

# Supplemental material

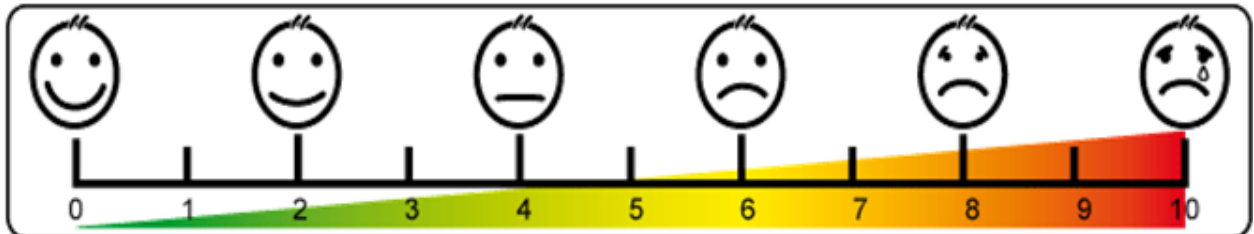
1. Patient's perception of the treatment questionnaire (English)
2. Patient's perception of the treatment questionnaire (Original, Danish)
3. CONSORT checklist
4. Protocol

## REMOTE ISCHEMIC CONDITIONING QUESTIONNAIRE (English translation)

### Patient's perception of the treatment

How much pain/discomfort did you experience during the treatment (remote ischemic conditioning)

(Mark an 'X' on the scale below)



1. Have you experienced any side effects during or after the treatment?

YES, please describe: \_\_\_\_\_

NO

2. If remote ischemic conditioning can protect against the development of certain cardiovascular diseases, would you be willing to continue the treatment after the trial is finished?

YES

NO

3. If you were to continue the treatment for 1 year, how often would you be willing to undergo the treatment?

2 times per day

1 time per day

Once every other day

2 times per week

Once a week

Less than once per week

4. How long time do you think each treatment should maximum last? (each treatment currently lasts 50 minutes)

- 1 hour
- 50 minutes
- 40 minutes
- 30 minutes
- 20 minutes or less

5. In the trial you have participated in, you could either be randomized to receive an *Active and working treatment* or a *Non-active and non-working placebo treatment*. Which form of treatment do you think you received?"

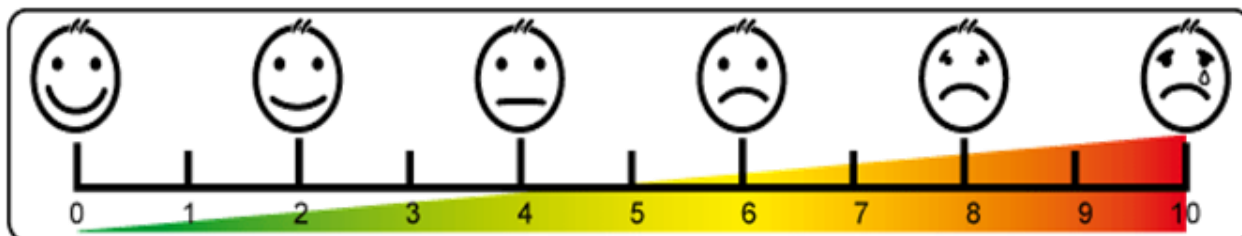
- The Active treatment
- The Non-active placebo treatment

**REMOTE ISKÆMISK KONDITIONINGS SPØRGESKEMA (Danish, Original)**

**Patientens opfattelse af behandlingen**

6. Hvor meget smerte/ubehag oplevede du under behandlingen (*afklemning af blodforsyningen til armen*)?

(Sæt kryds på nedenstående skala)



7. Har du under eller efter behandlingen (*afklemning af blodforsyningen til armen*) oplevet nogle bivirkninger til behandlingen?

JA, beskriv

Nej, ikke?: \_\_\_\_\_

NEJ

8. Såfremt *afklemning af blodforsyningen armen* kan beskytte mod udvikling af visse hjerte-kar sygdomme, ville du så være villig til at fortsætte med behandlingen efter at forsøget er færdigt?

JA

NEJ

9. Hvis du skulle fortsætte behandlingen i 1 år, hvor tit ville du være villig til at tage behandlingen?

2 gange dagligt

1 gang dagligt

Hver anden dag

2 gange om ugen

- 1 gang om ugen
- Mindre end 1 gang pr uge.

10. Hvor lang tid synes du hver behandling maksimalt måtte vare? (hver behandling varer nu 50 minutter)

- 1 time
- 50 minutter
- 40 minutter
- 30 minutter
- 20 minutter eller mindre

11. Man kan i forsøget du har været med i enten blive udtrukket til at blive behandlet med *én virksom* eller *én ikke-virksom* form af behandlingen. Hvilken for form af behandlingen blev du behandlet med?

- Den virksomme behandling
- Den ikke-virksomme behandling



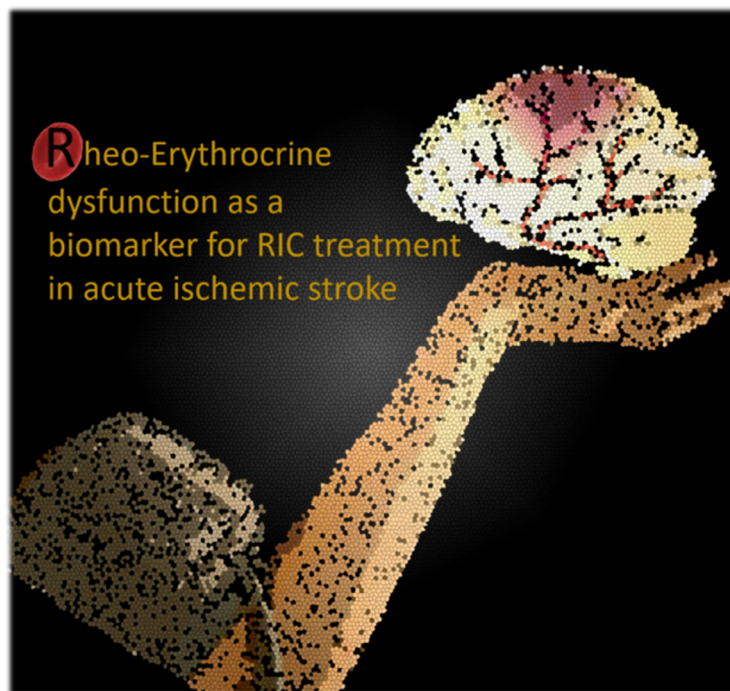
# CONSORT 2010 checklist of information to include when reporting a randomised trial\*

