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Protocol of an observational study for intraoperative assessment of the human cerebrovascular glycocalyx

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Abstract

Introduction

Adequate functioning of the blood-brain barrier (BBB) is important for brain homeostasis and normal neuronal function. Disruption of the BBB has been described in several neurological diseases. Recent reports suggest that an increased permeability of the BBB also contributes to increased seizure susceptibility in epilepsy patients.

The endothelial glycocalyx is coating the luminal side of the endothelium and can be considered as the first barrier of the BBB. We hypothesize that an altered glycocalyx thickness plays a role in the etiology of temporal lobe epilepsy (TLE), the most common type of epilepsy. Here, we propose a protocol that allows intraoperative assessment of the cerebrovascular glycocalyx thickness in TLE patients and assess whether its thickness is decreased in TLE-patients when compared to controls.

Methods and analysis

This protocol is designed as a prospective observational case-control study in patients who undergo resective brain surgery as treatment for TLE. Control subjects are patients without a history of epileptic seizures, who undergo a craniotomy or burr hole surgery for other indications. Intraoperative glycocalyx thickness measurements of the sublingual, cortical, and hippocampal microcirculation are performed by videomicroscopy using sidestream darkfield imaging. Demographic details, seizure characteristics, epilepsy risk factors, intraoperative hemodynamic parameters, and histopathological evaluation are additionally recorded.

Ethics and dissemination

This protocol has been ethically approved by the local medical ethical committee (ID: NL51594.068.14) and complies with the Declaration of Helsinki and principals of Good Clinical Practice. Informed consent is obtained before study enrollment and only coded data will be stored in a secured database, enabling audit trail. Results will be submitted to international, peer-reviewed journals and presented at international conferences.

Registration details

Registered at the Netherlands National Trial Register: NTR5568.

1. Introduction

With a prevalence of 4-10 per 1000, epilepsy is one of the most common neurological disorders [1]. The most frequent type of focal epilepsy is temporal lobe epilepsy (TLE). Although most patients have adequate seizure control by using antiepileptic drugs, it is estimated that 22.5 % - 30 % of patients are drug-resistant [2,3]. This implies that worldwide about 13 million patients suffer from drug-refractory epilepsy, which has a major impact, both medically and socioeconomically [3,4]. The development of new antiepileptic drugs has hardly reduced the number of drug-resistant patients, and therefore several different treatment alternatives have been explored over the past 25 years. Epilepsy surgery has been demonstrated to be a successful treatment alternative in selected patients. Furthermore, discovery of new drugs, targeted at specific underlying pathophysiologic mechanisms keeps holding a promise for improved treatment of drug-resistant epilepsy patients.

In this regard, important discoveries on microvasculature abnormalities in TLE-patients have been reported, such as loss of blood-brain barrier (BBB) integrity [5–7]. BBB opening induces extravasation of proteins, like albumin, which in turn activates transforming growth factor β (TGFβ) signalling in astrocytes, leading to astrocytic transformation [7,8]. This transformation is characterized by downregulation of inward rectifier potassium channels and astrocytic glutamate transporters, leading to impaired cerebral homeostasis, altered neurovascular coupling, enhanced neuronal excitability, and upregulation of proinflammatory cytokines [8,9]. Moreover, leucocyte extravasation due to increased BBB permeability, contributes to neuronal excitability and reorganization of local neuronal networks [9]. Thus, loss of BBB integrity results in increased seizure susceptibility and contributes to epileptogenesis.

The barrier function of the BBB is mainly determined by endothelial tight junctions and membrane transport mechanisms, whereas surrounding astrocytes and pericytes play a supplementary role [10]. As was recently pointed out, the endothelial glycocalyx could be a significant determinant of the BBB function as well [11]. The endothelial glycocalyx, further referred to as 'glycocalyx', is a gel-like layer lining the luminal surface of the endothelium. It has important barrier properties that reduce the interaction between endothelial cells and plasma cells and components. As a consequence, the glycocalyx limits leucocyte adhesion and protein extravasation [12–15].

The glycocalyx is a vulnerable layer that is easily disrupted resulting in a reduced thickness [16]. As part of the BBB, a disrupted glycocalyx would result in increased BBB permeability, and propagate leucocyte adhesion and extravasation. A number of laboratory and clinical studies have shown that both increased BBB permeability and leucocyte adhesion/extravasation play a role in epilepsy [7–9,17].

So far, no data are available on glycocalyx thickness in epilepsy. The cerebrovascular glycocalyx has only been evaluated preclinically [18–21]. Although, none of these studies have analyzed glycocalyx thickness in relation to neurological diseases. However, glycocalyx thickness can be assessed noninvasively as part of microcirculation imaging using sidestream darkfield (SDF) imaging [11]. A technique that has mainly been performed on the sublingual microcirculation.

Our hypothesis is that the cerebrovascular glycocalyx can be visualized clinically using SDF imaging, and that drug-resistant TLE-patients have a decreased cerebrovascular glycocalyx thickness in comparison to controls. In this paper, we present the study protocol according to the STROBE guidelines for case-control studies and SPIRIT protocol guidelines [22,23].

The primary objective of this case-control study is to visualize the cerebrovascular glycocalyx using SDF imaging in TLE-patients and control patients during brain surgery. Moreover, an eventual correlation between the cerebrovascular and sublingual glycocalyx dimensions is assessed.

2. Methods

2.1 Study setting and population

This is a multicenter prospective observational case-control study that involves assessment of the sublingual, cortical, and hippocampal glycocalyx. In conjunction, data are recorded regarding patient's medical history, current physiology, and cerebral pathology. Assessments take place during brain surgery, which is performed by the department of Neurosurgery of both the Maastricht University Medical Center+ (MUMC+) and the Zuyderland Medical Center (ZMC), respectively located in Maastricht and Heerlen, The Netherlands.

Patients with unilateral drug-resistant TLE that are designated for resective brain surgery, i.e. anterior temporal lobectomy and/or amygdalohippocampectomy, are possible study candidates. The diagnosis of unilateral TLE is based on thorough examination including a video-EEG, high-resolution MRI, neuropsychological examination and, when indicated, a PET- and or SPECT-scan, functional MRI, and eventually subdural or depth (stereo-EEG) electrode implantation. Subsequently, eligibility for resective surgery is assessed by a multidisciplinary team, consisting of neurologists, clinical neurophysiologists, neuropsychologists, neuroradiologists, and neurosurgeons.

The control group includes patients without a history of epileptic seizures, and who will undergo a craniotomy for intracranial tumor resection or neurovascular indications, like aneurysm clipping and arteriovenous malformation resection, or who will undergo burr hole surgery for tumor biopsy.

We have included an upper age limit of 60 years to avoid 'background' microvascular disease which is strongly age-related. Especially since tumor patients are already older, on average, than epilepsy patients, age could otherwise have been an important confounder. Control patients with vascular pathology, like aneurysms and arteriovenous malformation, are not known to have microcirculatory pathology. Moreover, cortical measurements are performed at a distance of the vascular pathology as allowed by the craniotomy, by example the superior temporal gyrus in a patient with an anterior cerebral artery aneurysm.

