Endothelial progenitor cells, potential biomarkers for diagnosis and prognosis of ischemic stroke: Protocol for an observational case control study

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Abstract

Ischemic stroke is a devastating, life altering event which can severely reduce patient quality of life. Despite years of research there have been minimal therapeutic advances. Endothelial progenitor cells (EPCs), stem cells involved in both vasculogenesis and angiogenesis, may be a potential therapeutic target. After a stroke, EPCs migrate to the site of ischemic injury to repair cerebrovascular damage, and their numbers and functional capacity may determine patients' outcome. This study aims to determine whether the number of circulating EPCs and their functional aspects may be used as biomarkers to identify the type (cortical or lacunar) and/or severity of ischemic stroke. The study will also investigate if there are any differences in these characteristics between healthy volunteers over and under 65 years of age. 100 stroke patients (50 lacunar and 50 cortical strokes) will be recruited in this prospective, observational case-controlled study. Blood samples will be taken from stroke patients at baseline (within 48 hours of stroke) and days 7, 30 and 90. EPCs will be counted with flow cytometry. The plasma levels of pro- and anti-angiogenic factors and inflammatory cytokines will also be determined. Outgrowth endothelial cells will be cultured to be used in tube formation, migration and proliferation functional assays. Primary outcome is disability or dependence on day 90 after stroke, assessed by the modified Rankin Scale. Secondary outcomes are changes in circulating

EPC numbers and/or functional capacity between patient and healthy volunteers, between patient subgroups and between elderly and young healthy volunteers. Recruitment started in February 2017 and as of September 2019, 167 participants have been recruited. Recruitment will end in November 2019. West Midlands - Coventry & Warwickshire Research Ethics Committee approved this study (REC number: 16/WM/0304) on September 8, 2016. Protocol version: 2.0. This study will determine whether the number of EPCs can be used as a prognostic or diagnostic marker for IS and is a step towards discovering if transplantation of EPCs may aid patient recovery.

Keywords: ageing; biomarkers; cortical stroke; endothelial progenitor cells; ischemic stroke; lacunar stroke; observational study; stem cells

INTRODUCTION

Stroke is the second leading cause of death worldwide and in the United Kingdom (UK) there are more than 100,000 cases of stroke diagnosed every year (Go et al., 2014). It is a devastating life-threatening condition with patient outcomes ranging between full recovery to needing life-long care. It is the leading cause of disability in the UK with an approximate 1.2 million stroke survivors. The cost to the UK economy including health and social care is estimated at £26 billion (StrokeAssociation, 2018). There are two major stroke sub-types, 85% of strokes are ischemic strokes (IS) with haemorrhagic stroke responsible for the remaining 15%. Depending on the area of the brain affected, neurological deficits can include amnesia, aphasia, dementia, dysphagia and hemiplegia (StrokeAssociation, 2019). The majority of sufferers are older, with ~66% of IS seen in people ≥65 years (Krishnamurthi et al., 2013). Ageing is the strongest risk marker for IS (Roger et al., 2012) with older people having greater mortality and poorer quality of life (Pohjasvaara et al., 1997). Other non-modifiable risk factors for IS include sex and race with coronary heart disease, diabetes and hypertension amongst a few of the most commonly

reported modifiable risk factors (Allen and Bayraktutan, 2008). Despite many years of research there are no clinical biomarkers to predict patient prognosis and no diagnostic markers which may indicate the type of IS a patient has suffered.

IS occurs when the blood vessels supplying the brain become narrowed or blocked, interrupting and reducing the blood supply to the central nervous system. This is usually in the form of an embolism or thrombus. Atheroscelerosis, small vessel disease and heart conditions such as atrial fibrillation and arterial dissection can all cause blood clots leading to IS (Hossmann, 2006). IS can be divided into lacunar and cortical subtypes. The lacunar subtype is associated with small blood vessel occlusions and infarcts deep within the white matter. They represent about 25% of all IS (Sudlow and Warlow, 1997). In contrast cortical strokes are associated with grey matter infarcts and large vessel occlusion.