Section/Topic	Item No	Checklist item	Reported on page No
<b>Title and abstract</b>			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
<b>Introduction</b>			
Background and objectives	2a	Scientific background and explanation of rationale	3
	2b	Specific objectives or hypotheses	3
<b>Methods</b>			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	4
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	4
Participants	4a	Eligibility criteria for participants	4
	4b	Settings and locations where the data were collected	4
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	4-7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	4-7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	6
Sample size	7a	How sample size was determined	6
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
<b>Randomisation:</b>			
Sequence generation	8a	Method used to generate the random allocation sequence	4
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	4
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	4
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	4
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	5

		assessing outcomes) and how	
Statistical methods	11b	If relevant, description of the similarity of interventions	5
	12a	Statistical methods used to compare groups for primary and secondary outcomes	6
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	6
<b>Results</b>			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	7
	13b	For each group, losses and exclusions after randomisation, together with reasons	7
Recruitment	14a	Dates defining the periods of recruitment and follow-up	7
	14b	Why the trial ended or was stopped	7
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	18 (Table 1)
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	7-8
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	7-8
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	7-8
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	7-8
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	7-8
<b>Discussion</b>			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	10
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	10
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	10
<b>Other information</b>			
Registration	23	Registration number and name of trial registry	4
Protocol	24	Where the full trial protocol can be accessed, if available	Appendix
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	12

\*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming; for those and for up to date references relevant to this checklist, see [www.consort-statement.org](http://www.consort-statement.org).

## RESEARCH PROTOCOL



### Rheo-Erythrocrine dysfunction as a biomarker for RIC treatment in acute ischemic stroke

## Project description

This study aims to investigate whether Remote Ischemic Conditioning improves rheo-erythrocrine dysfunction in acute ischemic stroke

<b>Project acronym</b>	<b>ENOS stroke trial</b> Rheo-Erythrocrine dysfuNction as a biOmarker for RIC treatment in acute ischemic Stroke
<b>Project type</b>	Pilot, randomized, patient-assessor blinded, sham-controlled study
<b>Sponsor/Principal Investigator</b>	Grethe Andersen, Senior Consultant, MD, DMSc, Professor of Neurology, Department of Neurology & Danish Stroke Center, Aarhus University Hospital, DK-8000, Denmark
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<b>Investigator (DK)</b>	Rolf Ankerlund Blauenfeldt, MD, PhD-student, Department of Neurology & Danish Stroke Center, Aarhus University Hospital, DK-8000, Denmark
<b>Investigator (US)</b>	Mohammad B. Khan, PhD, Research Scientist, (Research Faculty), Department of Neurology, Georgia Medical College, Georgia, USA
<b>Study Coordinator &amp; Investigator (DK)</b>	Maria Kjølhede Sørensen, stud. med., Aarhus University, Denmark

## *Abbreviations*

RIC	Remote Ischemic Conditioning
RBC	Red Blood Cell
NO	Nitric Oxide
-SNO	S-nitrosylation
EI	Elongation Index
DI	Deformability Index
mRS	Modified Rankin Scale
MRI	Magnetic Resonance Imaging
NIHSS	National Institutes of Health Stroke Scale
MRI	Magnetic Resonance Imaging
TIA	Transient ischemic attack
AE	Adverse effects
DSR	Danish Stroke Registry
CRF	Case report form
Stroke RIC	Automatic Remote Ischemic Conditioning Device
PVP	Polyvinylpyrrolidone
GPA	Glycophorin A
miRNA	Micro ribonucleic acid
EV	Extracellular vesicles, also known as exosomes
ELISA	enzyme-linked immunosorbent assay
MoCA	Montreal Cognitive Assessment
PASE	Physical activity in the elderly
SAE	Serious Adverse Event
SADE	Serious Adverse Device Effect
USADE	Unanticipated Serious Adverse Device Effect
ASADE	Anticipated Serious Adverse Device Effect
AIS	Acute Ischemic Stroke
ICH	Intracerebral hemorrhage

# Contents

1. Background.....	6
2. Project thesis .....	6
<i>Hypothesis</i> .....	6
<i>Primary endpoint</i> .....	6
<i>Secondary endpoints</i> .....	7
3. Methods.....	7
<i>Design</i> .....	7
<i>Patient selection</i> .....	7
<i>Informed consent</i> .....	8
<i>Method of blinding</i> .....	9
<i>Discontinuation of study participation</i> .....	9
<i>Time schedule</i> .....	9
4. Study procedures and assessment .....	9
<i>Blood samples</i> .....	10
<i>Intervention (Remote Ischemic Conditioning)</i> .....	11
<i>RBC – deformability - Ectacytometry</i> .....	11
<i>Erythrocrine dysfunction - Flow Cytometry</i> .....	12
<i>MicroRNA analysis</i> .....	12
<i>Extracellular vesicle analysis</i> .....	12
<i>Use of routine collected blood samples</i> .....	13
<i>Montreal Cognitive Assessment (MoCA)</i> .....	13
<i>PASE - physical activity in the elderly</i> .....	14
<i>Remote Ischemic Conditioning questionnaire</i> .....	14
5. Statistical analysis .....	14
<i>Primary endpoint analysis</i> .....	14
6. Ethical considerations .....	14
<i>Advantages and disadvantages of participating in the study</i> .....	15
7. Assessment of safety.....	15
<i>Emergency unblinding procedure</i> .....	15
<i>Adverse events</i> .....	15
<i>Adverse event (AE)</i> .....	16
<i>Serious Adverse Event (SAE)</i> .....	16
<i>Device Deficiency</i> .....	16
<i>Adverse Device Effect</i> .....	16

<i>Serious Adverse Device Effect (SADE)</i> .....	16
<i>Unanticipated Serious Adverse Device Effect (USADE)</i> .....	16
<i>Anticipated SADE (ASADE)</i> .....	17
<i>Event report and causality assessment</i> .....	17
<b>8. Data handling and record keeping</b> .....	<b>20</b>
<b>9. Further documentation</b> .....	<b>20</b>
<b>10. Economics</b> .....	<b>21</b>
<b>11. Publication</b> .....	<b>22</b>
<b>12. Source data access and monitoring</b> .....	<b>22</b>
<b>13. Appendix</b> .....	<b>23</b>
<i>Appendix 1: Baseline data and treatment characteristics</i> .....	23
<i>Appendix 2: Study flowchart</i> .....	24
<i>Appendix 3: Montreal Cognitive Assessment</i> .....	25
<i>Appendix 4: RIC questionnaire (Danish)</i> .....	26
<b>14. References</b> .....	<b>28</b>

## 1. Background

Stroke is a leading cause of death and disability worldwide(1, 2). Of all strokes, 85% are ischemic strokes caused by a thrombus or an embolus. The additional 15% are caused by hemorrhage(3). Currently the only approved treatments for ischemic strokes are thrombolysis given within 4.5 hours and thrombectomy performed within 6 hours of symptom onset – in some cases up to 24 hours(4-7). The majority of stroke patients are not however eligible for acute reperfusion therapy, mainly due to time constraints and late presentation. Novel neuroprotective strategies available for all stroke patients are thus urgently needed.