2.2 Eligibility criteria

Inclusion criteria are: mentally competent adults between 18 and 60 years of age who will undergo resective surgery for unilateral drug-resistant TLE, or for a tumor or vascular abnormality, or burr hole surgery for tumor biopsy (table 1). Exclusion from this study occurs in case of pregnancy, history of established hypertension, diabetes mellitus, hyperlipidemia, stroke or other cardiovascular disease, use of cardiovascular medication, or non-symptomatic signs of cerebral small vessel disease on brain MRI. Additionally, control patients in which no 'normal', 'non-compressed' and/or 'non-

edematous' cerebral cortex can be assessed during surgery or in whom a history of seizures is reported, are excluded.

2.3 Intervention protocol

The glycocalyx thickness is assessed intraoperatively at the following time points: 1. a sublingual measurement (M1) directly following the induction of general anesthesia, 2. a cortical measurement (M2) upon opening of the meninges allowing a direct view at the cortex and 3. a hippocampal measurement (M3) only in TLE-patients. The latter measurement is performed upon removal of the temporal neocortex allowing view at the hippocampus. At each time point, systolic and diastolic blood pressure, heart rate, pulse oxygen saturation, hemoglobin concentration, and hematocrit are additionally recorded.

Glycocalyx measurements are performed using an SDF video microscope. The camera consists of a central light guide with magnifying lens and concentric light emitting diodes. The diodes emit light at a wavelength of 530 nm, which is absorbed by (de)-oxyhemoglobin in erythrocytes. Consequently, erythrocytes appear black on a grayish background. This technique has mainly been performed on the sublingual microcirculation but has previously been used for cerebral microcirculation assessment as well [24–28]. However, the glycocalyx was not measured in these studies [24–27]. Recently, it was pointed out that SDF imaging, when combined with dedicated software, is the most suitable technique for clinical cerebrovascular glycocalyx visualization [11].

In order to reduce user-induced variations, image acquisition is trained. Using the camera with low pressure, minimizing movement artefacts and optimizing light intensity and focus are of particular importance to further reduce variability.

The glycocalyx analysis is based on the principle of the erythrocyte-endothelial exclusion zone. Since erythrocytes do not significantly compress the glycocalyx, there is an exclusion zone between the red blood cell column (RBCC) and the endothelium. GlycoCheck © software automatically selects approximately 3,000 microcirculatory vessel segments and measures the RBCC width at each segment. Based on the RBCC width distribution, the total perfused diameter (Dperf) is assessed by linear regression analysis of the 25^{th} and 75^{th} RBCC width percentiles [11]. The perfused boundary region (PBR) is the outermost luminal part of the glycocalyx that is only slightly permeable for erythrocytes. This region is calculated in a two-dimensional plane as follows: PBR = (Dperf – median RBCC) / 2. As an unstable or damaged glycocalyx is more accessible to erythrocytes, an increased RBCC and, consequently, increased Dperf and PBR values signify a damaged glycocalyx [28].

2.4 Outcome

The primary outcome of this study is cortical glycocalyx thickness in TLE patients and controls. The thickness is estimated by measuring the perfused boundary region (PBR) and the total perfused diameter (Dperf), both expressed in micrometers (µm).

As secondary outcome, sublingual, cortical, and hippocampal glycocalyx thickness in TLE-patients and controls are evaluated within and between groups. In addition, we aim to analyze the relation between these measures and epilepsy-specific risk factors (e.g. febrile seizures, traumatic brain injury), seizure characteristics, drug use (e.g. type of drugs, dosage), and histopathological outcome (e.g. degree of hippocampal sclerosis, type of cortical dysplasia). Finally, glycocalyx thickness values will be correlated to general demographic (e.g. age, gender, bodyweight, smoking status) and intraoperative clinical parameters.

2.5 Recruitment capacity, consent and timeline

We have calculated a sample size of 15 patients for each group, to be included over 4 years. At the MUMC+ and ZMC, all types of surgery required for inclusion are performed at least 15-20 times every year. Based on these surgery numbers, the likelihood to recruit the calculated sample size is high.

The study participants' time line is outlined below and an overview is also given in figure 1.

1. Recruitment

   Patients will visit the neurosurgical outpatient clinic preoperatively. During this visit, the neurosurgeon will ask the patients whether they are interested to take part in the study. If interested, they will receive the applicable patient information brochure (PIB). Within a few days, possible candidates will be informed more extensively by one of the investigators about the study objective, procedures, benefits, risks, and insurance. Participation decisions will be inquired at the day of admission to the hospital. Patients are allowed at least 3 days to consider participation for the study, starting from the moment of receiving the PIB. Participation is voluntarily and does not affect standard treatment in any way. Patients will be informed that they can decide to end their participation in the study at any time.

2. Informed consent

   At the day of hospital admission, patient's study participation is ultimately inquired. When patients agree on participation, the informed consent agreement will be signed in duplicate.

3. Data recording

   Following informed consent agreement, patient data will be recorded from the digital patient files. The digital patient files encompass the digital patient dossier at the MUMC+, ZMC and, when applicable, the file of the multidisciplinary epilepsy surgery work-up.

4. Intraoperative measurements

   A total of two or three glycocalyx measurements will be performed, as described in paragraph 2.3. All measurements are performed at the operating theatre when the patient is under general anesthesia. The hippocampal measurement is solely performed in TLE-patients.

5. End of study

   Directly following the final measurements, i.e. M2 in control-patients and M3 in TLE-patients, patients have reached the endpoint of this study.

2.6 Population size

We have calculated a population size of 15 patients per group. This number is based on a power of 80% to detect a difference of cortical glycocalyx thickness of at least 12% between groups with a standard deviation of 15%, at a significance level of 0.05 and an expected drop-out of 2 patients.

Since there is no literature on cerebrovascular glycocalyx thickness, we have reviewed and assessed the literature on variation in sublingual glycocalyx measurements. Intra-individual variation of sublingual glycocalyx thickness, assessed using SDF imaging, has been found to be ±5% [29]. A difference in PBR thickness of 9.6-12.5 % with a standard deviation of ± 15% has been found relevant when comparing a disease state to healthy controls [28–31]. Based on these studies, we have determined minimal glycocalyx difference and standard deviation. Due to the explorative nature of our study, clinical relevance of a 12% difference is indistinct.

2.7 Data processing

2.7.1 Procedures

This study complies with the Declaration of Helsinki, and will be conducted in accordance with the principals of Good Clinical Practice (GCP). Standardized processing files for obtaining informed consent, measurement procedures, reporting (serious) adverse events and recording patient and measurement data parameters in the electronic case report file (eCRF), are available. Investigators obtaining informed consent from the patient, performing glycocalyx measurements, and recording eCRF data will receive specific training beforehand.

