Stroke-on-a-chip

PROTOCOL TITLE

Stroke-on-a-chip

Protocol ID	
Short title	**Stroke-on-a-chip**
Version	**3**
Date	**November 13, 2020**
Coordinating investigator	***J. Hofmeijer, MD PhD*** ***Department of Clinical Neurophysiology, University of Twente & neurologist at*** ***Department of Neurology, Rijnstate*** Wagnerlaan 55, 6815 AD, Arnhem Tel: +31 (0)88 005 8877 E-mail: jhofmeijer@rijnstate.nl
Local Principal investigators	***J. Hofmeijer, MD PhD*** ***Department of Neurology, Rijnstate*** Wagnerlaan 55, 6815 AD, Arnhem Tel: +31 (0)88 005 8877 E-mail: jhofmeijer@rijnstate.nl ***M. Frega, PhD*** ***Department of Clinical Neurophysiology, University of Twente*** Drienerlolaan 5, 7522 NB, Enschede Tel: +31 (0)55 4894051 / 1116 E-mail: m.frega@utwente.nl
Sponsor (in Dutch: verrichter/opdrachtgever)	***Rijnstate Hospital*** ***Department of Neurology*** Wagnerlaan 55, 6815 AD Arnhem Tel: 088 005 8888

Subsidising party	***None***
Independent expert	***J.Molenaar, MD*** ***Department of Neurology, Rijnstate*** Wagnerlaan 55, 6815 AD, Arnhem Tel: +31 (0)88 005 8877 E-mail: jmolenaar@rijnstate.nl
Laboratory sites	***Clinical Neurophysiology, University of Twente*** Drienerlolaan 5, 7522 NB, Enschede Tel: +31 (0)55 4894051 / 1116
Pharmacy	***Not applicable***

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Sponsor ***M.J.A.M. van Putten*** ***University of Twente*** ***Clinical Neurophysiology*** Drienerlolaan 5, 7522 NB, Enschede		
Coordinating Investigator/Project leader/Principal Investigator ***J. Hofmeijer*** ***Clinical Neurophysiology, University of Twente & Department of Neurology, Rijnstate*** Wagnerlaan 55, 6815 AD, Arnhem		

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
AR	**Adverse Reaction**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics; in Dutch: officiële productinformatie IB1-tekst**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: To identify new, individualized, neuroprotective treatments for ischemic stroke, responses to ischemia of human neurons, including inter-individual variation, need clarification. By combining state of the art stem cell biology and organ-on-a-chip technology, we aim to derive neuronal networks of patients with brain infarcts and investigate the effects of simulated cerebral ischemia.

Objective: The primary objective is generation of functional neuronal networks from induced pluripotent stem cells (iPSCs) derived from patients with ischemic stroke. The secondary objectives are to measure neuronal network responses to simulated cerebral ischemia, estimate differences between patients and controls, and estimate variation amongst patients.

Study design: This will be a prospective, experimental, case-control study in human blood.

Study population: Ten adult patients with ischemic stroke will be included in this study. These patients have clearly documented cerebral infarcts. hiPSCs from five age-matched control subjects from our repository will be used as controls.

Study procedures: Collection of heparin diluted blood samples (30cc per subject). Blood samples will be used for derivation of neuronal networks in the laboratory. All subsequent experiments are with these neuronal networks in the lab. Patients will be treated according to local and national guideline for ischemic stroke. There will be no experimental / additional treatment for patients. Standard treatment or care will not be withheld.

Main study parameters/endpoints: Primary outcome measure is network functionality, where a network is considered functional when a minimum of 1/3 of the electrodes shows activity, with a minimum of 6 spikes per minute per active electrode, and a minimum of one synchronous event per minute at eight weeks after plating. Other study parameters include electrophysiological responses to simulated ‘cerebral ischemia’, and network and neuronal properties as studied by immunocytochemical analyses.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Risks of blood sampling are considered negligible.

