# Targeted Metabolic Profiling in Deep Vein Thrombosis

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## Clinical Queries

Clinical queries should be directed to Professor Alun H. Davies or Mr Kemal I Kemal who will direct the query to the appropriate person.

## Sponsor

Imperial College London is the main research Sponsor for this study. For further information regarding the sponsorship conditions, please contact the Head of Regulatory Compliance at:

Joint Research Compliance Office
Imperial College London & Imperial College Healthcare NHS Trust Room 215,
Level 2, Medical School Building, Norfolk Palace London
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## Funder

There will be no participants or investigator payments. The Graham- Dixon Charitable Trust has awarded £14 491 towards the consumables cost of this research for the duration of the project. Further grant applications will be submitted to fund the experimental analysis.

This protocol describes the "Targeted Metabolic Profiling in Deep Venous Thrombosis" study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

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## GLOSSARY OF ABBREVIATIONS

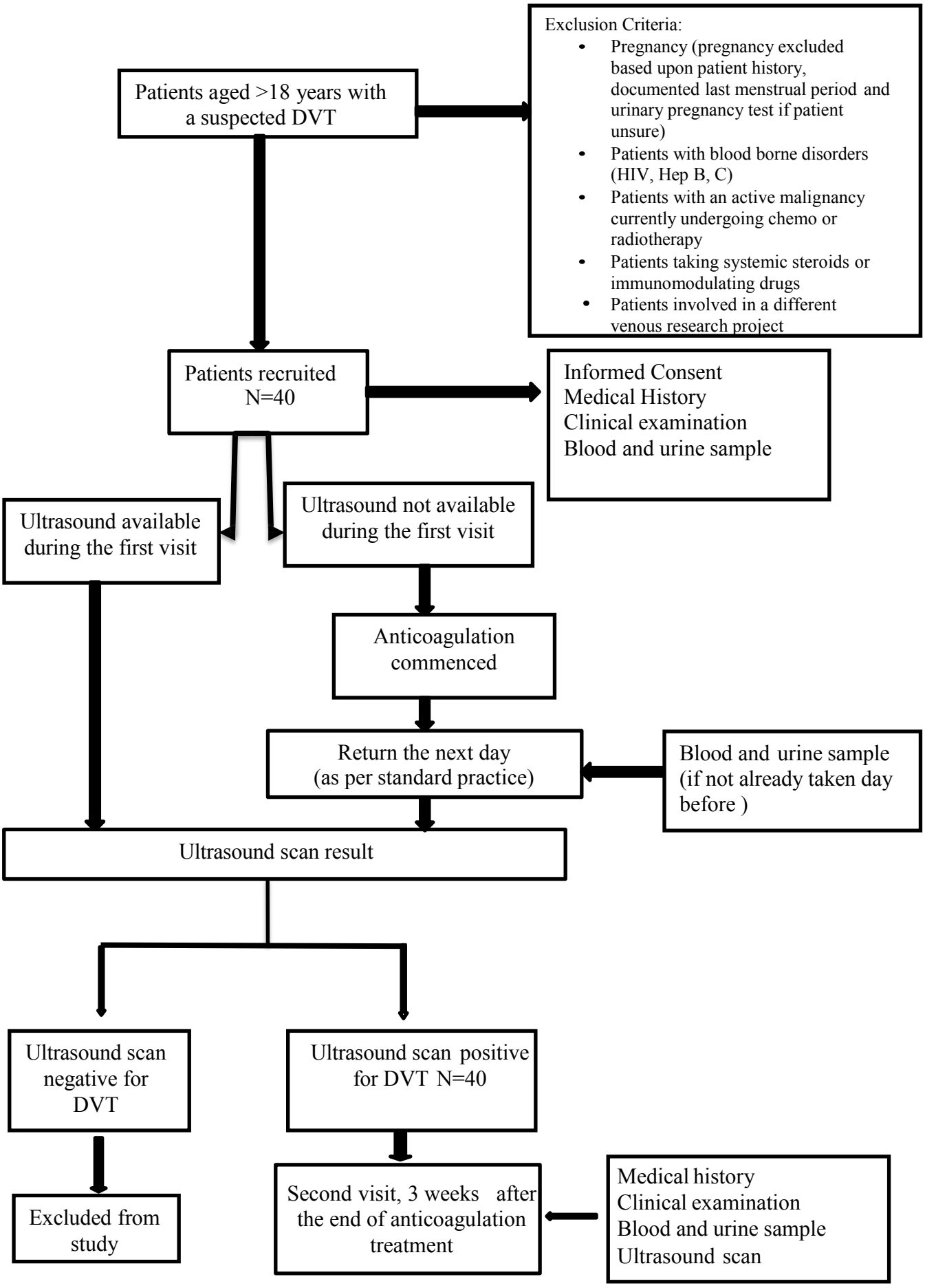
| | |
|---|---|
| DUS | Duplex ultrasound |
| DVT | Deep vein thrombosis |
| LMWH | Low molecular weight heparin |
| MS | Mass spectrometry |
| NMR | Nuclear magnetic resonance |
| DOAC | Direct oral anti-coagulant |
| VTE | Venous thromboembolism |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| RCF | Relative centrifugal force |
| PLS | Partial least squares |
| PCA | Principal component analysis |
| SAE | Serious adverse event |
| NRES | National Research Ethics Service |
| CSM | Section of Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London |
| OPLS | Orthogonal partial least squares |
| OPLS-DA | Orthogonal partial least squares - discriminant analysis |