Endothelial dysfunction is regarded as the main pathology that renders cerebral vessels susceptible to atherosclerosis and subsequent vascular events (Ludmer et al., 1986) and is therefore seen as a precursor to development of cardiovascular disease (Lakatta and Levy, 2003). It is also a predominate cause of lacunar strokes which results from the restriction of arterial blood flow deep within the brain (Lavallee et al., 2013). Abnormal endothelial function is also a primary effect of ageing, with advancing age shown to be associated with endothelial dysfunction in the absence of clinical disease (Vaitkevicius et al., 1993). One of the main causes of endothelial dysfunction appears to be reduced bioavailability in nitric oxide (NO) (Werns et al., 1989) partly through an increase in oxidative stress which can also be caused by ageing (Taddei et al., 2001). A healthy endothelium, vital in sustaining vascular homeostasis and regulating functions such as thrombosis, permeability and inflammation, is crucial in preventing vascular damage and the development of cardiovascular diseases such as IS.

IS has a complex pathogenesis. Following an IS there is a reduction in or compete lack of blood supply to an area of the brain. This results in the loss of oxygen and nutrients necessary for neuronal survival. The lack of oxygen stalls adenosine triphosphate (ATP) production which in turn disrupts cellular homeostasis and the transmembrane ionic gradient. This initiates a cascade of deleterious mechanisms including, acidosis, excitotoxicity, inflammation, oxidative stress, apoptosis and necrosis (Khoshnam et al., 2017). These mechanisms are not limited to neurones but also affect glia, astrocytes and endothelial cells of the blood-brain barrier (BBB). In some cases, once blood flow has been restored the central nervous system can experience reperfusion injury where the original ischemic injury is exacerbated. Reperfusion injury can present as haemorrhagic transformation and is associated with BBB dysfunction (Khatri et al., 2012).

Due to the complexity of IS pathogenesis and pathophysiology there are very few treatments. Currently, recombinant tissue plasminogen activator (r-tPA) is the only fully available medical therapy (Clark et al., 2000). It is complicated by a short therapeutic window of 4.5 hours (Wahlgren et al., 2008) which allows only ~5% of patients to receive therapy (Adeoye et al., 2011). Moreover, roughly 6% of patients administered with r-tPA develop intracerebral haemorrhage (O'Carroll and Aguilar, 2015). An alternative treatment available for IS is thrombectomy. However, it is a relatively new procedure and not fully available in all stroke units. It is also limited to IS presented with clots in a large artery, equivalent to only 10% of patients (Texakalidis et al., 2019). Most patients will also be treated with a combination of antiplatelets, anticoagulants, statins and hypertension lowering medication to prevent subsequent blood clots and therefore IS (Bansal et al., 2013).

Other stroke therapies have focussed on post stroke administration or diet supplementation of antioxidants to tackle oxidative stress (Shuaib et al., 2007; Ye et al., 2013) or calcium antagonists to prevent excitotoxicity (Zhang et al., 2019). However, despite the positive results in preclinical studies, the success has not been translated in clinical trials (Yang et al., 2015; Zhang et al., 2019). Even the current treatment r-tPA has been shown to increase inflammation and neuronal cell damage (Won et al., 2015). There have been recent advances in therapies which may be able to negate the r-tPA induced inflammatory response and contribute to neuronal survival such as selenium nanoparticles (Amani et al., 2019). However, such therapies are in their infancy and currently only being tested in animal models. Some advances have also been made in stem cell therapies, but again the potential shown in preclinical trials in experimental models have failed to translate into positive clinical trials. These trials have shown that the stems cells, predominantly derived from the bone marrow, are be safe but not efficacious (Borlongan, 2019). Therefore, continued research into therapeutic targets is vital.