1. INTRODUCTION AND RATIONALE

Ischemic stroke: individualized treatable targets needed

Acute ischemic stroke is the leading cause of chronic adult disability and the third leading cause of death in the Western world.[1,2] Epidemiological studies predict a rising incidence.[1] The only treatment of proven benefit to reduce neurological impairment is acute re-canalization by intravenous thrombolysis (IVT) or intra-arterial thrombectomy (IAT).[3,4] However, only ~30% of all patients are eligible for either IVT or IAT. Moreover, even with recanalization, recovery rates vary widely.[4] New experimental stroke research on identification of new, individualized treatable targets is one of the three goals set by the European Stroke Organization for 2030.[5]

Patient variation incompletely understood

Even if complete revascularization is obtained, recovery of patients with acute ischemic stroke varies from complete recovery to persistent neurological deficit.[6] Approximately one third recovers, whereas another third deteriorates with an increase of neurological deficit.[7] In 2-10%, space-occupying edema formation causes severe deterioration or death.[8] Variations in outcome cannot be fully explained by variations of (re)perfusion.[9] Electrophysiological and biochemical mechanisms that may contribute to secondary damage or recovery include synaptic failure,[10] cortical spreading depression,[11] excitotoxicity,[12] and formation of heat shock proteins.[9] All these contributors vary substantially between brain areas within one patient and between patients.[9–12]This indicates possible differences between patients with regard to brain responses to ischemia.

Neuroprotection: poor extrapolation from animal studies to patients

Over the past two decades, >1200 experimental studies have been reported providing strong proof of principle that high grade protection of ischemic brain tissue is an attainable goal. Effective treatments included ion channel blockage, neurotransmitter antagonism, and suppression of inflammation.[13] However, in >500 clinical trials, none of these therapies could be translated into clinical effectiveness in patients with ischemic stroke.[13] One important, but underexposed, factor hampering demonstration of efficacy in patients with brain infarcts is the large heterogeneity of patient groups, where the pathophysiology of either recovery or deterioration often remains enigmatic.[2] To identify new, individualized, neuroprotective treatment, inter-individual differences of neuronal responses to ischemia, and causes hereto, need clarification.

Proposed solution: human in vitro model

By combining state of the art stem cell biology and organ-on-a-chip technology, we aim to investigate the effects of simulated cerebral ischemia on human neurons from patients with brain infarcts. We will collect blood from patients with ischemic stroke, derive stem cells, and differentiate into neuronal networks. Herewith, we obtain patient specific neuronal networks that will possibly reflect the inherent vulnerability to ischemia and to neuroprotective medication of a specific patient. Neuronal responses to ischemia and medication will be

related to clinical and neurophysiological properties. Herewith we will obtain insight into patient-specific vulnerability to ischemia and probably identify specific treatment targets.

2. OBJECTIVES

Primary Objective:

To generate functional neuronal networks from induced pluripotent stem cells (iPSCs) derived from patients with ischemic stroke

Secondary Objectives:

To measure neuronal network responses to simulated cerebral ischemia, estimate differences between patients and controls, and estimate variation amongst patients.

Tertiary Objectives

To relate ischemic responses to patient characteristics and to neuronal properties

3. STUDY DESIGN

This will be a prospective, experimental, case-control study in human blood.

4. STUDY POPULATION

4.1 Population (base)

Ten patients with ischemic stroke will be included in this study. These patients should have clearly documented cerebral infarcts, confirmed by MRI or CT imaging. Patients will be included during follow up, at approximately six to eight weeks from stroke onset, or later.

hiPSCs from five age-matched control subjects from our repository will be used as controls.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a patient must meet all of the following criteria:

- Age ≥ 18y
- Clinical and radiological diagnosis of acute ischemic stroke
- Admission to stroke unit
- Capability to provide written informed consent

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Any relevant systemic disease that is expected to interfere with a patient outcome within six months, such as malignancy
- Any progressive neurodegenerative disease
- Severe aphasia (informed consent not possible)

4.4 Sample size calculation

Sample size calculation is hampered by lack of data. A sample size of ten patients and five controls is based on feasibility considerations: cell lines, culturing, and measurements of 15 subjects are possible within 2 years and provide a first estimation of the feasibility of the objectives.

