostic study following surgery and after tumour recurrence (n=60); cross platform chemical validation using GC-MS (n=30). The diagnostic model had a sensitivity of 85% and specificity of 84% for cancer diagnosis. Following surgery the biomarker normalised to control level, but levels significantly increased with recurrence (20). The volatile biomarkers for oesophago-gastric and colorectal cancers are different with no overlap.

Volatile Organic Compounds (VOCs)

VOCs emitted from the human body have been of interest to researchers for several decades. In 1971, Pauling et al (21), reported that breath and urine contained approximately 250 and 280 VOCs respectively in normal human subjects. VOCs are traditionally measured in food industry and counter-terrorism (22-24).

The analysis of exhaled breath for the non-invasive detection and monitoring of disease is an attractive and evolving field of clinical research. Routine clinical applications of breath testing include the alcohol breathalyser, ^{13}C urea breath test for H. pylori, nitric oxide in asthma, and hydrogen/methane test for bacteria overgrowth (25-29).

Alteration in VOC production in patients with cancer has been postulated to relate to oxygenation of cell membrane-based polyunsaturated fatty acids resulting from genetic and/or protein mutations within tumour cells and the increased relative prevalence of reactive oxygen species (30, 31). These VOCs produced from the cancer site within the body, travel within blood to reach pulmonary alveoli where they are exhaled, permitting an objective quantitative measurement (32). There have been early reports using VOCs to detect lung, breast, bladder, prostate and gastric cancers (33, 34). Reports on colorectal cancer had small patient cohorts and lacked validation (32, 35, 36).

Due to the complex composition of human breath a number of sophisticated mass spectrometer based technologies have been employed for the detection of exhaled VOCs including Gas Chromatography coupled with Mass Spectrometry (GC/MS), Proton Transfer Reaction-Mass Spectrometry (PTR-MS), Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS) and Ion Mobility Spectrometry (IMS). These instrumental methods all have their strengths and limitations for applications in breath analysis. We intend to use multiple analytical platforms in our proposed work as these techniques are complementary and will allow us to acquire more robust findings.

Thermal-desorption (TD) coupled to Gas Chromatography Mass Spectrometry (GC/MS) is a widely adopted method for measuring breath VOCs. Although this method requires the pre-concentration of breath volatiles prior to analysis, it allows for the comprehensive identification and semi-quantification of VOCs in unknown complex mixtures such as breath. It can separate and detect hundreds of compounds from a breath sample, allowing for VOC profiling and has therefore been used in the study of disease biomarkers for lung cancer (34, 37), breast cancer (33), colorectal cancer (32) and liver diseases (38, 39).

Selective Ion Flow Tube Mass Spectrometry (SIFT-MS) permits online, real-time VOC

quantification (40). SIFT-MS has been utilised in the study of VOCs in breath and urine from patients with conditions including cystic fibrosis and bladder cancer (41, 42). The SIFT-MS technique allows real-time detection and quantification of VOCs within biological samples such as exhaled breath without any sample preparation minimising diagnostic delay.

Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) is a relatively new instrumental tool that is gaining interest in breath research applications. Similarly to SIFT-MS, Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) is a direct injection-based technology, obviating problems related to pre-concentration and separation steps typical of GC-MS. The most important advantage guaranteed by the ToF mass analyzer over quadrupole-based instruments (GC-MS and SIFT-MS) is represented by the high time resolution. In a ToF analyser a complete mass spectrum is generated at each ionisation event and spectra can be recorded at a frequency of 1 Hz or lower. This improves the accuracy of measurement during *in vivo* studies; moreover, full spectral scans can be performed without compromising sensitivity, thus enabling simultaneous targeted and untargeted analysis.

The use of multi-platform analytical methods will enable both compound identification and quantification which are very important in the search and validation of biomarkers.

3.2 Rationale

Colonoscopy remains the gold-standard investigation for the assessment of patients with lower gastrointestinal symptoms and considered at risk of colorectal cancer. Colonoscopy is an expensive investigation that is uncomfortable for the patient and not without important risks including visceral perforation and bleeding.