Recently a new subset of stem cells, endothelial progenitor cells (EPCs) have become a focus of stroke research. EPCs are involved in maintaining appropriate endothelial function and have the ability to proliferate, differentiate and mature into endothelial cells (Yoder, 2012). These cells are present in the circulation and can also be mobilised from the bone marrow, after vascular injury, to home into the site of damage and repair the endothelium (Condon et al., 2004). EPCs are involved in maintaining the integrity and function of the brain vessels (Guo et al., 2017) and through their ability to induce both angiogenesis and vasculogenesis (Asahara et al., 1997) would be crucial to restoring blood flow after IS.

EPCs were first isolated from peripheral blood as mononuclear cells (MNCs) expressing the stem cell, CD34 and endothelial cell KDR, antigens (Asahara et al., 1997). These cells are rare

and make up approximately 0.1-2.0% of total MNCs in bone marrow, peripheral blood and cord blood (Esquiva et al., 2018). The phenotyping of these cells is controversial as a unique marker has yet to be identified (Fadini et al., 2012). The most widely accepted characterisation is the co-expression of the CD34, CD133 and KDR cell surface antigens (Liao et al., 2017). Characterisation is further complicated by different EPC sub populations observed when cultured. These sub populations can be broadly separated into early EPCs and late EPCs or outgrowth endothelial cells (OECs). Only OECs appear to have proliferative and tubulogenic potential and therefore are a target for transplantation (Hur et al., 2004).

Following IS, EPCs are mobilised to migrate from the bone marrow and home into the site of ischemia. Their mobilization is influenced by several signal transduction pathways, predominantly stromal cell-derived factor 1 (SDF-1) and C-X-C chemokine receptor type 4 (CXCR4). During ischemia CXCR4 expression on EPCs is enhanced and SDF-1 expression in injured brain tissue is up regulated, attracting the EPCs to the ischemic tissue (Chen et al., 2012). Endothelial NO synthase (eNOS) dependent signaling is an alternative mobilization pathway. After IS, eNOS is upregulated in EPCs stimulating them to move into the peripheral blood and increase NO which can relax blood vessels and promote blood flow to the injured area (Ohta et al., 2006). The homing of EPCs to damaged vessels is achieved through interactions between P-selectin, β_1/β_2 integrins, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Massberg et al., 2006). In ischemic tissue EPCs perform many functions. They can differentiate and mature into endothelial cells and become part of the new vasculature (Ingram et al., 2005; Hu et al., 2010), repair endothelial damage and through secretion of growth factors such as vascular endothelial growth factor (VEGF), SDF-1α and insulin like growth factor 1 (IGF-1) they can recruit more EPCs, induce

angiogenesis or vasculogensis and protect the neurovascular unit (Imitola et al., 2004; He et al., 2011).

There is strong preclinical evidence indicating at the therapeutic potential of EPCs. Studies with rat and mouse models of permanent and transient middle cerebral artery occlusion (MCAO) have shown EPC transplantation to increase angiogenesis, reduce infarct volume and improve long term neurological outcome (Esquiva et al., 2018). Furthermore, evidence from small trials in patients with myocardial infarction has also shown positive results from EPC transplantation, through improvements in left ventricular function (Liao et al., 2017).

However, despite the interest EPCs have generated, evidence of their levels and function during the acute, subacute and chronic phases of IS is sparse and inconsistent. Both an increase (Paczkowska et al., 2009) and decrease (Chu et al., 2008) in EPC numbers have been recorded for patients with acute stroke compared to healthy controls, with varying time frames of EPC release reported including increases in EPC numbers at 24 hours (Paczkowska et al., 2009) and on day 7 (Marti-Fabregas et al., 2013). Although some studies have shown associations between EPC number and patient outcome (Tsai et al., 2014). The most recent trial with EPCs has shown autologous transplantation to be safe and feasible in IS patients but study outcomes were predominantly statistically neutral (Fang et al., 2019). So far studies with EPCs and IS have been small, focused on correlating prognosis with either EPC number or functionality not both. Furthermore, the correlation between EPC number and days post IS in these studies is inconsistent. To date no study has been conducted which attempts to correlate both EPC number and functionality to prognosis, diagnosis and ageing. Seeing as most IS stroke and adverse vascular events are in people over 65 years of age, this is a vast oversight.

