PROTOCOL

Version 1.6 September 2014

Full title

Microbleeds and genetic risk factors to predict the risk of intracranial haemorrhage in patients treated with anticoagulation following cardioembolic stroke due to atrial fibrillation.

Short title

Clinical Relevance Of Microbleeds In Stroke (CROMIS-2)

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### Study sites and Investigators

#### Main Study Sites

The study co-ordinating centre will be based at University College Hospital and The National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG

Collaborating sites:

- Addenbrooke's Hospital
- Airedale General Hospital
- Altnagelvin Hospital
- Antrim Area Hospital
- Ashford and St Peter's Hospital
- Barnet and Chase Hospital
- Basingstoke and North Hampshire Hospital
- Bedford Hospital NHS Trust
- Bradford Royal Infirmary
- Bristol Royal Infirmary
- Charing Cross Hospital
- Chesterfield Royal Hospital
- City Hospital Birmingham
- Countess of Chester Hospital
- County Durham & Darlington
- Croydon University Hospital
- Darent Valley Hospital
Derby Hospital
Derriford Hospital
Doncaster Royal Infirmary
Dorset County Hospital
Epsom Hospital
Hampshire County Hospital
Hillingdon Hospital
Kent and Canterbury Hospital
Kings College Hospital
Kingston Hospital
Leeds General Infirmary
Leicester University Hospital
Lincoln County Hospital
Luton and Dunstable Hospital
Maastricht University Hospital
Medway Maritime Hospital
Mid Yorxls Hospital
North Middlesex University Hospital
North Tees University Hospital
Northwick Park Hospital
Nottingham City Hospital
Peterborough & Stamford Hospital
Pilgrim Hospital, Boston

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<tr>
<td>AF</td>
<td>Atrial Fibrillation</td>
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<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
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<td>INR</td>
<td>International Normalized Ratio</td>
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<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>ICH</td>
<td>Intracerebral haemorrhage</td>
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<tr>
<td>UCH</td>
<td>University College Hospital</td>
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<td>NHNN</td>
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Summary

Background

Over the last decade, increasing use of oral anticoagulants to prevent cardioembolic ischaemic stroke due to atrial fibrillation (AF) in an ageing population has led to a five-fold increase in the incidence of anticoagulant-related intracranial haemorrhage (ICH) - a rare but unpredictable and catastrophic complication. Cerebral microbleeds (CMBs) on magnetic resonance imaging (MRI) may predict ICH risk, as may genetic polymorphisms influencing brain small-vessel integrity or anticoagulation stability.

Aims

To establish the value of CMBs and genetic factors in predicting symptomatic ICH following best practice oral anticoagulation to prevent recurrent ischaemic stroke due to AF.

Methods

**CROMIS-2: Study I (AF)** - Prospective, multicentre, inception cohort study in 1425 patients with ischaemic stroke due to AF started on best practice oral anticoagulation. Patients will have genetic testing and standardized MRI including GRE at baseline, with follow-up by postal questionnaire (and clinical assessment or medical records surveillance after suspected events), and where possible there will be an in person clinical assessment at 2 years. We will compare the rate of symptomatic ICH between CMB and CMB-free patients and test for associations with plausible candidate genes. We aim to develop and validate a risk model to predict symptomatic ICH following best practice oral anticoagulation to prevent recurrent ischaemic stroke due to AF.

**CROMIS-2: Study II (ICH)** - An observational study of ICH investigating genetic, clinical and radiological risk factors associated with anticoagulant-related ICH. We will recruit patients admitted to participating centres with ICH (with a target of at least 300 anticoagulant-related ICH cases) and collect DNA to increase the power of the genetic
studies. We will collect clinical and imaging data from these ICH cases to investigate risk factors associated with anticoagulant-related ICH compared to non anticoagulant-related ICH.

**Expected outcomes**

A successful predictive model for ICH risk after best practice oral anticoagulation for AF will help to determine whether genetic or CMB screening should be used in clinical practice and future trials. New genetic, clinical and radiological risk factors associated with anticoagulant-related ICH will be identified.
Background

**Atrial Fibrillation and oral anticoagulation**

Atrial fibrillation (AF) is the commonest cause of cardioembolic stroke, and is associated with about 20% of all strokes. The lifetime risk for developing AF is one in four for men and women over the age of 40.1 AF affects about 12% of people over the age of 75, and its prevalence is increasing with an ageing population. If untreated, AF increases the risk of stroke five-fold, with the highest risk seen in elderly patients who have had a previous stroke or transient ischaemic attack (TIA). Oral anticoagulation reduces ischaemic stroke risk by about 65% in patients with AF: however, this benefit has to be balanced against an increased risk of intracranial haemorrhage (ICH). ICH is the most feared complication of oral anticoagulation, causing death or severe disability in up to 75% of patients.2 In one trial, the use of warfarin for AF in patients older than 75 years was associated with a higher risk of ICH which substantially reduced the overall benefit of warfarin.3 A recent observational inception cohort study of patients treated with oral anticoagulation (of whom a quarter had a previous history of stroke) reported a 2.5% (95% CI 1.1 to 4.7%) risk of ICH in 1 year.4 Because of an ageing population, and increased use of oral anticoagulation for AF, the incidence of oral anticoagulant-related ICH has increased five-fold over the past decade, and already accounts for about 15% of all ICH:5 this trend is set to continue and will be a huge healthcare, social and economic challenge for the future.

Because oral anticoagulation-related ICH is so often fatal or disabling, even a small increase in the absolute risk of ICH of only 1 to 2% per year could cancel out the benefit of oral anticoagulant treatment.6 Clinical risk factors for oral anticoagulant-related ICH include increasing age, previous stroke, and intensity of oral anticoagulation7 however, these are not sufficiently reliable for clinical application. It is a paradox that many elderly patients at the highest risk of ischaemic stroke are also at the highest risk
of ICH, often making it extremely difficult to balance the risks and benefits of oral anticoagulation.