A breath test is a non-invasive investigation. The use of a breath test as a triage investigation in symptomatic patients could identify high-risk patients who should be referred for colonoscopy at an earlier stage, increase the proportion of appropriate referrals from primary care, and improve NICE guidelines which may currently miss particularly younger patients (43). If a GP is presented with a patient with gastrointestinal symptoms that do not prompt referral under NICE guidelines, he/she would not need to watch-and-wait to see if symptoms worsen but could offer the test immediately. The GP would order a breath test in much the same way as routine blood tests, with a single breath collection assessing for individual or multiple cancers. A nurse can perform the test and send breath samples to a regional laboratory for analysis. A positive result would warrant immediate referral. A negative test would permit the GP to reassure the patient and offer retesting if symptoms persist. Furthermore if we could develop a breath test that can discriminate colonic adenomas, the test would have further a potential application within the screening programme.

A breath test could also be used to detect recurrence in patients post-resection for their colorectal cancer, allowing earlier detection and limiting invasive testing in those who are free of recurrence.

Previous pilot study data for colorectal cancer detection by our group produced a diagnostic model with a sensitivity of 85% and specificity of 84% for colorectal cancer. The next stage of this research and the subject of this current proposal is to assess the influence of lower gastrointestinal diseases on VOC exhaled breath and urine. The external validation of this VOC breath model in a large multi-centre study will provide this risk-stratification tool for future clinical practice.

4. STUDY AIMS

4.1 Aims

The primary aim of this study is to determine the diagnostic accuracy of the exhaled breath and urine test for the prediction of colorectal disease.

Secondary aims are:
(i) Refine the current diagnostic model using technologies that allow identification of additional biomarkers at a lower detection level for CRC.
(ii) Confirm the diagnostic accuracy of the test in a new and independent group of patients who have CRC compared to subjects with benign diseases or normal colon.
(iii) Confirm the prognostic accuracy of the test in detecting tumour recurrence after surgical removal of CRC.
(iv) Profile VOCs in patients with colonic adenomas and construct a diagnostic model.
(v) Profile VOCs from colonic air in a subset of patients, for use as a comparison against breath testing in order to improve understanding of underlying pathological mechanisms.
(vi) To assess the acceptability of using breath and urine testing

5. METHODOLOGY

5.1 Inclusion Criteria

Any patient who is:

- ≥ 18 years old and below 90 years of age, AND:
- undergoing a lower gastrointestinal endoscopy as part of their routine clinical care OR:
- seen in another hospital clinical setting with symptoms of lower gastrointestinal disease
- able to provide informed written consent

5.2 Exclusion Criteria

Any patient who:

- Lacks capacity or is unable to provide informed consent.
- Any patient below 18 years of age or over 90 years of age.

5.3 Study Design and Plan

Patients recruited from the endoscopy unit will have been referred by their responsible clinician to undergo a lower gastrointestinal endoscopy (which involves a telescope test to visualise the colon). Others may be attending for colonoscopy as part of the bowel cancer screening programme or as part of routine surveillance. When patients attend the endoscopy unit, a member of the research team will discuss the research project with the patient, explain that the study involves sampling of their breath and urine while they are waiting for their endoscopy. Patients may also be asked to consent to the use of the colonic air collected during colonoscopy for analysis. Patients will then be provided with a patient

information leaflet, (see Participant Information Leaflet version 3.0 24/04/2017). Should the patient agree to participate they will be asked to sign a written consent form (see Consent Form Version 2.0 15/02/2017).

Patients recruited from secondary care will be approached either before or after a routine appointment that they will be attending as part of their clinical care. This could include general colorectal clinics, pre-operative colorectal clinics, oncology clinics, theatres or on hospital wards.

The project will be explained to them and they will be provided with an information leaflet. If they agree to participate they will be asked to sign a written consent form in order to provide breath and urine samples for the research project. Consent for colonic air sampling is not applicable to the majority of secondary care patients, only to those having a colonoscopy. No additional invasive procedures will be undertaken for the purposes of this study. A subset of patients with known cancer who are due to have a cancer resection may be asked to provide additional breath and urine samples 6–8 weeks following surgical resection of their tumour and/or if their disease should recur.