2.7.2 Data management

Patient's demographic and clinical data are recorded in an eCRF at a secure encrypted database (by Castor EDC©), which enables audit trail and is GCP certified. Measurement procedures are trained and standardized as described in paragraph 2.3. Measurement data is collected at a secure encrypted

laptop and outcome is recorded in the eCRF. After verification of recorded data to source data by one the executive investigators, recorded data in the eCRF by Castor EDC© will be exported to a SPSS file for further statistical analysis.

Patients will be assigned an alphanumeric sequential study number to identify all clinical data. Patient's demographic data linked to the assigned study number is documented in a separate screening database held on a secure computer at both study sites. Source data, the code encrypting document, and coded data in the study database are locked and only accessible to the principal and executive researchers, and monitors. On completion of the study, the study database will be locked and data is securely archived for 15 years in accordance with local policy.

Due to the nature and short participation time period of this study, we expect full patient retention and adherence.

2.8 Safety

The principal investigator (OS) has overall responsibility for the conduct of this study including safety. Individual investigators will be responsible for reporting adverse events (AEs) and serious adverse events (SAEs) to the principal investigator. SAEs are defined as AEs resulting in death, life threatening events, prolonged hospital stays, or significant disability. There are no reported (S)AEs associated with the use of SDF imaging in the current literature. A possible attributable risk of glycocalyx measurement is sublingual, cortical, or hippocampal contusion due to pressure on the tissue during the measurement. We deem risk frequency and severity as low. Moreover, cortical or hippocampal local contusion will take place in non-eloquent and to-be-resected tissue. Postoperative consequences due to this contusion are unlikely. All events are reviewed by the principal investigator to decide if there is a causal link and, when applicable, appropriate action will be undertaken. SAEs will be reported to the local medical ethical committee (METC azM/UM) according to local policy. Liability and subject insurance is provided.

2.9 Statistical analysis

Data are presented as mean and standard deviation when normally distributed, and non-normally distributed data as median and interquartile range. Glycocalyx dimensions between groups are compared using the independent T-test or the Mann-Whitney test, as appropriate. Correlation between sublingual and cortical, sublingual/cortical and hippocampal glycocalyx thickness is calculated by Pearson's or Spearman's correlation coefficient, when data is normally or non-normally distributed, respectively. In TLE-patients, the cortical and hippocampal glycocalyx thickness results are correlated to seizure characteristics, epilepsy risk factors, anti-epileptic drug usage, and

histological findings of pathological examination by using Pearson's or Spearman's correlation coefficient or uni- and multivariate regression analysis, as appropriate. Demographic and clinical parameters are correlated to glycocalyx thickness results by using uni- and multivariate regression analysis. A p-value of < 0.05 will be considered statistically significant. Statistical analysis is performed using SPSS software.

Interim-analyses will be performed yearly and following data recording of 5 patients in each group. When a significant difference between the groups regarding the primary outcome is found, the study will be terminated prior to inclusion of 15 patients in each group. Interim-analyses are reported to the local medical ethical committee.

2.10 Monitoring and auditing

This study was classified as minimal-risk by the local data monitoring committee (CTCM). Monitoring visits include review of consent and study procedures according to study protocols, source data and audit trail verification, and the review of (serious) adverse event reporting. Monitoring is independent and performed at least once a year. Monitor evaluations are reported to the local medical ethical committee. Unannounced audits can be performed by the audit team of the CTCM.

2.11. Ethics, amendments, and dissemination

This research protocol has been approved by the local medical ethical committee (METC azM/UM) and has been assigned the following protocol ID: NL51594.068.14. Also, this study has been registered at the Netherlands National Trial Register (ID: NTR 5568). The NTR is acknowledged by the WHO and International Committee of Medical Journal Editors (ICMJE).

Substantive protocol amendments will be reported, reviewed, and approved by the METC azM/UM before application. In the currently presented protocol, a variety of substantive protocol amendments have already been incorporated. Substantive amendments were introduced due to lagging inclusion. The substantive amendments included, extending the study by the addition of the ZMC as a study center, enlarging the control-group with burr hole tumor biopsy and neurovascular surgery patients, and reducing sublingual measurements from three to one.

Results of this study will be evaluated yearly and following 5 participants in both groups. Results will be recorded using audit trails to increase reproducibility. Study protocol and results will be submitted to peer-reviewed journals and presented at international conferences.

3. Discussion

Microvascular injury, in particular increased permeability of the wall of the cerebral microcirculation, seems to play a role in the transformation of astrocytes and increased seizure susceptibility in epilepsy. It is hypothesized that leakage of albumin orchestrates this transformation leading to a disturbed potassium and glutamate metabolism and extracellular cerebral homeostasis [7,8]. Upregulation of proinflammatory cytokines as a response to leakage of leucocytes further contributes to the subsequent increased neuronal excitability [9]. However, it is still unclear why some patients do and some patients do not develop epilepsy following a first seizure. Heinemann et al. have brought up the hypothesis that lasting dysfunction of the cerebral microcirculation results in dysregulation of the normal network response to injury, leading to the development of epilepsy. Therefore, they have emphasized the urgent need for non-invasive clinical visualization of the cerebral microcirculation in order to evaluate local dynamical functioning and possible injury [8].

The glycocalyx is a significant component of the cerebral microcirculation and involved in the regulation of plasma cell adhesion, oxidative stress, and shear stress reduction [11,12]. The glycocalyx is easily disrupted, i.e. reduced in thickness [16]. Disruption is induced by local cytokine expression and ischemia, and results in inflammation, edema, oxidative stress and loss of vascular responsiveness [11]. Also, many vascular disease risk factors and specific diseases like lacunar stroke, sepsis, and renal failure are associated with decreased glycocalyx thickness [28,32–35].
The glycocalyx plays an important role in vascular wall permeability. Albumin extravasation is prohibited by the negatively charged glycocalyx. Whereas, leukocytes cannot attach to endothelial surface components including important cell-adhesion molecules, like ICAM-1 and VCAM-1, that reside within the glycocalyx. As a consequence, leucocyte adhesion and extravasation is limited. Therefore, a disturbed glycocalyx is suspected to play a significant role in increased BBB permeability including albumin and leucocyte extravasation, as is seen in epilepsy.

Assessment of the cerebrovascular glycocalyx offers the opportunity to gain greater insights in its thickness and function at the level of the cerebral microcirculation. Advances in microcirculation imaging by handheld SDF-based video microscopes enables real-time clinical assessment of the microcirculation. Several cerebral microcirculation parameters, but not the glycocalyx, have been evaluated using this technique [24–27]. It was recently pointed out that SDF imaging is the most eligible technique, when proper software is subjoined [11]. Using SDF imaging, we aim to visualize the cerebrovascular glycocalyx and measure its thickness in TLE-patients and controls. Subsequently,

cerebrovascular glycocalyx dimensions are compared to sublingual dimensions to establish whether glycocalyx dimensions are regulated at a systemic level.