**Cerebral microbleeds and intracerebral haemorrhage**

Cerebral microbleeds (CMBs) are small dark rounded areas on gradient-recalled echo T2*-weighted MRI (GRE T2*) and are a marker for bleeding-prone arterial pathology affecting cerebral small vessels. CMBs are found in about 30% of patients with ischaemic stroke, 60% of patients with ICH, and in up to 40% of the healthy population over the age of 80. CMBs appear to predict the risk of ICH in patients with stroke (ischaemic or haemorrhagic). We showed in a case-control study that lobar CMBs (suggesting possible cerebral amyloid angiopathy [CAA] – see figure) are a risk factor for aspirin-related ICH, and a recent systematic review of case-case comparisons showed that CMBs are much more common in oral anticoagulation-related ICH than “spontaneous” ICH. Another study reported CMBs in 87% of patients with ICH following warfarin treatment of AF, while a recent case-control study also reported more CMBs in warfarin-associated ICH than in matched warfarin users without ICH. The underlying causes of CMBs include pathology affecting brain small vessels, mainly CAA and hypertensive small vessel disease. There is increasing evidence that CAA carries a particularly high risk of warfarin-related ICH.

CMBs are being found more and more often as MRI is used more frequently in patients with stroke. A survey we conducted showed that many clinicians are withholding oral anticoagulant treatment in patients with CMBs, whilst others are ignoring them. The significance of CMBs for intracranial bleeding risk therefore needs to be urgently clarified to avoid patients being denied effective treatment due to concerns regarding CMBs; conversely, if high risk patients could be identified, they could have individualised antithrombotic treatment and be spared the devastating yet potentially avoidable hazard of ICH.
Hypothesis

Because oral anticoagulant associated ICH is associated with increased age and previous stroke, and often occurs with anticoagulation intensity within the therapeutic range\(^1\), it is likely that the mechanism underlying the high risk is related to individual patient factors, for example an age-related disorder of small brain blood vessels, such as cerebral amyloid angiopathy (CAA) or hypertensive small vessel disease. In keeping with this hypothesis, some studies suggest that leukoaraiosis - a confluent deep white matter abnormality seen as low attenuation on computed tomography (CT) or high signal on T2-weighted MRI, which is a marker of small vessel disease - increases the risk of oral anticoagulant-related ICH.\(^{19,20}\) CMBs are associated with leukoaraiosis: however, because they provide direct evidence of leakage of blood from pathologically fragile small vessels, we hypothesize that microbleeds are a better predictor of oral anticoagulant-associated ICH than leukoaraiosis alone.

Oral anticoagulant-associated intracerebral haemorrhage and genetics

There is increasing interest in genetic factors that might predispose to oral anticoagulant-associated ICH, with two plausible mechanisms: first, some genes may influence the sensitivity to oral anticoagulant and stability of anticoagulation\(^2\) and second, other genes may make cerebral small vessels more likely to bleed by affecting the integrity of the arterial wall.\(^1\)

Why is there a need to perform this study now?

The few available previous studies on CMBs and ICH risk have many limitations. Most available data is from cross-sectional studies in Asian cohorts so the findings cannot clearly show causative relationships and may not be generalisable to other populations. No previous studies have fully investigated the effect of CMB burden i.e. whether the number and/or location of CMBs affects the risk of ICH on oral anticoagulant treatment, which may be important given that the severity of leukoaraiosis seems to show a graded
relationship to the risk of oral anticoagulant-related ICH, and CAA (in which lobar CMBs are a hallmark) seems to be a particular risk factor. Indeed, no large prospective studies of CMBs of any sort in ischaemic stroke have been completed in Europe. The best way to definitively investigate the real value of CMBs in assessing the risk of oral anticoagulation-related ICH is with a large prospective observational inception cohort study. Given the substantial recent increase in the rate of antithrombotic-related ICH in the UK (associated with lobar haemorrhage attributed to CAA) an adequately powered multicentre study is now an urgent priority. In our survey of 22 stroke centres in the UK, >90% of respondents wish to take part in such a study, subject to funding for MRI scans (see list of centres).
Questions the research will set out to answer

Primary Questions

(1) Does the presence of CMBs help predict the risk of symptomatic oral anticoagulant-related ICH in patients who are anticoagulated following cardioembolic stroke due to non-valvular AF?

(2) Do the burden (number) and distribution of CMBs at baseline influence the risk of ICH in this cohort?

Secondary Questions

(3) In patients anticoagulated after ischaemic stroke due to non-valvular AF, are CMBs associated with an increased risk of recurrent TIA, ischaemic stroke or death?

(4) Are genetic polymorphisms related to the integrity of brain small vessels or anticoagulant metabolism associated with an increased risk of ICH?

(5) Are CMBs a better predictor of oral anticoagulant-related ICH than clinical risk factors and/or leukoaraiosis on MRI scans?

(6) Can a useful risk prediction model incorporating clinical, imaging and genetic factors be developed to assess the risk of best practice oral anticoagulant-related ICH?

(7) Can we identify new genetic, clinical or radiological risk factors associated with anticoagulant-related ICH?
Study Design

Study I: CROMIS-2 (AF)

Prospective cohort study of patients anticoagulated after cardioembolic stroke

Observational inception cohort study (n=1425) of patients throughout the UK (79 hospitals) started on best practice oral anticoagulant (without prior use) for presumed cardioembolic ischaemic stroke due to non-valvular AF with follow up for the occurrence of ICH, ischaemic stroke and cognitive function for an average of two years. Our main baseline exposures (risk factors of interest) are the presence of CMBs on MRI, and genetic polymorphisms in candidate genes with potential functional relevance to ICH risk.

Study II: CROMIS-2 (ICH)

Observational and genetics study of intracerebral haemorrhage

We will also recruit 600 patients admitted to participating centres with ICH (with a target of at least 300 anticoagulant-related ICH cases) and collect DNA to increase the power of the genetic studies. We will collect clinical and imaging data from these ICH cases to investigate risk factors associated with anticoagulant-related ICH compared to non anticoagulant-related ICH.