Patients attending for colonoscopy will be fasted for a minimum of six hours prior to their breath sample collection. This is normal routine practice for patients undergoing endoscopy and therefore will not alter their treatment pathway. All breath samples will be retrieved prior to endoscopy. In other secondary care settings patients will not be specifically asked to fast prior to giving breath or urine samples but note will be made of how long they have fasted for. Patients will be asked to give a number of exhalations into a breath sampling device until a defined volume of breath has been sampled. This involves performing normal tidal breathing whilst wearing a face mask, and takes around 2 minutes on average. Patients will also be asked to perform a single deep nasal inhalation followed by complete exhalation via their mouth into a secure GastroCHECK steel breath bag (500 mL) via a 1 mL Luer-Lok syringe (Terumo Europe, Leuven, Belgium). Patients at St Mary's Hospital will also be asked to breathe directly into a mass spectrometer machine. The mass spectrometer is a self-contained mobile device which can be easily moved between clinical areas within St Mary's Hospital. Sampling by this method will involve a single deep nasal inhalation followed by complete exhalation via their mouth into a plastic cylinder connected to a tube leading to the mass spectrometer itself. We expect the breath sampling process to take no longer than 5-10 minutes in total. Patients will be requested to pass urine into a standardised 60ml urine specimen vial, which will be immediately sealed. 20ml of urine will be aliquoted into a standard 60ml specimen vial.

Of patients attending for colonoscopy, a subset will be asked to consent to have air from their colon collected. This air comes up from the colon through the colonoscope suction channels. Suctioning of air is a routine part of the examination, but for this study we will be collecting it for analysis. Collection is done via a small suction pump connected to a fine bore tube that is passed through the suction channel of the colonoscope. Sampling in this way will add no more than a few minutes (<4 minutes) on to the procedure time.

The VOCs from the breath/colonic air will be trapped onto adsorbent tubes inserted into the breath taking device. In the case of colonic air sampling, an adsorbent tube is attached to the pump end of the fine bore disposable suction tube. After sampling the adsorbent tube is removed from the device, capped and transported back to the laboratory at St Mary's Hospital London for analysis. Samples will be transported by courier or by a member of the research team. The sample(s) shall undergo analysis via our mass spectrometry instruments, at St Mary's Hospital London.
Breath samples collected in steel bags will be immediately disposed of following analysis within 6 hours of the sample being taken. Breath VOCs sampled on adsorbent tubes will be

analysed within 48 hours of collection. The sample constituents are destroyed within the mass spectrometer for detection meaning that no clinical material will remain after analysis by GC-MS. Urine samples will be stored within a registered tissue bank (REC Ref no. 04/Q0403/119, custodian Prof G Hanna) in accordance with the Human Tissue Act and disposed of following analysis.

This study has been specifically designed to ensure that any disruption to patient's routine care and management is kept to an absolute minimum.

6. PROCEDURES

6.1 Study Timetable

January 2017 – March 2017
Research & Development and ethical approval.

April 2017 – March 2022
Participant recruitment and sample collection. Laboratory analysis of samples, write up and dissemination of results.

6.2 Recruitment and Informed Consent Procedures

A member of the research team will identify and approach patients when they attend for a lower gastrointestinal endoscopy. All patients attending for colonoscopy within the specified age range are potentially eligible for this study. If the patient is in agreement, a member of the research team will explain the research project and provide a written information leaflet (see Participant Information Leaflet version 3.0 24/04/2017). Patients will be given adequate time to decide if they wish to participate and should they agree they will complete a written consent form (see Consent Form Version 2.0 15/02/2017).

A member of the research team will identify patients when they attend for a clinic appointment by discussion with the Consultant clinician responsible for the clinic. A member of the research team will approach patients when they attend for their clinic or pre-assessment appointment prior to planned surgery. Patients will have the research project explained to them and will be provided with a written information leaflet (see Participant Information Leaflet version 3.0 24/04/2017). Patients will be provided with adequate time to decide if they wish to participate and should they agree they would complete a written consent form (see Consent Form Version 2.0 15/02/2017).

All of the above would occur at one of the patient's routine clinical attendances and would not require any additional patient attendances.