Furthermore, no studies have been conducted in IS patients which comprehensively examine the biochemical profile of patient blood plasma. Various signaling molecules affect the mobilisation, recruitment and homing of EPCs to sites of vascular injury and changes induced by ischemic injury or ageing may also affect the generation and function of EPCs. For example, growth factors such as SDF-1 (Ceradini et al., 2004), VEGF (Hattori et al., 2001) and granulocyte-colony stimulating factor (GCSF) (Powell et al., 2005) have been shown to affect EPC functionality. eNOS, the predominant source of vascular NO and has also been linked to the mobilization of EPCs (Aicher et al., 2003). Since NO bioavailability is impaired with ageing and IS, it is probable that EPC recruitment will also be adversely affected (Ozuyaman et al., 2005). Furthermore, an increase in oxidative stress, inflammation (Cesari et al., 2008) and age (Heiss et al., 2005), risk factors for IS, have also been reported to impair EPC function.

Therefore, we propose a comprehensive study which will examine EPC number, function and response to signalling molecules in the blood of IS patients. We hypothesis that the patient's number and functional capacity of EPCs may determine their clinical outcome. Therefore, a patient's inherent number and activation of EPCs may be a unique prognostic marker of their IS disease. Furthermore, due to the broadly different aetiologies of cortical and lacunar IS, with lacunar strokes associated with small vessel disease and therefore vascular abnormality and endothelial dysfunction (Wardlaw, 2005), we hypothesis the number of EPCs may also prove to be a diagnostic marker in differentiating between these IS subtypes. By looking at all these factors together we will gain a better understanding of the role of EPCs in IS which may then be used in future therapy, either as transplantation of EPCs themselves or through medication of activating signalling molecules.

Objectives

This study aims to investigate whether the number and functional capacity of EPCs present in the peripheral blood of IS patients can used as a prognostic and/or diagnostic marker. To this end patients will be recruited into two IS subgroups of lacunar and cortical strokes and blood samples will be taken at different time points to represent the acute, sub-acute and chronic phase of the disease. This study also aims to investigate whether ageing has an effect on EPC number and functionality. Therefore, healthy volunteers (HVs) will also be split into two subgroups of younger and older than 65 years of age. Finally, the study will investigate the biochemical profile of patient and healthy volunteer blood plasma to see if any inflammatory cytokines, or angiogenic factors correlate with EPC number or functionality.

Methods and Design

Design

This is a single-center, prospective, observational case-controlled study investigating three central objectives, the number of EPCs present in the peripheral blood circulation, the functionality of these EPCs and the biochemical profile of the blood plasma. These objectives will be investigated at four different time points of IS, days 0 (within 48 hours of IS symptom onset), 7, 30 and 90. To do this a 30 mL sample of blood will be taken per participant and time point and then split between the three different objectives (**Figure 1**). In cases where less than 30 mL of blood is collected the counting of EPCs with flow cytometry will be given priority. Researchers conducting these experiments will be blind to patient and healthy volunteer subgroups.

1. 6 mL of blood will be used to count circulating EPCs using flow cytometry. EPCs are defined as cells simultaneously expressing the KDR, CD133 and CD34 cell surface markers (Peichev et al., 2000).

2. The remaining blood will be used to isolate and then culture the mononuclear cells. Any EPCs present in the mononuclear cell population will be encouraged to mature into OECs by culturing cells under conditions which favor EPC growth such as fibronectin coated flasks and media supplemented with endothelial growth factors. To verify the cells obtained as OECs, cells will be stained with DiI conjugated acetylated low density lipoprotein (DiI-AcLDL) and FITC conjugated *Ulex europaeus* agglutinin (FITC-lectin) (Medina et al., 2010). Cells positive for both markers will be considered OECs and used in downstream analysis. The functional capacity of these cells will then be assessed in tube formation, proliferation and migration assays. NO levels and eNOS activity will also be measured in these cells.
3. Plasma will also be extracted from the blood simultaneously. The levels of pro- and anti-inflammatory cytokines and promoters and inhibitors of angiogenesis will be measured. Total antioxidant capacity and NO levels in plasma will also be measured.