5. TREATMENT OF SUBJECTS

Patients will be treated according to local and national guideline for ischemic stroke. There will be no experimental / additional treatment. Standard treatment or care will not be withheld.

6. INVESTIGATIONAL PRODUCT

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

All study parameters will be collected from neuronal networks *in vitro*. Determinants will be collected from patients, all in the context of current care.

8.1.1 Main study parameter/endpoint

Primary outcome measure is network functionality, where a network is considered functional when a minimum of 1/3 of the electrodes shows activity, with a minimum of 6 spikes per minute per active electrode, and a minimum of one synchronous event per minute at eight weeks after plating.

8.1.2 Secondary study parameters

Electrophysiological responses to simulated 'cerebral ischemia', i.e. reduction of oxygen and glucose levels in the medium at eight to ten weeks after plating, as described previously.[14]

- Percentage reduction in network activity, functional connectivity, and synaptic responses (i.e. network responses to electrical stimulation of one electrode).
- Speed of reduction in network activity, functional connectivity, and synaptic responses.
- Extent of restoration of network activity, functional connectivity, and synaptic responses after restoration of oxygen and glucose levels.

8.1.3 Tertiary study parameters (potential factors that associate with ischemic responses)

Network and neuronal properties as studied by immunocytochemical analyses at eight weeks after plating.

- Excitation-inhibition ratio defined as the number of AAV-positive (excitatory) and AAV-negative (inhibitory) synapses
- Synaptic density, defined as the number of synaptophysin-positive puncta per neuron
- Apoptosis as visualized by Caspase 3/7 staining
- Cell death as visualized with propidium iodide

8.1.4 Determinants (potential clinical predictors of ischemic responses)

Demographic, baseline clinical and radiological parameters, and functional recovery of patients will be collected in the context of current care. Potential predictors of ischemic responses include age, sex, medical history, cardiovascular risk factors, stroke severity, medication, radiological stroke characteristics, and signs of small vessel disease. Also, data on final infarct size and functional recovery (modified Rankin Scale at three months) will be collected.

8.2 Randomisation, blinding and treatment allocation

Not applicable.

8.3 Study procedures

Procedures in patients

All patients with acute brain infarcts will be admitted to a stroke unit to receive diagnosis and treatment as usual, as described in national and local stroke unit protocols. Standard CT or MRI, CT-angiography, CT-perfusion, and measures of neurological impairment and functional recovery are collected in the context of current care.

Patients will be informed about the study after admission on the stroke unit or at the time of follow up by the treating physician. If the patient gives permission, the treating physician will contact the research coordinator or study nurse. The research coordinator or study nurse will then contact the patient and provide additional information, both orally and written. The patient will be able to read the information carefully, discuss with family and ask questions. Patients will have at least one week to consider participation. . Inclusion will take place at the time of follow up, approximately six to eight weeks after infarction.

After oral and written informed consent, 30cc of venous blood in heparin anticoagulant (3x10cc) will be collected, handled, and stored according to local guidelines. Blood samples from patients will be collected during routine follow up at the hospitals outpatient department. Blood samples will be collected during routine blood sampling, if possible. If not, there will be one additional puncture. There will be no additional hospital visit.

Procedures in blood samples

Blood samples will be transported to University of Twente, CNPH lab, for further work-up and analysis. In short, we will generate iPSCs lines by exposing lymphoblast to reprogramming factors using a non-integrating episomal plasmid system to acutely express the Yamanaka reprogramming factors.[15,16] hiPSC clones will be validated for pluripotency and genomic integrity through a standardized battery of quality control tests. iPS cells will be directly derived into excitatory and inhibitory neurons as published previously.[17] Physiological ratios of excitatory and inhibitory neurons will be grown on Multiwell-MEAs for non-invasive, continuous recording of network functioning through extracellular electrodes. In parallel, cultures plated on coverslips will be fixed in 4% paraformaldehyde in 0.1M PBS at pH 7.4 for immunocytochemical staining (Figure 1). Staining protocols are available on request.

