Predicting experimental success: A retrospective case-control study using the rat intraluminal thread model of stroke

Lisa Liebenstund, DVM[1]; Mark Coburn, Prof[1]; Christina Fitzner, Dipl-Stat[1,2]; Antje Willuweit, PhD[3]; Karl-Josef Langen, Prof[3,4]; Jingjin Liu, MD[1]; Michael Veldeman, MD[5]; Anke Höllig, MD[5]*

1 Department of Anesthesiology, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; e-mail: lliebenstund@ukaachen.de; mcoburn@ukaachen.de; Journal@fitzworld.de; jingjin.liu@rwth-aachen.de.
2 3CARE, Cardiovascular Critical Care & Anesthesia Research, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; e-mail: Journal@fitzworld.de.
3 Institute of Neuroscience and Medicine, Medical Imaging Physics (INM-4), Forschungszentrum Jülich GmbH, Jülich, Germany; e-mail: a.willuweit@fz-juelich.de; k.j.langen@fz-juelich.de.
4 Department of Nuclear Medicine, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; email: k.j.langen@fz-juelich.de.
5 Department of Neurosurgery, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; e-mail: mveldeman@ukaachen.de; ahoellig@ukaachen.de.

* Correspondence: ahoellig@ukaachen.de, telephone number: 0049 241 80 35698 (AH)

Received: date; Accepted: date; Published: date

Portions of this work were presented in abstract form at the “45. Jahrestagung der Sektion Intrakranieller Druck, Hirndurchblutung und Hydrozephalus der Deutschen Gesellschaft für Neurochirurgie (DGNC) e.V”. 08. – 09. November 2019 Mainz.

Abstract: The poor translational success rate of preclinical stroke research may partly be due to inaccurate modelling of the disease. We provide data on transient middle cerebral artery occlusion (tMCAO) experiments including detailed intraoperative monitoring to elaborate predictors indicating experimental success (ischemia without occurrence of confounding pathologies).

The tMCAO monitoring data (bilateral cerebral blood flow - CBF, heart rate - HR, mean arterial pressure - MAP) of 16 animals with an “ideal” outcome (MCA-ischemia) and 48 animals with additional or other pathologies (subdural hematoma or subarachnoid hemorrhage) were checked for their prognostic performance (receiver operating characteristic curve and area under the curve - AUC).

Animals showing a decrease in the contralateral CBF at the time of MCA occlusion suffered from unintended pathologies. Implementation of baseline MAP in addition to baseline HR (AUC 0.83, 95% CI 0.68 to 0.97) increased prognostic relevance (AUC 0.89, 95% CI 0.79 to 0.98). Prediction performance improved when two additional predictors referring differences in left and right CBF were considered (AUC 1.00, 95% CI 1.0 to 1.0).

Our data underline the importance of periinterventional monitoring to verify a successful experimental performance in order to ensure a disease model as homogeneous as possible.

Keywords: tMCAO; rat model; stroke animal model; periinterventional monitoring; experimental quality assurance

1. Introduction

Despite extensive clinical and experimental research in the field of ischemic stroke hardly any findings take the decisive step from bench to bedside (Endres, Engelhardt et al. 2008). Basically there are various pitfalls and problems which have to be addressed in order to produce clinically relevant and reproducible results (Tymianski 2015). One aspect is the documentation and reporting of studies, including results and limitations. Clinical scientists started to implement clear and structured reporting guidelines in order to provide transparent and complete reporting of randomized clinical trials (RCT), resulting in the publication of the Standardized Reporting of Trials (SORT) statement in 1994, which has been developed further in 1996 (Consolidated Standards of Reporting Trials (CONSORT) Statement) as a result of the merge with the at the same time developed Asilomar Guideline (by the Asilomar Working Group on Recommendations for Reporting of Clinical Trials in the Biomedical Literature) (1994, 1994, Begg, Cho et al. 1996). In line with this effort to increase the transparency and improve the quality of studies with regards to preclinical research several calls (Landis, Amara et al. 2012) and recommendations such as the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations (initially published in 1999) (Stroke Therapy Academic Industry 1999) (with their updates from 2009 (Fisher, Feuerstein et al. 2009) and 2019 (Savitz, Baron et al. 2019)) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments, published in 2010) (Kilkenny, Browne et al. 2010) criteria and have been published. The IMPROVE guidelines also provide a detailed guide for the performance of ischemia models (Percie du Sert, Alfieri et al. 2017).

Another aspect is the experimental methodology itself: The most common model in preclinical stroke research is the MCAO (middle cerebral artery occlusion) model once established by Koizumi et al.(Koizumi, Yoshida et al. 1986) and later modified by Longa et al. (Longa, Weinstein et al. 1989). It is a legitimate objection to ask if the common animal models actually reflect the disease exhaustively, if statistical instruments (as effect sizes) are used properly, if end-points are chosen reasonably (prior to study start) and if the complexity of the pathology actually is considered (Endres, Engelhardt et al. 2008, Sughrue, Grobelny et al. 2010, Hossmann 2012, Landis, Amara et al. 2012, Mergenthaler and Meisel 2012). From a technical point of view, however, one also has to consider if the model is applied correctly or if there might be scope for improvement: Reproducibility of preclinical data is a major issue (Prinz, Schlange et al. 2011, Llovera, Hofmann et al. 2015).

Unfortunately, due to heterogeneous experimental setups, studies using MCAO model bear the risk of inconsistent outcomes and limited reproducibility (Braeuninger and Kleinschnitz 2009). The extent of the ischemic damage varies highly due to the variable cerebrovascular anatomy of different rat strains (Oliff, Weber et al. 1995, Bardutzky, Shen et al. 2005, Walberer, Stolz et al. 2006), different types of filaments (Bouley, Fisher et al. 2007, Zhao, Mayhan et al. 2008) or anesthesia (Zausinger, Baethmann et al. 2002, Zhao, Mayhan et al. 2008). Further, the methods applied to verify correct MCAO (resulting in cerebral ischemia) vary highly: During the intervention, indirect methods are available in order to ascertain correct MCAO (e.g. via measurement of cerebral blood flow (CBF) (Engelhorn, Doerfler et al. 2005, Bleilevens, Roehl et al. 2013) or - with certain limitations - electroencephalography (EEG) (Schmid-Elsaesser, Zausinger et al. 1998, Hungerhuber, Zausinger et al. 2006)); afterwards, histological staining of brain sections (Kramer, Dang et al. 2010, Rousselet, Kriz et al. 2012) or MRI (Iskander, Knight et al. 2013, Trotman-Lucas, Kelly et al. 2017) provide evidence for ischemia. The impact of

ischemia -to some degree- can be described by neurological deficits using neurological severity assessment scores (Bederson, Pitts et al. 1986, Garcia, Wagner et al. 1995) or behavioral tests(Schallert 2006, Encarnacion, Horie et al. 2011). However, one major drawback of the intraluminal method is the occurrence of model-immanent confounding pathologies and complications: incomplete MCAO (Zausinger, Baethmann et al. 2002), early, late or missing reperfusion (Livnat, Barbiro-Michaely et al. 2010), and subarachnoid hemorrhage (Longa, Weinstein et al. 1989, Schmid-Elsaesser, Zausinger et al. 1998). Particularly with regard to time- and labour-consuming long-term experiments a reliable and prompt surveillance of the desired vessel occlusion excluding any secondary damages is of great importance because MRI is not always available or feasible and the additional lesions at the time of sacrifice may no longer be recognizable and therefore results may be biased.

We present data on a selection of MCAO experiments including detailed monitoring of bilateral CBF, heart rate and systolic/diastolic and mean artery blood pressure. The aim of our secondary analysis is the identification of predictors and their optimal combination to anticipate a successful induction of ischemic stroke, thus the early detection of confounding pathologies such as subarachnoid hemorrhage (SAH) and subdural hematoma (SDH).

2. Materials and Methods

The experiments were performed at the Medical Faculty of the RWTH Aachen University, Aachen, Germany, in accordance with German legislation governing animal studies (Tierschutzgesetz, Tierschutz-Versuchstierverordnung) and in accordance with the ARRIVE guidelines. The protocol (reference number 84-02.04.2013.A418) was approved by the State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Recklinghausen, Germany.

This report was guided by the TRIPOD (Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis) recommendations (Moons, Altman et al. 2015).

In the framework of a randomized controlled animal study, funded by the Deutsche Forschungsgemeinschaft (reference number CO 799/9-1), we performed a retrospective analysis. Results of the underlying study have partially already been published elsewhere (Liu, Nolte et al. 2019).

2.1. Animals

Male *Wistar* rats (Charles River, Sulzfeld, Germany) were housed for at least one week before surgery with free access to food and water on a 12-hour light/dark cycle according to the FELASA health monitoring recommendations (Mahler Convenor, Berard et al. 2014).

2.2. Induction of Focal Cerebral Ischemia

Animals underwent two hours (h) of transient middle cerebral artery occlusion (tMCAO) using the intraluminal thread technique as described previously (Liu, Nolte et al. 2019). Briefly, general anesthesia was applied with an intraperitoneal combination of 0.15 mg/kg body weight (BW) medetomidin (Domitor®, Pfizer GmbH, Berlin, Germany), 2 mg/kg BW midazolam (Midazolam-ratiopharm®, Ratiopharm GmbH, Ulm, Germany) and 0.005 mg/kg BW fentanyl (Rotexmedica GmbH, Trittau,

Germany). 10 ml/kg BW saline was administered every hour of anesthesia as a subcutaneous depot for fluid substitution.

After loss of righting reflex all animals were intubated endotracheally and mechanically ventilated (RUS-1321-RA, Small Animal Ventilator, Föhr Medical Instruments GmbH, Seeheim-Jugenheim, Germany). Protective eye lubricant (Bepanthen®, Bayer Vital GmbH, Leverkusen, Germany) was administered to both eyes, body temperature was maintained at 37-37.5°C with a feedback-controlled warming plate (TCAT-2LV Controller, Physitemp Instruments Inc, Clifton, NJ, USA) during the entire surgical intervention and three electrocardiographic needle electrodes were placed for continuous heart rate monitoring. Surgical anesthesia was judged regularly by the pedal withdrawal reflex.