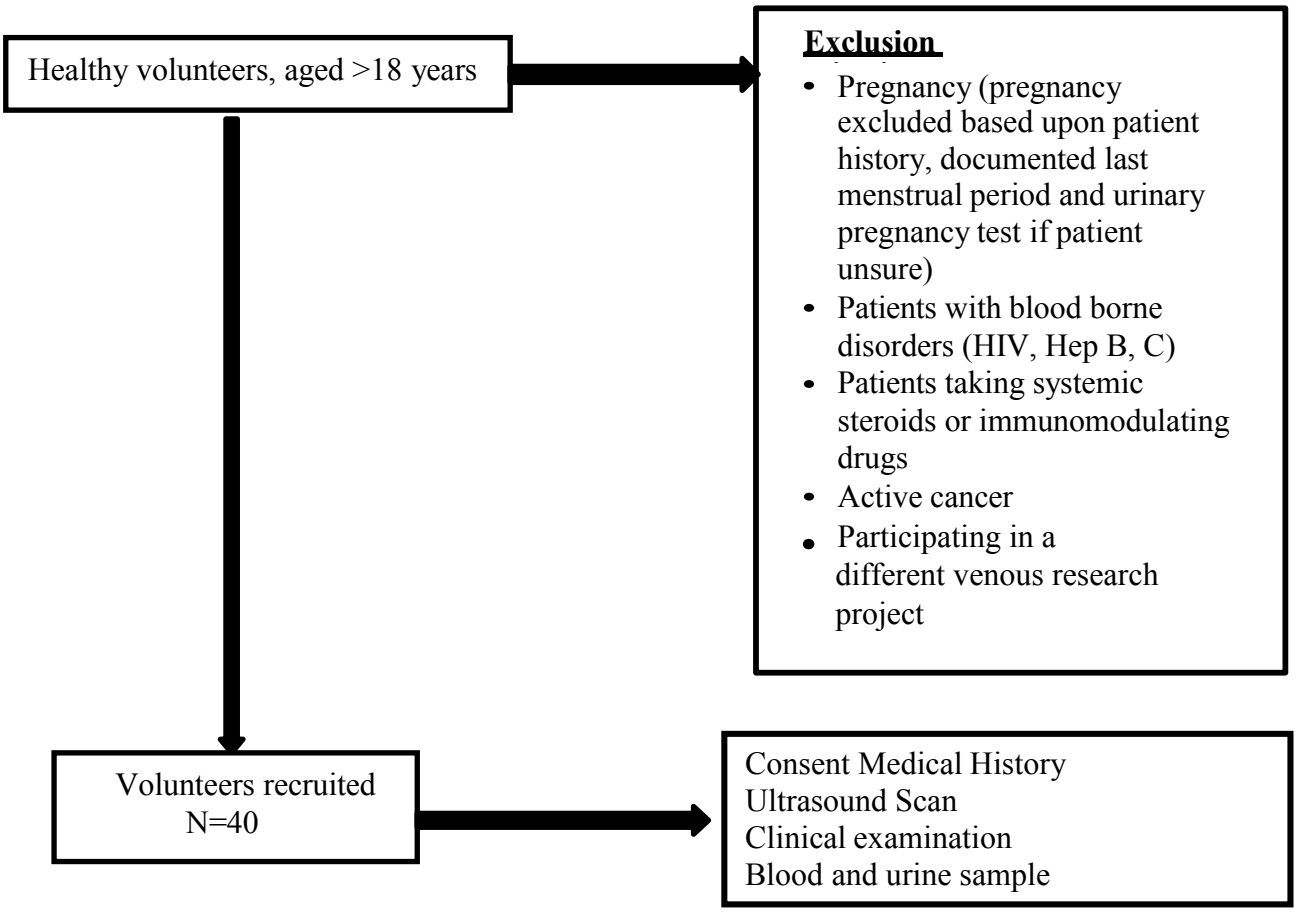
## KEYWORDS
Deep vein thrombosis, metabonomics, biomarkers