Remote Ischemic Conditioning (RIC) is a simple intervention in which transient ischemia is induced in an extremity by repetitive inflation-deflation of a blood pressure cuff. It remains uncertain exactly how the protective effect of RIC is transmitted and communicated between the extremity and the brain. Both humoral, immunological and neuronal pathways seem to be involved (4, 8-10). Treatment with RIC and has proven to be a safe, feasible and low-cost treatment in clinical settings(9-12).

Biomarkers of the RIC treatment is a new area of stroke research and are important to establish in order to assess and predict responders of the conditioning treatment. Rheo-erythrocyte dysfunction of the Red Blood Cell (RBC) is a novel biomarker in both ischemic strokes in general and on the effect of RIC. Red Blood Cells with a diameter of 6-8  $\mu\text{m}$  must be highly deformable in order to deliver oxygen to brain tissue by travelling through micro vessels with a diameter of just 2-3  $\mu\text{m}$ . RBC's can carry nitric oxide as NO<sub>2</sub>-/s-nitrosylated proteins. These proteins improve RBC deformability and induce hypoxic vasodilation thereby improving passage through the microvasculature(13-18). RBC's also express Erythrocyte Nitric Oxide Synthase 3, which regulate the rheo-erythrocyte function. Erythrocyte Nitric Oxide Synthase 3 is activated by shear stress and provide an extra source of NO for hypoxic vasodilation(19-22). Preliminary data have shown that experimental stroke on mice seems to cause a rheo-erythrocyte dysfunction of the RBC's leading to a loss of deformability. The RBC's become rigid, which can lead to occlusion of micro vessels in the brain and further ischemic damage. Loss of deformability can be measured as a reduced Elongation Index (EI) by ektacytometry and may be attenuated by RIC(23).

## 2. Project thesis

### *Hypothesis*

RIC improves rheo-erythrocyte dysfunction in acute ischemic stroke as compared to sham RIC.

### *Primary endpoint*

Rheo-erythrocyte dysfunction in RBC's (deformability and Erythrocyte Nitric Oxide Synthase 3) will serve as a biomarker of the conditioning response and predictor of the clinical outcome in stroke patients.

## Secondary endpoints

- RBC deformability presentation across stroke subtypes
- RBC deformability in relation to infarct size/stroke severity
- RBC erythrocrine dysfunction presentation across stroke subtypes
- RBC erythrocrine dysfunction in relation to infarct size/stroke severity
- Difference in 7 days cognitive impairment between treatment groups.
- RBC erythrocrine dysfunction and deformability as a marker for difference in 7 days cognitive impairment
- Circulating microRNA and/or extracellular vesicle profile of RIC-induced neuroprotection
- Circulating microRNA and/or extracellular vesicle profile as a marker for difference in 7 days cognitive impairment
- Circulating microRNA and/or extracellular vesicle profile as a marker for RBC erythrocrine dysfunction and deformability

## 3. Methods

### Design

Pilot, randomized, patient-assessor blinded, sham-controlled study on stroke patients admitted to the Stroke Unit at Aarhus University Hospital.

### Patient selection

The study population is selected according to the criteria in table 1:

**Table 1. Inclusion and exclusion criteria for stroke patients and controls**

Stroke patients Inclusion/Exclusion criteria	
Inclusion criteria	Exclusion criteria
Onset to randomization < 48h Age 18-80 Independent in daily living (mRS 0-2) Legal competent Ambulatory Documented ischemic stroke on baseline MRI	Prior stroke, dementia or other known neurological condition Pregnancy* Contraindications to MRI Investigators discretion Known upper extremity peripheral arterial stenosis Diabetes

Controls Inclusion/Exclusion criteria	
Inclusion criteria	Exclusion criteria
Independent in daily living (mRS 0-2) Ambulatory Age 18-80 Legal competent Non vascular diagnosis (e.g. epilepsy, migraine etc.)	Prior stroke, dementia or other known neurological condition Pregnancy* Contraindications to MRI Investigators discretion Known upper extremity peripheral arterial stenosis Diabetes

*\*Women of child-bearing age will be asked about their use of safe birth control methods (contraceptive pill, intrauterine devices both hormonal and non-hormonal, hormonal implants, hormonal depot injection and transdermal hormonal patch). A safe birth control method shall be used throughout the study period. A pregnancy test will be performed (ruling out pregnancy) on women of child-bearing age before study participation is possible.*

### *Informed consent*

Patients who fulfill the study criteria will be invited to participate in the study. It will be endeavored to create a calm and safe environment for this conversation either in the patient room or in an interview room at the ward. We will prioritize the presence of nearest relatives at the time of study information and inclusion. Furthermore, we will inform the patient on the right to 24-hour consideration time before entering the study. All information will be given orally as well as in writing. A patient can only participate in the study if he/she has signed the consent forms ("*samtykkeerklæring og fuldmættserklæring*")

A patient can withdraw from the study at any time. Withdrawal can also be due to the principal investigator's discretion. In case the patient cannot be reached by telephone, every effort will be made to contact the patient or to document the outcome regarding new vascular events via registries/electronic health records. If the patient withdraws from the study, the date and the reason for the patient's withdrawal will be recorded. The patient is encouraged to provide information about his or her reason(s) for withdrawal and any experienced adverse effects (AE) during the study.

The study will follow the course of the flow chart (please refer to appendix 2). A total of 45 patients will be included in the study: 15 will receive stroke-RIC, 15 will receive sham-RIC and 15 will constitute the control group. The initial RIC treatment regime will be applied as soon as possible and less than 48 hours after stroke onset. If patients are discharged from the Stroke Unit before day 7, they will continue the RIC treatment at home according to written instructions. On day 7 patients will return to the Stroke Unit for further tests (Table 2 – study procedures) and return the RIC device. The control group will consist of patients with putative stroke/TIA's ending up with a non-vascular diagnosis upon examination in the outpatient clinic or at the stroke unit. In addition to the study procedures all patients will receive standard treatment.

#### *Method of blinding*

Patients and the assessors of endpoints are blinded. No information regarding randomization status will be recorded in the patient record.

#### *Discontinuation of study participation*

A patient can withdraw from the study at any time. Patients can be withdrawn from the study at the principal investigator's discretion. If the patient withdraws from the study, the date and the reason for the patient's withdrawal will be recorded. The patient is encouraged to provide information about his or her reason(s) for withdrawal and any experienced adverse effects (AE) during the study.

#### *Time schedule*

The study is scheduled to start 1<sup>st</sup> of February 2020 and end upon 31<sup>st</sup> of January 2021. The inclusion period is scheduled to 5 months.

## 4. Study procedures and assessment

Baseline data (demographics) and process indicators are collected from the Danish Stroke Registry (DSR). Additional information regarding medical history, treatment characteristics, use of medication, clinical and physiological data during the hospitalization will be obtained from the electronic health records. This includes information about clinical examinations, scan results and biochemical results. Data are recorded in an electronic case report form (CRF) at discharge from the stroke unit. For a complete list of registered data, see *Appendix 1*.