Reasons for the chosen study design

We considered conducting a randomised trial of best practice oral anticoagulant versus aspirin in patients with CMBs, but concluded that reliable observational data are needed first to confirm that CMBs predict ICH, and to determine the incidence and effect of CMB numbers on risk, before considering a randomised trial.
Detailed plan of methods to answer the questions

Sample size and recruitment of subjects

Study I: CROMIS-2 (AF)

Prospective cohort study of patients anticoagulated after cardioembolic stroke

Based on our statistical sample size calculations (see below) we will recruit a total of 1425 patients from UK centres in the Stroke Research Network over 36 months. All eligible patients with first or recurrent ischaemic stroke and TIA in whom it is decided that best practice oral anticoagulant treatment is to be commenced will be invited to participate from acute stroke units and outpatient stroke clinics. If 40 hospitals participate, this is a target recruitment rate of about 8 patients per year per participating hospital. The Scottish National Stroke Audit showed that 41% of inpatients discharged alive with ischaemic stroke and in AF were anticoagulated; in the National Sentinel Audit for Stroke in England (2006) 34% of patients in atrial fibrillation were taking oral anticoagulants on discharge. If we therefore assume that 34% of stroke patients with atrial fibrillation are discharged on oral anticoagulants, that atrial fibrillation accounts for 20% of all ischaemic strokes, and that each participating hospital admits about 240 ischaemic strokes per year, we expect at least 16 inpatients to be eligible for the study at each centre per year. With at least as many TIA and stroke patients assessed as outpatients, we expect over 30 patients to be eligible per centre per year. Our minimum target of 8 patients per centre per year is realistic to allow for non-participation, recruitment into other studies, and patients who are already taking oral anticoagulants prior to screening for the study.
Study II: CROMIS-2 (ICH)

Observational and genetic study of intracerebral haemorrhage

We will recruit 600 patients treated at participating hospitals with ICH. Of these patients, 300 ICH cases will be related to anticoagulant use. We will also recruit at least 300 ICH cases not related to anticoagulant use during the study period. Of an estimated 60 patients with ICH admitted per centre per year, we expect each participating hospital to recruit at least 2-3 patients with anticoagulant-associated ICH per year, giving a cohort of approximately 240-400 (target 300) cases of anticoagulant-associated ICH over 3 years. Patients seen in outpatient clinics or from existing databases may also be recruited, at centres where these are available.

Detailed clinical and imaging (CT or MRI) data, and blood for DNA analysis, will be collected from all ICH cases.

Baseline data for CROMIS-2 (AF) and CROMIS-2 (ICH) will be entered by the local study team into an electronic case report form (CRF) and submitted to the central study co-ordinating centre at UCL.

Follow up

In CROMIS-2 (AF), patients will be followed up at 6, 12, and 24 months by a postal questionnaire sent from the co-ordinating centre at UCL. At these time points, data collected will include an INR monitoring, stroke event and outcome self-reporting questionnaire. If the questionnaire is returned and indicates the patient may have had a stroke or other potentially significant medical event since last assessed, further data regarding the event will be obtained by the research team at the centre where the patient was recruited by inspection of medical notes (including GP and hospital records).

The co-ordinating centre will send out a regular list of outstanding patient data. For non-responders, the address of the patient will then be checked with the GP by the
local recruiting centre research team, who will contact the co-ordinating centre to resend the questionnaire. If data is not received back, the research team at the relevant local recruiting centre will then contact the patient (having confirmed they are still alive and living at the same address with the GP) to obtain the follow up data by telephone.

Patients may also be invited for an in person follow-up visit (which may include neuropsychological assessments, blood tests and/or brain scans) at the local recruiting centre at 1 year and 2 years. Further yearly follow up may continue for a maximum of 4 years for the first included patient. We expect that this will lead to a mean follow up of 2 years, taking into account the fact that recruitment is likely to be slower initially and allowing for drop outs from treatment. Beyond 2 years, the annual assessment will use a similar postal questionnaire to the follow up at 6, 12 and 24 months.

At each follow-up time point, GP surgeries or anticoagulant clinics will also be contacted by postal questionnaire from the co-ordinating centre, requesting INR values and to ask them to inform the co-ordinating centre if any stroke events or bleeding events occur during the follow up period. Similar protocols to chase missing data will be used as described above.

The co-ordinating centre will automatically receive notification of death of any patient in the study from the NHS Information Centre (IC).

Where notification is received from the patient or local study team that patients have been admitted to hospital, the central study team will request all available information from the local study team, including medical records and investigation reports including any brain imaging. They will request access to data where necessary from Hospital Episode Statistics Service.

Clinical events will be classified by an adjudication panel at the co-ordinating centre as stroke (ischaemic or ICH), TIA, other vascular event, serious bleeding event according to standardized criteria (below), or other diagnosis. Symptomatic bleeding will be recorded with major haemorrhage defined as: fatal, hospitalisation with transfusion of 2
units of red blood cells, or involvement of a critical site (i.e. intracranial, retroperitoneal, intraspinal, intraocular, pericardial, or atraumatic intra-articular haemorrhage). For symptomatic ICH events, potential contributory factors (e.g. falls, frailty) will be recorded. Where patients are admitted with an ICH event, any histological samples taken as part of standard clinical care (e.g. at neurosurgery) will be reported clinically and made available to the research team. Further analysis of these samples may be undertaken by the central research team at UCL.

In CROMIS-2 (ICH) patients will be followed up by postal questionnaire at 6 months.

All included patients will have a “CROMIS-2 (AF)” or “CROMIS-2 (ICH)” sticker put on their medical notes, and the local research and/or clinical team will be encouraged to send copies of all follow up correspondence to the central study team at UCL.