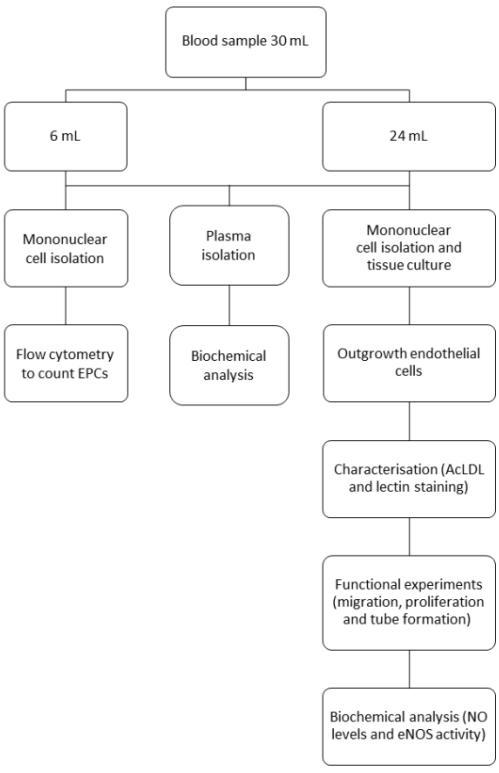

Figure 1 Experimental processing.

Patient population

Patients admitted to Nottingham University Hospitals Stroke Service will be recruited for the study into lacunar (n = 50) and cortical (n = 50) stroke subgroups. Blood samples and independent outcome assessments, modified Rankin Scale (mRS), Barthel Index (BI) and National Institutes of Health Stroke Scale (NIHSS), will be performed at baseline (within 48 hours of stroke) and on days 7, 30 and 90. Blood samples will be processed as in **Figure 1**. The patient pathway is summarized in **Figure 2** and a study assessment schedule is shown in **Table 1**. Medical treatment of eligible patients will be left to the discretion of the attending physician. To encourage participation and retain patients at follow up transport costs will be paid for. The present study allows co-enrolment.

Inclusion criteria

- Within 48 hours of symptom onset
- ≥ 65 years or older
- Anterior circulation IS
- Independence prior to stroke (mRS < 3)
- Ability to give informed consent (directly or via consultee)

Exclusion criteria

- < 65 years of age
- Posterior circulation IS
- Primary intracerebral haemorrhage
- Transient ischemic attack