## STUDY SUMMARY

| | |
|---|---|
| **TITLE** | Targeted Metabolic Profiling in Deep Venous Thrombosis |
| **DESIGN** | Prospective observational case-control study |
| **AIMS** | Identification of novel biomarkers for Deep Vein Thrombosis using biometabolomic platforms |
| **OUTCOME MEASURES** | Deep Vein Thrombosis biomarkers |
| **POPULATION** | 40 patients diagnosed with a confirmed DVT and 40 healthy non-DVT controls |
| **ELIGIBILITY** | Disease - Adults, willing and able to consent, presenting with confirmed DVT.<br>Controls - Adults, able and willing to consent, without a DVT diagnosis.<br>Exclusion criteria - pregnancy, Hepatitis B, C or HIV positive, active cancer, steroid treatment |
| **DURATION** | 28 months |

**FLOWCHART-REFERENCE DIAGRAM**




Healthy volunteers, aged >18 years
Exclusion
Pregnancy (pregnancy excluded based upon patient history, documented last menstrual period and urinary pregnancy test if patient unsure)
Patients with blood borne disorders (HIV, Hep B, C)
Patients taking systemic steroids or immunomodulating drugs
Active cancer
Participating in a different venous research project
Volunteers recruited N=40
Consent Medical History
Ultrasound Scan
Clinical examination
Blood and urine sample

# 1. INTRODUCTION

## 1.1 BACKGROUND AND RATIONALE FOR CURRENT STUDY

Deep venous thrombosis (DVT) is a blood clot, usually affecting the legs, causing pain, swelling, and redness. The clot damages the veins, which can result in chronic pain, swelling and ulceration. This is called the post-thrombotic syndrome, which impacts heavily on patients' life and work. If the clot dislodges and travels to the lungs, it becomes a pulmonary embolus (PE), which can be life threatening. Together, DVT and PE affect 500,000 people in Europe every year, representing the most common preventable cause of hospital acquired death [1,2]. They are expensive conditions not only due to their treatment costs, but also secondary to the resulting loss of work days and productivity.