**Number of centres involved and involvement of the UK Stroke Research Network (SRN)**

A final total of 79 hospitals are taking part. Both CROMIS-2: Study I (AF) and CROMIS-2: Study II (ICH) have been adopted by the Stroke Research Network.
**Subject inclusion and exclusion criteria**

**Study I: CROMIS-2 (AF)**

**Inclusion criteria:**

- Adult (≥18y; no upper limit) patients with a clinical diagnosis of non-valvular AF (verified by ECG) with intention to treat with best practice oral anticoagulants (e.g. warfarin)

- Previous ischaemic stroke or TIA diagnosed by treating clinician

- All patients must be able to have GRE MRI before (or within 1 week) of starting best practice oral anticoagulant

**Exclusion criteria:**

- Any MRI contraindications

- Previous use of oral anticoagulation

- Definite contra-indication to oral anticoagulation

- Serious head injury (resulting to loss of consciousness)
Study II: CROMIS-2 (ICH)

Inclusion criteria:

- Adult (>18y) patients treated at participating centres with confirmed ICH (confirmed on CT or MRI scans) with or without a history of anticoagulant use at the time of the ICH

Exclusion criteria:

- Known underlying structural cause for ICH (e.g. arteriovenous malformation, tumour, cavernoma, intracranial aneurysm, haemorrhagic transformation of an infarct)

- Major head trauma (causing loss of consciousness and though to be sufficient to have caused the ICH) in previous 24 hours

Data monitoring

A Study Steering Committee to oversee the organisation of the study. The Study Steering Committee and investigators will review the progress of the study and unblinded data at regular intervals, and monitor the completeness and quality of data. Major outcome events (ICH, any stroke or TIA or death) will be adjudicated by a separate end point committee blinded to the baseline microbleed imaging findings.

Ethical considerations

This is an observational study. The only additions to routine care, required for CROMIS-2 (AF) are: an MRI scan if this is not routine practice at the centre, a blood test, additional follow up postal questionnaires/telephone calls, and possible face to face assessments at 1 and 2 years. The risks of MRI are extremely low, so long as patients in whom MRI is contra-indicated are excluded as required by the protocol. The patients in the study are likely to benefit from the additional follow up required by the protocol.
Should it become clear from either our own data or from other studies, that CMBs are a clearly significant risk for future ICH on oral anticoagulation, then the Data Monitoring Committee will advise the Study Steering Committee, who will meet to discuss any requirement to discontinue or modify the study.

The only addition to routine care required for CROMIS-2(ICH) are: a blood test and the additional follow up postal questionnaire/telephone calls.

**Data to be collected:**

**Clinical data:**

**Study I: CROMIS-2 (AF)**

Detailed clinical and demographic information will be recorded which may include ECG, echocardiography, blood pressure, blood test results including LFTs, CHADS2 score, National Institute of Health Stroke Scale (NIHSS) where appropriate, modified Rankin Score, Quality of Life measures, Cognitive screen (including the Montreal Cognitive Assessment [MoCA], the IQ-CODE short form). Patients may be invited back at 1 year for a further cognitive assessment using the Montreal Cognitive Assessment [MoCA].

For patients unable to attend in person psychological testing by telephone may be offered by the local study team or CROMIS-2 central team.

Blood will be taken and DNA will be extracted from consenting patients at baseline and stored in a repository at UCL Institute of Neurology, Queen Square.

EDTA plasma, citrated plasma and serum may also be taken for biomarker substudies and which can be kept for up to 4 weeks at -40°C and indefinitely at -70°C, prior to transport to the co-ordinating centre.

The target INR (International Normalized Ratio) – usually 2.5 (range 2.0-3.0) - will be decided as per standard clinical care by the local anticoagulant service.
Initial hospital INR readings (where available) will be entered into the electronic CRF by local research practitioners. Other INR readings will be entered into a postal questionnaire sent to patient’s addresses from the co-ordinating centre, by patients or carers. Patients will also be asked to report on any bleeding events using a standardized questionnaire / diary. The clinical service responsible for the participants' anticoagulation control (GP surgeries or anticoagulant clinics) will also be contacted by questionnaire to request INR values and whether any stroke events or bleeding events occur during the follow up period. Outstanding data will be monitored by the central research team, who will contact the local study team to chase missing data.

No additional blood tests for INR will be requested as part of the study. The percentage of time in range (TIR) will be calculated using linear interpolation. The proportions of INR tests within, below and above the therapeutic range will be determined. Data available from the first 6 weeks of treatment will be analysed separately, as oral anticoagulant control is known to be poor during this period. As the risk of ICH increases significantly above INRs of 3.5 and exponentially above INRs of 4.0, the incidence of INR values above these critical limits will also be monitored.

The clinical responsibility for oral anticoagulant control remains with the participating centre local clinical anticoagulant clinic team. The maintenance dose will be defined as the mean dose administered during therapeutic oral anticoagulation.

**Study II: CROMIS-2 (ICH)**

Detailed clinical and demographic information will be recorded including measures of stroke severity, demographic variables and vascular risk factors. We will record the National Institute of Health Stroke Scale (NIHSS) modified Rankin Score, Quality of Life measures (EQ5D+C), Cognitive screen (Montreal Cognitive Assessment [MoCA]) and IQ-CODE short form. Blood will be taken and DNA extracted from consenting patients at baseline and stored in a repository at Queen Square.
Blood or serum may also be taken for biomarker substudies and frozen for storage at -70°C subject to funding.

Patients in selected centres (including UCL Institute of Neurology) may be invited to have fundoscopy and retinal photography as part of a substudy (subject to funding).

**Imaging data:**

In study I: CROMIS-2 (AF), all patients will have T2* GRE MRI (with standardized sequence parameters recommended by the central study team) at baseline, as soon as possible after stroke or TIA attributed to AF (ideally before, but within 1 week after anticoagulation is started) with other vascular MRI sequences (Axial T1, Axial T2 FSE, Coronal FSE Flair and DWI). We will also acquire 3D GRE susceptibility-weighted images (SWI) at as many sites as possible, including UCH / NHNN; this method has increased sensitivity to detect CMBs. Other advanced MRI sequences (e.g. diffusion tensor imaging, cerebral perfusion MRI) may be obtained as part of a substudy, subject to funding, at selected centres.