Bilateral laser Doppler flowmetry (moorVMS-LDF2, Moor Instruments Ltd., Axminster, Devon, United Kingdom) was used to measure CBF over both MCA supplied territories during the entire experiment. A polyethylene catheter (Portex Polythene Tubing, SIMS Portex Ltd., Kent, United Kingdom) was inserted into tail artery for blood pressure measurement.

After exposure of the distal left common carotid artery (CCA) a nylon filament with a silicone-coated tip (0.38 ± 0.02 mm diameter) was introduced into the internal carotid artery (ICA) and advanced until resistance was felt and the left CBF (lCBF) measurement showed a significant drop simultaneously (t0). Two hours after MCAO, brain perfusion was restored by removal of the filament (t120). All parameters analyzed (heart rate: HR; lCBF; right cerebral blood flow: rCBF; mean arterial pressure: MAP; systolic/diastolic pressure) were continuously recorded with a data acquisition system (PowerLab, ADInstruments, Spechbach, Germany). Baseline measurement (BL) was taken 10-15 min after final setup and before surgical approach to the neck vessels; measured values were picked up every 5 min starting with the drop of CBF representing MCAO (t0) and ending 30 min after reperfusion (t150).

Animals were sacrificed either three hours (3h group), seven (7d group) or 30 days after MCAO according to the original study. Animals from the 30 days observation group were not included in the presented analysis. After euthanasia the brains were immediately removed. The evaluation of the brains was carried out by an experienced neurosurgeon. The brains were carefully rinsed off immediately after removal, examined under the microscope and photographed. With the help of the subsequent TTC-staining (3h group) and hematoxylin-eosin staining (all animals, after 4% paraformaldehyde fixation, microscopical assessment), a reliable diagnosis could be made depicting the ischemia and blood distribution.

2.3. Outcome Groups and Experiments Included

The analysis is based upon an original study (DFG CO 799/9-1) including 203 tMCAO procedures (three observation times: 3 h, 7 d and 30 d). During the project a considerable amount of procedures resulting in SAH or SDH was observed. In order to quantify the value of periinterventional monitoring with respect to optimal tMCAO performance (resulting in ischemia), the present analysis was initiated. We did not include experiments from the 30 d observation group, as pathologies as SAH are no more detectable. Thus, we limited ourselves to animals (observation times: 3 h or 7 d) with a clear diagnosis based on macroscopic and microscopic examination.

Out of the initial data pool of 203 tMCAO experiments, 16 animals were selected to represent the "ideal" group (outcome group I; ischemia in the territory supplied by the middle cerebral artery; observation times: 3h: n = 3; 7d: n = 13), whereas all animals with "undesired" results detected after euthanasia (such as additional SDH and/or SAH; n = 48; observation time: 3 h) formed outcome group II. Outcome group I was defined by the following inclusion criteria: macroscopic and microscopic confirmation of MCA-ischemia, exclusion of undesired pathologies (such as SAH or SDH) via macroscopic inspection and microscopic evaluation, full periinterventional monitoring (bilateral CBF measurement, blood pressure, heart frequency and temperature control) applied. Outcome group II was defined accordingly: confirmation of initially undesired pathology (such as SAH or SDH, which may be observed in addition to MCA-ischemia or independent from MCA-ischemia), full periinterventional monitoring (bilateral CBF measurement, blood pressure, heart frequency and temperature control) applied. Details of the animals are shown in Figure 1. Only animals with a clear diagnosis based on macroscopic and microscopic examination and corresponding documentation of vital parameters were chosen.

One animal (SAH and MCA-ischemia) was euthanized before the end of the observation period because of apnea, but all collected data were analyzed. Another animal (SAH) was euthanized due to a bleeding complication during the further experimental course but baseline and t0 values were saved. Further, from four animals only incomplete monitoring data not covering the entire procedure (≤ 2h after MCA-occlusion) were available.

2.4. Tissue preparation

After the initial photo documentation, the brains were either TTC-stained (every second slice, 3h group) or immediately fixed in 4% paraformaldehyde. Then, the fixed sections (each 2 mm thick) were cut and embedded. From the start of each block a 2-µm section was harvested for hematoxylin-eosin staining and the infarct volume measurement.

2.4.1. Infarct volume measurement

Sections were stained with routine hematoxylin-eosin, then visualized and photographed with an Axiovert 200 M microscope (ZEISS, Oberkochen, Germany; × 10 objective). The images were analyzed using ZEN software (ZEISS, Oberkochen, Germany). The sections were analyzed with regards to confirmation of ischemia, identification of undesired pathologies and measurement of infarct volume (outcome group I). The infarct volume was assessed using an indirect method: The volume of the non-lesioned volume of the ischemic hemisphere was subtracted from the total volume of the contralateral hemisphere. Afterwards the volume was normalized to the volume of the contralateral hemisphere (same section).

2.5. Statistical Analysis Methods

Continuous variables are described by mean and standard deviation. Correlation between continuous variables was described by Pearson's correlation coefficient and corresponding 95 % confidence intervals (CI). We have captured the course of the left CBF through two different variants: The difference between mean lCBF before (t0 to < t120) and the mean after occlusion (t120 to t150)

(Δ_1lCBF) and the difference between time point t0 and t120 (Δ_3lCBF). The course of the right CBF were also captured by two variants: the difference between rCBF at baseline and at t0 (Δ_2rCBF) and the mean of all time points (t0 to t150) (mean_rCBF). All single predictors were analyzed by univariate logistic regression with respect to experimental success.

Multiple logistic regression (PROC LOGISTIC) was used to investigate if the specific variables (heart rate at baseline: BL_HR; mean arterial pressure at baseline: BL_MAP; Δ_1lCBF, Δ_2rCBF) predict the occurrence of unwanted pathologies (SAH, SDH or a mixed type). The prognostic performance is described by receiver operating characteristic curve (ROC) and corresponding area under the curve (AUC) including confidence interval. Here, the AUC corresponds to the c-index. For each model we reported R^2, Brier Score and Akaike information criterion (AIC). We used leave-one-out cross validation imputed in PROC LOGISTIC as internal validation and compared results using corresponding Mann-Whitney-U-Test.

If single values in the time course of CBF were missing, the summary measures for each rat were calculated for the available cases. Further missing values were reported.

We assessed a 5 % significance level for each model. Statistical analyses were performed using SAS Software (version 9.4, SAS Institute Inc., Cary, NC, USA). Graphs were created using R Software (R Development Core Team 2018) and GraphPad Prism (version 8.3.0; La Jolla, USA).

.

3. Results

3.1. Comparison of outcome groups

In order to identify predictors indicating undesired results at an early stage of the experiment we compared two groups of animals representing the extremes (“ideal” vs. “undesired” outcome): Outcome group I incorporated 16 animals with MCA-ischemia, whereas outcome group II was composed of 48 animals presenting” undesired” results such as SAH or SDH. The specific diagnoses are presented in Figure 1.

For outcome group I the infarct volume was assessed. A median infarct volume of 30.5% (±13.5%) was found. The most common findings among the “undesired” results (outcome group II) were SAH (n = 9), SDH (n = 7) and SAH additional to MCA-ischemia (n = 22). The courses of bilateral CBF (Fig. 2 +3), MAP (Fig. 4) and HF (Fig. 5) according to the specific diagnoses are depicted below. Results of blood gas analyses have been partially published elsewhere (Liu, Nolte et al. 2019).

3.2. Description and correlation of single predictors

Particularly the courses of the CBF showed distinct patterns according to the final diagnoses: In outcome group I (MCA-ischemia) a sharp decrease of lCBF up to 75 % indicated occlusion of MCA whereas rCBF remained relatively stable (Figure 2 + 3). When the filament was withdrawn after 120 minutes lCBF increased immediately (in some cases even above baseline) and was then followed by a gradual decrease ipsilaterally. Right CBF showed no or just minor increase after withdrawal.

A steep decrease of the rCBF was seen when ischemia was accompanied by SAH or SAH alone was detected (Figure 3).

Both baseline HR (outcome group I: 263.3 ± 76.3 bpm; outcome group II: 198 ± 42.8 bpm; p = 0.0028) and baseline MAP (outcome group I: 90.4 ± 44.5 mmHg; outcome group II: 144.2 ± 38.8 mmHg; p = 0.0013) differed significantly between the two outcome groups (univariate logistic regression, Figure 6). Additionally, Δ_1lCBF (outcome group I: 40.5 ± 37.4 %; group II: -2.8 ± 15 %; p = 0.0005) and Δ_2rCBF (outcome group I: 2.7 ± 10.6 %; outcome group II: 54.9 ± 26.9 %; p = 0.0132) were selected as specific predictor variables (see Figure 6). The initial weight of both outcome groups did not differ significantly (outcome group I: 349.3 ± 36.7 g; outcome group II: 363.7 ± 42.8 g, p = 0.2348).

Moderate up to strong correlations were found for: BL_HR and Δ_2rCBF (Pearson r = -0.40, 95 % CI -0.59 to -0.16), BL_HR and Δ_1lCBF (r = 0.34, 95 % CI 0.09 to 0.55), whereas an inverse correlation was seen for BL_MAP and Δ_1lCBF (r = -0.41, 95 % CI -0.60 to -0.17) (Figure I Supplemental material).

