SUPPLEMENTAL MATERIAL

**The PASTIS trial. Testing tadalafil for possible use in vascular cognitive impairment**

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**Expanded Materials & Methods**

Standard Protocol Approvals, Registrations, and Patient Consents

The trial received ethical approval from the UK National Research Ethics Service, London-Brent Committee on 6th May 2015 (REC reference: 15/LO/0714). Written informed consent was obtained from all participants or their next of kin. The trial was prospectively registered in the European Union Clinical Trials Register (EudraCT number 2015-001235-20; registered 13/05/2015) and in the ClinicalTrials.gov database (NCT02450253; registered 18/05/2015).

Trial design – Randomisation and Treatment

All participants were recruited as part of a phase-II double-blind crossover trial: Perfusion by Arterial spin labelling following Single dose Tadalafil In Small vessel disease (PASTIS)[1]. Participants were enrolled by members of the trial team (MMHP, RG, ST, RW) and randomised to order of treatment (tadalafil 20 mg, placebo; oral administration). The randomisation list was generated in advance by Sharp Clinical Services, Crickhowell, Powys, UK. Randomisation was in blocks as detailed in the Client Study Information form kept in the Sponsor Site File. Random allocation was implemented as sequentially numbered participant packs, each containing two identical child-resistant, tamper-evident bottles, one holding a tadalafil 20mg capsule and one a matched placebo capsule, both over-encapsulated. Each participant received on two separate occasions, visit#1 and visit#2, a placebo dose and a tadalafil 20mg dose which were identical in size, shape, weight and colour.

Two study visits were performed at least 7 days apart, with blood pressure measurement, MRI scanning and a battery of cognitive tests up to 3 h before and 3-5 h after dosing (see Fig.1A). Participants, care providers and those assessing outcomes were all blind to treatment allocation.

Trial Endpoints

The primary endpoint was change in CBF in subcortical brain tissue. This was assessed in three tissue types: DGM, NAWM and WMH. Change in CBF for cortical grey matter was a secondary endpoint.

Power analyses

We aimed to detect a treatment effect of at least 15% in subcortical CBF, with 90% statistical power using a two-tailed paired t-test at the 0.05 significance level. We assumed average CBF (± SD) of 70 (±15) ml/100g/min in grey matter and 30 (±10) ml/100g/min in subcortical white matter. We estimated that a sample size of N=24 would be required in grey matter and N=54 in white matter[1].

Setting of the Study

The trial commenced on 4th September 2015. Participants were recruited from St George’s University Hospitals NHS Foundation Trust and local Participant Identification Centre sites. All patient visits, data management and trial coordination were performed at the St George’s site. PASTIS was adopted onto the UK NIHR Clinical Research Network Portfolio (CRN Study ID# 18978). The trial ended when the pre-determined recruitment target was met (25 January 2018).

Study population

All data were from a cohort of older adults without known diagnosis of dementia, with radiological and clinical evidence of symptomatic SVD. Demographic details are summarized in Table 1. Inclusion criteria were as follows. 1, radiological evidence of SVD, defined as: MRI evidence of lacunar infarct(s) up to 15 mm maximum diameter and/or confluent deep WMH (grade 2 or higher on the Fazekas scale[2]). 2, clinical evidence of cerebral small vessel disease defined as either: lacunar stroke syndrome with symptoms lasting at least 24 hours, occurring at least 6 months prior to visit#1; or: transient ischaemic attack lasting less than 24 hours with limb weakness, hemi-sensory loss or dysarthria at least 6 months previously and with diffusion-weighted MRI performed acutely showing lacunar infarction. If MRI was not performed within 10 days of TIA, a lacunar infarction in an anatomically appropriate position as demonstrated on a subsequent MRI was also deemed eligible. 3, Age at least 50 years. 4, imaging of the carotid arteries with Doppler ultrasound, CT angiography or MR angiography in the previous 12 months, demonstrating less than 70% stenosis in both internal carotid arteries or less than 50% stenosis in both internal carotids if measured in previous 12-60 months. Exclusion criteria included: known diagnosis of dementia; cortical infarction (more than 15 mm maximum width); systolic BP below 90 mmHg; diastolic BP below 50 mmHg; creatinine clearance less than 30ml/min; stroke or TIA within the previous 6 months; concomitant use of PDE5i. A full list of exclusion criteria is given in the published protocol[1].

Clinical Assessments

Participants attended an initial screening visit (“Visit 0”) and completed an eligibility check. Informed consent was documented. During the screening visit, education level and Montreal Cognitive Assessment (MoCA) scores were recorded (Table 1). Following consent, participants attended two study visits (Visit#1, Visit#2) at least 7 days apart [1]. At each study visit, participants underwent systolic/diastolic blood pressure (SBP/DBP) measurements, a cognitive test battery[1] and brain MRI scanning. SBP/DBP measurements were taken from each participant for each visit, first on arrival after resting, then again after MRI scanning, using a validated Omron MX3Plus machine.