7. STATISTICAL CONSIDERATIONS

Both univariate and multivariate data analysis techniques will be applied to the results to 1) identify VOC components with the best discriminating ability between the groups and 2) to develop a multivariate discriminant analysis model.

Mann-Whitney U test will be used to compare the measured metabolite concentrations between the study groups. A p value <0.05 will be taken as the level to indicate statistical significance.

Non-parametric (Kruskal-Wallis) ANOVA test will be used to compare the measured metabolite levels between the study groups. Statistically significant metabolites will be selected using False Discovery Rate (FDR) adjusted p value threshold such that less than 5% of discovered candidate markers are false positives (i.e. q-value <0.05).
The FDR strategy will be based on Benjamini–Hochberg procedure.

Receiver operating characteristic (ROC) curves will be used to determine the accuracy of a diagnostic test in classifying those with and without colorectal disease. The metabolite concentrations that exhibit significant differences (q<0.05) between the cancer and positive controls (including adenomatous polyps) will be included as variables for the proposed diagnostic test. The linear discriminant analysis based on multivariate logistic regression will be subsequently used to derive a combination of the metabolite concentrations for sample classification. The optimum threshold for group discrimination will be calculated to achieve the highest possible classification accuracy. The predictive capacity of the model will be assessed on independent sample set, as described below.

Validation
The Breath and Urine metabolite model for the prediction of colorectal disease developed in the previous studies will be used to assign risk of disease in a prospective cohort. Investigators undertaking metabolite sampling and analysis will be blinded to the results of the endoscopy. Comparison of predicted disease risk and actual endoscopy findings will then be made, and the overall diagnostic accuracy (sensitivity, specificity, positive and negative predictive value, ROC curve analysis) for this non-invasive diagnostic investigation will be determined. Colonic air VOC profiles will be used for validation of paired breath results, and to gain further understanding of the mechanism of VOC production in humans.

8. ETHICAL CONSIDERATIONS

8.1 Declaration of Helsinki and Good Clinical Practice

The study will conform to the spirit and letter of the declaration of Helsinki and in accordance with the Good Clinical Practice Guidelines

8.2 Ethical Committee Review

The study protocol will be used when approval has been obtained from a UK Research Ethics Committee. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the Trust R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions. Additionally, in order to sample breath from any patient who is having a colonoscopy via the UK Bowel Cancer Screening Programme (BCSP) pathway, there also has to be specific approval from the UK BCSP Research Advisory committee. Formal approval has been gained for an initial 100 BCSP patients to be approached for this study, as a pilot, with a view to further approval after this period (BCSP ID189, approval given on 18/1/17). Further approval will therefore be sought for the recruitment of additional BCSP patients, after 100 patients have been approached, as per BCSP panel request.

8.3 Consent

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed

participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. All participants are free to withdraw at any time from the protocol without giving reasons and without prejudicing further treatment.

9. SAFETY CONSIDERATIONS AND REPORTING

This is not a study of an investigational medicinal product and therefore all untoward occurrences will be adverse events rather than adverse reactions.

9.1 *Adverse Event*

An adverse event is defined as any untoward medical occurrence affecting a participant. The terms '*mild, moderate or severe'* are used to describe the intensity of a specific event or reaction. An event would be considered '*serious'* based upon participant/event outcome or action criteria as defined below.

9.2 *Serious Adverse Event (SAE)*

A serious adverse event (SAE) will be considered any untoward medical occurrence/effect that:

- Results in death
- Is life-threatening
- Requires hospitalisation or prolongation of existing inpatient's hospitalisation.
- Results in persistent or significant disability or incapacity.
- Is otherwise considered medically significant by the investigator

'Life threatening' refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically may have caused death if it had been more severe.

9.3 *Unexpected Adverse Event*

An unexpected adverse event is considered an adverse event of which the nature and severity is not consistent with the expected consequences of sample collection.

9.4 *Expected Adverse Events*

We do not believe any risks or adverse events are associated with patients providing samples of urine and breath or in the consent process for these procedures. All samples will be disposed of following analysis. All hospital records will be handled with strict confidentiality in accordance with the Data Protection Act 1998. All other risks will be in accordance with usual clinical practice. These include risk of endoscopic perforation, patient discomfort during endoscopy, and local bruising following intravenous cannulation. There are no other serious adverse events caused by sample collection and tissue biopsy.