- Prior IS within the last 3 months

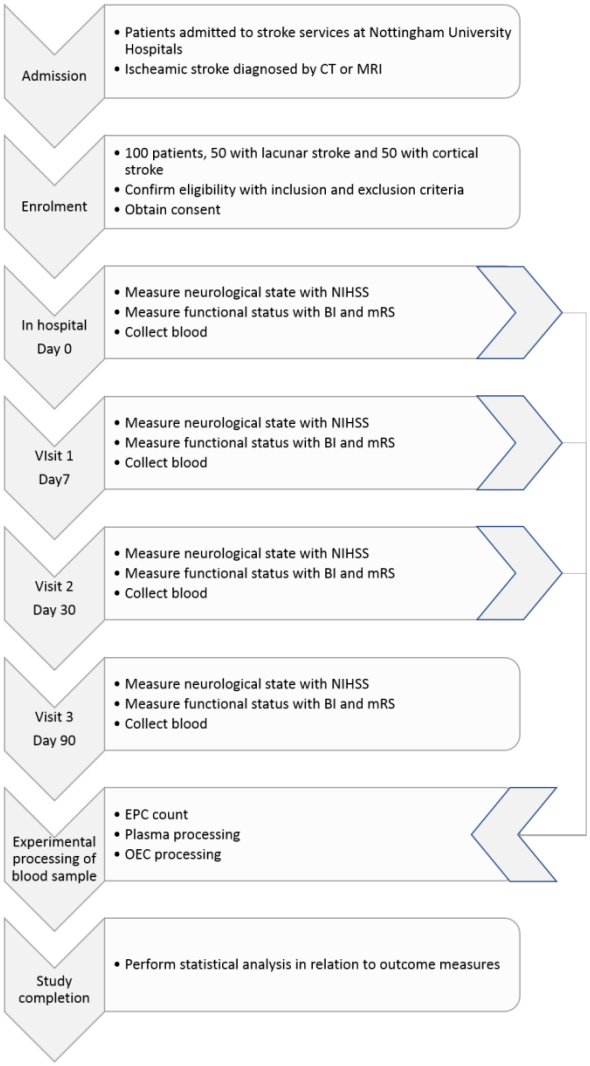

Figure 2 Patient pathway.

Table 1 Patient study assessment schedule

Assessment	Baseline	Day 7	Day 30	Day 90
Consent	X			
Contact details	X			
Living circumstances	X			
Lifestyle	X			
Medical history	X			
Medication history	X	X	X	X
mRS (primary outcome)	X	X	X	X
NIHSS	X	X	X	X
BI	X	X	X	X
Blood sample	X	X	X	X

Healthy volunteers

100 HVs will be recruited into two subgroups of, 50 individuals ≥ 65 years (elderly) and 50 individuals between 18 and 64 years (young). As it is unlikely that the EPC characteristics would vary within 3 months, a blood sample will be taken only once. Blood samples will be processed as in **Figure 1**. The HV pathway is summarized in **Figure 3** and a study assessment schedule in shown in **Table 2**.

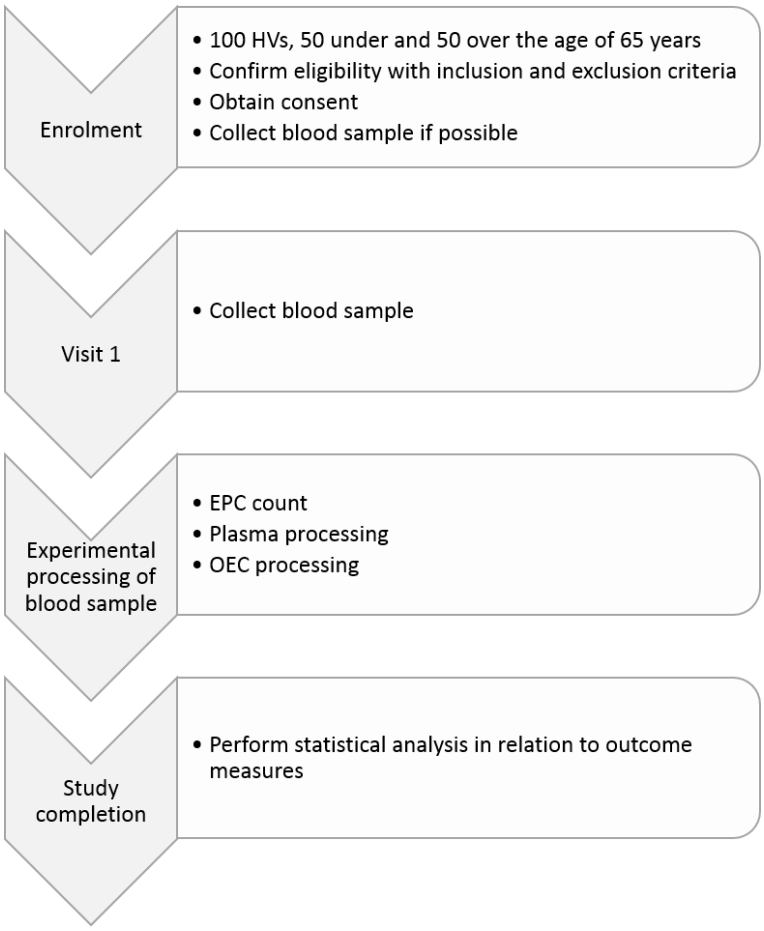

Figure 3 Healthy volunteer pathway.