3.3. Prediction of confounding pathologies

Using only single predictors, BL_HR yielded the lowest AUC of 0.83 (95 % CI 0.68 to 0.97), whereas the AUC was slightly higher when baseline MAP was used (0.84; 95 % CI 0.73 to 0.95). The AUC of Δ_1lCBF and Δ_2rCBF proved even higher with 0.95 (95 % CI 0.89 to 1.00) and 0.97 (95 % CI 0.93 to 1.00) respectively. Due to low AUC values baseline systolic (BL_SYS), diastolic blood pressure (BL_DIAS), Δ_3lCBF and mean_rCBF were excluded as predictors from further analysis (Table II Supplemental material). Adding a second parameter (BL_HR and BL_MAP) to the model increased the AUC (0.89, 95 % CI 0.79 to 0.98). The average area under the ROC using BL_HR + Δ_1lCBF + Δ_2rCBF was 0.99 (95 % CI 1.0 to 1.0). Prediction performance improved when all four predictors (BL_HR + BL_MAP + Δ_1lCBF + Δ_2rCBF) were taken into account (AUC 1.00, 95 % CI 1.0 to 1.0), indicating perfect discrimination (details of the prediction model see table 1). Small Brier scores (close to zero) indicate better forecasts: In our model this score ranged from 0.13 using one predictor to 0.00 using four predictors. The entire statistics including all performance measures, odds ratios and cross validation are demonstrated in detail in the Supplemental material.

4. Discussion

Our data underline the importance of perioperative monitoring for the intraluminal thread model of stroke in rats: There is a clear predictive value of bilateral CBF, HR and MAP measurement with regards to undesired results such as SAH or SDH. Bilateral CBF measurement offers simultaneous surveillance of both MCA-territories and - as far as our experience goes - indicated occurrence of SAH clearly. Higher initial HR and low MAP at baseline were associated with the successful induction of MCA-ischemia.

4.1. Bilateral CBF Monitoring via LDF

Yet in 1998, with regards to the MCAO model Schmidt-Elsaesser and colleagues reported occurrence of SAH in up to 30% of the cases and stressed the importance of bilateral CBF measurement as a sensitive tool in order to identify SAH (Schmid-Elsaesser, Zausinger et al. 1998). However, there is still an enormous amount of publications on experiments using the MCAO model without perioperative monitoring. Bearing in mind the “1,026 experimental treatments in acute stroke” from 2006 (O'Collins, Macleod et al. 2006) it has to be mentioned that - from a methodological point of view - there is not only a lack of modelling risk factors (such as age) and confounding diseases (e.g. atherosclerosis) (Endres,

Engelhardt et al. 2008) but also an absence of a sophisticated periprocedural surveillance in order to assure experimental success as undesired pathologies in addition or without ischemia may falsify the experimental result, particularly if unperceived. In contrast to the presented data of Woitzik and Schilling (Woitzik and Schilling 2002), who advocated the use of a unilateral CBF measurement, our data did not allow a distinct discrimination of MCA-ischemia, ischemia plus SAH, SAH and SDH by analyzing left CBF exclusively even though a partial recovery has been observed. Further, by examining a relatively large cohort we were able to detect various complications which all may be discriminated from MCA-ischemia by analyzing CBF bilaterally with an AUC of 1.0. Thus, by bilateral CBF measurement a reliable control of the experimental results is granted, which is essential particularly in cases of long-term experiments where the chance to identify undesired pathologies such as SAH macroscopically or histologically diminishes by time. Strikingly, the results of a survey within the scientific community on "Phase III Preclinical Trials in Translational Stroke Research" revealed that 38% of the participants do not attach importance to the periprocedural CBF monitoring (Boltze, Wagner et al. 2016). Thus, the authors conclude that a "broader awareness of the necessity to thoroughly control stroke induction" is needed (Boltze, Wagner et al. 2016).

4.2. Blood Pressure and Heart Rate Monitoring

Basically, several guidelines recommend to monitor blood pressure closely (Liu, Zhen et al. 2009, Howells, Porritt et al. 2010). The successful induction of stroke in the intraluminal thread model has not been analyzed as a function of baseline MAP or HR up to now. Our results suggest that the successful induction of ischemic strokes is more likely in the presence of lower preischemic MAP values; whereas undesired pathologies like SAH or SDH occur more frequently when preischemic MAP values are higher.

The different baseline MAP and HR values in the outcome groups might be due to the inter-individual sensitivity of animals to anesthetic and analgesic drugs (Davis 2008). These variations can be attributed to differences in age, weight, genetic background of the strain (inbred or outbred), sex, age, body temperature, nutritional/health status (e.g. acid-base metabolism, hemodynamics, pulmonary function) circadian rhythm and endocrine factors (Avsaroglu, van der Sar et al. 2007, Davis 2008).

Of note, the filament model generally shows a high incidence of reperfusion-associated parenchymal hematomas in spontaneously hypertensive rats (SHR) (Henning, Latour et al. 2008), which have been established in order to bring autonomic and cardiovascular effects of stroke into focus and, thus, mimic the pathophysiological human background more specifically. In spontaneously hypertensive animals significantly more frequent vascular injury or hemorrhagic infarction after 3 hours of transient MCAO compared to normotensive rats is reported (Guan, Kozak et al. 2011). This vascular vulnerability and preexisting damage in animals with higher blood pressure resulting in endothelial dysfunction is a possible explanation for the detected susceptibility of the rats to unintended lesions (Yao and Nabika 2012).

Blood pressure and heart rate naturally interact and thus, in any case of hypotension a compensatory rise of HR can be detected (Groth, Blume et al. 2003). Therefore, the correlation of HR and MAP is obvious. The specific rise of HR may occur in answer to hypotension induced by anesthesia;

individual reactions in response to analgesic and anesthetic medications are common (Davis 2008) and may explain the variability of physiological parameters.

4.3. Limitations

Limitations of this study arise from its retrospective character: We processed selected data from an animal study in order to perform this subanalysis to compare the "extreme" results (ideal vs. undesired outcome). Observation times differed within the cohort, but no animals attributed to a long-term observation were included. Furthermore, the applied method of CBF measurement is biased by confounding effects such as moving artifacts and heterogeneous vascular supply. Blood gas analyses (usually at least three measurements during procedure) are not available for all of the animals included. Further, our results are limited to our specific setting (male Wistar rats, injectable anesthetics, ventilated animals and transient occlusion of the MCA). Finally, we cannot propose a final prediction model yet; further research and external validation is required.

4.4. Translational Approach and Recommendations

Considering the enormous efforts and financial investments in preclinical stroke research the translational power remains disappointing. Various aspects may contribute to the translational failure. There are fundamental concerns, if the models used actually reflect the pathology adequately (Hossmann 2012, Mergenthaler and Meisel 2012) and if the experimental methodology (including realistic estimation of effect sizes, sample size planning as well as quality assurance and reporting) is considered responsibly (Braeuninger and Kleinschnitz 2009, Sughrue, Grobelny et al. 2010, Landis, Amara et al. 2012, Llovera, Hofmann et al. 2015, Boltze, Wagner et al. 2016). Beyond that, the predominant inclusion of young adult rats does not reflect human epidemiology of ischemic stroke. Same applies for the health status of laboratory animals: there are several animal models resembling our diverse and ageing society stroke research should not omit. Therefore, an important issue is the implementation of sex and age balanced animals (Endres, Engelhardt et al. 2008, McCullough, de Vries et al. 2014). Further, the methodological quality of preclinical trials varies (Philip, Benatar et al. 2009). But the entire debate is also cross-linked with other aspects such as science policy, funding strategies and scientific culture (acknowledging also negative trials and experimental failures) (Begley, Buchan et al. 2015, Tymianski 2015).

Our data confirms and supports the concrete recommendations for practical application given by the IMPROVE and several other guidelines (Liu, Zhen et al. 2009, Howells, Porritt et al. 2010, Percie du Sert, Alfieri et al. 2017): Among other things, the highest possible reproducibility of a study is achieved by implementing various intraoperative monitoring procedures (Percie du Sert, Alfieri et al. 2017). Based on our data, we confirm the recommendation of the IMPROVE guidelines and advise to monitor cardiovascular and respiratory parameters as continuously as possible from the induction until the discharge of anesthesia (Percie du Sert, Alfieri et al. 2017). This includes surveillance of heart rate, blood pressure, respiratory rate, and blood gases. Intubation and mechanical ventilation offer the possibility of immediate correction in case of deviations of the vital parameters. Invasive and non-invasive alternatives should be carefully selected, depending on laboratory conditions, experience of

the experimenters, experimental design, etc. The maintenance of body temperature via a feedback system and strict control of the depth of anesthesia are mandatory.

To control the correct induction of vessel occlusion, we consider bilateral CBF measurement in the intraluminal thread model to be an indispensable tool. It provides an immediate indication of the success of the intervention during the ongoing experiment and thus offers the possibility of a direct decision on whether to include or exclude the animals. In addition to the exclusion of undesired pathologies that falsify the results, there is a saving of time, work and money on the scientific side that should not be underestimated. The CBF measurement is also beneficial to animal welfare, as it avoids unnecessary suffering of the animals in the presence of undesirable pathologies in accordance with the refinement of experimental procedures (Tannenbaum and Bennett 2015).

In addition, behavioral test may help to distinguish animals with only small lesions from those with larger infarction during the further course (Bernard, Balkaya et al. 2016, Metz 2016). However, the specific tests have to be chosen carefully and usually it is advisable to apply a battery of tests instead of a single one (Balkaya, Trueman et al. 2018). There are several pitfalls such as the misinterpretation of improvement through real functional recovery from (learned) compensation (Boltze, Lukomska et al. 2014). Computer-assisted and automated systems may offer an additional objective evaluation (Balkaya, Trueman et al. 2018).

Further, it has to be mentioned that between the rat strains and even within a single rat strain (dependent on the breeder) cerebrovascular anatomy –and therefore experimental results- may vary substantially (Fox, Gallacher et al. 1993, Oliff, Weber et al. 1995, Walberer, Stolz et al. 2006). Thus, also the experimental planning has to be undertaken carefully as even the change of a breeder may influence the extent of ischemia.

We addressed a selected subtopic evaluating the quality assurance applying the tMCAO model. Of note, the model itself is debated controversially (Hossmann 2012), but is still one of the most common stroke models. Undesired results such as SAH are common and a reliable detection of these pathologies is essential to minimize falsification of the experimental results and produce research as relevant as possible. Therefore, it is of utmost importance to make every effort to ensure the desired experimental result (ischemia).

Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

5. Conclusions

In conclusion, we show the predictive value of periprocedural monitoring (CBF, MAP and HR) applying the intraluminal thread model to detect a successful induction of stroke reliably. It is an important issue to assure experimental quality and eliminate falsification of the experimental results by inclusion of undesired pathologies (such as SAH).

Considering the poor translational power of preclinical stroke research every effort has to be made to improve experimental methodology and transparency.

Supplementary Materials: Supplementary materials can be found at XXX

Author Contributions AH conceived and designed the study; JL and MV performed the experiments; LL acquired the data; LL, CF and AH analyzed the data; KJL and AW helped to elaborate the experimental setting; MC contributed to data interpretation; LL wrote and drafted the first version of the manuscript; all authors revised the manuscript substantially; all authors reviewed the final draft of the manuscript. and approved the final article.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (CO 799/9-1).

Acknowledgments: In this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare that there is no conflict of interest.

Abbreviations

AIC	Akaike information criterion
AUC	Area under the curve
BL	baseline
CBF	Cerebral blood flow
CCA	common carotid artery
CI	Confidence interval
HR	Heart rate
ICA	Internal carotid artery
l	left
MAP	Mean arterial pressure
MCA	Middle cerebral artery
r	right
ROC	receiver operating characteristic curve
SD	Standard deviation
SAH	Subarachnoid hemorrhage
SDH	Subdural hematoma
tMCAO	Transient middle cerebral artery occlusion

References

(1994). "Call for comments on a proposal to improve reporting of clinical trials in the biomedical literature. Working Group on Recommendations for Reporting of Clinical Trials in the Biomedical Literature." Ann Intern Med **121**(11): 894-895.

(1994). "A proposal for structured reporting of randomized controlled trials. The Standards of Reporting Trials Group." JAMA **272**(24): 1926-1931.

Avsaroglu, H., A. S. van der Sar, H. A. van Lith, L. F. van Zutphen and L. J. Hellebrekers (2007). "Differences in response to anaesthetics and analgesics between inbred rat strains." Lab Anim **41**(3): 337-344.

Balkaya, M. G., R. C. Trueman, J. Boltze, D. Corbett and J. Jolkkonen (2018). "Behavioral outcome measures to improve experimental stroke research." Behav Brain Res **352**: 161-171.

Bardutzky, J., Q. Shen, N. Henninger, J. Bouley, T. Q. Duong and M. Fisher (2005). "Differences in ischemic lesion evolution in different rat strains using diffusion and perfusion imaging." Stroke **36**(9): 2000-2005.

Bederson, J. B., L. H. Pitts, M. Tsuji, M. C. Nishimura, R. L. Davis and H. Bartkowski (1986). "Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination." Stroke **17**(3): 472-476.

Begg, C., M. Cho, S. Eastwood, R. Horton, D. Moher, I. Olkin, R. Pitkin, D. Rennie, K. F. Schulz, D. Simel and D. F. Stroup (1996). "Improving the quality of reporting of randomized controlled trials. The CONSORT statement." JAMA **276**(8): 637-639.

Begley, C. G., A. M. Buchan and U. Dirnagl (2015). "Robust research: Institutions must do their part for reproducibility." Nature **525**(7567): 25-27.

Bernard, R., M. Balkaya and A. Rex (2016). Behavioral Testing in Rodent Models of Stroke, Part I. Rodent Models of Stroke. U. Dirnagl, Humana Press, New York, NY**:** 199-223.

Bleilevens, C., A. B. Roehl, A. Goetzenich, N. Zoremba, M. Kipp, J. Dang, R. Tolba, R. Rossaint and M. Hein (2013). "Effect of anesthesia and cerebral blood flow on neuronal injury in a rat middle cerebral artery occlusion (MCAO) model." Exp Brain Res **224**(2): 155-164.

Boltze, J., B. Lukomska and J. Jolkkonen (2014). "Mesenchymal stromal cells in stroke: improvement of motor recovery or functional compensation?" J Cereb Blood Flow Metab **34**(8): 1420-1421.

Boltze, J., D. C. Wagner, N. Henninger, N. Plesnila and C. Ayata (2016). "Phase III Preclinical Trials in Translational Stroke Research: Community Response on Framework and Guidelines." Transl Stroke Res **7**(4): 241-247.

Bouley, J., M. Fisher and N. Henninger (2007). "Comparison between coated vs. uncoated suture middle cerebral artery occlusion in the rat as assessed by perfusion/diffusion weighted imaging." Neurosci Lett **412**(3): 185-190.

Braeuninger, S. and C. Kleinschnitz (2009). "Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems." Experimental & Translational Stroke Medicine **1**(1): 8.

Davis, J. A. (2008). "Mouse and rat anesthesia and analgesia." Curr Protoc Neurosci **Appendix 4**: Appendix 4B.

Encarnacion, A., N. Horie, H. Keren-Gill, T. M. Bliss, G. K. Steinberg and M. Shamloo (2011). "Long-term behavioral assessment of function in an experimental model for ischemic stroke." J Neurosci Methods **196**(2): 247-257.

Endres, M., B. Engelhardt, J. Koistinaho, O. Lindvall, S. Meairs, J. P. Mohr, A. Planas, N. Rothwell, M. Schwaninger, M. E. Schwab, D. Vivien, T. Wieloch and U. Dirnagl (2008). "Improving outcome after stroke: overcoming the translational roadblock." Cerebrovasc Dis **25**(3): 268-278.

Engelhorn, T., A. Doerfler, M. Forsting, G. Heusch and R. Schulz (2005). "Does a relative perfusion measure predict cerebral infarct size?" AJNR Am J Neuroradiol **26**(9): 2218-2223.

Fisher, M., G. Feuerstein, D. W. Howells, P. D. Hurn, T. A. Kent, S. I. Savitz, E. H. Lo and S. Group (2009). "Update of the stroke therapy academic industry roundtable preclinical recommendations." Stroke **40**(6): 2244-2250.

Fox, G., D. Gallacher, S. Shevde, J. Loftus and G. Swayne (1993). "Anatomic variation of the middle cerebral artery in the Sprague-Dawley rat." Stroke **24**(12): 2087-2092; discussion 2092-2083.

Garcia, J. H., S. Wagner, K. F. Liu and X. J. Hu (1995). "Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation." Stroke **26**(4): 627-634; discussion 635.

Groth, W., A. Blume, P. Gohlke, T. Unger and J. Culman (2003). "Chronic pretreatment with candesartan improves recovery from focal cerebral ischaemia in rats." J Hypertens **21**(11): 2175-2182.

Guan, W., A. Kozak and S. C. Fagan (2011). "Drug repurposing for vascular protection after acute ischemic stroke." Acta Neurochir Suppl **111**: 295-298.

Henning, E. C., L. L. Latour, J. M. Hallenbeck and S. Warach (2008). "Reperfusion-associated hemorrhagic transformation in SHR rats: evidence of symptomatic parenchymal hematoma." Stroke **39**(12): 3405-3410.

Hossmann, K. A. (2012). "The two pathophysiologies of focal brain ischemia: implications for translational stroke research." J Cereb Blood Flow Metab **32**(7): 1310-1316.

Howells, D. W., M. J. Porritt, S. S. Rewell, V. O'Collins, E. S. Sena, H. B. van der Worp, R. J. Traystman and M. R. Macleod (2010). "Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia." J Cereb Blood Flow Metab **30**(8): 1412-1431.