9.5 *Reporting Unexpected Adverse Events*

Given the nature of this investigation, it is unlikely that any will occur. Nonetheless, should an unexpected adverse events occur, the investigators will make an assessment of severity and report the event to the Research Ethics Committee and the Sponsor as appropriate. Usually, this will mean within 24 hours in the case of severe or moderate events, and within 14 days for mild events.

9.5.1 *Assessment of Intensity*

Mild: The subject is aware of the event or symptom, but the event or symptom is easily tolerated.
Moderate: The subject experiences sufficient discomfort to interfere with or reduce their usual level of activity.
Severe: Significant impairment of functioning; the subject is unable to carry out usual activities and/or the subject's life is at risk from the event.

9.6 *Urgent Safety Measures*

Should it be necessary to undertake urgent safety measures to ensure the safety and protection of the participants the CI will take these measures immediately. The CI would inform the sponsor and Main research Ethics Committee of this event immediately via telephone and in writing within 3 days in the form of a substantial amendment.

10. DATA HANDLING AND RECORD KEEPING

Information related to study participants will remain confidential and be managed in accordance with the Data Protection Act, NHS Caldecott Principles, The Research Governance Framework for Health and Social Care, and the conditions of Research Ethics Committee Approval.

All case record forms will be held in the site file, which will be kept in a locked drawer in a locked office at the study site. They will be destroyed after 10 years. All digital data will be stored on a password-protected NHS computer on site at St Mary's Hospital, under the control of the Chief Investigator. Data will be erased securely after 10 years.

11. SPECIMEN ANONOMISATION AND STORAGE

Samples will be anonymised with all patients being assigned a study number that will be added to the specimen during storage. Patients would not be identifiable from this study number. These study numbers will be linked to the digital database stored on a password-protected NHS computer on site at St Mary's Hospital, under the control of the Chief Investigator.

Breath samples collected in steel bags will be disposed of immediately following analysis. Breath/colonic air VOCs sampled on to adsorbent tubes will be released by thermal desorption into the analytical instrument where they will be destroyed during mass spectrometric detection. Urine samples will be stored within -80C freezers used solely for the storage of human samples within a secure laboratory within the research department at St Mary's hospital. The only people who will have access to this tissue bank are key members of the research team. These samples will be added to Professor Hanna's current tissue bank for which local ethical approval is already in place for sample collection at St Mary's Hospital (REC Ref no. 04/Q0403/119). Urine samples will be stored until study completion. Analysed samples will be destroyed by incineration following analysis and surplus samples will be stored in the current tissue bank until study completion in case a repeat analysis is required.

12. SPECIMEN ANALYSIS

Selective Ion Flow Tube Mass Spectrometry (SIFT–MS)
SIFT-MS permits online, real-time Volatile Organic Compound (VOC) quantification. SIFT-MS has been utilised in the study of VOCs in breath and urine from patients with conditions including cystic fibrosis and bladder cancer. The principle of SIFT-MS is selected precursor ions are formed in a microwave discharge source and are selected according to their mass-to-charge ratio, m/z, by a mass filter and injected into a helium carrier gas where they are convected as a thermalised swarm along a flow tube. H_3O^+, NO^+, O_2^+ precursor ions are used to ionize the trace gases in an air sample that is introduced into the helium at a known flow rate, these ions selectively ionise VOCs present within the sample resulting in characteristic product ions. By measuring the count rate of both precursor ions and the characteristic product ions at the downstream detection system, a real-time quantification is achieved, realising the absolute concentration of trace and volatile compounds at the parts-per-billion by volume or parts-per-million by volume. The SIFT-MS technique allows real-time detection and quantification of Volatile Organic Compounds within biological samples such as exhaled breath without any sample preparation minimising diagnostic delay. This is particularly advantageous within the clinical environment where samples can be retrieved and real-time VOC measurements made with negligible concern for sample degradation.