Changes to Methods after trial commencement

Amendments were made to the published protocol[1]:

* to perform some cognitive testing in Visit 0 (from 02 September 2015).
* Eligibility criteria were adjusted to allow lower age limit of 50 and lower Creatinine Clearance of 30ml/min (from 29 October 2015).

Blood sampling and analyses

At the end of each study visit, and at least 3 h post dosing, a blood sample (approximately 5 ml) was taken for full blood count. A second blood sample was taken (5 ml) for subsequent analysis of tadalafil concentration. Blood was taken in purple capped EDTA tubes, inverted to mix, and centrifuged at room temperature at 3000 RPM for 5 minutes to remove cellular material. Plasma (approximately 1.5-2.0 ml) was decanted into a labelled plastic cryovial, then transferred to a designated -80 oC freezer. Plasma tadalafil concentration was measured by LC-MS-MS assay (ASI Bioanalytics Ltd, London UK, <https://www.bioanalytics.co.uk/>).

The trial was subject to an ICH-Good Clinical Practice inspection by the Medicines and Healthcare Products Regulatory Agency (MHRA), the statutory regulator in the UK in September 2019 which identified breaches of ICH-GCP associated with sample analysis. The analytical method used was a forensic toxicology procedure rather than a method which had been validated against the European Medicines Agency Bioanalytical Method Validation guidance. Though tadalafil has high freeze/thaw stability [3] the impact of the storage of samples and freeze/thaw cycles, along with other assessments stated in the European Medicines Agency guidance, on tadalafil plasma concentration were not determined during this trial. All tadalafil concentrations reported here were derived from first analysis, so were not subject to repeated freeze/thaw effects. The maximum duration between sample storage at -80 oC and analysis was 892 days (median 454 days, IQR 366-586 days).

An additional ICH-GCP breach identified that plasma tadalafil levels were analysed prematurely in fifteen participants, resulting in the chief investigator (AHH) being unblinded to the treatment group for these individuals. The trial had been designed as a double-blinded randomized control trial which meant that no members of the research team should have been aware of IMP regime of any of the subjects during the trial. As the chief investigator had no direct role in patient assessment or data acquisition and performed none of the data analyses reported, this was not considered to have compromised the trial outcomes or conclusions and the study continues to remain double-blinded (i.e. patient-blinded and clinician-blinded).

Magnetic Resonance Image Acquisition

Whole-brain perfusion MRI was acquired using a 3T scanner (Achieva Dual TX MRI scanner, Philips Medical Systems, Eindhoven, Netherlands) at St George’s University Hospitals NHS Foundation Trust. Whole brain T1-weighted, Fluid Attenuated Inversion Recovery (FLAIR), susceptibility-weighted imaging (SWI) and pseudo-continuous arterial spin labelling (pCASL) images (which included a proton-density weighted image) were acquired. All MRI data were acquired from brain scans performed on a Tuesday or Thursday, pre-dosing scans between the hours of 10:00 and 12:00 and post-dosing scans 14.00-17.00.

*T1-weighted MRI.* Whole brain sagittal 3D T1-weighted images were acquired to enable tissue segmentation with the following protocol: Turbo Field Echo (TFE) sequence with an inversion pre-pulse, TFE factor 240 in multi-shot mode with 3000 ms shot interval, 8° flip angle, TR 7.9 ms, TE 3.8 ms, 1mm×1mm×1.5mm acquired resolution with interpolation to 1 mm isotropic resolution, 1 average and SENSE factor 2 for a 3 minutes 47 seconds acquisition time.

*FLAIR MRI.*2D T2-weighted axial FLAIR images were acquired to detect WMH using the following protocol: T2 weighted turbo-spin-echo sequences with selective fat suppression (TSE-SPIR), TR 11000ms, TE 120ms, TI 2800ms, 0.65mm×1.00mm acquired resolution interpolated to 0.45×0.45 mm over 24 thick slices (5 mm thickness), with 2 averages and a 1.75 SENSE factor for a 3 minutes 51 seconds acquisition time.