In this study, we will determine whether glycocalyx thickness is reduced in TLE-patients when compared to controls. Included TLE-patients have undergone thorough examination and were selected by a multidisciplinary team. Naturally, genuinely healthy controls for intracranial cerebrovascular glycocalyx assessment are not available, making the selected patients the most suitable candidates as controls. Since an intact hemodynamic circulation is required for SDF-imaging, post-mortem patients cannot be included as controls.

The upper age cut-off of 60 years is included to reduce the influence of microcirculatory degeneration which is associated to increasing age. As tumor patients are, on average, older patients than epilepsy patients, this could otherwise have been an important confounder. In order to limit the risk of vascular abnormalities of the visualized and assessed vessels, patients will only be included as controls when non-compressed and/or non-edematous cerebral cortex can be assessed during surgery. In case of patients undergoing surgery for vascular abnormalities, the cortical measurements are performed at the furthest distance away of the abnormality, as is allowed by the craniotomy.

Typical pathological findings in TLE include hippocampal sclerosis characterized by neuronal cell loss, reactive astrogliosis, mossy fiber sprouting, and granular cell dispersion [5]. Unfortunately, assessment of the hippocampal glycocalyx can only be performed in TLE-patients, as the hippocampus will rarely be exposed in the control subjects. Hippocampal glycocalyx thickness of TLE-patients will be compared to cortical glycocalyx thickness and cannot be compared to hippocampal controls.

When clinically relevant differences in glycocalyx dimensions are found, future research to determine glycocalyx component variation, glycocalyx permeability determinants and mechanisms of disruption and repair, is required. Subsequently, repair of the glycocalyx could be a selective and efficient, yet hypothetical, target for modification of increased BBB permeability. This would open a new field of pharmacological interventions for currently drug-resistant epilepsy patients.

Table 1: Overview of inclusion and exclusion criteria.

Inclusion criteria	
TLE*-patients	**Control-patients**
Mentally-competent patients between 18 and 60 years of age.	Mentally-competent patients between 18 and 60 years of age.
Patients undergoing resective surgery for unilateral drug-resistant TLE of which the epileptic focus is located in non-eloquent area.	Patients undergoing an elective craniotomy for intracranial tumor resection or neurovascular surgery, or undergoing burr hole surgery for tumor biopsy, in non-eloquent area.
Exclusion criteria	
Applicable to all patients	
Patients who are pregnant, or have known diabetes mellitus, hyperlipidemia, history of stroke or other cardiovascular diseases, or use of cardiovascular medication.	
Patients who have silent signs of cerebral small vessel disease on brain MRI**.	
Applicable to control-patients only	
Patients in whom no 'normal', 'non-compressed' and/or 'non-edematous' cerebral cortex can be assessed intraoperatively.	
Patients in whom a history of seizures is reported.	

*TLE: temporal lobe epilepsy; **MRI: magnetic resonance imaging.

Author's contribution

R.H.L. Haeren: executive investigator and concept, drafting, design and revising of protocol.

H. Vink: concept and critically revising of protocol.

J. Staals: design and critically revising of protocol.

M.A.M.J. van Zandvoort: concept and critically revising of protocol.

J. Dings: concept and design of protocol.

J.J. van Overbeeke: design and critically revising of protocol.

G. Hoogland: concept and design of protocol.

K. Rijkers: executive investigator, and concept, drafting and design of protocol.

O.E.M.G. Schijns: principal investigator, and concept, design and critically revising of protocol.

Disclosure

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Conflict of interest

H. Vink is Chief Science Officer at GlycoCheck BV.

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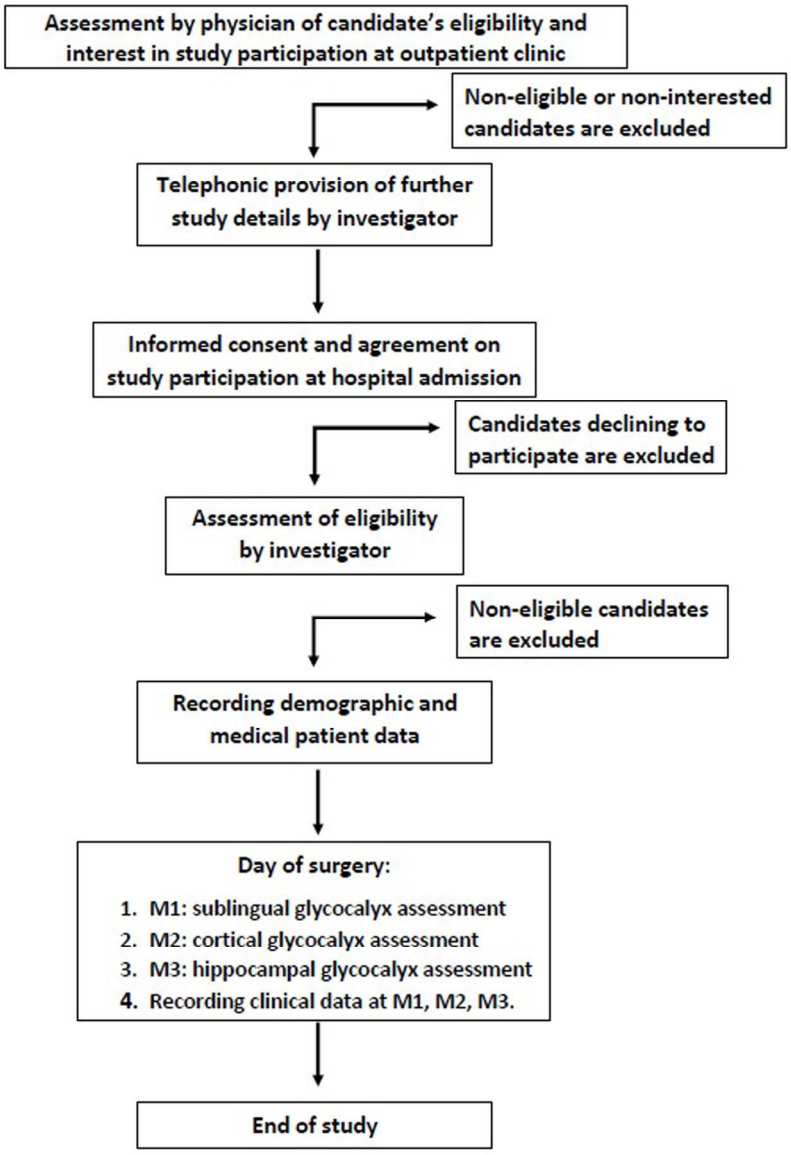

Study inclusion and intervention timeline for patients and controls.

127x161mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls
		Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed
		Case-control study—For matched studies, give matching criteria and the number of controls per case N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed
		Case-control study—If applicable, explain how matching of cases and controls was addressed N/A
		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol for intraoperative assessment of the human cerebrovascular glycocalyx

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Protocol for intraoperative assessment of the human cerebrovascular glycocalyx

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Abstract

Introduction

Adequate functioning of the blood-brain barrier (BBB) is important for brain homeostasis and normal neuronal function. Disruption of the BBB has been described in several neurological diseases. Recent reports suggest that an increased permeability of the BBB also contributes to increased seizure susceptibility in epilepsy patients.