**Table 2. Study procedures for patients with acute ischemic stroke.**

	Enrollment	Pre-allocation	Post-allocation			Follow-up
Time points			0h	+2h*	Day 0-7 (8AM and PM)	+7days
Informed consent	X					
Randomisation		X				
<b>Intervention</b>						
Remote Ischemic Conditioning (RIC/Sham)			X		X ——— X	
<b>Blood samples</b>						
Blood samples (2x3,0 mL EDTA + 2x3,5 mL citrate)		X		X		X
RBC deformability		X		X		
RBC erythrocyte dysfunction		X		X		X
Biobank samples (EVs and microRNA)		X		X		X
<b>Clinical evaluation</b>						
NIHSS		X				X
PASE questionnaire		X				
MoCA		X				X
RIC questionnaire						X
<b>End of study</b>						X

RIC: Remote Ischemic Conditioning. NIHSS: National Institute of Health Stroke Scale. PASE: Physical Activity scale for the elderly. RBC: Red Blood Cells. EVs: Extracellular Vesicles. microRNA: Micro ribonucleic acid. For patients without a vascular diagnosis study participation will exclude RIC/Sham-RIC treatment and no RIC questionnaire.

\*2hours after completion of first RIC/Sham-RIC treatment

### Blood samples

Study blood samples will be drawn at the ward in a total of 3 times (Table 2 - Study procedures). Each sample set will consist of 2x3,0 mL EDTA + 2x3,5 mL citrate. Ektacytometry and flowcytometry will be performed on fresh whole-blood samples, excess material (plasma) will be placed in a research biobank and used for nitrite and EV/microRNA analysis. Furthermore, we will use biochemical lab results taken as part of routine care at admission.

Analysis of blood samples will be performed during the study and until 2 years after inclusion of the last patient (no later than 31<sup>st</sup> of January 2023). After the last analysis and no later than 31<sup>st</sup> of January 2023 the blood samples will be destroyed.

If routine blood samples are taken, we will endeavor to include the baseline study blood samples in these in order to minimize discomfort for the patient. If, however, the routine blood samples cannot be drawn within less than 2 hours after inclusion, the study personnel will draw them. The blood samples will be safely stored at Aarhus University Hospital. No samples or biological material will be sent abroad. Research documents are stored for 15 years, after which they are destroyed

Blood samples	Accepted time frame
Baseline samples	< 2 hours from inclusion
+2-hour samples	1-3 hours from completed baseline RIC
+7 days samples	< 4 hours from completed 08 AM RIC

#### *Intervention (Remote Ischemic Conditioning)*

The investigational device used to induce RIC is an automatic Remote Ischemic Conditioning device (Stroke-RIC) – a blood pressure cuff for application on an upper arm. It is preprogrammed for 5 cycles of RIC. Each of these rounds consist of 5 minutes of inflation followed by 5 minutes of deflation. For the RIC device cuff pressure will be 200 mmHg unless the systolic blood pressure exceeds 175 mmHg in which case a pressure of 35mmHg will be added to the systolic pressure.

Patients allocated to the sham-RIC group will go through the same program as the intervention group, except the sham-RIC device cuff pressure will be only 20 mmHg during inflation.

The devices are designed and developed in corporation with Aarhus University, The faculty of Biomedicine technologies, 8200, Aarhus N, Denmark, Seagull Aps, 4160, Herlufmagle, Denmark and the department of Neurology, Aarhus University Hospital, 8000-DK, Aarhus C.

RIC/sham-RIC protocol	Accepted time frame
Baseline RIC	< 2 hours from inclusion
RIC twice daily for 7 days – 08.00 PM and AM	06.00 to 10.00 PM and 06.00 to 10.00 AM

#### *RBC – deformability - Ectacytometry*

The rheological properties of RBCs are quantified using a Deformability or Elongation Index (DI or EI). A higher EI at the optimum viscosity (300 Osmolality) indicates highly deformable RBCs indicative of better microcirculation, while a lower EI indicates rigid RBC's. Briefly, 6 uL of heparinized or EDTA treated fresh blood is mixed with 600-uL of polyvinylpyrrolidone (PVP) solution (300 Osm) and transferred to a disposable kit. The kit is placed inside the laser-assisted

ectacytometer for automated read out, data and image collection as per the vendors instructions. In a similar approach aggregation and shear stress will be measured using 0,5 mL of blood. With the current knowledge on RBC deformability it is impossible to predict a risk of developing specific diseases in the future.

#### *Erythrocrine dysfunction - Flow Cytometry*

Analytical flow cytometry for Erythrocyte Nitric Oxide Synthase 3 phosphorylation (pNOS3Ser1177) and s-nitrosylation (-SNO) in RBCs: Red blood cells are separated from 50-uL of freshly fixed blood samples, using a cocktail of monoclonal antibodies to RBC-specific markers (Glycophorin A (GPA)) and Hemoglobin. To assess the functional features, after fixation and permeabilization, RBCs are incubated with antibodies conjugated to fluorochrome either directly or through secondary antibodies to s-nitrosocysteine (-SNO), pNOS3Ser1177 and AMPK $\alpha$ 1. A titration study is currently ongoing to determine the optimal antibody concentration. Next, RBC samples are run through a flow cytometer (Cytoflex S, Beckman Coulter, Colorado, USA), and data is collected using CellQuest software to process for FC analysis. With the current knowledge on the Erythrocrine dysfunction it is impossible to predict a risk of developing specific diseases in the future.

#### *MicroRNA analysis*

MicroRNAs will be identified with Illumina next-generation sequencing using the TruSeq Small RNA Sample Preparation kit (Illumina) which allows for the addition of unique barcode sequences to each sample. Such barcoding allows pooling and simultaneous sequencing of multiple samples in a single-sequencing run on the Illumina NextSeq500 sequencer thereby significantly reducing the cost of sequencing. Pooling of multiple samples will generate adequate data amounts for detailed miRNA profiling, yet at limited costs. Data generated from the NextSeq500 sequencer will be filtered based on sequence quality as part of our established bioinformatics pipeline. This will also include matching the filtered data to annotated RNA databases such as miRNA sequences from miRBase ([mirbase.org](http://mirbase.org)). The output from our bioinformatic pipeline will be quantitative miRNA expression levels for each sample, which will form the basis for a miRNA differential analysis where miRNAs with statistically significant expression changes will be found.

The microRNA (and extracellular vesicles) analysis is not a mapping of the genome. The microRNA profile is a snapshot of microRNA activity at the specific time of the blood sampling. Levels of microRNA are changing constantly and with the current knowledge on the topic it is impossible to predict a risk of developing specific diseases in the future. Cryotubes **not** used to for microRNA/exosome analysis will be destroyed.

