RESEARCH ARTICLE

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, 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. The Bayraktutan Dunhill Medical Trust EPC Study was registered in ClinicalTrials.gov (NCT02980354) on November 15, 2016. This study will determine whether the number of EPCs can be used as a prognostic or diagnostic marker for ischemic strokes and is a step towards discovering if transplantation of EPCs may aid patient recovery.

Key Words: *ageing; biomarkers; cortical stroke; endothelial progenitor cells; ischemic stroke; lacunar stroke; observational study; stem cells*

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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 (Stroke Association, 2018). There are two major stroke subtypes, 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. Atherosclerosis, 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 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. 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 blood-brain barrier 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 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 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 mononuclear cells 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 subpopulations 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 C-X-C chemokine receptor type 4 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 nitric oxide 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 nitric oxide (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, β1/β2 integrins, intracellular adhesion molecule-1 and vascular cell adhesion molecule-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, SDF-1α and insulin like growth factor 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 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), vascular endothelial growth factor (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 hypothesize 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 etiologies of cortical and lacunar IS, with lacunar strokes associated with small vessel disease and therefore vascular abnormality and endothelial dysfunction (Wardlaw, 2005), we hypothesize 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, subacute 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.

I. Blood (6 mL) 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).

II. 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 and FITC conjugated Ulex europaeus agglutinin (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.

III. 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.

Study 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 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 old 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

Healthy volunteers

100 HVs will be recruited into two subgroups of, 50 individuals ≥ 65 years old (elderly) and 50 individuals between 18 and 64 years old (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**.

Inclusion criteria

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

Exclusion criteria

- Previous history of stroke

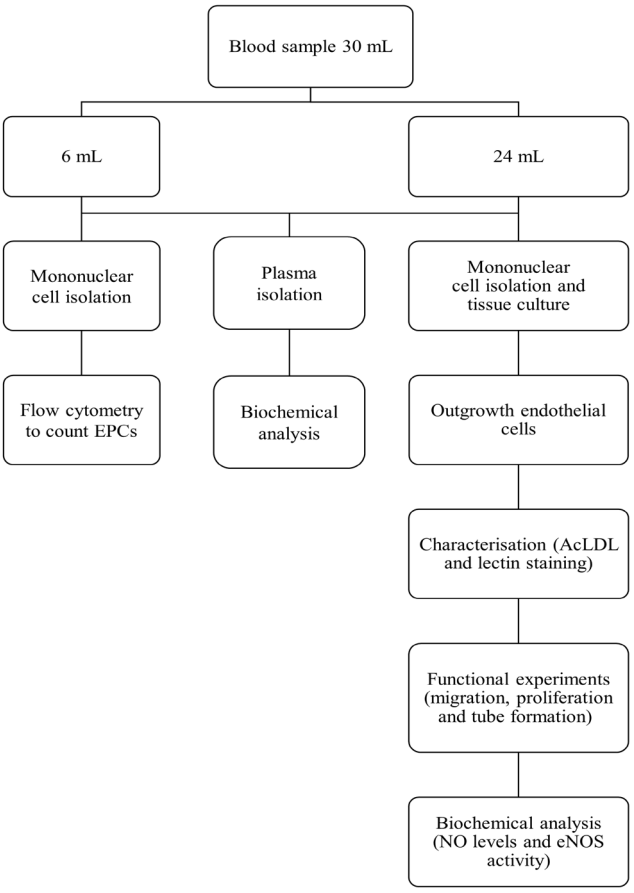

Figure 1 Experimental processing.
AcLDL: Acetylated low density lipoprotein; eNOS: endothelial NO synthase; NO: nitric oxide.

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
Barthel index	X	X	X	X
Blood sample	X	X	X	X

mRS: Modified Rankin Scale; NIHSS: National Institutes of Health Stroke Scale.