The endothelial glycocalyx is coating the luminal side of the endothelium and can be considered as the first barrier of the BBB. We hypothesize that an altered glycocalyx thickness plays a role in the etiology of temporal lobe epilepsy (TLE), the most common type of epilepsy. Here, we propose a protocol that allows intraoperative assessment of the cerebrovascular glycocalyx thickness in TLE patients and assess whether its thickness is decreased in TLE-patients when compared to controls.

Methods and analysis

This protocol is designed as a prospective observational case-control study in patients who undergo resective brain surgery as treatment for TLE. Control subjects are patients without a history of epileptic seizures, who undergo a craniotomy or burr hole surgery for other indications. Intraoperative glycocalyx thickness measurements of the sublingual, cortical, and hippocampal microcirculation are performed by videomicroscopy using sidestream darkfield imaging. Demographic details, seizure characteristics, epilepsy risk factors, intraoperative hemodynamic parameters, and histopathological evaluation are additionally recorded.

Ethics and dissemination

This protocol has been ethically approved by the local medical ethical committee (ID: NL51594.068.14) and complies with the Declaration of Helsinki and principals of Good Clinical Practice. Informed consent is obtained before study enrollment and only coded data will be stored in a secured database, enabling audit trail. Results will be submitted to international, peer-reviewed journals and presented at international conferences.

Registration details

Registered at the Netherlands National Trial Register: NTR5568.

1. Introduction

With a prevalence of 4-10 per 1000, epilepsy is one of the most common neurological disorders [1]. The most frequent type of focal epilepsy is temporal lobe epilepsy (TLE). Although most patients have adequate seizure control by using antiepileptic drugs, it is estimated that 22.5 % - 30 % of patients are drug-resistant [2,3]. This implies that worldwide about 13 million patients suffer from drug-refractory epilepsy, which has a major impact, both medically and socioeconomically [3,4]. The development of new antiepileptic drugs has hardly reduced the number of drug-resistant patients, and therefore several different treatment alternatives have been explored over the past 25 years. Epilepsy surgery has been demonstrated to be a successful treatment alternative in selected patients. Furthermore, discovery of new drugs, targeted at specific underlying pathophysiologic mechanisms keeps holding a promise for improved treatment of drug-resistant epilepsy patients.

In this regard, important discoveries on microvasculature abnormalities in TLE-patients have been reported, such as loss of blood-brain barrier (BBB) integrity [5–7]. BBB opening induces extravasation of proteins, like albumin, which in turn activates transforming growth factor β (TGFβ) signalling in astrocytes, leading to astrocytic transformation [7,8]. This transformation is characterized by downregulation of inward rectifier potassium channels and astrocytic glutamate transporters, leading to impaired cerebral homeostasis, altered neurovascular coupling, enhanced neuronal excitability, and upregulation of proinflammatory cytokines [8,9]. Moreover, leucocyte extravasation due to increased BBB permeability, contributes to neuronal excitability and reorganization of local neuronal networks [9]. Thus, loss of BBB integrity results in increased seizure susceptibility and contributes to epileptogenesis.

The barrier function of the BBB is mainly determined by the endothelium. Endothelial cells are interconnected by tight junctions and adherent junctions to prevent paracellular diffusion [10]. As a consequence, the endothelium forms a continuous cell membrane layer along the cerebral capillaries. Solutes and nutrients are transported by transport proteins expressed on the endothelial cells [10,11]. Endothelial cells thus restrict and actively controls the passage of substances from the blood to the brain in order to tightly regulate cerebral homeostasis. The pericytes and astrocyte foot processes form a complex network surrounding the endothelial cells to induce and maintain endothelial barrier properties. As was recently pointed out, the endothelial glycocalyx could be a significant determinant of the BBB function as well [12]. The endothelial glycocalyx, further referred to as 'glycocalyx', is a gel-like layer lining the luminal surface of the endothelium. It has important barrier properties that reduce the interaction between endothelial cells and plasma cells and components. As a consequence, the glycocalyx limits leucocyte adhesion and protein extravasation [13–16].

The glycocalyx is a vulnerable layer that is easily disrupted resulting in a reduced thickness [17]. As part of the BBB, a disrupted glycocalyx would result in increased BBB permeability, and propagate leucocyte adhesion and extravasation. A number of laboratory and clinical studies have shown that both increased BBB permeability and leucocyte adhesion/extravasation play a role in epilepsy [7–9,18].

So far, no data are available on glycocalyx thickness in epilepsy. The cerebrovascular glycocalyx has only been evaluated preclinically [19–22]. None of these studies have analyzed glycocalyx thickness in relation to neurological diseases. However, glycocalyx thickness can be assessed noninvasively as part of microcirculation imaging using sidestream darkfield (SDF) imaging [12]. To date, this technique has mainly been performed to assess the sublingual microcirculation.

Our hypothesis is that the cerebrovascular glycocalyx can be visualized clinically using SDF imaging, and that drug-resistant TLE-patients have a decreased cerebrovascular glycocalyx thickness in comparison to controls. In this paper, we present the study protocol according to the STROBE guidelines for case-control studies and SPIRIT protocol guidelines [23,24].

The primary objective of this case-control study is to visualize the cerebrovascular glycocalyx using SDF imaging in TLE-patients and control patients during brain surgery. Moreover, an eventual correlation between the cerebrovascular and sublingual glycocalyx dimensions is assessed.

2. Methods

2.1 Study setting and population

This is a multicenter prospective observational case-control study that involves assessment of the sublingual, cortical, and hippocampal glycocalyx. In conjunction, data are recorded regarding patient's medical history, current physiology, and cerebral pathology. Assessments take place during brain surgery, which is performed by the department of Neurosurgery of both the Maastricht University Medical Center+ (MUMC+) and the Zuyderland Medical Center (ZMC), respectively located in Maastricht and Heerlen, The Netherlands.

Patients with unilateral drug-resistant TLE that are designated for resective brain surgery, i.e. anterior temporal lobectomy and/or amygdalohippocampectomy, are possible study candidates. The diagnosis of unilateral TLE is based on thorough examination including a video-EEG, high-resolution MRI, neuropsychological examination and, when indicated, a PET- and or SPECT-scan, functional MRI, and eventually subdural or depth (stereo-EEG) electrode implantation. Subsequently, eligibility for resective surgery is assessed by a multidisciplinary team, consisting of neurologists, clinical neurophysiologists, neuropsychologists, neuroradiologists, and neurosurgeons.

The control group includes patients without a history of epileptic seizures, and who will undergo a craniotomy for intracranial tumor resection or neurovascular indications, like aneurysm clipping and arteriovenous malformation resection, or who will undergo burr hole surgery for tumor biopsy.