#### *Extracellular vesicle analysis*

Extracellular vesicles (EVs, also known as exosomes) will be isolated from plasma samples before characterization of surface markers and content. EVs are isolated by a number of different

techniques, ultra-centrifugation, precipitation, size exclusion chromatography among others. While protein characterization will be done using classical molecular biological techniques such as ELISA and Western blots in addition to array techniques, EV-array, that utilizes a panel of antibodies directed against known EV surface markers. These analyses might be supported by proteomic analysis of all proteins as well as post-translational modifications such as phosphorylations and glycosylations. To broaden the feasibility of finding stroke type specific EV surface markers, we will utilize recombinant antibody library techniques to find novel disease binders with the potential of diagnosing stroke types in blood samples.

In addition, the nucleic acid (DNA and RNA including miRNA) content of EVs will be analyzed using next generation sequencing (NGS), qRT-PCR, and other nucleic acid detection techniques. Both the protein data and the nucleic acid data will be subjected to bioinformatic analysis using different pipelines and analysis tools depending on the dataset and the purpose of the analysis. A number of validated published databases will be used for annotation and comparison. EVs do not contain genomic DNA and the analysis are therefore not a mapping of the subject's genome. EV secretion and nucleic acid content in these are thought to change rapidly in the body due to external and internal signals and are considered a snapshot at the specific time of blood sampling. With the current knowledge on the topic it is impossible to predict a risk of developing specific diseases in the future.

#### *Use of routine collected blood samples*

The following biochemical entities will be used (lab results performed as a part of standard operating procedure): C-reactive protein, Glucose, HBA1c, Potassium, Natrium, Calcium, Albumin, Creatinine, eGFR (estimated Glomerular Filtration Rate), Total WBC (White Blood Cell count/Leukocytes), Hemoglobin, Erythrocytes, Erythrocytes (Volume Fraction, EVF), Erythrocytes distribution widths (RDW), Erythrocyte median cell volume (MCV), Hemoglobin concentration (MCHC and MCH), Reticulocyte count, Platelet count, Iron, Transferring, Ferritin, International Normalized Ratio (INR), Activated Partial Tromboplastin Time (APTT), Cholesterol (Total, HDL, LDL, Triglyceride).

#### *Montreal Cognitive Assessment (MoCA)*

The Montreal Cognitive Assessment (MoCA) is a brief screening tool originally designed to identify mild cognitive impairment. MoCA is a 1-page (healthcare administered), 30-point test, administrable in  $\approx$ 10 minutes, which evaluates different domains: visuospatial abilities, executive functions, short-term memory recall, attention, concentration, working memory, language, and orientation to time and space (appendix 4).

### *PASE - physical activity in the elderly*

Participants will be asked to complete the physical activity in the elderly (PASE) questionnaire during the acute hospital admission. PASE is a 12-item questionnaire, which quantifies the amount of PA over a 7-day period. The PASE questionnaire was developed with the purpose of assessing the level of PA in middle-aged and elderly individuals. The PASE score is calculated by taking the average number of hours spent on an activity (sports, occupational activity, household activities, and leisure time activities) per day over a 7-day period multiplied by an activity coefficient. Item scores are added to reveal the PASE score. The score may range from zero to more than 400.

### *Remote Ischemic Conditioning questionnaire*

In the RIC questionnaire the study participants are asked to reveal the level of discomfort (if any) experienced during the investigational treatment and whether they knew which treatment they received (Sham/Sham-RIC) despite the blinded study design. Control subjects will not be interviewed with the RIC questionnaire. The questionnaire is administered by study personnel. (Appendix 4)

## 5. Statistical analysis

Sample size estimation is based on yet unpublished data on elongation index in experimental stroke models. The maximum difference in IE between stroke and non-stroke (experimental models) was observed between 16 and 48 hours from stroke onset (IE of 0,43 and 0,58 at maximum deformability, 3000sm). Thus, a sample size of 12 stroke patients and 12 patients without stroke will be required to detect a deference of 0,15 on the elongation index with a power 90% (significance level 5%). To account for dropouts (estimated to maximum 3 per arm) and the additional study arm of RIC treated stroke patients a total of 45 patients will be included - 15 stroke patients (RIC), 15 stroke patients treated with sham-RIC and 15 patients (non-stroke controls). The maximum number of included patients will be 45.

### *Primary endpoint analysis*

Differences in RBC deformability, RBC NOS3, plasma nitrite and other biomarkers over time (baseline, +2 hours and +7 days post RIC) between RIC groups (yes/no) will be examined using mixed models. Fixed effects in the model are RIC group, time, and the two-factor interaction between RIC and time. Subjects nested within RIC groups will be considered a random effect.

## 6. Ethical considerations

The RIC intervention is associated with mild to moderate discomfort and minimal risk for adverse events (10-12). The additional blood samples will however result in slight discomfort associated with venipuncture. The number of additional venipunctures will range from one to three, depending the number of routine planned blood samples. Every effort will be made to have study

blood samples taken when routine samples are taken. Use of sham is necessary for methodological reasons and is not associated with any risk/discomfort.

#### *Advantages and disadvantages of participating in the study*

- a. Potential advantages: Participants diagnosed with acute ischemic stroke will receive extra follow-up including an extra neurological examination.
- b. Potential disadvantages: mild/moderate discomfort and minor bleeding in the skin can occur on the RIC treated arm. The RIC treatment has otherwise proven safe and without serious adverse effects. Sham-conditioning will not cause discomfort or side effects. Extra blood withdrawals will cause discomfort for the patient.

Results from this pilot study will help establish whether a rheo-erythrocrine dysfunction in the RBC's is a biomarker for stroke and RIC. The establishment of a biomarker will help predict responders to RIC and serve as a preliminary investigation of a novel stroke biomarker and to better understand causes of microcirculatory dysfunction in stroke.

The pilot study will need approval from The Central Denmark Region Committees on Health Research Ethics and the Danish Medicines Agency before the research project begins.

## 7. Assessment of safety

### *Emergency unblinding procedure*

All on call neurologists will have 24/7 telephone access to study investigators who can perform emergency unblinding if necessary. All changes will leave an audit-trail.

### *Adverse events*

Patients are monitored in the stroke unit with NIHSS scoring and Scandinavian Stroke Scale (SSS) at close intervals, and adverse events are treated according to clinical guidelines.

During the acute in-hospital phase the patients will be asked if they have experienced any deterioration of health or new symptoms during or after treatment with the investigational device. Furthermore, all patients will have a 24/7 contact number to the stroke center and a contact to a study research physician and will be instructed to report any deterioration of health or new symptoms. All discharged patients (and relatives) will be given contact details to study personnel and be instructed to report any deterioration of health or new symptoms. The patients with acute ischemic stroke will once again be asked for adverse events at the interview on day 7. End of study is the interview on day 7.

**Adverse events and adverse device events are defined** according to ISO 14155:2011 and European Commission guideline on medical devices (MEDDEV 2.7/3 revision 3).

#### *Adverse event (AE)*

Any untoward medical occurrence, unintended disease or injury or any untoward clinical signs (including an abnormal laboratory finding) in subjects, users or other persons whether or not related to the investigational medical device.