Imaging analysis for CMBs and other imaging markers of cerebrovascular disease will take place at the co-ordinating centre using appropriate validated rating scales and, where appropriate, automated segmentation or quantitative measures. Scans from other centres will be sent to the co-ordinating centre in anonymized digital form (DICOM format) according to a standardized protocol, via portable media or web-based transfer.

The main baseline factor recorded will be the presence or absence of CMBs, but the number and volume of CMBs may be quantified by a semi-quantitative method using in house software. CMB burden will be rated on a scale based on the number of CMBs. White matter changes, including leukoaraiosis, will be graded using a standard validated rating scale. The presence, number and anatomical distribution (lobar vs. deep) of CMBs, and the severity of leukoaraiosis, will be quantified centrally by an observer blinded to clinical details. New methods of automated or semi-automated analysis may be developed and used.
In Study II: CROMIS-2 (ICH), all brain imaging undertaken as part of standard clinical care will be collected and analysed in the study. We expect that MRI including GRE T2* will be obtained in many cases as a routine investigation in the diagnosis of spontaneous ICH. However, MRI imaging is NOT essential for CROMIS-2 (ICH). Available Computed tomography (CT) data will also be included. Scans from other centres will be sent to the co-ordinating centre in anonymized digital form (DICOM format) according to a standardized protocol, via portable media or web-based transfer.

**Genetics data:**

The basic initial study design will be a candidate gene approach (see table) and a subsequent genome-wide case-control association study (GWAS) – subject to funding - to investigate genetic polymorphisms associated with oral anticoagulant-associated ICH. We will compare the genotype of oral anticoagulant-associated ICH (n~300) with our internal non-ICH controls (n>900) and >2000 controls drawn from external cohort data, including the Wellcome Trust 1958 cohort and our own bank of UK and CEPH ethnically diverse controls. Two vials of Blood in EDTA will be collected and transported to Queen Square by post.

The sample may remain at ambient temperature for up to 1 week before arriving at Queen Square; if a longer delay is anticipated the sample must be frozen prior to transfer (samples may be kept for up to 4 weeks at -40°C or indefinitely at -70°C). At the co-ordinating centre, DNA will be extracted from each patient and the DNA stored at -70 °C.

We will genotype the samples for putative polymorphic risk factors which may include those examples in the table below, but which will depend on the prevailing knowledge about genetic risks for bleeding at the time of analysis. This will be carried out using Polymerase Chain Reaction (PCR) Restriction Fragment Length Polymorphism (RFLP) or sequencing analysis. We will use a case-control chi square analysis to investigate these candidate genes. Over the lifetime of the study, further ICH candidates will be identified and these will be incorporated. The Genome wide association study (GWAS) will be...
carried out when all samples have been collected (years 3 and 4) and, given the rapid evolution of array and sequencing technology, we will use the best form of analysis available at that time. We will visually examine all the files in GenomeViewer tool within BeadStudio v3.1 Genotyping module (Illumina Inc., San Diego, CA), where log R ratio and a B allele frequency will be assessed, allowing visualization of copy number changes and homozygosity. Analysis of whole genome Single Nucleotide Polymorphism (SNP) genotype data. The data will be analyzed by the appointed clinical research fellow and Dr Houlden, with input and supervision from Prof Hardy. We will use PennCNV to collate the copy number variation (CNV) data into Excel tables, look for smaller insertions/deletions and carry out chi-squared tests for associations with disease phenotype. For each SNP generated from the whole genome analysis, we will compute summary statistics and association tests using the PLINK toolset. Analysis with STRUCTURE will be carried out to identify discernible difference in the population substructure between ICH and control cases. Statistical analysis of association with ICH will be done for all genotypes, irrespective of Hardy-Weinberg disequilibrium or minor allele frequency. However, each SNP will be required to have a call rate greater than or equal to 95% and a minor allele frequency (MAF) greater than or equal to 0.01. Attention will be paid to the SNPs close to the candidate genes which may include the following (Table, below).
Table: some potential candidate genes for anticoagulant-related ICH 21,30

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>ApoE e2/3/4 and promoter</td>
</tr>
<tr>
<td>ACE</td>
<td>I/D</td>
</tr>
<tr>
<td>SERPINE I 4G 5G</td>
<td>4G/5G</td>
</tr>
<tr>
<td>Collagen type IV, Alpha-1 (COL4A1)</td>
<td>Tagging SNPs across the gene (haplotype)</td>
</tr>
<tr>
<td>VKORCI</td>
<td>Tagging SNPs across the gene (haplotype)</td>
</tr>
</tbody>
</table>

In SNPs that are associated with ICH phenotype, we will seek to confirm the finding with sequencing and RFLP analysis and plan to replicate the findings in cohorts from the United States and Europe in future collaborations. We will make the data and DNA available to other collaborators on request. We will validate any positive findings in our patient population against publically available ethnically diverse population genome data to ensure that any significant findings are not confounded by ethnic differences between our case cohort compared to the control cohorts. We will also investigate correlations between allelic variations and anticoagulant dose and stability of best practice oral anticoagulation.

**Genetic power calculations** With 300 cases of oral anticoagulant-related ICH and >2,000 controls, our study will achieve 96% power with a target α p-value of 0.0000001, assuming ~99% genotyping success in case samples (which our laboratory has been achieving with the Illumina platform) and approximate minor allele frequency (MAF) = 0.3 in cases. This calculation assumes an approximate relative risk maximum for oral anticoagulant-related ICH of 2.0 for the additive risk model (based on MAF dosage per disease locus).31 These power calculations were undertaken by Dr Michael A. Nalls
We expect to pool our data with other international stroke genetics research groups including the International Stroke Genetics Consortium (within which Professor John Hardy is a collaborator), to increase the power of analyses to detect smaller gene variant effects.