We have included an upper age limit of 60 years to avoid 'background' microvascular disease which is strongly age-related. Especially since tumor patients are already older, on average, than epilepsy patients, age could otherwise have been an important confounder. Control patients with vascular pathology, like aneurysms and arteriovenous malformation, are not known to have microcirculatory pathology. Moreover, cortical measurements are performed at a distance of the vascular pathology as allowed by the craniotomy, by example the superior temporal gyrus in a patient with an anterior cerebral artery aneurysm. Cortical measurements in the oncological control patients will also be performed at the furthest distance away of the abnormality possible, as allowed by the craniotomy.

2.2 Eligibility criteria

Inclusion criteria are: mentally competent adults between 18 and 60 years of age who will undergo resective surgery for unilateral drug-resistant TLE, or for a tumor or vascular abnormality, or burr hole surgery for tumor biopsy (table 1). Exclusion from this study occurs in case of pregnancy, history of established hypertension, diabetes mellitus, hyperlipidemia, stroke or other cardiovascular disease, use of cardiovascular medication, or non-symptomatic signs of cerebral small vessel disease on brain MRI. Additionally, control patients in which no 'normal', 'non-compressed' and/or 'non-

edematous' cerebral cortex can be assessed during surgery or in whom a history of seizures is reported, are excluded.

2.3 Intervention protocol

The glycocalyx thickness is assessed intraoperatively at the following time points: 1. a sublingual measurement (M1) directly following the induction of general anesthesia, 2. a cortical measurement (M2) upon opening of the meninges allowing a direct view at the cortex and 3. a hippocampal measurement (M3) only in TLE-patients. The latter measurement is performed upon removal of the temporal neocortex allowing view at the hippocampus. At each time point, systolic and diastolic blood pressure, heart rate, pulse oxygen saturation, hemoglobin concentration, and hematocrit are additionally recorded.

Glycocalyx measurements are performed using an SDF video microscope. The camera consists of a central light guide with magnifying lens and concentric light emitting diodes. The diodes emit light at a wavelength of 530 nm, which is absorbed by (de)-oxyhemoglobin in erythrocytes. Consequently, erythrocytes appear black on a grayish background. This technique has mainly been performed on the sublingual microcirculation but has previously been used for cerebral microcirculation assessment as well [25–29]. However, the glycocalyx was not measured in these studies [25–28]. Recently, it was pointed out that SDF imaging, when combined with dedicated software, is the most suitable technique for clinical cerebrovascular glycocalyx visualization [12].

In order to reduce user-induced variations, image acquisition is trained. Using the camera with low pressure, minimizing movement artefacts and optimizing light intensity and focus are of particular importance to further reduce variability.

The glycocalyx analysis is based on the principle of the erythrocyte-endothelial exclusion zone. Since erythrocytes do not significantly compress the glycocalyx, there is an exclusion zone between the red blood cell column (RBCC) and the endothelium. GlycoCheck © software automatically selects approximately 3,000 microcirculatory vessel segments and measures the RBCC width at each segment. Based on the RBCC width distribution, the total perfused diameter (Dperf) is assessed by linear regression analysis of the 25^{th} and 75^{th} RBCC width percentiles [12]. The perfused boundary region (PBR) is the outermost luminal part of the glycocalyx that is only slightly permeable for erythrocytes. This region is calculated in a two-dimensional plane as follows: PBR = (Dperf – median RBCC) / 2. As an unstable or damaged glycocalyx is more accessible to erythrocytes, an increased RBCC and, consequently, increased Dperf and PBR values signify a damaged glycocalyx [29].

2.4 Outcome

The primary outcome of this study is cortical glycocalyx thickness in TLE patients and controls. The thickness is estimated by measuring the perfused boundary region (PBR) and the total perfused diameter (Dperf), both expressed in micrometers (µm).

As secondary outcome, sublingual, cortical, and hippocampal glycocalyx thickness in TLE-patients and controls are evaluated within and between groups. In addition, we aim to analyze the relation between these measures and epilepsy-specific risk factors (e.g. febrile seizures, traumatic brain injury), seizure characteristics, drug use (e.g. type of drugs, dosage), and histopathological outcome (e.g. degree of hippocampal sclerosis, type of cortical dysplasia). Finally, glycocalyx thickness values will be correlated to general demographic (e.g. age, gender, bodyweight, smoking status) and intraoperative clinical parameters.

2.5 Recruitment capacity, consent and timeline

We have calculated a sample size of 15 patients for each group, to be included over 4 years. At the MUMC+ and ZMC, all types of surgery required for inclusion are performed at least 15-20 times every year. Based on these surgery numbers, the likelihood to recruit the calculated sample size is high.

The study participants' time line is outlined below and an overview is also given in figure 1.

1. Recruitment

   Patients will visit the neurosurgical outpatient clinic preoperatively. During this visit, the neurosurgeon will ask the patients whether they are interested to take part in the study. If interested, they will receive the applicable patient information brochure (PIB). Within a few days, possible candidates will be informed more extensively by one of the investigators about the study objective, procedures, benefits, risks, and insurance. Participation decisions will be inquired at the day of admission to the hospital. Patients are allowed at least 3 days to consider participation for the study, starting from the moment of receiving the PIB. Participation is voluntarily and does not affect standard treatment in any way. Patients will be informed that they can decide to end their participation in the study at any time.

2. Informed consent

   At the day of hospital admission, patient's study participation is ultimately inquired. When patients agree on participation, the informed consent agreement will be signed in duplicate.

3. Data recording

   Following informed consent agreement, patient data will be recorded from the digital patient files. The digital patient files encompass the digital patient dossier at the MUMC+, ZMC and, when applicable, the file of the multidisciplinary epilepsy surgery work-up.

4. Intraoperative measurements

   A total of two or three glycocalyx measurements will be performed, as described in paragraph 2.3. All measurements are performed at the operating theatre when the patient is under general anesthesia. The hippocampal measurement is solely performed in TLE-patients.

5. End of study

   Directly following the final measurements, i.e. M2 in control-patients and M3 in TLE-patients, patients have reached the endpoint of this study.

2.6 Population size

We have calculated a population size of 15 patients per group. This number is based on a power of 80% to detect a difference of cortical glycocalyx thickness of at least 12% between groups with a standard deviation of 15%, at a significance level of 0.05 and an expected drop-out of 2 patients.

Since there is no literature on cerebrovascular glycocalyx thickness, we have reviewed and assessed the literature on variation in sublingual glycocalyx measurements. Intra-individual variation of sublingual glycocalyx thickness, assessed using SDF imaging, has been found to be ±5% [30]. A difference in PBR thickness of 9.6-12.5 % with a standard deviation of ± 15% has been found relevant when comparing a disease state to healthy controls [29–32]. Based on these studies, we have determined minimal glycocalyx difference and standard deviation. Due to the explorative nature of our study, clinical relevance of a 12% difference is indistinct.