#### *Serious Adverse Event (SAE)*

Adverse event that:

- a) led to a death, injury or permanent impairment to a body structure or a body function.
- b) led to a serious deterioration in health of the subject, that either resulted in: - a life-threatening illness or injury, or - a permanent impairment of a body structure or a body function, or - in-patient hospitalization or prolongation of existing hospitalization, or - in medical or surgical intervention to prevent life threatening illness.

#### *Device Deficiency*

Inadequacy of an investigational medical device related to its identity, quality, durability, reliability, safety or performance. This could be malfunctions, use error, or inadequacy in the information supplied by the manufacturer.

#### *Adverse Device Effect*

Adverse event related to the use of an investigational medical device. This includes any adverse event resulting from insufficiencies or inadequacies in the instructions for use, the deployment, the implantation, the installation, the operation, or any malfunction of the investigational medical device. This includes any event that is a result of a use error or intentional abnormal use of the investigational medical device.

#### *Serious Adverse Device Effect (SADE)*

Adverse device effect that has resulted in any of the consequences characteristic of a serious adverse event.

#### *Unanticipated Serious Adverse Device Effect (USADE)*

Serious adverse device effect which by its nature, incidence, severity or outcome has not been identified in the current version of the risk analysis report.

*Anticipated SADE (ASADE)*

An effect which by its nature, incidence, severity or outcome has been previously identified in the risk analysis report

*Event report and causality assessment*

The relationship between the use of the medical device and the occurrence of each adverse event will be assessed and categorized. All events will be registered in the electronic CRF and reported to the authorities at the interim analysis. A yearly safety report, containing a list of all SAEs/SADEs and near-miss incidents, thorough evaluation of individual events, risk/benefit analysis of the investigational medical device and safety conclusions, will be submitted to the Danish Health and Medicines Authority and the Independent Ethics Committee by the Sponsor as required by Danish law.

*Exceptions to and special considerations about reporting procedures are stated below:*

Acute stroke is an acute life-threatening disease with a high risk of neurological deterioration and mortality. The natural history of stroke is associated with a high risk of complications (stroke in progression, hemorrhagic transformation, dysphagia, cardiac arrhythmia, pneumonia, and other serious infections). These *complications* all relate to the index stroke (occurring *before* randomization) and is foreseeable. If an event occurs that based on the assessment by the investigator is a result of the natural history of the disease it will not be reported by the sponsor to the Danish medicines agency and regional ethics committee within 7 days/2 days (see list of foreseeable events/complications below).

All expected serious adverse events will be reported to the Danish Medicines Agency (and regional ethics committee) every 3 months.

All *new* serious events (Serious Adverse Events), complications that are not foreseeable or device near incidents or malpractice will be reported by the sponsor to the authorities (within 7 days, or 2 days if there is a risk of event reoccurrence) according to ISO 14155:2011 and European Commission guideline on medical devices (MEDDEV 2.7/3 revision 3). Investigators will report the event to sponsor (within 24 hours). Sponsor will as fast as possible and no later than 7 days, or 2 days if there is a risk of event reoccurrence, report the event to the Danish Medicines Agency and regional ethics committee.

All Serious Adverse Device Effect (SADE) will be reported to the Danish Medicines Agency (and regional ethics committee) within 7 days, or 2 days if there is a risk of event reoccurrence

## List of foreseeable adverse events/complications

Foreseeable events/complications that will not be reported by the sponsor to the Danish Medicines Agency and Regional Ethics Committee within 7 days/2days.

Patient Population	Event	Frequency	Mitigation/treatment
TIA and AIS	Recurrent stroke.	10% at 90 days <sup>a</sup>	Standard medical treatment, intravenous thrombolysis/mechanical thrombectomy according to patient characteristics
AIS	Symptomatic intracerebral hemorrhage (related to IV tPA or EVT)	7% <sup>b</sup>	Antithrombotic/anticoagulation therapy cessation. Antifibrinolytic agent. Bloodpressure control.
AIS, ICH	Early (<24hours) and late (>24hours) neurological deterioration ( $\Delta$ NIHSS $\geq$ 2 or death)	7-22% <sup>c</sup>	According to etiology
AIS, ICH	Aspiration pneumonia	5-20% <sup>d</sup>	Antibiotic treatment according to hospital SOP
AIS, ICH	Urinary tract infections	12% <sup>e</sup>	Antibiotic treatment according to local hospital SOP
AIS, ICH	Severe infections (sepsis)	13% <sup>f</sup>	Antibiotic treatment according to local hospital SOP
AIS, ICH	Cardiac arrhythmia	29% <sup>g</sup>	Depending on arrhythmia type. Treatment according to cardio.dk
AIS, ICH	Acute myocardial infarction	6% <sup>h</sup>	Treatment according to cardio.dk and conference call with cardiologist
AIS, ICH	Deep venous Thrombosis(DVT)/pulmonary embolism(PE)	DVT: 8% <sup>i</sup> PE:1% <sup>j</sup>	Treatment according to cardio.dk
AIS, ICH	Falls (in-hospital phase)	14-65% <sup>k,l</sup>	Treatment according to type of injury
AIS, ICH	Seizure	AIS:3-10% <sup>m</sup> ICH:8% <sup>n</sup>	Treatment according to local SOP
AIS/ICH	Mortality 30 days	AIS:10-20% <sup>m</sup> ICH:30% <sup>n</sup>	

- a) Wang Y et al. Clopidogrel with Aspirin in Acute Minor Stroke or Transient Ischemic Attack. NEJM. 2013; 369:11-19
- b) Berkhemer OA. A Randomized Trial of Intraarterial Treatment for Acute Ischemic Stroke. NEJM. 2015; 372:11-20
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- h) Johnston KC et al. Medical and neurological complications of ischemic stroke: experience from the RANTTAS trial. RANTTAS Investigators. Stroke. 1998;29(2):447
- i) Bembenek J. Early stroke-related deep venous thrombosis: risk factors and influence on outcome. J thromb Thrombolysis. 2011 Jul; 32(1):96-102
- j) Pongmoragot J. et al. Pulmonary Embolism in Ischemic Stroke: Clinical Presentation, Risk Factors, and Outcome. J Am Heart Ass. 2013 Dec;2(6)
- k) Davenport RJ, Dennis MS, Wellwood I, Warlow CP. Complications after acute stroke. Stroke. 1996; 27: 415–420.
- l Teasell R, McRae M, Foley N, Bhardwaj A. The incidence and consequences of falls in stroke patients during inpatient rehabilitation: Factors associated with high risk. Arch Phys Med Rehabil. 2002; 83: 329–333.
- m) Bladin C, Alexandrov AV, Bellavance A et al: Seizures After Stroke: A Prospective Multicenter Study. Arch Neurol. 2000;57(11):1617-1622.
- n) Woo K-M, Yan S-Y, Cho K-T. Seizures after Spontaneous Intracerebral Hemorrhage. J. Korean Neurosurg Soc. 2012 Oct;52(4):312-319
- o) Schwartz J et al. Incorporating Stroke Severity Into Hospital Measures of 30-Day Mortality After Ischemic Stroke Hospitalization. Stroke. 2017. Ahead of print: <https://doi.org/10.1161/STROKEAHA.117.017960>
- p) Lichtman JH et al. 30-Day Mortality and Readmission after Hemorrhagic Stroke among Medicare