*pCASL MRI.*Our pCASL protocol was developed based on the consensus recommendations of the ISMRM Perfusion study group and European consortium for ASL in dementia[4] using the Philips product pCASL sequence in the scanner 5.3 software release. A 64×64 acquisition matrix with 16 slices was used to acquire data with 4 x 4 mm in-plane resolution with 6mm slice thickness and 1 mm slice gap (hence, approximate voxel size 4mm×4mm×6mm). Background tissue suppression and SPIR fat suppression were applied to improve the contrast to noise of the blood perfusion signal. A total of 140 volumes (alternating with and without the spin labelling inversion pulse) were acquired in two separate 10 minute acquisitions using SENSE 2.3, and TE 8ms and TR 4300ms with a labelling duration (τ) = 1800ms and post labelling delay = 2000ms. A fixed labelling distance of 85 mm from the centre of the imaging block was used with the labelling slice positioned below the cerebellum at an angle perpendicular to the carotid arteries (visualized by time of flight angiography). Proton density weighted images were acquired using the pCASL sequence without the inversion pulse and background suppression, but with fat suppression and an increased TR 5000ms to reduce T1 weighting effects (TE 9ms with 8 averages). Supplemental Fig. S1 provides an outline of the MRI data analysis pipeline.

Computation of CBF maps

The pCASL data acquisitions at each visit were corrected for subject movement using the FMRIB software library function *eddy\_correct* ([fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL](https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL)). An average pCASL map was then separately computed for each pCASL data acquisition. The average pCASL maps and the second proton density weighted image were aligned to the initial proton density weighted image in each scan session using the FSL Linear Image Registration Tool (flirt) [5]. These transformations were applied to the motion-corrected pCASL data to ensure all proton density weighted and pCASL images were aligned in the same space. The aligned proton density weighted images were averaged and CBF was computed using *oxford\_asl* (part of the FSL-BASIL toolset, [fsl.fmrib.ox.ac.uk/fsl/fslwiki/BASIL](https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BASIL)). Cerebral blood flow in each voxel was calculated using the standard equation for pCASL[4]:

ml/100g/min (Equation 1)

where SIcontrol and SIlabel are the time-averaged signal intensities in the pCASL control and label images, respectively, and SIPD is the signal intensity of a proton density weighted image. Standard values were inserted into Equation 1 for the brain/blood partition coefficient, λ=0.9 ml/g, the labeling efficiency, τ=0.85, longitudinal relaxation time of arterial blood T1,blood=1650 ms at 3T. An example of a pCASL map is shown in Fig.2.

WMH delineation

WMHs were delineated on each axial slice of the Visit#1 FLAIR images using commercially available JIM software v7.0 (Xinapse Systems Ltd, West Bergholt, Essex, UK). WMH were defined as hyperintense regions, which were (1) not due to presence of blood vessels and (2) not less than 10 mm2 in size and (3) not a narrow band, one pixel wide, along the edge of the ventricles. A binary WMH image was generated and the total WMH volume (in mm3) was computed for each participant. All WMH maps used here were produced by a single operator, blind to treatment allocation and to all clinical details (FAHH). A second, blinded operator (MMHP) independently produced maps for a subset of participants (n=51) and inter-operator agreement was good (WMH volume Intraclass Correlation Coefficient 0.855 [95% CI: 0.760, 0.915]).

Tissue Segmentation

For each scanning session, T1-weighted images in native space were segmented into grey matter, white matter and cerebrospinal fluid (CSF) tissue probability maps (Fig.2). This was performed using a modified form of the standard Statistical Parametric Mapping (SPM Version 12, <https://www.fil.ion.ucl.ac.uk/spm/>) geodesic shooting segmentation and normalization procedure, described in our previous paper[6]. This procedure captures population-specific features (e.g. enlarged ventricles) and allows superior delineation of deep grey matter structures compared to the standard SPM pipeline. The binary WMH mask derived from JIM software was co-registered into native T1-weighted space so as to repair the tissue probability maps for any misclassification caused by WMHs.

Native space T1-weighted and native space FLAIR images were skull-stripped using the brain extraction tool within FMRIB software library (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BET>) then co-registered to the average proton density-weighted image by a process of boundary-based registration (using FSL epi-reg). These 12-parameter linear transformations were used to align the corrected T1-weighted tissue probability maps and the binary WMH map to the CBF maps. A tissue mask in the average proton density-weighted image space was computed. Each voxel in the CBF map was provisionally assigned to either grey matter, normal appearing white matter, WMH or CSF, based on the maximum tissue probability.