Thermal Desorption (TD) and Gas Chromatography Mass Spectrometry (GC/MS)
Thermal desorption coupled to GC/MS is one of the most widely used techniques for breath biomarker discovery work for a number of reasons. Firstly, VOCs in exhaled breath are present at very low concentrations of parts-per-million (ppm) to parts-per-trillion (ppt) and pre-concentration is often necessary to allow for detection. This can be achieved by adsorption onto sorbent beds (tubes packed with tenax, carbon molecular sieve, or graphitized carbon) followed by release through thermal desorption. The coupling of a thermal desorber to a GC/MS system allows for the trapped VOCs to be released and rapidly injected on to the GC column for analysis. GC/MS is a very well established analytical technique that allows for the successful separation, identification, and semi-quantification of trace VOCs in unknown complex mixtures such as breath. This technique has allowed for the identification of over 800 compounds in breath (44). Although this technique does not allow for real-time analysis it provides a much more comprehensive analysis of the VOC profile. It will complement results obtained with SIFT-MS and can allow for cross-platform validation.

Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS)
Similarly to SIFT-MS, Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) is a direct injection-based technology, obviating problems related to pre-concentration and separation steps typical of GC-MS. In PTR-MS the ion beam generated at the source does not undergo selection by means of a mass filter as in SIFT-MS so the detection limits are on average two orders of magnitude lower than those obtained with SIFT-MS. However, equipped with a Time-of-Flight (ToF) mass analyzer, a higher mass resolution is achieved, signals represented by a single mass peak in a quadrupole-generated spectrum, can be resolved in two or more distinct mass peaks, generally resulting in an improved discrimination performance. In addition to this, accurate masses can be determined with at least two decimal digits; this means that the data generated by PTR-ToF-MS, especially when integrated with GC-MS, can be used for compound identification and thus permit the discovery of new biomarkers. The most important advantage guaranteed by the ToF mass analyzer over quadrupole-based instruments (as those in GC-MS and SIFT-MS) is represented by the high time resolution. In a ToF analyzer a complete mass spectrum is generated at each ionisation event and spectra can be recorded at a frequency of 1 Hz or lower. This improves the accuracy of measurement during *in vivo* studies; moreover, full

spectral scans can be performed without compromising sensitivity, thus enabling simultaneous targeted and untargeted analysis.

13. DEVICES AND TECHNIQUES

13.1 *Devices*

A device that collects and concentrates breath VOCs on adsorbent tubes in one easy step will be used to collect breath (Owlstone's CE-marked hand-held breath sampler, ReCIVA, Owlstone Ltd, Cambridge, UK). This device has been designed for easy patient use, it contains a pump of filtered air to ensure clean air sampling, it allows for the concentration of a defined volume, and is more economical than other sampling methodologies available. A steel bag will also be used for the collection of breath. For direct sampling into the mass spectrometer, the patient blows into a disposable cardboard tube attached to the inlet to the machine, discarded between each patient. All other devices are those used within the patient's routine clinical practice including endoscopic equipment and universal sampling containers. These will all be used in accordance with routine clinical practice.

14. MONITORING AND AUDITING

The study will be monitored and audited by the Sponsor, in accordance with usual protocols.

15. STUDY COMMITTEES

a. *Study Steering Committee*

This will comprise of Professor George Hanna, Professor Wendy Atkins, Professor Brian Saunders, Dr Jonathan Hoare

16. FINANCE AND FUNDING

Equipment utilised for sample collection will be funded as part of Professor Hanna's research funding. Laboratory analysis of the biological specimens (breath, colonic air and urine samples) obtained will also be funded through Professor Hanna's research group with external funding from HCA Hospitals. A grant from the Rosetrees Trust (ref: A1361) has already been approved to fund an analytical chemist to work on this study.

17. INDEMNITY

The nominated sponsor of our research study is Imperial College London, Research and Development Department, Room 501B, Laboratory Block, Charing Cross Hospital, Fulham Palace Road, London, W6 8RF. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.
Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study. Medical co-investigators will also be covered by their own medical defence insurance for non-negligent harm.

18. DISSEMINATION OF RESEARCH FINDINGS

Results from this study will be presented in a peer-reviewed journal. Authorship will follow international guidelines.

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