## List of anticipated adverse device effects

RIC device	Frequency	Mitigation/treatment
Local petechiae	4-5% <sup>a</sup>	none
Discomfort/pain	20-30%	Treatment stopped if the non-competent patient verbally or non-verbally demonstrates intolerable discomfort or if the competent patient verbally wants to discontinue the treatment
Near events (device related)	None known in the literature	
Acute limb ischemia (upper extremity) – theoretical complication.	None known in the literature	Treatment stopped. Treatment according local hospital SOP's for acute limb ischemia.  Known atherosclerotic stenosis in upper extremities are <b>an exclusion criterion</b> .  See Investigators Brochure for further details.
Sham RIC device		
None known		

a) Hausenloy D et al. Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. New England J. Medicine. 2015 Oct;373(15):1408-17

Treatment and mitigation of any adverse events will be according to regional guidelines. Adverse events as a result of standard treatment will be handled according to local guidelines. Adverse

events may also occur as a suspected reaction to RIC/Sham RIC – in these cases, the conditioning stimulus will be stopped immediately, and appropriate treatment will be initiated. In cases of SAE and device related near events all patients will be followed until resolution of symptoms/signs and/or all clinically relevant treatment have been performed and/or until the symptoms/signs are in a stable phase.

## 8. Data handling and record keeping

All study data are recorded in an electronic CRF with blinded data and identification via a study identification number. The study will apply to the specifications of act on processing of personal data. The database(e-CRF) is handled by REDCAP at Aarhus University, Aarhus, Denmark. Patient participation will be recorded in the medical record. Data will be stored at the Department of Neurology, Aarhus University Hospital, for 15 years, after which the documents will be shredded. Blood-samples in the research biobank will be stored until completion of the trial (no later than 31. january 2023).

Methodological experts from collaborators at Medical College Georgia, Augusta, USA (Dr. Hess' and Dr. Baban's lab) will remotely supervise the technical aspects of flowcytometry and ektacytometry and assist in the interpretation of results (only anonymized flowcytometry and ektacytometry data). Flowcytometry and ektacytometry results reflects only snapshots of the function and deformability of the red blood cells and change rapidly.

During the entire study period the local GCP unit will perform quality assurance control including source data verification. A complete list of all source data will be made and approved by the local GCP unit before study initiation. The investigator permits direct access to all source data/documents (including electronic patient record) at monitoring visits, audits and/or inspections by the regional ethics committee and Danish Medicines Agency.

## 9. Further documentation

- The study will respect and protect the patients' physical and mental integrity as well as their privacy. The data protection regulations as well as the law on data protection will abide.
- When the patient signs the consent form, the patient grants the study personnel access to information within the medical record necessary for the completion of the study. It also allows supervisory authorities in the Danish Medicines Agency access to the medical record in order to inspect and monitor the study. Information regarding previous admissions as

well as diagnoses are retrieved from national registers including the National Patient Registry, the Danish Stroke Registry and the Central Person Register.

- The study will be notified to the Region's Internal List of research projects
- The study is monitored by the GCP-unit Aalborg/Aarhus.
- The study is covered by the Danish Patient Compensation Association.

## 10. Economics

- Initiator: Grethe Andersen, Professor, Senior Consultant, MD, DMSc, Department of Neurology & Danish Stroke Center, Aarhus University Hospital.
- The ENOS trial has received funding from the Lundbeck Foundation on 140.000 dkr. (study investigator salary and current expenses)
- Equipment and supplies are supplied by the "mother trial", RESIST, which received funding from the following:
  - The Danish foundation Vilhelm Petersens mindelegat has supported the RESIST study with 1.240.000 dkr (research nurse salary and "stroke RIC" devices).
  - The Danish foundation TrygFonden has supported the RESIST study with 4.000.000 dkr (research personnel salaries, current expenses and investigational devices).
  - Aarhus University has supported the RESIST study with 550.000 dkr (RESIST study investigator salary)
  - Novo Nordisk has supported the RESIST study with 2.600.000 dkr (Rheo-erythrocrine dysfunction in stroke)
- Financial supporters have no influence on study design, data collection, analysis/interpretation of results or publication of results.
- The devices (Stroke-RIC and Sham-RIC) are produced by Seagull Aps. The company has no influence on study design, data collection, analysis/interpretation of results or publication of results.
- Financial contributions will be deposited into a fund account administered by Aarhus University Hospital's financial department. None of the physicians nor nurses involved in the study will benefit economically from their participation. Participation in the study is not linked to any remuneration.

## 11. Publication

Results, both positive, negative and inconclusive will be published in scientific papers and during scientific meetings/congresses.

## 12. Source data access and monitoring

Trial-related audits and/or monitoring will be provided by direct access to source data/documents. Local monitors from the Unit for Good Clinical Practice, Aarhus University, will perform the audit and monitoring. Before enrollment can start a trial monitoring plan must be available. The audit and monitoring process will involve a 100% monitoring of signed consent forms (“samtykkeerklæringer og fuldmagtserklæringer”) and serious adverse events.