DVT is diagnosed by clinical examination, risk scoring, usually via the Wells Score, and a blood test called D dimer, a fibrin degradation product. D-dimer is specific but not very sensitive. In other words, when the test is negative, it is unlikely that a DVT is present. However, many conditions can raise D-dimer levels, making it less useful when positive [3]. Duplex ultrasound can confirm the presence of clot but this can be difficult to visualize or is often not seen at all. The clot can take time to form and patients may not experience symptoms immediately. This is a problem for treatment, as new, clot-busting medication works best in the first 2 weeks after a DVT and it is currently impossible to tell when the thrombus formed [4].

Because of the importance of DVT, and ongoing issues regarding its diagnosis, ageing and prognosis, numerous research groups have been exploring novel candidate biomarkers to help develop a more accurate diagnostic and prognostic marker for DVT. The Section of Vascular Surgery and of Computational and Systems Medicine (CSM) at Imperial College, have had a longstanding collaboration working towards this end using metabonomics.

Metabonomics is defined as: *'the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification'*. In other words, it examines the end products of cellular metabolism via platforms such as Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS), which are both high throughput analytical platforms. These enable the qualification (untargeted) and quantification (targeted) of different molecules in a given sample, to a degree of detail superior to that offered by other omic technologies (e.g. proteomics). Metabonomics has demonstrated clear applications in vascular disease, including chronic venous disease [5], atherosclerosis [6], venous leg ulceration [7], aneurysmal disease and stroke [8].

Untargeted preliminary work on deep venous thrombosis has revealed a metabolic signature for DVT using a murine animal model [9], and promising pilot data in a human study. The aim of the proposed study is to validate previous findings in a separate patient cohort and perform quantification of the metabolites identified in the pilot studies via targeted NMR and MS experiments, continuing the exploration of novel biomarkers for DVT.

# 2. STUDY OBJECTIVES

Primary outcome:

- Quantification of metabolites of interest and comparison of their concentrations in DVT and control participants

Secondary outcomes:

- Comparison of metabolite presence and concentration with other endogenous biomarkers associated with DVT, including D-dimer and P selectin.
- Validation of previously identified metabolites in the human DVT pilot study
- Assessment of the response to DVT treatment following anticoagulation
- Identification of candidate biomarkers to explore thrombus ageing in future studies

### 2.1 STUDY OUTCOME MEASURES

Primary Outcome:

- Identification of diagnostic deep vein thrombosis metabolites

Secondary Outcome:

- Validation of previously identified molecules/chemicals in a separate population
- Quantification of identified molecules in DVT patients and controls
- Comparison of identified metabolite concentrations to known DVT biomarkers (e.g. D dimer)
- Identification of potential molecules to study in future work with respect to thrombus ageing
- Longer term, genetic testing will be performed to compare the results to the metabonomic analysis

# 3. STUDY DESIGN

**Type of study**
The study is a prospective observational case-control study, recruiting 40 DVT patients and 40 healthy subjects. Patients diagnosed with a DVT will be recruited at the sites of Imperial College Healthcare NHS Trust.

**Summary**
Forty patients diagnosed with a DVT will be recruited. Participation in the study will not interfere with the decision making and diagnostic investigations by the direct care team. A second control group of 40 healthy volunteers will be also examined. Healthy subject recruitment will take place at the same sites. A patient information leaflet will be provided to participants for informed consent purposes.

A member of the research team will explain the study to prospective participants. Both patients and volunteers will be given sufficient time to read the information sheet and make an informed decision regarding participation in the study; this will be confirmed by the participant signing a consent form. The study involves a comprehensive history and clinical examination, recording medication taken and collection of blood and urine samples. The samples will be labeled anonymously with a study number and transferred to the Imperial

College laboratories in South Kensington. Sample transfer will be performed securely according to established departmental standard operating procedures (SOPs). A blood sample will be stored for future DNA analysis to complement the findings from the metabonomic assays. No DNA analysis will be performed for this particular project.