Computation of CBF in whole brain tissue  
For the alignment of the T1-weighted tissue segmentation images to the low-resolution pCASL images it was necessary to apply a further segmentation step. This tissue segmentation procedure has not been previously applied to ASL data and employs a novel application of a tissue segmentation algorithm to CBF maps[7]. It is designed to assign voxels with high CBF values to grey matter and low CBF values to white matter segments. The distribution of CBF values within the grey matter and white matter tissue masks computed in the Tissue Segmentation section (above) were entered as empirical priors to a Hidden Markov random field model and segmentation (FMRIB's Automated Segmentation Tool, FAST)[7] to provide an improved segmentation of grey and white matter tissue from the CBF maps. This technique reduces the effects of partial volume and tissue classification errors at the boundary between grey and white matter tissue caused by the large pCASL image voxel size and the relative difference between voxel sizes of the native pCASL and T1-weighted images. In particular, this method assigns voxels with high CBF values at the grey/white matter tissue boundary to the grey matter segment and voxels with low CBF values at the grey/white matter tissue boundary to the white matter segment. To avoid misclassification of CSF and WMH regions, voxels in these regions were not entered into the FAST segmentation step. For each participant at each scan session, median CBF values were calculated for total grey matter, NAWM and WMH (example in Fig.2).

Computation of CBF in deep grey matter structures

Cerebral deep grey matter structures were segmented on native space T1-weighted images using Freesurfer (Freesurfer Version 5.3.0, <https://surfer.nmr.mgh.harvard.edu/fswiki/>). The binary segmentations of the caudate, putamen and thalamus were aligned to the CBF maps by application of the affine transformation computed in Tissue Segmentation (see above). Median CBF values were calculated for all voxels in each of these three anatomical deep grey matter structures across the left and right cerebral hemispheres. The average of these three median values is reported for CBF in deep grey matter (DGM).

Counting cerebral microbleeds in susceptibility weighted scans

All participants were assessed for the presence of cerebral microbleeds (CMBs) using recognised criteria, the BOMBS rating protocol (Cordonnier et al. 2009). Susceptibility weighted (SW) scans were examined visually. CMBs were defined as hypointense foci up to 10 mm in greatest diameter. All microbleed assessments were performed by a single experienced observer (Mr A Shtaya FRCS). SW scans were available for 60 participants, among whom 40 (67%) had at least 1 CMB, 11 (18%) had only 1 CMB, 12 (20%) had 2-3 CMBs and 17 (28%) had more than 3 CMBs (range 4-49).



**Supplementary Figure S1. MRI data analysis workflow**.

FAST, JIM, SPM and freesurfer are all software packages. Most processes are automated. The exception is semi-automatic WMH delineation using JIM software, to produce user-defined WMH maps.

Abbreviations. ASL: arterial spin labelling; CBF: cerebral blood flow; CSF: cerebrospinal fluid; FLAIR: Fluid Attenuated Inversion Recovery; FMRIB: Functional Magnetic Resonance Imaging of the Brain; FSL: FMRIB software library; GM: grey matter; WM: white matter; WMH: white matter hyperintensities.

**Supplemental Table S1. Table of Adverse Events in the PASTIS trial**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Partici-pant No. | Age (y) | Sex | Group 1 or 2 | Treatment at onset of AE | Duration of AE | Description of AE |
| 6 | 75 | f | 2 | Placebo | 2 d | Headache and vomited after visit 1 at home; had passed by the next morning. Withdrew from the trial. |
| 13 | 72 | m | 2 | Placebo | 2 d | Had a cold and did not tolerate first MRI scan, study visit abandoned. |
| 15 | 61 | f | 1 | Placebo | 1 day | Had headache lasting 2 mins after lunch. |
| 17 | 69 | f | 2 | Placebo | 1 day | Diabetes mellitus type 1 (all adult life); had a hypoglycaemic event during visit 1. Resolved after a sugary drink and fruit. |
| 22 | 77 | f | 1 | Placebo | 6 days | Had a chest infection treated by GP with antibiotics between visits 1 and 2. |
| 33 | 59 | f | 2 | Tadalafil | 5 days | COPD Asthma (lifelong); had lower respiratory tract infection. |
| 33 | 59 | f | 2 | Tadalafil | 3 days | Left knee pain (psoriatric arthritis). |
| 37 | 73 | f | 1 | Placebo | 11 days | Sore throat and cough and feeling unwell. Cancelled visit 2 due to inability to lie still with cough. Re-enrolled as #51. |
| 41 | 56 | m | 2 | Placebo | 1 day | Felt flushed and slightly faint for 5 mins, starting about 30 mins after treatment. Recovered spontaneously and felt better after a few hours. |
| 49 | 57 | m | 2 | Placebo | 1 day | Panic attack in MRI scanner during visit 1. Withdrew from the trial. |
| 59 | 72 | f | 1 | Tadalafil | 1 day | Felt lightheaded after first MRI scan on visit 1. Had a sandwich and felt better in 10 mins. |

**Abbreviations**: AE: adverse event. Note that two AEs relate to the same participant (#33).

**Supplemental References**

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