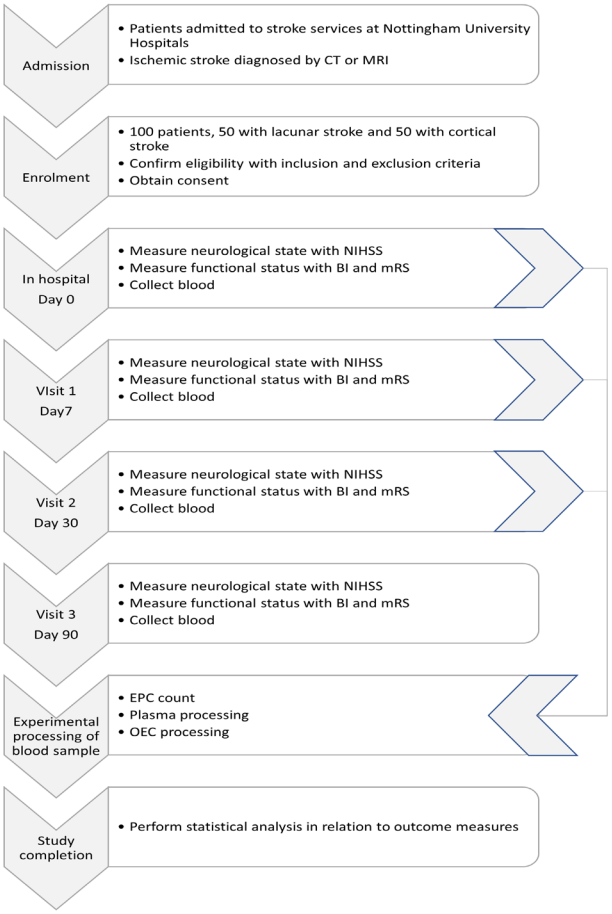

Figure 2 Patient pathway.
BI: Barthel Index; CT: computerized tomography; EPC: endothelial progenitor cell; MRI: magnetic resonance imaging; mRS: modified Rankin Scale; NIHSS: National Institutes of Health Stroke Scale; OEC: outgrowth endothelial cell.

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

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.

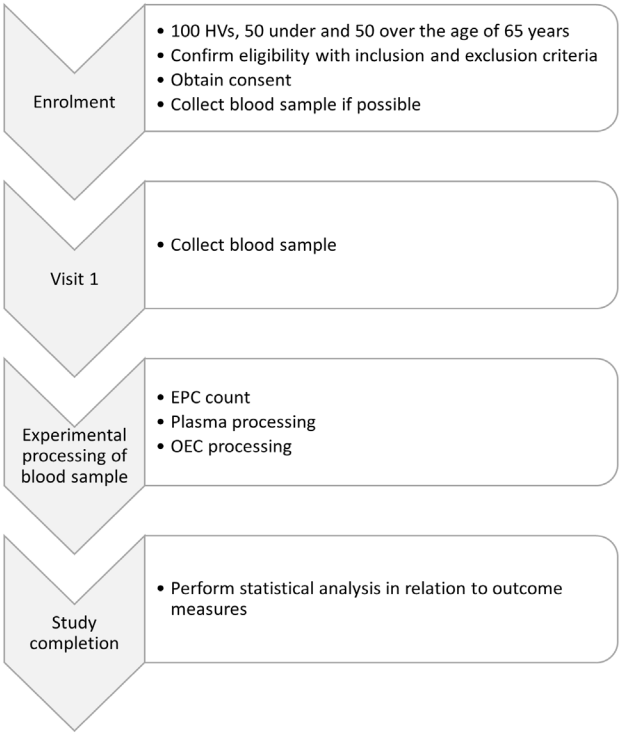

Figure 3 Healthy volunteer pathway.
EPC: Endothelial progenitor cell; HVs: healthy volunteers; OEC; outgrowth endothelial cell.

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 earli-

er 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 (H0) is that the severity of stroke assessed by the mRS on day 90, will not be affected by EPC number. The alternative hypothesis (H1) 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 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 elderly 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.