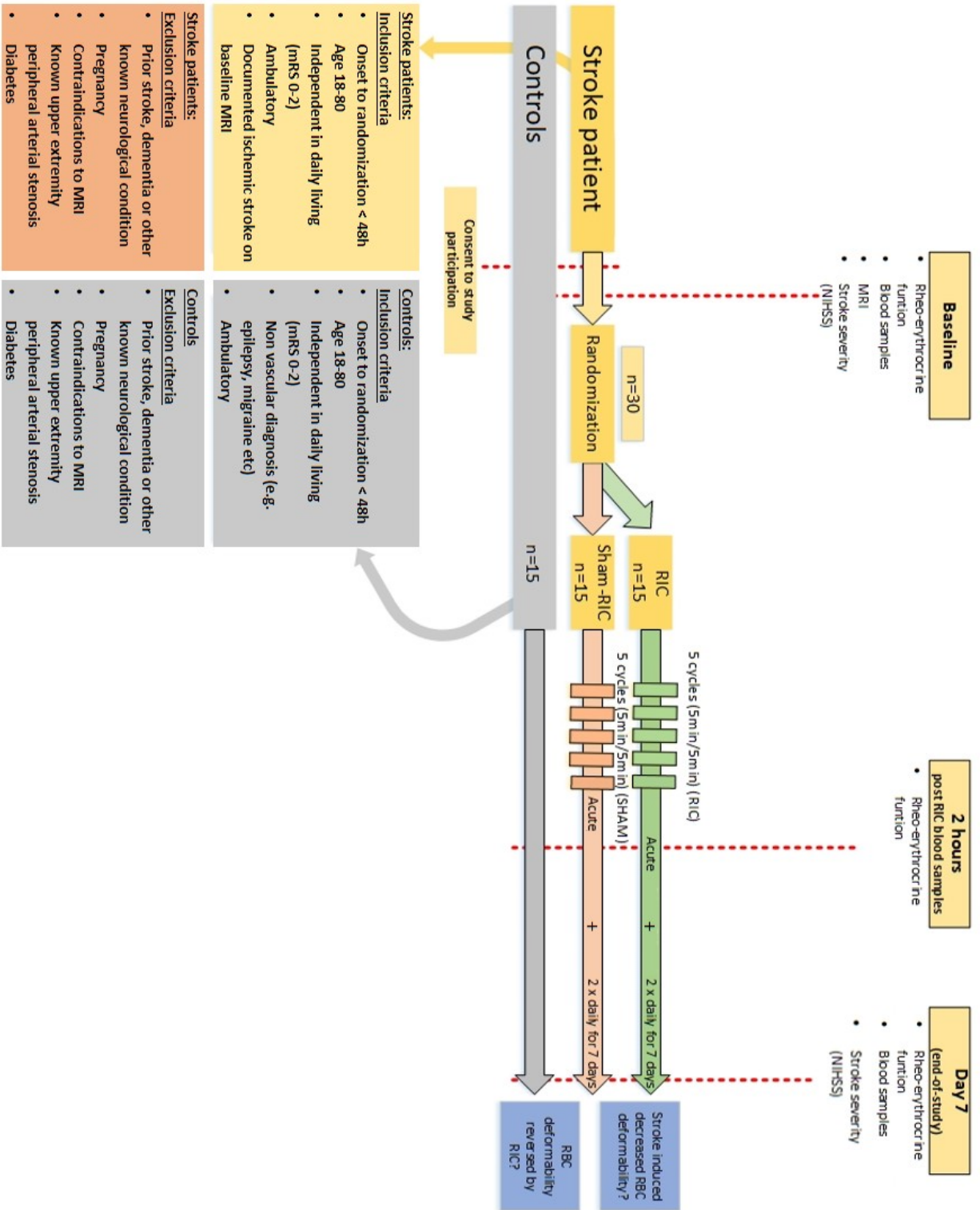
## 13. Appendix

### Appendix 1: Baseline data and treatment characteristics

Age	Platelet inhibitor treatment
Male/female	Anticoagulation therapy
<b>Medical History</b>	New oral anticoagulation treatment (NOAC)
Hypertension	Opioid treatment
Smoking	SSRI treatment
Alcohol	<b>Clinical and physiological data</b>
Hyperlipidemia	Modified Rankin Scale prestroke
Previous myocardial infarction <i>Yes: within 3 months, yes: more than 3 months, no, unknown</i>	NIHSS (Baseline)
Angina pectoris	Symptom onset (time)
Recent angina pectoris (< 4 weeks) <i>If yes, date for latest episode</i>	Time of admission
Atrial fibrillation	RIC compliance data
Previous ischemic stroke	<ul style="list-style-type: none"> <li>• Time (start/stop) for each RIC/sham cycle</li> <li>• Bloodpressure and pulse at every RIC/sham RIC treatment</li> </ul>
Stroke < 3 month <i>If yes, date</i>	
Previous TIA	NIHSS (7 days)
Recent TIA < 4 weeks <i>If yes, date for latest episode</i>	Stroke etiology (TOAST)
Previous ICH	Montreal Cognitive assessment at baseline-and +7 days
Peripheral artery disease	RIC questionnaire at +7 days
Physical activity (PASE interview)	<b>IV tPA/EVT treated AIS</b>
<b>Medication on admission</b>	Treatment initiation (time) and treatment characteristics
Statins	<b>Quality indicators (Danish Stroke Registry)</b>
Calcium channel blockers	
ACE inhibitors/angiotensin receptor blockers	
Beta-blockers	

Baseline data obtained from treating physician, database of Danish stroke center (ddsc.dk), Danish stroke registry and electronic health records.

Appendix 2: Study flowchart



## Appendix 3: Montreal Cognitive Assessment

### Montreal Cognitive Assessment (MOCA)

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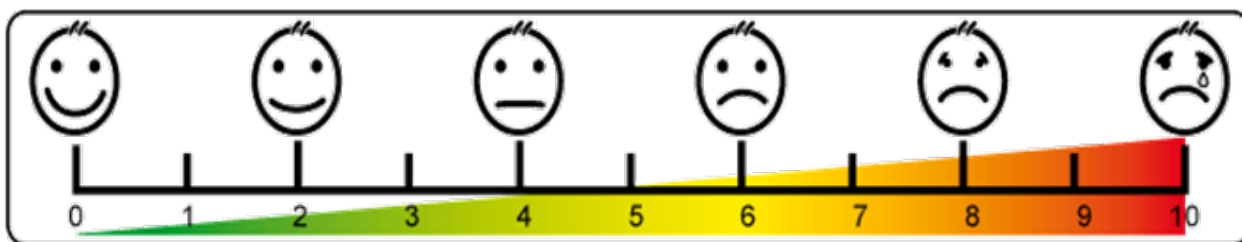
Appendix 4: RIC questionnaire (Danish)

*Example, minor changes may occur. Only patients with ischemic stroke.*

**REMOTE ISKÆMISK KONDITIONINGS SPØRGESKEMA**

***Patientens opfattelse af behandlingen***

1. Hvor meget smerte/ubehag oplevede du under behandlingen (*afklemning af blodforsyningen til armen*)?  
(Sæt kryds på nedenstående skala)



2. Har du under eller efter behandlingen (*afklemning af blodforsyningen til armen*) oplevet nogle bivirkninger til behandlingen?

JA, beskriv hvilke?: \_\_\_\_\_

NEJ

3. Såfremt *afklemning af blodforsyningen armen* kan beskytte mod udvikling af visse hjerte-kar sygdomme, ville du så være villig til at fortsætte med behandlingen efter at forsøget er færdigt?

JA

NEJ

4. Hvis du skulle fortsætte behandlingen i 1 år, hvor tit ville du være villig til at tage behandlingen?

2 gange dagligt

1 gang dagligt

Hver anden dag

- 2 gange om ugen
- 1 gang om ugen
- Mindre end 1 gang pr uge.

5. Hvor lang tid synes du hver behandling maksimalt måtte vare? (hver behandling varer nu 50 minutter)

- 1 time
- 50 minutter
- 40 minutter
- 30 minutter
- 20 minutter eller mindre

6. Man kan i forsøget du har været med i enten blive udtrukket til at blive behandlet med *én virksom* eller *én ikke-virksom* form af behandlingen. Hvilken for form af behandlingen blev du behandlet med?

- Den virksomme behandling
- Den ikke-virksomme behandling

Tak for din hjælp. Spørgeskemaet afleveres til forsøgspersonalet

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