2.7 Data processing

2.7.1 Procedures

This study complies with the Declaration of Helsinki, and will be conducted in accordance with the principals of Good Clinical Practice (GCP). Standardized processing files for obtaining informed consent, measurement procedures, reporting (serious) adverse events and recording patient and measurement data parameters in the electronic case report file (eCRF), are available. Investigators obtaining informed consent from the patient, performing glycocalyx measurements, and recording eCRF data will receive specific training beforehand.

2.7.2 Data management

Patient's demographic and clinical data are recorded in an eCRF at a secure encrypted database (by Castor EDC©), which enables audit trail and is GCP certified. Measurement procedures are trained and standardized as described in paragraph 2.3. Measurement data is collected at a secure encrypted

laptop and outcome is recorded in the eCRF. After verification of recorded data to source data by one the executive investigators, recorded data in the eCRF by Castor EDC© will be exported to a SPSS file for further statistical analysis.

Patients will be assigned an alphanumeric sequential study number to identify all clinical data. Patient's demographic data linked to the assigned study number is documented in a separate screening database held on a secure computer at both study sites. Source data, the code encrypting document, and coded data in the study database are locked and only accessible to the principal and executive researchers, and monitors. On completion of the study, the study database will be locked and data is securely archived for 15 years in accordance with local policy.

Due to the nature and short participation time period of this study, we expect full patient retention and adherence.

2.8 Safety

The principal investigator (OS) has overall responsibility for the conduct of this study including safety. Individual investigators will be responsible for reporting adverse events (AEs) and serious adverse events (SAEs) to the principal investigator. SAEs are defined as AEs resulting in death, life threatening events, prolonged hospital stays, or significant disability. There are no reported (S)AEs associated with the use of SDF imaging in the current literature. A possible attributable risk of glycocalyx measurement is sublingual, cortical, or hippocampal contusion due to pressure on the tissue during the measurement. We deem risk frequency and severity as low. Moreover, cortical or hippocampal local contusion will take place in non-eloquent and to-be-resected tissue. Postoperative consequences due to this contusion are unlikely. All events are reviewed by the principal investigator to decide if there is a causal link and, when applicable, appropriate action will be undertaken. SAEs will be reported to the local medical ethical committee (METC azM/UM) according to local policy. Liability and subject insurance is provided.

2.9 Statistical analysis

Data are presented as mean and standard deviation when normally distributed, and non-normally distributed data as median and interquartile range. Glycocalyx dimensions between groups are compared using the independent T-test or the Mann-Whitney test, as appropriate. Correlation between sublingual and cortical, sublingual/cortical and hippocampal glycocalyx thickness is calculated by Pearson's or Spearman's correlation coefficient, when data is normally or non-normally distributed, respectively. In TLE-patients, the cortical and hippocampal glycocalyx thickness results are correlated to seizure characteristics, epilepsy risk factors, anti-epileptic drug usage, and

histological findings of pathological examination by using Pearson's or Spearman's correlation coefficient or uni- and multivariate regression analysis, as appropriate. Demographic and clinical parameters are correlated to glycocalyx thickness results by using uni- and multivariate regression analysis. A p-value of < 0.05 will be considered statistically significant. Statistical analysis is performed using SPSS software.

Interim-analyses will be performed yearly and following data recording of 5 patients in each group. When a significant difference between the groups regarding the primary outcome is found, the study will be terminated prior to inclusion of 15 patients in each group. Interim-analyses are reported to the local medical ethical committee.

2.10 Monitoring and auditing

This study was classified as minimal-risk by the local data monitoring committee (CTCM). Monitoring visits include review of consent and study procedures according to study protocols, source data and audit trail verification, and the review of (serious) adverse event reporting. Monitoring is independent and performed at least once a year. Monitor evaluations are reported to the local medical ethical committee. Unannounced audits can be performed by the audit team of the CTCM.

2.11. Ethics, amendments, and dissemination

This research protocol has been approved by the local medical ethical committee (METC azM/UM) and has been assigned the following protocol ID: NL51594.068.14. Also, this study has been registered at the Netherlands National Trial Register (ID: NTR 5568). The NTR is acknowledged by the WHO and International Committee of Medical Journal Editors (ICMJE).

Substantive protocol amendments will be reported, reviewed, and approved by the METC azM/UM before application. In the currently presented protocol, a variety of substantive protocol amendments have already been incorporated. Substantive amendments were introduced due to lagging inclusion. The substantive amendments included, extending the study by the addition of the ZMC as a study center, enlarging the control-group with burr hole tumor biopsy and neurovascular surgery patients, and reducing sublingual measurements from three to one.

Results of this study will be evaluated yearly and following 5 participants in both groups. Results will be recorded using audit trails to increase reproducibility. Study protocol and results will be submitted to peer-reviewed journals and presented at international conferences.

3. Discussion

Microvascular injury, in particular increased permeability of the wall of the cerebral microcirculation, seems to play a role in the transformation of astrocytes and increased seizure susceptibility in epilepsy. It is hypothesized that leakage of albumin orchestrates this transformation leading to a disturbed potassium and glutamate metabolism and extracellular cerebral homeostasis [7,8]. Upregulation of proinflammatory cytokines as a response to leakage of leucocytes further contributes to the subsequent increased neuronal excitability [9]. However, it is still unclear why some patients do and some patients do not develop epilepsy following a first seizure. Heinemann et al. have brought up the hypothesis that lasting dysfunction of the cerebral microcirculation results in dysregulation of the normal network response to injury, leading to the development of epilepsy. Therefore, they have emphasized the urgent need for non-invasive clinical visualization of the cerebral microcirculation in order to evaluate local dynamical functioning and possible injury [8].

The glycocalyx is a significant component of the cerebral microcirculation and involved in the regulation of plasma cell adhesion, oxidative stress, and shear stress reduction [12,13]. The glycocalyx is easily disrupted, i.e. reduced in thickness [17]. Disruption is induced by local cytokine expression and ischemia, and results in inflammation, edema, oxidative stress and loss of vascular responsiveness [12]. Also, many vascular disease risk factors and specific diseases like lacunar stroke, sepsis, and renal failure are associated with decreased glycocalyx thickness [29,33–36].
The glycocalyx plays an important role in vascular wall permeability. Albumin extravasation is prohibited by the negatively charged glycocalyx. Whereas, leukocytes cannot attach to endothelial surface components including important cell-adhesion molecules, like ICAM-1 and VCAM-1, that reside within the glycocalyx. As a consequence, leucocyte adhesion and extravasation is limited. Therefore, a disturbed glycocalyx is suspected to play a significant role in increased BBB permeability including albumin and leucocyte extravasation, as is seen in epilepsy.