The DVT group will have a repeat ultrasound study, performed by a vascular research fellow on a dedicated (non-NHS) machine 3 weeks after anticoagulation treatment has ended. They will also have repeat urine and blood samples taken. None of the research interventions will have an impact on the clinical care the patients will receive.
A letter explaining the purpose of the study will be sent to the General Practitioner if the participant wishes. All clinical and research information will remain confidential. Consent forms will be stored in a locked room accessible only to the researchers involved in the study. Any clinical data recorded electronically will be stored in an anonymised fashion using study numbers and saved in encrypted, password protected files.

**Patient Groups:**

- Group 1: Patients with DVT confirmed by lower limb venous duplex ultrasound (DUS) (n =40)
- Group 2: Healthy volunteers with no DVT confirmed on DUS (n = 40)

**PARTICIPANT ENTRY:**

**3.1 INCLUSION CRITERIA**

- >18 years of age
- Willing/able to give written informed consent
- Patients with DVT and age and sex matched controls confirmed on duplex ultrasound

**3.2 EXCLUSION CRITERIA**

- Pregnancy (excluded based upon patient history, documented last menstrual period and urinary pregnancy test if patient unsure)
- Patients with blood borne disorders (HIV, Hepatitis B, C)
- Patients on systemic steroids and immunomodulating drugs
- Patients involved in a different venous research project or who have recently been involved with a venous research project

**3.3 WITHDRAWAL CRITERIA**
Participants can withdraw from the study at any point without giving any reason.

## 3.4 Study procedures, sample collection and preparation

**Clinical data:**
**First Visit (Group 1 – DVT)**: Patient history (including medications, dietary habits and last meal) and clinical examination will be performed. A Wells' score will be recorded (to test probability of DVT) and the presence of varicose veins noted. DUS will be performed on the affected limb by a vascular scientist, and by the clinical research fellow (CRF) to the asymptomatic limb. Blood and urine sampling will be performed. A pregnancy test will also be performed in all females with child bearing potential. The CRF will be trained by Mary Ellis, Lead Vascular Scientist at Imperial College Healthcare NHS Trust, in performing DUS examinations. The anatomical extent of the acute thrombus will be assessed using LET (Lower Extremity Thrombus) scoring.

Occasionally it is not possible to carry out the DUS on the day of presentation; in this case, the patient will be recruited and samples taken before they return home. This may be before or after administration of parenteral anticoagulation. The patient will then return to hospital the next day to have the scan – participation in the study will be confirmed following the scan results. If the patient is excluded from this study we will retain the blood and urine samples taken for future DNA analysis on an established biobank.

**First Visit (Group 2 – controls):** History (including medications, dietary habits and last meal) and clinical examination will be performed. DUS to both legs will be performed by a CRF or vascular scientist to ensure no DVT is present. Blood and urine sampling will be performed and stored as per departmental SOPs.

**Sample preparation and storage:**
All sample collection will be performed according to departmental SOPs.
*Blood:*
Blood tests will be performed via aseptic technique by a trained healthcare professional. Samples collected at clinical sites will be placed in containers and labelled anonymously using a study participant number. Blood will be transferred into two 13 x 100 mm (5mL) plastic serum tubes (Becton, Dickinson and Company, New Jersey, USA), allowed to stand for 30 minutes at room temperature, centrifuged at 4500RCF for 10 minutes; this will cause serum separation. The serum fraction will generate a minimum of three 0.4mL aliquots, which will be transferred to 1.5 ml Eppendorf® tubes, which will be immediately placed on dry ice and transferred to a -80°C freezer.

*Urine:*
Patients will be asked to provide a mid-stream urine sample into a 50mL Falcon tube (Becton, Dickinson and Company, New Jersey, USA). Four 1mL aliquots of urine will be transferred to 1.5 ml Eppendorf® tubes, which will be immediately placed on dry ice and then transferred to a -80°C freezer.

**Sample separation:**
Sample separation and analysis will take place at the Section of Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London. We will isolate components from the blood and store these (e.g. DNA, RNA, plasma, serum, blood precursor cells, etc.) for use in future research (for a maximum of 10 years) for the patient group.