Hungerhuber, E., S. Zausinger, T. Westermaier, N. Plesnila and R. Schmid-Elsaesser (2006). "Simultaneous bilateral laser Doppler fluxmetry and electrophysiological recording during middle cerebral artery occlusion in rats." J Neurosci Methods **154**(1-2): 109-115.
Iskander, A., R. A. Knight, Z. G. Zhang, J. R. Ewing, A. Shankar, N. R. Varma, H. Bagher-Ebadian, M. M. Ali, A. S. Arbab and B. Janic (2013). "Intravenous administration of human umbilical cord blood-derived AC133+ endothelial progenitor cells in rat stroke model reduces infarct volume: magnetic resonance imaging and histological findings." Stem Cells Transl Med **2**(9): 703-714.
Kilkenny, C., W. J. Browne, I. C. Cuthill, M. Emerson and D. G. Altman (2010). "Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research." PLoS Biol **8**(6): e1000412.
Koizumi, J., Y. Yoshida, N. T. and G. Ooneda (1986). "Experimental studies of ischemic brain edema. 1. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area." Jpn J Stroke **8**: 1-8.
Kramer, M., J. Dang, F. Baertling, B. Denecke, T. Clarner, C. Kirsch, C. Beyer and M. Kipp (2010). "TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses." J Neurosci Methods **187**(1): 84-89.
Landis, S. C., S. G. Amara, K. Asadullah, C. P. Austin, R. Blumenstein, E. W. Bradley, R. G. Crystal, R. B. Darnell, R. J. Ferrante, H. Fillit, R. Finkelstein, M. Fisher, H. E. Gendelman, R. M. Golub, J. L. Goudreau, R. A. Gross, A. K. Gubitz, S. E. Hesterlee, D. W. Howells, J. Huguenard, K. Kelner, W. Koroshetz, D. Krainc, S. E. Lazic, M. S. Levine, M. R. Macleod, J. M. McCall, R. T. Moxley, 3rd, K. Narasimhan, L. J. Noble, S. Perrin, J. D. Porter, O. Steward, E. Unger, U. Utz and S. D. Silberberg (2012). "A call for transparent reporting to optimize the predictive value of preclinical research." Nature **490**(7419): 187-191.
Liu, J., K. Nolte, G. Brook, L. Liebenstund, A. Weinandy, A. Hollig, M. Veldeman, A. Willuweit, K. J. Langen, R. Rossaint and M. Coburn (2019). "Post-stroke treatment with argon attenuated brain injury, reduced brain inflammation and enhanced M2 microglia/macrophage polarization: a randomized controlled animal study." Crit Care **23**(1): 198.
Liu, S., G. Zhen, B. P. Meloni, K. Campbell and H. R. Winn (2009). "Rodent Stroke Model Guidelines for Preclinical Stroke Trials (1st Edition)." J Exp Stroke Transl Med **2**(2): 2-27.
Livnat, A., E. Barbiro-Michaely and A. Mayevsky (2010). "Mitochondrial function and cerebral blood flow variable responses to middle cerebral artery occlusion." J Neurosci Methods **188**(1): 76-82.
Llovera, G., K. Hofmann, S. Roth, A. Salas-Perdomo, M. Ferrer-Ferrer, C. Perego, E. R. Zanier, U. Mamrak, A. Rex, H. Party, V. Agin, C. Fauchon, C. Orset, B. Haelewyn, M. G. De Simoni, U. Dirnagl, U. Grittner, A. M. Planas, N. Plesnila, D. Vivien and A. Liesz (2015). "Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia." Sci Transl Med **7**(299): 299ra121.
Longa, E. Z., P. R. Weinstein, S. Carlson and R. Cummins (1989). "Reversible middle cerebral artery occlusion without craniectomy in rats." Stroke **20**(1): 84-91.
Mahler Convenor, M., M. Berard, R. Feinstein, A. Gallagher, B. Illgen-Wilcke, K. Pritchett-Corning and M. Raspa (2014). "FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units." Lab Anim **48**(3): 178-192.
McCullough, L. D., G. J. de Vries, V. M. Miller, J. B. Becker, K. Sandberg and M. M. McCarthy (2014). "NIH initiative to balance sex of animals in preclinical studies: generative questions to guide policy, implementation, and metrics." Biol Sex Differ **5**: 15.
Mergenthaler, P. and A. Meisel (2012). "Do stroke models model stroke?" Dis Model Mech **5**(6): 718-725.
Metz, G. A. S. (2016). Behavioral Testing in Rodent Models of Stroke, Part II. Rodent Models of Stroke. U. Dirnagl, Humana Press, New York, NY**:** 225-241.
Moons, K. G., D. G. Altman, J. B. Reitsma, J. P. Ioannidis, P. Macaskill, E. W. Steyerberg, A. J. Vickers, D. F. Ransohoff and G. S. Collins (2015). "Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD): explanation and elaboration." Ann Intern Med **162**(1): W1-73.
O'Collins, V. E., M. R. Macleod, G. A. Donnan, L. L. Horky, B. H. van der Worp and D. W. Howells (2006). "1,026 experimental treatments in acute stroke." Ann Neurol **59**(3): 467-477.
Oliff, H. S., E. Weber, G. Eilon and P. Marek (1995). "The role of strain/vendor differences on the outcome of focal ischemia induced by intraluminal middle cerebral artery occlusion in the rat." Brain Res **675**(1-2): 20-26.
Percie du Sert, N., A. Alfieri, S. M. Allan, H. V. Carswell, G. A. Deuchar, T. D. Farr, P. Flecknell, L. Gallagher, C. L. Gibson, M. J. Haley, M. R. Macleod, B. W. McColl, C. McCabe, A. Morancho, L. D. Moon, M. J. O'Neill, I. Pérez de Puig, A. Planas, C. I. Ragan, A. Rosell, L. A. Roy, K. O. Ryder, A. Simats, E. S. Sena, B. A. Sutherland, M. D. Tricklebank, R. C. Trueman, L. Whitfield, R. Wong and I. M. Macrae (2017). "The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements Of in Vivo Experiments)." J Cereb Blood Flow Metab **37**(11): 3488-3517.
Philip, M., M. Benatar, M. Fisher and S. I. Savitz (2009). "Methodological quality of animal studies of neuroprotective agents currently in phase II/III acute ischemic stroke trials." Stroke **40**(2): 577-581.

Prinz, F., T. Schlange and K. Asadullah (2011). "Believe it or not: how much can we rely on published data on potential drug targets?" Nat Rev Drug Discov **10**(9): 712.
R Development Core Team (2018). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.
Rousselet, E., J. Kriz and N. G. Seidah (2012). "Mouse model of intraluminal MCAO: cerebral infarct evaluation by cresyl violet staining." J Vis Exp(69).
Savitz, S. I., J. C. Baron and M. Fisher (2019). "Stroke Treatment Academic Industry Roundtable X: Brain Cytoprotection Therapies in the Reperfusion Era." Stroke **50**(4): 1026–1031.
Schallert, T. (2006). "Behavioral tests for preclinical intervention assessment." NeuroRx **3**(4): 497-504.
Schmid-Elsaesser, R., S. Zausinger, E. Hungerhuber, A. Baethmann, H. J. Reulen and J. H. Garcia (1998). "A Critical Reevaluation of the Intraluminal Thread Model of Focal Cerebral Ischemia: Evidence of Inadvertent Premature Reperfusion and Subarachnoid Hemorrhage in Rats by Laser-Doppler Flowmetry." Stroke **29**(10): 2162-2170.
Stroke Therapy Academic Industry, R. (1999). "Recommendations for standards regarding preclinical neuroprotective and restorative drug development." Stroke **30**(12): 2752-2758.
Sughrue, M. E., B. T. Grobelny, A. F. Ducruet, R. J. Komotar, J. Mocco, R. R. Sciacca and E. Sander Connolly (2010). "Data presentation in rodent stroke studies and the predictive value of confidence intervals." J Clin Neurosci **17**(1): 11-15.
Tannenbaum, J. and B. T. Bennett (2015). "Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose." J Am Assoc Lab Anim Sci **54**(2): 120-132.
Trotman-Lucas, M., M. E. Kelly, J. Janus, R. Fern and C. L. Gibson (2017). "An alternative surgical approach reduces variability following filament induction of experimental stroke in mice." Dis Model Mech **10**(7): 931-938.
Tymianski, M. (2015). "Neuroprotective therapies: Preclinical reproducibility is only part of the problem." Sci Transl Med **7**(299): 299fs232.
Walberer, M., E. Stolz, C. Muller, C. Friedrich, C. Rottger, F. Blaes, M. Kaps, M. Fisher, G. Bachmann and T. Gerriets (2006). "Experimental stroke: ischaemic lesion volume and oedema formation differ among rat strains (a comparison between Wistar and Sprague-Dawley rats using MRI)." Lab Anim **40**(1): 1-8.
Woitzik, J. and L. Schilling (2002). "Control of completeness and immediate detection of bleeding by a single laser-Doppler flow probe during intravascular middle cerebral artery occlusion in rats." J Neurosci Methods **122**(1): 75-78.
Yao, H. and T. Nabika (2012). "Standards and pitfalls of focal ischemia models in spontaneously hypertensive rats: with a systematic review of recent articles." J Transl Med **10**: 139.
Zausinger, S., A. Baethmann and R. Schmid-Elsaesser (2002). "Anesthetic methods in rats determine outcome after experimental focal cerebral ischemia: mechanical ventilation is required to obtain controlled experimental conditions." Brain Res Brain Res Protoc **9**(2): 112-121.
Zhao, H., W. G. Mayhan and H. Sun (2008). "A modified suture technique produces consistent cerebral infarction in rats." Brain Res **1246**: 158-166.

Figures

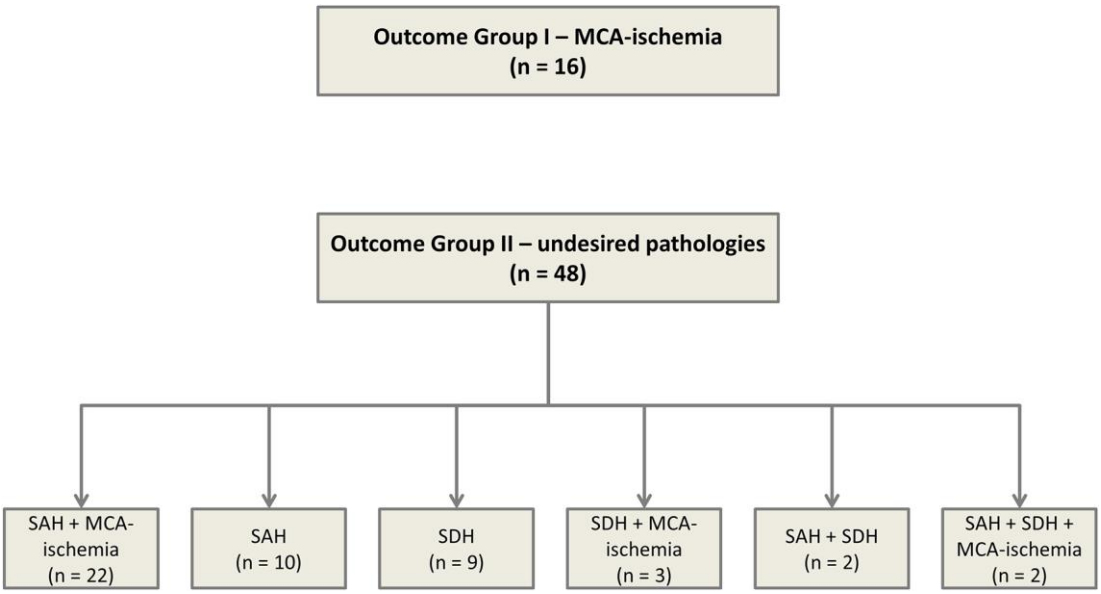

Figure 1. Flowchart of animals enrolled: subarachnoid hemorrhage (SAH); subdural hematoma (SDH); middle cerebral artery (MCA)