**Histopathological samples acquired as a part of routine care**

In patients undergoing surgical evacuation of ICH at any point during their participation in CROMIS-2(AF) or CROMIS-2(ICH), it is expected that any histological samples taken as part of standard diagnostic clinical care will be reported clinically and the samples made available to the CROMIS-2 research team at the co-ordinating centre. Further analysis of these samples may be undertaken by the central research team at UCL. Similarly any tissue samples obtained from a post-mortem investigation (but only if performed as part of standard clinical care) will be requested to be made available to the CROMIS-2 co-ordinating centre.
Sub-study (selected centres only): research post mortem and brain banking

At centres with appropriate local infrastructure, expertise, funding, and with appropriate procedures in place, consent will be sought for future research post mortem and brain banking from patients enrolled in both CROMIS-2 (AF) and CROMIS-2 (ICH). All consent procedures at centres participating in this aspect of the study must follow the code described in the Human Tissue Act. At participating centres, patients in CROMIS-2 (AF) who die during the follow up will already have given valid informed consent for a post mortem examination and removal and storage of tissue. In CROMIS-2 (ICH) consent will be sought where possible and appropriate from patients during life, according to the standard consent procedure. Where this is not possible, the healthcare professional will seek consent from a person with parental responsibility, or a partner, relative or close friend.

Anyone in this study seeking consent for a research hospital post-mortem must be experienced and well informed, with a thorough knowledge of the procedure. They will have been trained in dealing with bereavement, in explaining the purpose and procedures and will have witnessed a post-mortem examination. Those seeking consent may include members of the clinical team involved in the care of the patient before death, and may also include someone closely involved with the pathology department, such as an Anatomical Pathology Technologist (APT) or a specialist nurse.

At all centres participating in the post mortem brain banking study, local protocols must be in place to ensure that the consent process is correct and that the decision has been properly recorded. A specific template will be used for this purpose.
Clinical outcome measures - Study I: CROMIS-2 (AF)

**Primary outcome:** Symptomatic intracranial haemorrhage (confirmed on brain imaging). Intracranial haemorrhage includes any bleeding within the skull, regardless of the site. We will record the incidence of different haemorrhage subtypes (intracerebral, subdural, extradural, subarachnoid).

**Secondary outcomes:** Ischaemic stroke, TIA, death of any cause, subdivisions of intracranial haemorrhage (intracerebral, subarachnoid, subdural, extradural haemorrhage), any major haemorrhagic events other than ICH, cognitive function, dementia, Quality of Life and long term physical disability.

**Summary of what will happen to participants, and when**

**Study I: CROMIS-2 (AF)**

Participants will all have been seen at a hospital, with a clinical diagnosis of stroke or TIA following which oral anticoagulation has been recommended by the treating team. Participants will be invited to take part in the study by an appropriately trained member of the research or clinical team. This will usually be a research nurse, or practitioner, or clinical researcher or treating clinician (junior doctor or consultant). Once participants have had a chance to consider the information about the study and agree to take part, they will have an interview where details of their medical history are recorded. For patients unable to consent for themselves, the guidance in the Mental Capacity Act will be followed, and a personal or nominated consultee will be approached. In such cases, medical information will be obtained either from the participant, personal consultee, GP or medical records as appropriate. Participants will have a bedside assessment of their physical and cognitive function using a questionnaire. All participants will have a blood test for genetic and where possible, biomarker analysis. They will also have an MRI scan of the brain including specific sequences to detect microbleeds. Some patients may be invited to have fundoscopy and retinal photography, a straightforward, non-invasive procedure that will take about 15 to 30 minutes. Participants will then be treated.
according to standard clinical care, with no additional procedures as part of the study. Information about best practice oral anticoagulant control will be recorded in the CRF whilst in hospital by the study team.

After discharge from hospital, patients (or carers / consultees) will be asked to send information about any blood tests of best practice oral anticoagulant control that they have to the central study team. This will be done by postal questionnaire. Patients will also be asked to contact their local study team if they are admitted to hospital for any reason or suffer any bleeding complications related to oral anticoagulation. This information will then be sent to the central study team by the local research team. Planned follow up will be at 6, 12 and 24 months using structured questionnaire to assess for any further stroke or TIA events or other clinical or medication changes. Patients will be contacted by postal questionnaire, or if this is not possible, will be contacted for a brief telephone interview. A cognitive questionnaire may also be completed where possible, at selected centres at 1 year and a final in person follow up visit.

INR values will also be requested from the patient’s GP and/or anticoagulant clinic by postal questionnaire or by telephone.

**Study II: CROMIS-2 (ICH)**

Participants treated in participating hospitals will be invited to take part in the study by an appropriately trained member of the research or clinical team. This will usually be a research nurse or practitioner, or clinical researcher or treating clinician. An invitation letter will be used where appropriate. Once participants have had a chance to consider the information about the study and agree to take part, they will have an interview where details of their medical history are recorded. For patients unable to consent for themselves, the guidance in the Mental Capacity Act will be followed, and a personal or nominated consultee will be approached. In such cases, medical information will be obtained either from the participant, personal consultee, GP or medical records as appropriate. Participants will have a bedside assessment of their physical and cognitive
function using a questionnaire. All participants will have a blood test for genetic and where possible, biomarker analysis. Participants will then be treated according to standard clinical care (which may include a brain scan), but with no additional procedures as part of the study. Patients in CROMIS-2 (ICH) will be followed up by postal questionnaire at 6 months only.