For the control group, blood and urine samples will be discarded at the end of this project. For any participant in the patient group, whose duplex scan demonstrates that they do not

have a DVT then they will be withdrawn from the study and all blood/urine samples collected for the study will be discarded.

**Metabolite Profiling:**

Targeted NMR and ultra performance liquid chromatography (UPLC-MS) platforms will be employed for the metabolic profiling of urine and serum. UPLC-MS analysis will be performed on a Waters ACQUITY UPLCTM system (Waters, Milford, MA), coupled with a Waters XEVO G2 Mass Spectrometer (Waters MS Technologies, Manchester, UK). Internal standards available at CSM will be used for quantification purposes. All samples will be analysed in both positive and negative electrospray ionisation (ESI) MS modes. 1H NMR spectra will be acquired on a Bruker DRX-600 spectrometer (Bruker Biospin, Germany) operating at 600.13 MHz, with a probe temperature of 300 K.

**Metabolites to study:**
Detection and quantification of metabolites in human blood serum and urine will be completed using targeted MS and NMR metabolic profiling approaches. MS and NMR are the two main analytical techniques employed for metabolic profiling, providing complementary information. All endogenous metabolic profiling studies result in complex multivariate data sets that require pre-processing, use of visualization software and chemometric methods for interpretation. The MS and NMR spectra from the samples will be analysed in order to identify metabolite features whose intensities and quantity is significantly different between the disease and control groups. These important metabolites will be classified and ultimately identified. The identification of molecules is a challenging procedure. Consultation with in-house metabolite databases and online databases is the first step in metabolite structural elucidation. These databases contain information regarding thousands of endogenous and drug metabolites, including MS spectra, in some cases MS/MS spectra and experimental and predicted metabolite 1H- and 13C-NMR data. Well-characterized metabolites may be identified through database searches but for unambiguous metabolite identification, co-chromatography and comparison of MS/MS data with the authentic compound are necessary.

**DNA: extraction and storage**
Blood sample for DNA will be preferentially taken during the first visit. A 5ml EDTA sample will be obtained for DNA extraction and storage. Extraction will be by standard DNA extraction columns (Qiagen, Germany). DNA will be aliquoted and stored at -80C. If indicated by the metabonomic analysis then the samples may be used for sequencing of specific genes. However, it is intended that if differences between patients and controls are identified, a submission for whole genomic sequencing will also be submitted. The rapidly falling costs of sequencing and increasing resource allocated to this analysis, make this a practical and important subsequent step.

## 4. ASSESSMENT AND FOLLOW-UP

The patients in Group 1 with confirmed DVT, receiving anticoagulation treatment according to standard care, will be reviewed 3 weeks after the end of their treatment. They will then have repeat urine and serum samples taken. Both limbs will be scanned again by the clinical research fellow to assess the deep venous system and check for clot resolution .

The clinical study will be complete when all patients have been recruited, followed up and the last sample has been taken. The laboratory portion of the study will be complete once all samples have been analysed.

# 5. STATISTICS AND DATA ANALYSIS

**Sample size**
The sample size of 40 patients in each group has been determined following consultation with Professor Elaine Holmes, Head of the Section of Computational and Systems Medicine, and based upon the results of the previous preliminary study, which revealed a statistically significant difference between the DVT and control groups in a sample size of 40 patients in each group. This will suffice to validate the previous results and quantify metabolite(s) of interest.

**Data Analysis and Statistics**
Following NMR and MS experiments, raw data is generated in the form of spectra. Prior to the application of multivariate approaches, both NMR and MS datasets will undergo a number of key pre-processing steps including binning, peak picking, alignment and normalization.