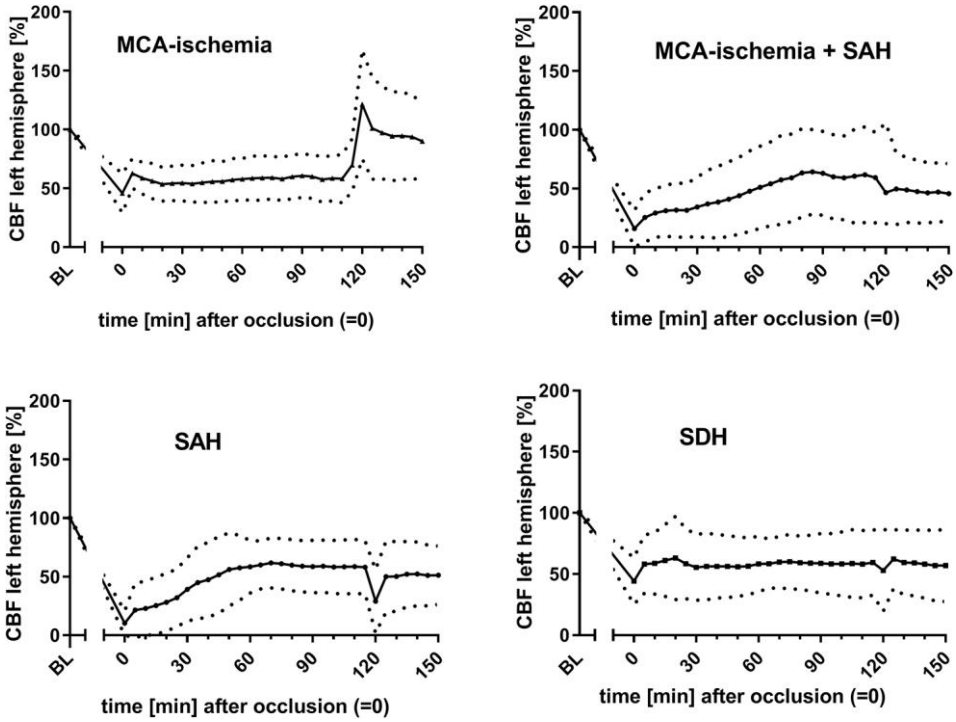

Figure 2 Courses of left CBF (mean as continuous lines ±SD as dotted lines) dependent on outcome; CBF – cerebral blood flow; BL – baseline; MCA-ischemia - ischemia in the middle cerebral artery territory; SAH – subarachnoid hemorrhage; SDH – subdural hematoma

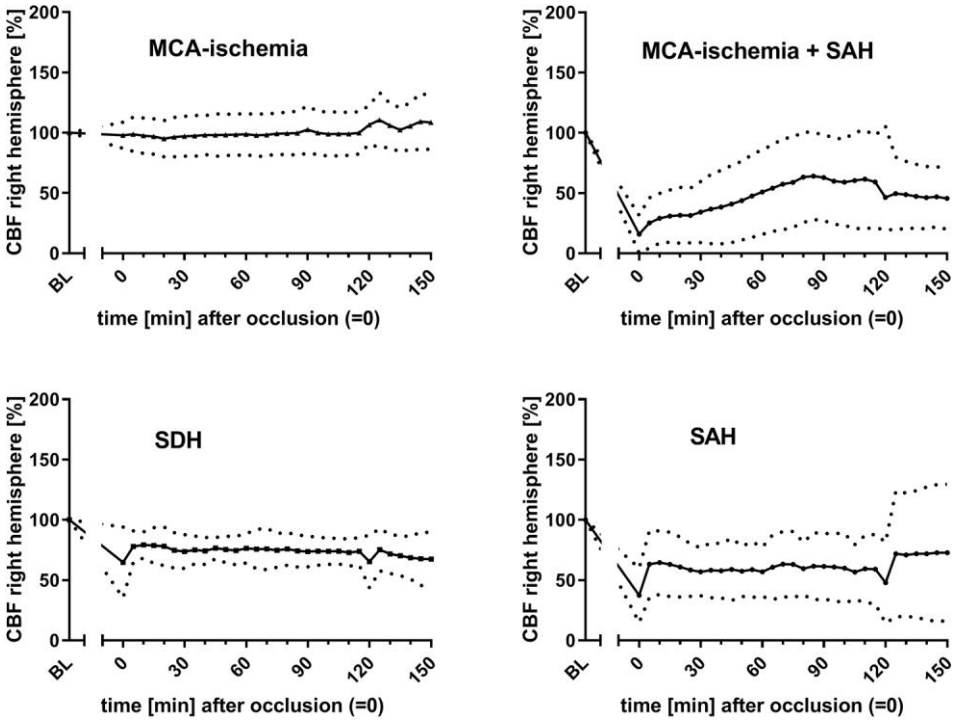

Figure 3 Courses of right CBF (mean as continuous lines ±SD as dotted lines) dependent on outcome; CBF – cerebral blood flow; BL – baseline; MCA-ischemia - ischemia in the middle cerebral artery territory; SAH – subarachnoid hemorrhage; SDH – subdural hematoma

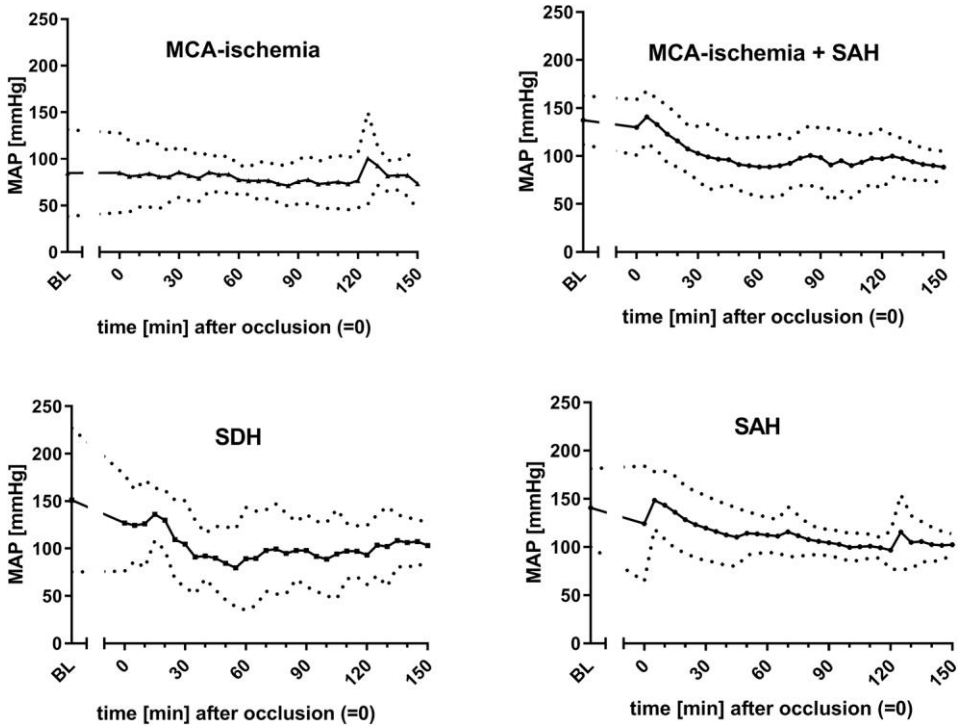

Figure 4 Courses of MAP (mean as continuous lines ± SD as dotted lines) dependent on outcome; MAP – mean arterial pressure; BL – baseline; MCA-ischemia - ischemia in the middle cerebral artery territory; SAH – subarachnoid hemorrhage; SDH – subdural hematoma

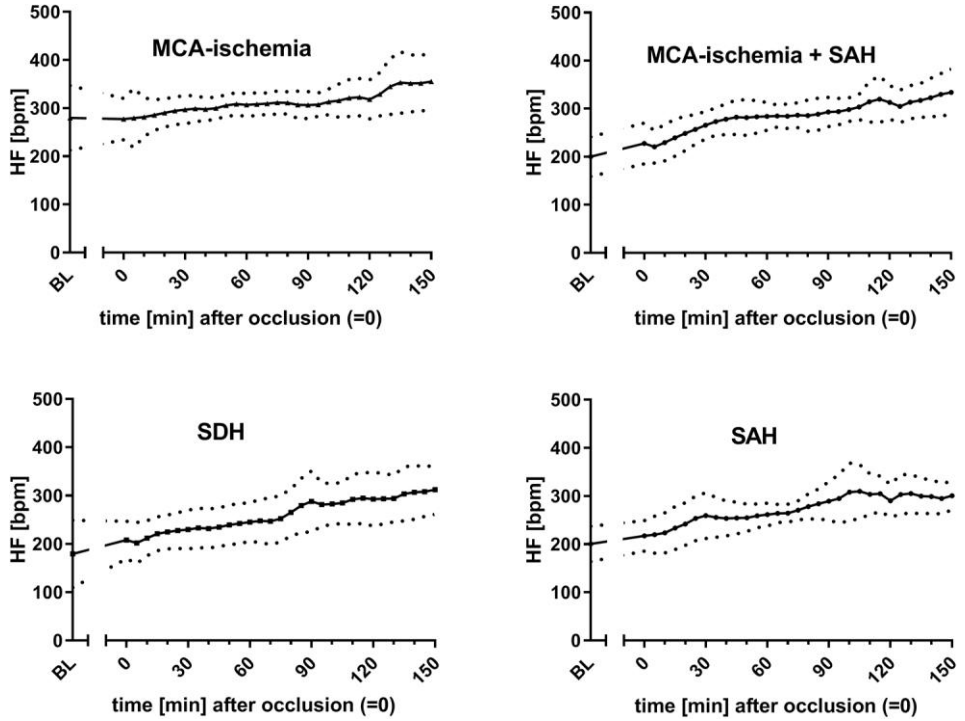

Figure 5 Courses HF (mean as continuous lines ±SD as dotted lines) dependent on outcome; HF – heart frequency; BL – baseline; MCA-ischemia - ischemia in the middle cerebral artery territory; SAH – subarachnoid hemorrhage; SDH – subdural hematoma