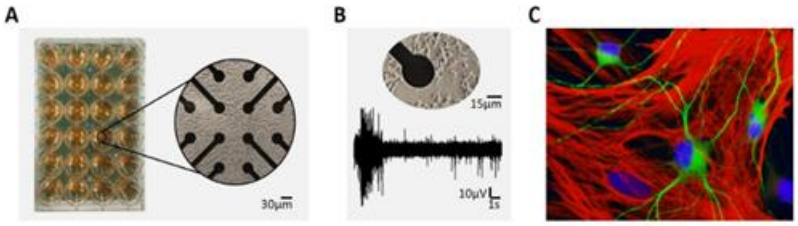

Figure 1. Examples. A) Multi-well multi-electrode array (MEA) plate; enlargement of one well with electrodes and neuronal network. B) Enlargement of micro-electrode with surrounding cultured neurons (upper) and spikes and burst recorded from this electrode (lower panel). C) Neurons (MAP2 staining, green) and astrocytes (GFAP staining, red) derived from hiPSCs of a healthy subject.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for medical reasons.

8.5 Replacement of individual subjects after withdrawal

Subjects will not be replaced after withdrawal.

8.6 Follow up of individual subjects after withdrawal

Subjects will be followed up after withdrawal.

8.7 Premature termination of the study

Not applicable.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

Not applicable.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the study procedures.

We request permission to only report AEs that are related to blood sampling, such as bleeding or hematoma.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

We request permission to only report SAEs that are related to blood sampling, such as bleeding or hematoma.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

9.5 Safety Committee

Not applicable

10. STATISTICAL ANALYSIS

10.1 Primary study parameter

Network activity and functionality are measured and quantified by standard equipment and software, and presented in a descriptive way. Networks are considered functional when at least 1/3 of all electrodes shows activity, with a minimum of 6 spikes per minute per active electrode, and a minimum of one synchronous event per minute at eight weeks after plating.

Fifteen networks belonging to three independent batches will be cultured from each patient and control. The fraction of cultures that develops functional networks will be calculated.

10.2 Secondary study parameters

Electrophysiological responses to ischemia will be measured with our standard experimental set up.

Differences with regard to electrophysiological responses to ischemia will be analysed between patients and controls with parametric or non-parametric statistical tests, depending on normality of the data.

Variance of electrophysiological responses to ischemia will be analysed by means of two-way ANOVA and post hoc Bonferroni correction.

- Between patients, i.e. inter-patient variance
- Between networks from one individual subject, i.e. intra-patient variance

If responses to ischemia differ between patients, inter-patient variance should be larger than intra-patient variance.

10.3 Other study parameters

In order to identify clinical factors possibly associated with of ischemic response, simple and logistic regression analyses will be applied. Covariates that show possible associations with ischemic responses ($P<0.10$) will be conceived as possible predictors. However, our sample size will be too small to identify independent predictors. If our approach will be successful, identification of independent predictors will be studied in a larger patient group.

In order to identify neuronal properties that are possibly associated with ischemic responses, simple logistic regression analyses will be applied as well.

10.4 Interim analysis (if applicable)

Not applicable

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (7th revision, Fortaleza, 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO) and other guidelines.

11.2 Recruitment and consent

Consecutive patients who meet the inclusion criteria will be asked for informed consent at the stroke unit or out-patient department of participating hospitals by the treating doctor, coordinating / principle investigator, or dedicated study nurse. The patient information letter and informed consent form are attached to this file. There will be sufficient time for consideration, at least half a day.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable: minors or incapacitated patients will not be included

11.4 Benefits and risks assessment, group relatedness

No relevant additional risks are expected for patients or controls.