Author contributions: *KR drafted the manuscript and processes the blood samples. OO processes the blood samples. UB searched literature*

and conceived the study and is the principle 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.*

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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 their images and other clinical information to be reported in the journal. The participants understand that their 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.*

Plagiarism check: *Checked twice by iThenticate.*

Peer review: *Externally peer reviewed.*

Open peer reviewer: *Idiris Altun, KSÜ University Medical Faculty, Turkey.*

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.*

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West Midlands - Coventry & Warwickshire Research Ethics Committee
The Old Chapel
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NG1 6FS

Telephone: 0207 104 8069

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

08 September 2016

Dr Ulvi Bayraktutan
University of Nottingham
Stroke, Division of Clinical Neuroscience
Clinical Sciences Bulding, Hucknall Road
Nottingham
NG5 1PB

Dear Dr Bayraktutan

Study title:	**ENDOTHELIAL PROGENITOR CELLS: POTENTIAL BIOMARKERS FOR DIAGNOSIS AND PROGNOSIS OF ISCHAEMIC STROKE**
REC reference:	**16/WM/0304**
Protocol number:	**16057**
IRAS project ID:	**206919**

Thank you for your submission of 1 September 2016, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Rachel Nelson at NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net

Confirmation of ethical opinion

The Committee advised that in each case where it is applicable, the age ranges should be stated as 1-64 and 65 and older, and not as 18-65. This is a recommended action point only and the changes can be made without the need for resubmission.

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for

medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [cover letter]	1.0	13 June 2016
Covering letter on headed paper [cover letter]	2	30 August 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [University of Nottingham Clinical Trials Insurance]		14 June 2016
GP/consultant information sheets or letters [GP letter]	1.0	10 June 2016
IRAS Application Form [IRAS_Form_16062016]		16 June 2016
IRAS Application Form XML file [IRAS_Form_14062016]		14 June 2016
IRAS Application Form XML file [IRAS_Form_16062016]		16 June 2016
Letter from funder [Award letter]		10 March 2016
Letter from sponsor [sponsor letter]		14 June 2016
Other [Schedule of Events]	1.0	13 June 2016
Other [Statement of Activities]	2.0	15 August 2016

Other [itemised responses]	1.0	15 August 2016
Other [requested letter - non-emergency research]	1.0	15 August 2016
Participant consent form [Participant consent form]	2.0	15 August 2016
Participant consent form [consultee advice form]	2.0	15 August 2016
Participant consent form [consent after consultee]	2.0	15 August 2016
Participant consent form [Consent form for HVs]	2.0	15 August 2016
Participant information sheet (PIS) [PIS]	2.0	15 August 2016
Participant information sheet (PIS) [PIS after consultee]	2.0	15 August 2016
Participant information sheet (PIS) [consultee PIS]	2.0	15 August 2016
Participant information sheet (PIS) [Healthy Volunteer IS]	2.0	15 August 2016
Referee's report or other scientific critique report [referees comments]		12 February 2016
Referee's report or other scientific critique report [responses to referees]		28 February 2016
Research protocol or project proposal [PROTOCOL]	2.0	15 August 2016
Summary CV for Chief Investigator (CI) [CV for Ulvi Bayraktutan]	1.0	10 June 2016
Validated questionnaire [Barthel Index]		12 June 2016
Validated questionnaire [Modified Rankin Scale]		12 June 2016
Validated questionnaire [NIH Stroke Scale]		12 June 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form

available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/WM/0304	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Dr Helen Brittain (Chair)
Chair

Email:NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Angela Shone*
Dr Maria Koufali, Nottingham University Hospitals NHS Trust

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	√
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	√
	2b	All items from the World Health Organization Trial Registration Data Set	√
Protocol version	3	Date and version identifier	√
Funding	4	Sources and types of financial, material, and other support	√
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	√
	5b	Name and contact information for the trial sponsor	√
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	√
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	√
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	√
	6b	Explanation for choice of comparators	√
Objectives	7	Specific objectives or hypotheses	√

Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	√
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	√
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	√
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	√
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	√
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	√
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	√
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	√
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	√
Methods: Assignment of interventions (for controlled trials)			
Allocation:			

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	√
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	√
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	√
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	√
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	√
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	NA

Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	√
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	NA
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	√
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	√
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	√
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	√
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA

Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	√
	31b	Authorship eligibility guidelines and any intended use of professional writers	NA
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	√
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	√
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	NA

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.