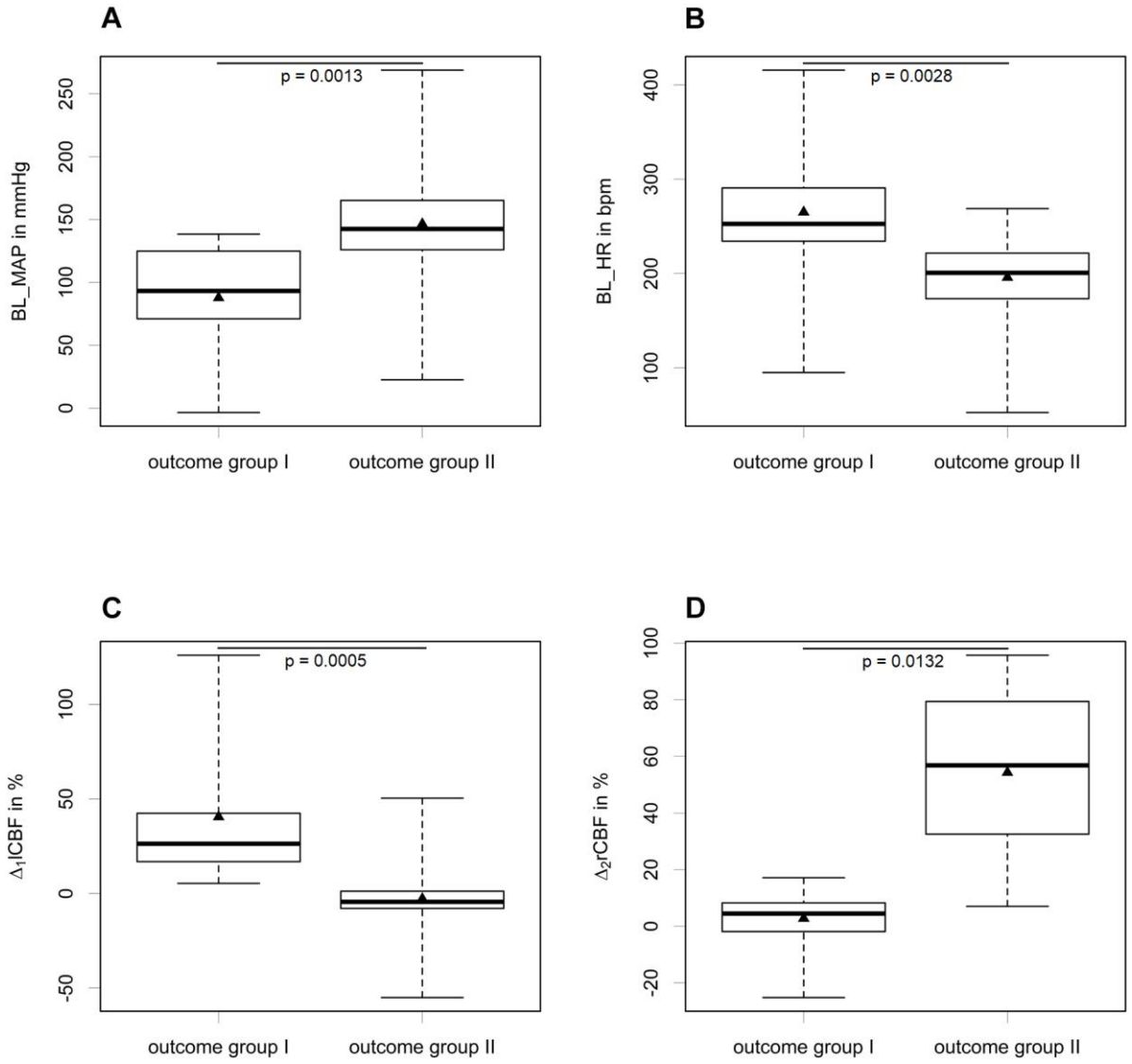

Figure 6 Figures of boxplots comparing **a** baseline mean arterial pressure (BL_MAP) in mmHg of outcome group I (n = 16) and II (n = 48) **b** baseline heart rate (BL_HR) in bpm of outcome group I (n = 16) and II (n = 48) **c** Δ_1lCBF (difference between mean left cerebral blood flow (lCBF) before (t0 to < t120) and the mean after occlusion (t120 to t150)) in % of outcome group I (n = 15) and II (n = 44) and **d** Δ_2rCBF (difference between right cerebral blood flow (rCBF) at baseline and at t0) in % of outcome group I (n = 15) and II (n = 45); for all parameters outcome groups I and II differ significantly (BL_MAP: p = 0.0013, BL_HR: p = 0.0028, Δ_1lCBF: p = 0.0005, Δ_2rCBF: p = 0.0132); mean is expressed as a triangle

Table

Table 1 Multiple logistic regression of the four included predictors baseline heart rate (BL_HR), baseline mean arterial pressure (BL_MAP); difference between mean left cerebral blood flow before (t0 to < t120) and the mean after occlusion (t120 to t150) (Δ_1lCBF) and the difference between right cerebral blood flow at baseline and at t0 (Δ_2rCBF); area under the curve (AUC); corresponding 95% confidence interval (95%-CI); R-squared (R^2); Brier Score

number of predictors	included predictors	AUC	95%-CI	R^2	Brier Score
1	BL_HR	0.83	(0.68,0.97)	0.23	0.13
	BL_MAP	0.84	(0.73,0.95)	0.26	0.13
	Δ_1lCBF	0.95	(0.89,1.00)	0.42	0.09
	Δ_2rCBF	0.97	(0.93,1.00)	0.52	0.07
2	BL_HR and BL_MAP	0.89	(0.79,0.98)	0.38	0.1
	BL_HR and Δ_1lCBF	0.96	(0.92,1.00)	0.48	0.07
	BL_MAP and Δ_1lCBF	0.97	(0.93,1.00)	0.5	0.07
	BL_MAP and Δ_2rCBF	0.99	(0.98,1.00)	0.61	0.03
	BL_HR and Δ_2rCBF	0.98	(0.95,1.00)	0.56	0.05
	Δ_1lCBF and Δ_2rCBF	1.00	(0.99,1.00)	0.63	0.02
3	BL_HR, BL_MAP and Δ_1lCBF	0.98	(0.96,1.00)	0.56	0.05
	BL_HR, BL_MAP and Δ_2rCBF	1.00	(0.99,1.00)	0.62	0.03
	BL_HR, Δ_1lCBF and Δ_2rCBF	0.99	(1.00,1.00)	0.67	0
	BL_MAP, Δ_1lCBF and Δ_2rCBF	1.00	(0.99,1.00)	0.66	0.01
4	BL_HR, BL_MAP, Δ_1lCBF and Δ_2rCBF	1.00	(1.00,1.00)	0.68	0

predictor variables	outcome group I (n = 16)			outcome group II (n = 48)		
	n	mean ± SD	median [Q1 - Q3]	n	mean ± SD	median [Q1 - Q3]
BL_HR	16	263.27 ± 76.25	251.95 [232.8 - 290.72]	48	198.03 ± 42.77	202.76 [176.89 - 221.6]
BL_DIAS	16	87.61 ± 43.7	96 [68.96 - 127.57]	48	138.81 ± 38.08	133.74 [118.83 - 154.85]
BL_SYS	16	95.86 ± 46.4	104.29 [73.07 - 134.32]	48	154.98 ± 42.59	149.01 [132.16 - 178.18]
BL_MAP	16	90.36 ± 44.53	98.77 [71.01 - 129.82]	48	144.2 ± 38.8	140.41 [121.66 - 162.83]
Δ_1lCBF	15	40.5 ± 37.4	26.26 [16.23 - 43.56]	44	-2.81 ± 15.02	-4.43 [-7.9 - 1.21]
Δ_2rCBF	15	2.71 ± 10.59	4.42 [-2.43 - 8.27]	45	54.86 ± 26.93	58.23 [32.76 - 79.49]
Δ_3lCBF	15	-75.46 ± 49.39	-55.94 [-89.58 - -42.34]	44	-23.65 ± 47.31	-11.99 [-32.53 - -2.6]
mean_rCBF	15	99.63 ± 16.43	101.52 [88.3 - 112.71]	45	75.31 ± 39.1	66.9 [52.21 - 82.8]
BL_weight	16	349.31 ± 36.73	347 [320 - 365]	48	363.65 ± 42.76	356.5 [333 - 385.5]

Table S1 Description of all possible predictors. Baseline heart rate (BL_HR) in bpm; baseline diastolic pressure (BL_DIAS) in mmHg; baseline systolic pressure (BL_SYS) in mmHg; baseline mean arterial pressure (BL_MAP) in mmHg; difference between mean left cerebral blood flow (CBF) before (t0 to < t120) and the mean after occlusion (t120 to t150) (Δ_1lCBF); difference between right CBF at baseline and at t0 (Δ_2rCBF); difference between time point t0 and t120 of left CBF (Δ_3lCBF); the mean of all time points of left CBF (t0 to t150) (mean_rCBF); initial weight in gram (BL_weight); standard deviation (SD); interquartile range (Q1-Q3)

single predictor	n	beta coefficient of predictors			performance			multivariable model
		log (OR)	SE	p-value	AUC	R^2	Brier Score	
BL_SYS	64	0.034	0.011	0.0017	0.83	0.25	0.13	excluded
BL_DIAS	64	0.038	0.012	0.0011	0.84	0.26	0.13	excluded
BL_MAP	64	0.037	0.012	0.0013	0.84	0.26	0.13	included
BL_HR	64	-0.028	0.009	0.0028	0.83	0.23	0.13	included
Δ_1lCBF	59	0.021	0.008	0.0086	0.89	0.16	0.15	included
Δ_2rCBF	60	0.193	0.078	0.0132	0.97	0.52	0.07	included
Δ_3lCBF	59	0.021	0.008	0.0086	0.95	0.42	0.09	excluded
mean_rCBF	60	-0.018	0.009	0.0442	0.81	0.08	0.18	excluded
BL_weight	64	0.009	0.008	0.2348	0.59	0.02	0.18	excluded