Spectral data is impossible to assess to the naked eye; therefore, multivariate statistical techniques must be employed to determine group differences (e.g. between disease and controls) and identify spectral areas responsible for driving the difference (representing metabolites of interest). This is performed by using software that presents data points and groupings in a visual manner. This permits identification of clusters, outliers, trends and batch effects. Multivariate statistics include unsupervised (i.e. principal component analysis [PCA]) and supervised methods (i.e. partial least squares [PLS], orthogonal-PLS [OPLS] and OPLS discriminant analysis [OPLS-DA]). Following multivariate analysis, metabolites will be identified and pathway analysis performed using in house and external tools (Chemspider, KEGG).


Fig 1. **Workflow in metabolic phenotyping.** Metabolite analysis includes digitalisation of findings into spectra, which will be compared to known metabolite lists and will be correlated to biological pathways in order to identify possible biomarkers.

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study, including the follow-up period.

# 6. ADVERSE EVENTS

## 6.1 DEFINITIONS

**Adverse Event (AE):** any untoward medical occurrence in a patient or clinical study subject.

**Serious Adverse Event (SAE):** any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is 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 might have caused death if it were more severe*
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**

- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

### 6.2 REPORTING PROCEDURES

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

#### 6.2.1 Non serious AEs

All such events, whether expected or not, should be recorded.

#### 6.2.2 Serious AEs

An SAE form should be completed and faxed to the Chief Investigator within 24 hours. However, hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the ……..REC where in the opinion of the Chief Investigator, the event was:

- 'related', ie resulted from the administration of any of the research procedures; and
- 'unexpected', ie an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator must also notify the Sponsor of all SAEs.

Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

**Contact details for reporting SAEs :**
**Please send SAE forms to:**
**Professor Alun H. Davies**
**Professor of Vascular Surgery**
**Academic Section of Vascular Surgery**
**Department of Surgery & Cancer**
**Imperial College London**
**Room 4E04, 4 East**
**Charing Cross Hospital**
**Fulham Palace Road**
**London, W6 8RF**
**Tel: 020 3311 7320 (Mon to Fri 09.00 – 17.00)**

# 7. REGULATORY ISSUES

### 7.1 ETHICAL APPROVAL

The Chief Investigator has obtained approval from the London Surrey NHS Research Ethics Committee). The study must also receive confirmation of capacity and capability from each participating NHS Trust before accepting participants into the study or any research activity is carried out. 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.

### 7.2 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. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases, the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

### 7.3 CONFIDENTIALITY

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

### 7.4 INDEMNITY

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

### 7.5 SPONSOR

Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

### 7.6 FUNDING

The funding will be internal. There will be no participants or investigator payments. The Graham-Dixon Charitable Trust has awarded £14 491 towards the consumables cost of this research. Further grant applications will be submitted.

### 7.7 AUDITS

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

# 8. STUDY MANAGEMENT

The day-to-day management of the study will be co-ordinated through the Clinical Research Fellow Mr Kemal Kemal, under the supervision of the Chief Investigator Professor Alun H. Davies.

# 9. PUBLICATION POLICY

The aim of this study is to generate results for dissemination at international meetings and peer reviewed articles in scientific journals. Authorship shall be determined by contribution to the various aspects of the study. All authors shall have the ability to review and comment on the manuscript of any work prior to submission. The study shall also form the basis of the higher degree submission of Mr Kemal Kemal at Imperial College London.

# 10. REFERENCES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## Appendix 1. Summary of investigations and assessments

| **Exam** | 1st visit | 2rd visit (if applicable) |
|---|---|---|
| Informed consent | X | |
| History, physical exam | X | X |
| Blood and urine sample | X | X |
| Venous duplex ultrasound | X | X |

Group1:
1st visit: medical history, examination blood and urine sample
Patients will also undergo a venous duplex ultrasound examination either on the same day or the day after.
All patients of group 1 will be asked to return 3 weeks after the end of their treatment During this 2nd visit the medical history will be revised, leg inspection and leg ultrasound will be performed and a blood and urine sample will be repeated.

Group 2 (volunteers):
Only 1st visit.