For further details of follow-up procedures please see detailed description in follow-up section, pages 20-22.
Specific points on methodology

Statistical Power Calculations

A prospective inception cohort study of patients with AF started on warfarin showed a rate of ICH of 2.5% in the first year of treatment.\textsuperscript{4} In this cohort acute stroke patients were not included, so we expect the total ICH rate in our cohort to be at least 2.5% per year. The relative risk for ICH of having CMBs is not well established. The largest prospective data in an ischaemic stroke cohort investigated for CMBs published to date\textsuperscript{33} found that of 908 patients with acute ischaemic stroke (96% not taking warfarin), 15/908 (1.7%) suffered ICH at an average follow-up time of 27 months. Of patients with MBs, 4.4% developed ICH, whilst 0.6% of patients without CMBs developed ICH. Thus the relative risk of the finding of CMBs for subsequent ICH is 4.4 / 0.6 = 7.3. If a similar relative risk of 7.3 is found in our study, and if the average risk of ICH matches the previously reported rate of 2.5% per annum, then we would expect a rate of ICH at 2 years follow up of 6.5% in patients with microbleeds, compared with 0.9% without CMBs: this difference would be clinically important and would tip the risk-benefit judgement in favour of avoiding or reducing the intensity of oral anticoagulation, or substituting an antiplatelet agent in patients with CMBs. If we assume that 20% of our patients will have CMBs,\textsuperscript{8} then to have power 0.9 ($\alpha$=0.05) to detect the difference in ICH rate between the groups, as outlined above, with a relative risk attributable to CMBs of 7.3, 739 patients would be needed (sample size calculated with Dr Constantinos Kallis, Medical Statistician, UCL Institute of Neurology, using Stata 10 [StataCorp]). We plan to recruit 1000 patients, to allow for a maximum drop out rate (due to death of unrelated causes or intolerance of oral anticoagulant) of ~30%; we will, however, follow-up all enrolled patients for a median of 2 years.

These estimated risks suggest that we will observe 30 events in total over a two year period (20 in patients with CMBs, and 10 in those without). The ‘rule of 10’ for developing risk models (e.g. Harrell, Regression Modeling Strategies, 2001) suggests that this will allow us to develop a risk model with three predictor variables in total. Any
more than this may result in over-fitting, though shrinkage methods may be employed to guard against this.

A risk model based solely on CMBs would have a sensitivity of 67% and a specificity of 82% for predicting an ICH within 2 years. The positive predicted value would be 14%. A risk model with more variables should improve on these values.
SAMPLE SIZE CALCULATIONS

Based on our sample size calculations we will recruit a total of 1425 patients from UK centres over 47 months. We expect that 20% of our cohort will have CMBs and that 2% will have an ICH within 2 years. If we assume a conservative relative risk of 4, in line with recent estimates (28), then we would expect the rate of ICH at 2 years follow-up to be 5.0% in patients with CMBs, compared with 1.25% in patients without CMBs. This difference would be clinically important and would tip the risk-benefit judgement in favour of avoiding or reducing the intensity of oral anticoagulation, or substituting an antiplatelet agent in patients with CMBs. To detect such a difference as statistically significant at the 5% level with 90% power would require 1425 patients. The best current evidence for the relative risk associated with CMBs in Caucasian populations is 3.9 (33), so we have calculated the power for a range of risk ratios, with all other assumptions kept the same (Figure). The figure suggests that we would still have 80% power to detect a statistically significant effect if the true relative risk was as low as 3.3. Attrition will also reduce power. However, we will still have 80% power even if attrition was as large as 28% (based on a relative of risk of 4).
Figure. Power of the study (without attrition) across a range of risk ratios, based on an overall event rate of 2% over two-year follow-up and 20% of patients having CMBs.

The anticipated ICH event rate of 2% over 2 years taking into account attrition suggests that we will observe up to 30 ICH events in total. The ‘rule of 10’ for developing risk models suggests that this will allow us to develop a risk model with just three predictor variables (45), though more will be possible through use of modern regression techniques (46). It is anticipated that a risk model based solely on CMBs would have a sensitivity of 50% and a specificity of 81% for predicting an ICH within 2 years. A risk model based on more predictors should improve on these values. We expect to use existing summary AF prediction risk scores (incorporating multiple variables, e.g. HAS-BLED) as a single predictor variable to allow us to assess the additional value of including CMBs as a predictor.
**Statistical analysis**

**ANALYSIS SUMMARY**

We plan to compare the rate of ICH between the CMB and CMB-free groups using the log-rank test and will investigate whether the number of CMBs is associated with the risk of ICH using Cox regression. In addition, Cox regression will be used to develop a risk prediction model for ICH. Potential risk factors for the model will be pre-specified in the Statistical Analysis Plan and variable selection methods may be used to reduce the number of predictors in the risk model. Penalised estimation, such as ridge or lasso(46), may be used to guard against over-fitting. Cross-validation, used in conjunction with calibration slopes and the c-index, will be used to internally validate the model and assess calibration, discrimination and predictive accuracy. Missing data, and the reasons for it, will be investigated. Imputation may be used if deemed necessary.
ANALYSIS DETAILS

General Principles

The assumptions underpinning each method will be checked. For example, residuals will be checked for normality where appropriate. The use of transformations or non-parametric methods will be considered if assumptions do not hold. The impact of missing data will be explored in all analyses; sensitivity analyses/multiple imputation will be performed as appropriate. Regression models with interaction terms will be used to perform pre-specified subgroup analyses; the results from these will considered as exploratory because the study is not powered for these. The STROBE guidelines will be followed regarding the reporting of the results of this cohort study.

Flow diagram

A Consort-style flow diagram will be produced to show the numbers of patients:

• Potentially eligible for the study
• Examined for eligibility
• Confirmed eligible
• Included in the study
• Completing each stage of follow-up
• Analysed

Patient Characteristics

Baseline patient characteristics will be described using means (SDs) or medians (interquartile range) for continuous measures, and proportions for categorical measures. These values will be presented by CMB group (with/without) at baseline.

In particular, the following variables will be described (Table 1):

a) Demographic information including age and sex.
b) Clinical information including presence, number and location of CMBs.