Table S2 Results of univariate logistic regression. Baseline heart rate (BL_HR); baseline diastolic pressure (BL_DIAS); baseline systolic pressure (BL_SYS); baseline mean arterial pressure (BL_MAP); difference between mean left cerebral blood flow (CBF) before (t0 to < t120) and the mean after occlusion (t120 to t150) (Δ_1lCBF); difference between right CBF at baseline and at t0 (Δ_2rCBF); difference between time point t0 and t120 of left CBF (Δ_3lCBF); the mean of all time points of left CBF (t0 to t150) (mean_rCBF); initial weight (BL_weight); logarithm of estimated odds ratio (log(OR)); standard error (SE); area under the curve (AUC); R-squared (R^2); Brier score

number of predictors	included predictors	n	beta coefficients of predictors										model fitting information (AIC)	results of cross validation		
			Intercept		BL_HR		BL_MAP		Δ_1lCBF		Δ_2rCBF			AUC CV	95%-CI	Mann-Whitney-U
			log (OR)	SE	log (OR)	SE	log (OR)	SE	log (OR)	SE	log (OR)	SE				
1	BL_HR	64	7.3924	2.1666	-0.0278	0.0093	-		-		-		59.05	0.8	(0.65,0.96)	0.012
	BL_MAP	64	-3.3585	1.3924	-		0.037	0.0115	-		-		56.95	0.8	(0.68,0.93)	0.0013
	Δ_1lCBF	59	2.3845	0.5953	-		-		-0.1183	0.0339	-		38.59	0.94	(0.88,0.99)	0.1005
	Δ_2rCBF	60	-2.1559	0.9173	-		-		-		0.1932	0.078	27.89	0.95	(0.9,1.00)	0.1397
2	BL_HR and BL_MAP	64	1.3039	2.0886	-0.0265	0.0096	0.047	0.0153	-		-		47.27	0.85	(0.74,0.97)	0.0341
	BL_HR and Δ_1lCBF	59	10.7438	4.2256	-0.0381	0.0177	-		-0.0963	0.034	-		33.85	0.94	(0.88,1.00)	0.1205
	BL_MAP and Δ_1lCBF	59	-1.7308	1.6319	-		0.0326	0.0133	-0.1233	0.0409	-		32.14	0.95	(0.9,1.00)	0.0531
	BL_MAP and Δ_2rCBF	60	-18.361	10.739	-		0.1108	0.065	-		0.294	0.1531	16.32	0.98	(0.95,1.00)	0.2214
	BL_HR and Δ_2rCBF	60	2.5931	2.3293	-0.0231	0.0118	-		-		0.2447	0.1113	24.37	0.96	(0.92,1.00)	0.1016
	Δ_1lCBF and Δ_2rCBF	59	-1.3935	2.2317	-		-		-0.2552	0.1286	0.2743	0.1807	13.51	0.98	(0.96,1.00)	0.1814
3	BL_HR, BL_MAP and Δ_1lCBF	59	4.7206	5.0304	-0.0424	0.0211	0.0558	0.0257	-0.1117	0.0414	-		26.24	0.96	(0.91,1.00)	0.115
	BL_HR, BL_MAP and Δ_2rCBF	60	-7.3086	10.368	-0.0313	0.0282	0.0846	0.055	-		0.2696	0.1384	16.72	0.98	(0.96,1.00)	0.1613
	BL_HR, Δ_1lCBF and Δ_2rCBF	59	39.0673	36.031	-0.1995	0.1714	-		-0.662	0.4946	0.779	0.5899	8.97*	0.98	(0.94,1.00)	0.2258
	BL_MAP, Δ_1lCBF and Δ_2rCBF	59	-24.4921	30.512	-		0.1593	0.2029	-0.4358	0.5334	0.3298	0.3393	11.66	0.97	(0.92,1.00)	0.2383
4	BL_HR, BL_MAP, Δ_1lCBF and Δ_2rCBF	59	5.3979	77.764	-0.1165	0.1533	0.1214	0.4413	-0.569	0.5119	0.4553	0.4206	10.26*	0.92	(0.83,1.00)	0.0638

Table S3 Results of all multivariable logistic regression models. Baseline heart rate (BL_HR); baseline diastolic pressure (BL_DIAS); baseline systolic pressure (BL_SYS); baseline mean arterial pressure (BL_MAP); difference between mean left cerebral blood flow (CBF) before (t0 to < t120) and the mean after occlusion (t120 to t150) (Δ_1lCBF); difference between right CBF at baseline and at t0 (Δ_2rCBF); logarithm of estimated odds ratio (log(OR)); standard error (SE); area under the curve cross validation (AUC CV); Akaike information criterion (AIC); corresponding 95% confidence interval (95%-CI); p-values of Mann-Whitney-U-test (Mann-Whitney-U)

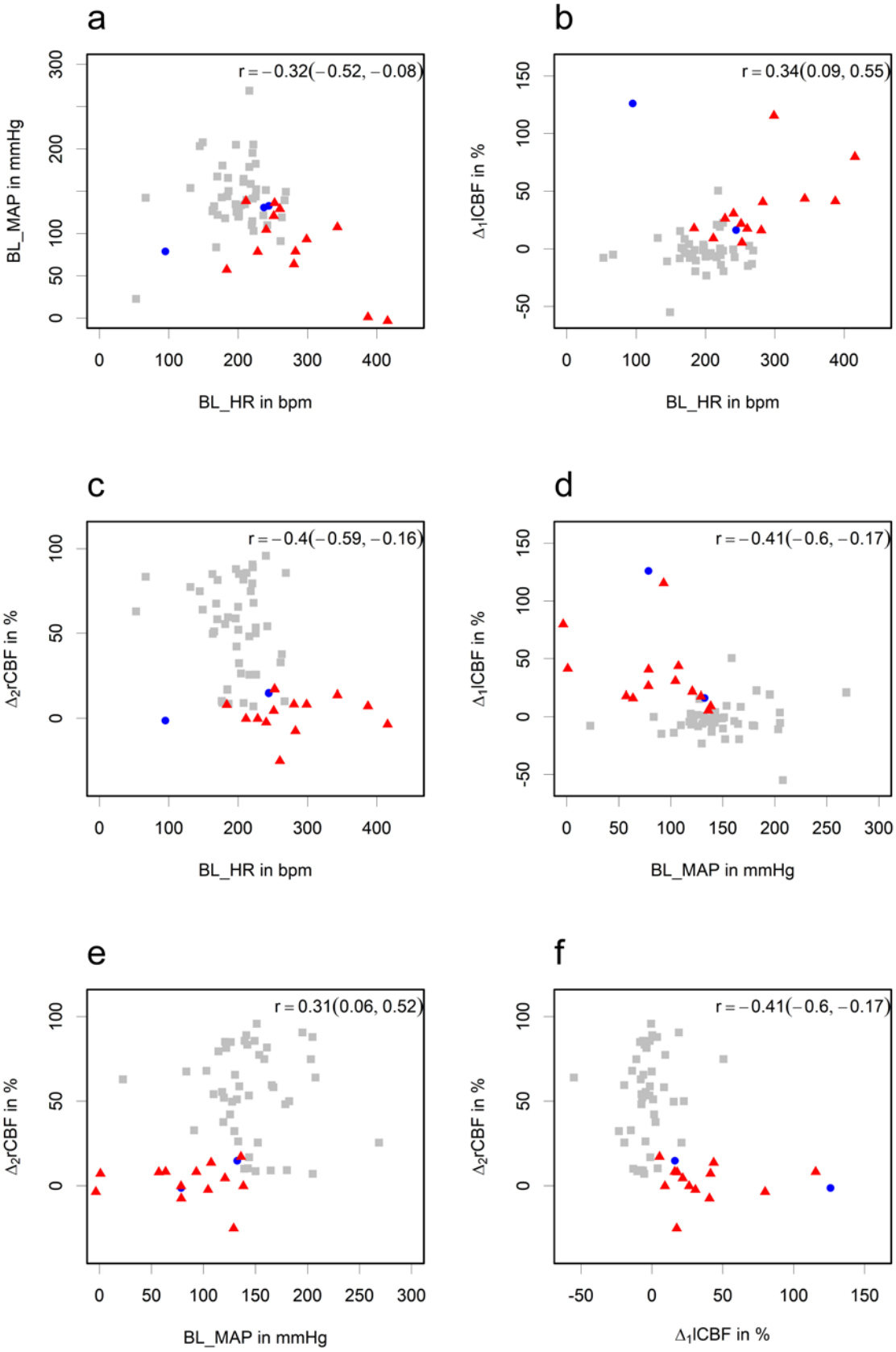

Figure S1. Correlation of **a** BL_MAP and BL_HR (n = 64) **b** Δ_1lCBF and BL_HR (n = 59) **c** Δ_2rCBF and BL_HR (n = 60) **d** Δ_1lCBF and BL_MAP (n = 59) **e** Δ_2rCBF and BL_MAP (n = 60) and **f** Δ_2rCBF and Δ_1lCBF (n = 59).

Baseline heart rate (BL_HR) in bpm; baseline mean arterial pressure (BL_MAP) in mmHg; difference between mean left cerebral blood flow before (t0 to < t120) and the mean after occlusion (t120 to t150) (Δ_1lCBF) in %; difference between right cerebral blood flow at baseline and at t0 (Δ_2rCBF) in %; data of animals sacrificed after 3 hours (outcome group I) are expressed as blue circles; data of animals sacrificed after 7 days (outcome group I) are expressed as red triangles; data of animals sacrificed after 3 hours (outcome group II) are expressed as grey squares

*The maximum likelihood estimate does not exist because of complete separation [1]. The information of the included predictors allow to separate completely outcome group I from outcome group II in this data set. Here, focus is on prediction not on specific estimate of the model. We only want to show the additional benefit of considering all possible predictors.

1. Albert A, Anderson JA. On the existence of maximun likelihood estimates in logistic regression models. *Biometrika*. 1984;71:1-10