Table 2 Healthy volunteer study assessment schedule

Assessment	Baseline
Consent	X
Contact details	X
Living circumstances	X
Lifestyle	X
Medical history	X
Medication history	X
Blood sample	X

Inclusion criteria

- No previous history of stroke.
- Ability to give informed consent.

Exclusion criteria

- Previous history of stroke.

Outcome measures

Primary outcomes

- Detection of disability or dependence in daily activities on day 90 after IS assessed by the mRS.

Secondary outcomes

Changes in EPC numbers and functional capacity between

- Stroke patients and HVs,
- Patients with lacunar or cortical stroke at abovementioned time points and
- Elderly and young HVs.

Other outcomes

Changes in

- plasma angiogenic factor levels,
- plasma total antioxidant capacity and NO levels,
- plasma pro- and anti-inflammatory cytokine levels, and
- EPC eNOS activity and NO levels

between stroke patients and HVs.

Adverse events

As this is an observational study, no adverse event is anticipated. Adverse events of venepuncture will be treated according to standard practice.

Ethics approval and consent

West Midlands - Coventry & Warwickshire Research Ethics Committee approved this study (REC number: 16/WM/0304) on September 8, 2016 (**Additional file 1**). This study is sponsored by the University of Nottingham. Written informed consent was obtained from all participants including the patients for their anonymised information to be published in this study. Protocol version: 2.0. This study followed the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidance for protocol reporting (**Additional file 2**). The Bayraktutan Dunhill Medical Trust EPC Study was registered in clinicaltrials.gov (NCT02980354) on November 15, 2016.

Lack of capacity will be determined by the attending physician. Potential participants will be provided with a Participant Information Sheet and given 24 hours to consider whether to consent but will be allowed to consent at an earlier time if they prefer (**Additional files 3 and 4**). It will be explained to the potential participant that entry into the study is entirely voluntary and that their treatment and care will not be affected by their decision and that they can withdraw at any time. In cases where stroke patients regain capacity, they will be re-consented for their ongoing participation in the study.

Participants may be withdrawn from the study in cases of disease development and withdrawal of consent. If withdrawn, it will be explained to participants that their data collected so far

cannot be erased and may be used in the final analyses. If a participant who has previously given informed consent loses capacity to consent during the study the participant would be withdrawn from the study.

Study data and monitoring body

A trial steering committee will meet on a regular basis to check progress of the study and any issues arising. The minimum required information for the purposes of the study will be collected. All paper data will be held securely, in a locked room or cabinet and access to the information will be limited to the study staff and investigators and relevant regulatory authorities. Electronic data including the study database will be held securely and password protected. All data including the participant's medical records and hospital notes will be treated confidentially in the same way as all other confidential medical information.

Study data will be monitored to confirm informed consent, source data and data storage and transfer procedures. Furthermore, entries on study forms will be verified by inspection against the source data. A sample of study forms (10% or as per the study risk assessment) and their corresponding database entry will be checked on a regular basis for verification of all entries made. If corrections are needed a full audit trail and justification will be required. Study data and evidence of monitoring systems will be made available for inspection when required.

Sample size estimates

The null hypothesis (H_0) is that the severity of stroke assessed by the mRS on day 90, will not be affected by EPC number. The alternative hypothesis (H_1) is that the severity of stroke will be linked to the number of EPC and that patients with higher numbers and functional capacity of EPCs will recover better. Assuming overall significance of $P = 0.025$, power (1-beta) = 0.90

and EPC number difference of 9 cells/mL with a SD of 11, a sample size of 38 is required for each group. Permitting for failures of patient attendance on days 30 and 90 (~15% for each time point) and possibility of patients' illness/death during the study (~5%), 50 patients will be recruited for each of the lacunar and cortical stroke subgroups. Previous studies of similar or smaller size had adequate power to distinguish meaningful changes in EPC counts (Ghani et al., 2005).