Assessment of the cerebrovascular glycocalyx offers the opportunity to gain greater insights in its thickness and function at the level of the cerebral microcirculation. Advances in microcirculation imaging by handheld SDF-based video microscopes enables real-time clinical assessment of the microcirculation. Several cerebral microcirculation parameters, but not the glycocalyx, have been evaluated using this technique [25–28]. It was recently pointed out that SDF imaging is the most eligible technique, when proper software is subjoined [12]. Using SDF imaging, we aim to visualize the cerebrovascular glycocalyx and measure its thickness in TLE-patients and controls. Subsequently,

cerebrovascular glycocalyx dimensions are compared to sublingual dimensions to establish whether glycocalyx dimensions are regulated at a systemic level.

In this study, we will determine whether glycocalyx thickness is reduced in TLE-patients when compared to controls. Included TLE-patients have undergone thorough examination and were selected by a multidisciplinary team. Naturally, genuinely healthy controls for intracranial cerebrovascular glycocalyx assessment are not available, making the selected patients the most suitable candidates as controls. Since an intact hemodynamic circulation is required for SDF-imaging, post-mortem patients cannot be included as controls.

The upper age cut-off of 60 years is included to reduce the influence of microcirculatory degeneration which is associated to increasing age. As tumor patients are, on average, older patients than epilepsy patients, this could otherwise have been an important confounder. In order to limit the risk of vascular abnormalities of the visualized and assessed vessels, patients will only be included as controls when non-compressed and/or non-edematous cerebral cortex can be assessed during surgery. In the control group, the cortical measurements are performed at the furthest distance away of the abnormality, as is allowed by the craniotomy.

Typical pathological findings in TLE include hippocampal sclerosis characterized by neuronal cell loss, reactive astrogliosis, mossy fiber sprouting, and granular cell dispersion [5]. Unfortunately, assessment of the hippocampal glycocalyx can only be performed in TLE-patients, as the hippocampus will rarely be exposed in the control subjects. Hippocampal glycocalyx thickness of TLE-patients will be compared to cortical glycocalyx thickness and cannot be compared to hippocampal controls.

There are some important variables that could affect the glycocalyx thickness that are not directly assessed in this study. These variables concern hemodynamic variations, intravascular volume variations and the effect of different anesthetics. Due to the explorative nature of this study, we have not included these variables, although post-hoc analyses will be performed to determine confounding. Another reason for exclusion of these variables is that this would necessitate additional preoperative (awake) and intraoperative sublingual measurements of the glycocalyx. At first, these measurements were included in our study protocol. But due to anxiety for the preoperative measurement, and the risks for sterility of the surgery due to the intraoperative measurements, these sublingual measurements were excluded in an amendment (see paragraph 2.11). The possible effects of intraoperative hemodynamic variation and anesthesia on glycocalyx thickness could however be an interesting future study. When clinically relevant differences in glycocalyx dimensions are found, future research to determine glycocalyx component variation, glycocalyx permeability

determinants and mechanisms of disruption and repair, is required. Subsequently, repair of the glycocalyx could be a selective and efficient, yet hypothetical, target for modification of increased BBB permeability. This would open a new field of pharmacological interventions for currently drug-resistant epilepsy patients.

Figure legends:

Figure 1: Overview of inclusion and exclusion criteria.

Author's contribution

R.H.L. Haeren: executive investigator and concept, drafting, design and revising of protocol.

H. Vink: concept and critically revising of protocol.

J. Staals: design and critically revising of protocol.

M.A.M.J. van Zandvoort: concept and critically revising of protocol.

J. Dings: concept and design of protocol.

J.J. van Overbeeke: design and critically revising of protocol.

G. Hoogland: concept and design of protocol.

K. Rijkers: executive investigator, and concept, drafting and design of protocol.

O.E.M.G. Schijns: principal investigator, and concept, design and critically revising of protocol.

Disclosure

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Conflict of interest

H. Vink is Chief Science Officer at GlycoCheck BV.

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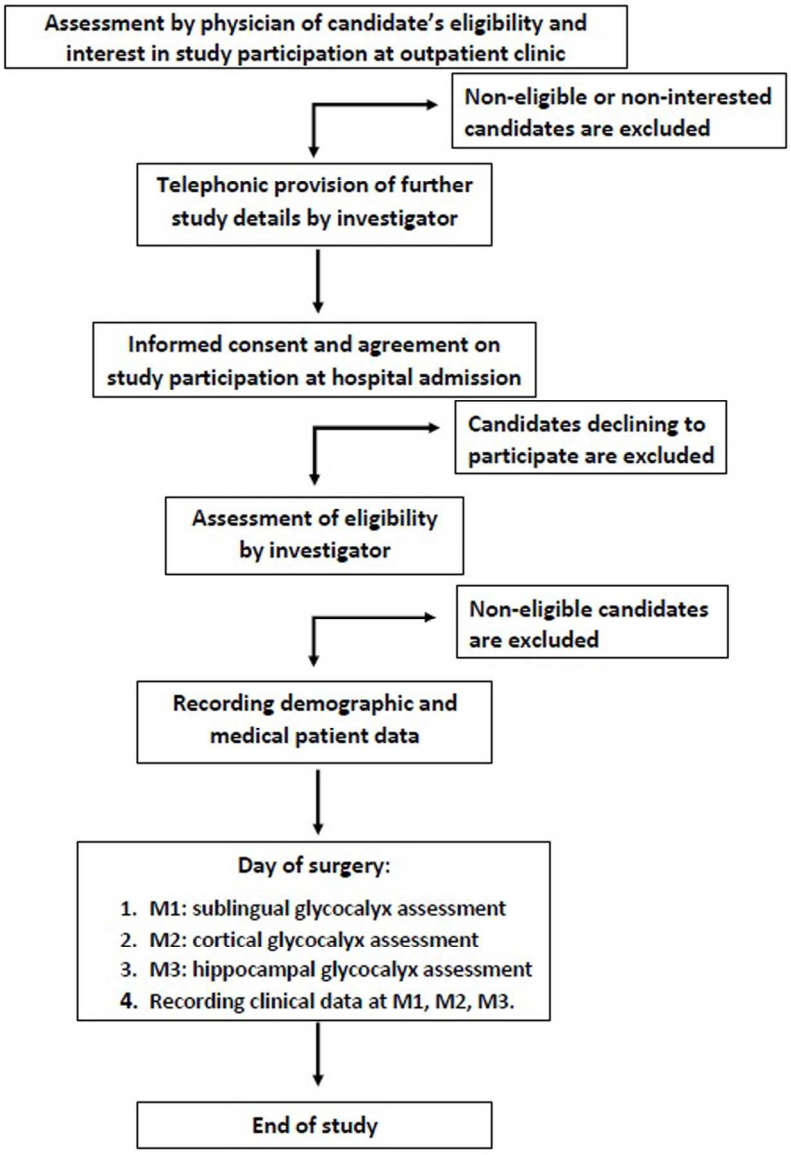

Study inclusion and intervention timeline for patients and controls.

127x161mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls
		Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed
		Case-control study—For matched studies, give matching criteria and the number of controls per case N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed
		Case-control study—If applicable, explain how matching of cases and controls was addressed N/A
		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.