11.5 Compensation for injury

Since no relevant additional risks are expected, we request exemption from the requirement of a liability insurance for patients and controls.

11.6 Incentives (if applicable)

Not applicable

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

All patients will receive a study number by which all data will be coded. The study coordinator and the principal investigators will have access to the source data, if necessary. The code will be safeguarded by the study coordinator. The study data will be stored digitally and saved for 15 years.

12.2 Monitoring and Quality Assurance

Not applicable

12.3 Amendments

Relevant amendments will be notified to the METC that gave a favourable opinion. Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit. Of note, neuronal cultures will be kept and studied thereafter.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final

study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

Publications will be by both principle investigators and engaged PhD or master students, if applicable. The collaborating investigators will adhere to the CCMO guideline on publication, as published on https://www.ccmo.nl/publicaties/publicaties/2002/03/15/ccmo-notitie-publicatiebeleid. Details on the publication policy are kept in a separate agreement.

13. STRUCTURED RISK ANALYSIS

Not applicable

14. REFERENCES

1. Hachinski V, Donnan GA, Gorelick PB, et al. Stroke: working toward a prioritized world agenda. Stroke J Cereb Circ. 2010;41:1084–1099.

2. GBD 2016 Neurology Collaborators. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2019;18:459–480.

3. Hacke W, Kaste M, Bluhmki E, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med. 2008;359:1317–1329.

4. Berkhemer OA, Fransen PSS, Beumer D, et al. A randomized trial of intraarterial treatment for acute ischemic stroke. N Engl J Med. 2015;372:11–20.

5. Norrving B, Barrick J, Davalos A, et al. Action Plan for Stroke in Europe 2018–2030. Eur Stroke J. 2018;3:309–336.

6. Jansen IGH, Mulder MJHL, Goldhoorn R-JB, MR CLEAN Registry investigators. Endovascular treatment for acute ischaemic stroke in routine clinical practice: prospective, observational cohort study (MR CLEAN Registry). BMJ. 2018;360:k949.

7. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. Circulation. 2011;123:e18–e209.

8. Hofmeijer J, Algra A, Kappelle LJ, van der Worp HB. Predictors of life-threatening brain edema in middle cerebral artery infarction. Cerebrovasc Dis Basel Switz. 2008;25:176–184.

9. del Zoppo GJ, Sharp FR, Heiss W-D, Albers GW. Heterogeneity in the penumbra. J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab. 2011;31:1836–1851.

10. Hofmeijer J, van Putten MJAM. Ischemic cerebral damage: an appraisal of synaptic failure. Stroke J Cereb Circ. 2012;43:607–615.

11. Lauritzen M. Pathophysiology of the migraine aura. The spreading depression theory. Brain J Neurol. 1994;117 (Pt 1):199–210.

12. Wang Y, Denisova JV, Kang KS, Fontes JD, Zhu BT, Belousov AB. Neuronal Gap Junctions Are Required for NMDA Receptor–Mediated Excitotoxicity: Implications in Ischemic Stroke. J Neurophysiol. 2010;104:3551–3556.

13. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology. 2008;55:363–389.

14. Hofmeijer J, Mulder ATB, Farinha AC, van Putten MJAM, le Feber J. Mild hypoxia affects synaptic connectivity in cultured neuronal networks. Brain Res. 2014;1557:180–189.

15. Brouwer M, Zhou H, Nadif Kasri N. Choices for Induction of Pluripotency: Recent Developments in Human Induced Pluripotent Stem Cell Reprogramming Strategies. Stem Cell Rev. 2016;12:54–72.

16. Fujimori K, Tezuka T, Ishiura H, et al. Modeling neurological diseases with induced pluripotent cells reprogrammed from immortalized lymphoblastoid cell lines. Mol Brain. 2016;9:88.

17. Frega M, van Gestel SHC, Linda K, et al. Rapid Neuronal Differentiation of Induced Pluripotent Stem Cells for Measuring Network Activity on Micro-electrode Arrays. J Vis Exp JoVE. Epub 2017 08.