Statistical analyses

All analysis will be undertaken with SPSS statistics software (version 26, IBM, Portsmouth, UK). Continuous variables will be reported as the mean ± SD. Categorical data variables will be displayed as frequency counts and percentages. Continuous variables, such as circulating EPC levels will be analyzed by independent *t*-test between groups. Circulating EPC levels at different time points (baseline and on days 7, 30 and 90 post-stroke) will be compared using the repeated measures of analysis of variance. Scheffe's multiple comparison will be used to analyze the intra-individual courses of parameters over time. These will then be compared among patients with lacunar and cortical strokes. Multiple logistic regression analyses will be used to determine the independent impact of different predictive variables on functional outcome and neurological deficits. Data will be corrected for individual factors such as gender and medications known to affect EPC characteristics such as statins (Shao et al., 2008).

Dissemination Policy

Once all statistical analysis have been performed, data will be published in an appropriate journal. Results will also be disseminated to trial participants.

Discussion

IS is a devastating condition with the majority of sufferers ≥65 years. Currently there is only one approved therapy, r-tPA and despite continuing research into IS, prognostic and diagnostic markers are yet to be identified. EPCs, which are capable of inducing angiogenesis and vasculogenesis, may prove to be effective diagnostic markers and offer therapeutic potential in the future. This study aims to elucidate the role of EPCs in IS and ageing. The study has been carefully designed through strict patient inclusion and exclusion criteria, recruitment of old and young HVs and the identification of many primary and secondary outcomes to optimise the blood donated. Measuring the number and function of EPCs will provide a better understanding of how these cells may help repair the damaged cerebrovasculature. Furthermore, identifying the biochemical profile of key angiogenic and inflammatory factors in plasma will give insight into any underlying elements which may affect the number and/or function of EPCs. This study aims to comprehensively look at the role of these cells in relation to the type of IS, patient outcome, ageing and pathophysiological factors which affect the function of EPCs.

Trial Status

Patient recruitment began in February 2017 and will end in November 2019. Recruitment is ongoing and as of September 2019 a total of 81 patients have been recruited with 43 in the cortical subgroup and 38 in the lacunar subgroup. A total of 86 HVs have been recruited, 49 in the young subgroup and 37 in the old subgroup. Primary outcome analysis will be completed by November 2019. Data collection will finish in January 2020. The study and data analysis will finish in February 2020.

Additional files

Additional file 1: Ethical approval documentation.
Additional file 2: SPIRIT checklist.

Additional file 3: Patient consent form.
Additional file 4: Healthy volunteer consent form.

Author contributions: KR drafted the manuscript and processes the blood samples. OO processes the blood samples. UB researched literature and conceived the study and is the chief investigator. NS and PB are co-investigators. All authors reviewed, edited and approved the final version of the manuscript.
Conflicts of interest: The authors declare that they have no conflicts of interest.
Funding: This work was supported by a grant to Dr Ulvi Bayraktutan from The Dunhill Medical Trust (R459/0216).
Institutional review board statement: Ethical approval was obtained from West Midlands - Coventry & Warwickshire Research Ethics Committee approved this study (REC number: 16/WM/0304) on September 8, 2016.
Declaration of participant consent: The authors certify that they will obtain all appropriate participant consent forms. In the forms, the participants will give their consent for the participants' images and other clinical information to be reported in the journal. The participants understand that the participants' names and initials will not be published and due efforts will be made to conceal their identity.
Reporting statement: This study followed the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidance for protocol reporting.
Biostatistics statement: The statistical methods of this study will be reviewed by the biostatistician of the Division of Clinical Neuroscience, Stroke, University of Nottingham, UK.
Data sharing statement: Anonymized individual participant data (IPD) will be available 12 months after publication of all relevant research findings. Anonymized IPD underlying the results presented in these articles including tables, figures or supplementary material, will be shared as per the regulations of the University of Nottingham indicated on https://www.nottingham.ac.uk/fabs/rgs/research-data-management/data-sharing-and-archiving/sharing-data.aspx. All requests concerning data sharing should be made to the chief investigator via email ulvi.bayraktutan@nottingham.ac.uk. Personal results will also be available to participants upon request. If requested, study protocols, forms and outputs of statistical analysis will be available. Enquiries can be made to the chief investigator at the email address above.

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