These characteristics will be presented separately according to whether CMBs are present at baseline. Confidence intervals and statistical tests (e.g. t-tests and chi-squared tests) will be used as appropriate to investigate whether there are differences between patients with and without CMBs. The number of patients with missing data on variables of interest will also be indicated.

A figure will also be presented showing the number of patients from each centre in the study. This will be broken down CMB status at baseline. The amount of follow-up time available will also be summarised (Table 2).

**Outcome data**

Study participants will be described with respect to their outcome data.

In particular, the following variables will be described:

a) Number and timings of ICH events.

b) Any stroke, cardiac event, death or major bleeding.

Patients with ICH and without ICH events will be described separately with respect to baseline characteristics (Table 3). Confidence intervals and statistical tests (e.g. t-tests and chi-squared tests, though see below) will be used as appropriate to investigate whether there are differences between patients with and without ICHs. The number of patients with missing data on variables of interest will also be indicated.

**Primary Analyses**

The primary analysis will be a comparison of the rate of ICH for patients with and without CMBs at baseline (Table 3). This will be carried out using either a chi-squared or Fisher’s exact test if every patient (who does not have an ICH event) has full follow-up data. Otherwise, a log-rank test will be used. If the latter, the proportional hazards assumption will be investigated. Non-ICH deaths will be regarded as censoring events for this analysis though this assumption will be investigated through sensitivity analyses.
The primary analysis will use all patients but additional analyses may be performed on those patients that actually received oral coagulation. Adjusted analyses will be carried out using either logistic or Cox regression models as appropriate. Adjustment variables will include age and hypertension measured at baseline (Table 4).

**Secondary Analyses**

The secondary analyses will be a comparison of the secondary outcomes for patients with and without CMBs at baseline.

**Risk Modelling**

A risk model that aims to predict the risk of ICH will be developed and validated. The model will be developed using either logistic or Cox regression, depending on the completeness of the follow-up data (Table 5). The risk model will be developed using variables derived from the CMB data, as well as additional variables (measured at baseline). The completeness of a variable will be a factor when considering whether to incorporate it in the regression model.

Due to the anticipated very small number of ICH events, care will need to be taken regarding over-fitting of the risk model. Therefore, few predictors will be included in the model (ideally three) and shrinkage methods will be used to re-calibrate the model. Variable selection, including pre-screening, may be used if many predictors are available for inclusion in the risk model. Relatively large P-values (e.g. P=0.2) will be used with these procedures. If necessary, penalised regression methods (e.g. lasso) will be used instead of standard regression methods to avoid over-fitting.

Bootstrapping methods will be used to validate the model. Calibration will be assessed using (Miller/Cox/van Houwelingen) calibration slopes, discrimination will be quantified using the c-index/ROC area and D-statistic, and predictive accuracy will be quantified using the Brier score. The sensitivity, specificity and positive predicted value (PPV) of the risk model will also be calculated.
**EXAMPLE TABLES**

**Table 1: Characteristics of patients with and without cerebral microbleeds at baseline**

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>With CMB (N = )</th>
<th>Without CMB (N = )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years ± SD (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, N (%)</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Imaging Characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence (% range)</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

...
Table 2: Available follow-up information on patients

<table>
<thead>
<tr>
<th>Information available</th>
<th>With CMB (N = )</th>
<th>Without CMB (N = )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline only, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 months, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 months, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 months, N (%)</td>
<td></td>
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</tr>
</tbody>
</table>

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Sponsor Protocol no: 11/0116  

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### Table 3: Characteristics of patients with and without intracerebral haemorrhage

<table>
<thead>
<tr>
<th></th>
<th>With ICH (N = )</th>
<th>Without ICH (N = )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age in years ± SD (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, N (%)</td>
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<td>...</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Imaging Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence (%, range)</td>
<td></td>
<td></td>
<td></td>
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<td>...</td>
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</tbody>
</table>

* at baseline
### Table 4: Logistic regression analyses to investigate the association between ICH and the presence of CMBs, adjusted for age and hypertension

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of CMBs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 5: Logistic regression analyses to predict ICH using CMB information and other variables

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of CMBs</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>...</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* Note: these odds ratios / coefficients may need to be recalibrated / shrunk to optimise predictive abilities
Table 6: Values of performance measures to assess risk prediction model

<table>
<thead>
<tr>
<th></th>
<th>Development</th>
<th>Bootstrap adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
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<td></td>
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**Recruitment strategy and methods to ensure maximum completeness of data**

We have simplified the protocol to make recruitment and follow-up as straightforward as possible. The study has been adopted as a multicentre study by the Stroke Research Network, allowing research practitioners (if available and agreed locally) to provide service support for the study, including assisting with recruitment, blood taking, organising MRI and follow-up. Additional funding is available to support the research costs of this locally, including payments per patient recruited and additional costs of the MRIs, to allow recruitment and follow-up. The trial co-ordinator will be responsible for
the completeness of all follow-up data. In person follow-up data collection will be undertaken by SRN research practitioners or the research fellows. The study team will undertake regular site visits to ensure satisfactory recruitment and follow-up.

**Reporting and dissemination**

Results will be fed back regularly during the study to The Stroke Association and British Heart Foundation. The results will be presented at scientific meetings and in scientific papers. A study website accessible to the public will be set up providing up to date information on study progress.

**Details of insurance**

University College London holds insurance against claims from participants for injury caused by their participation in this clinical study. Participants may be able to claim compensation if they can prove that UCL has been negligent. However, if this clinical study is being carried out in a hospital, the hospital continues to have a duty of care to the participant of the clinical study. University College London does not accept liability for any breach in the hospital’s duty of care, or any negligence on the part of hospital employees. This applies whether the hospital is an NHS Trust or otherwise.

Hospitals selected to participate in this clinical study must provide clinical negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary can be provided on request.
References


Short title: CROMIS-2


32. Details available at http://pngu.org/~purcell/