SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

NEUROPATHOLOGICAL STUDIES IN CEREBROVASCULAR DISEASES

CELL DEATH MECHANISMS AND HAEMORRHAGIC TRANSFORMATION IN ISCHAEMIC STROKE, PROGNOSTIC FACTORS IN CEREBRAL HAEMORRHAGE

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DOCTORAL SCHOOL OF NEUROSCIENCES

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The Examination takes place at room 312, Department of Neurology, Faculty of Medicine, University of Debrecen, 9th of June 2016, 11:00 AM.

Head of the **Defense Committee**: Miklós Antal, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 9th of June 2016, 13:00.

1. INTRODUCTION

Although the rate of clinical autopsies has been declining drastically for decades, it remains an important tool of quality control in clinical practice. It serves to determine the exact cause of death, reveals unexpected complications of disease processes including adverse or any other effects of treatment as well as validates the cause of death for epidemiological statistics. Autopsies make important contribution to the under- and postgraduate training in medicine. Clinico-pathological studies are of major importance, because (agreeing with the Agency for Healthcare Research and Quality U.S. Department of Health and Human Services) 'clinical diagnoses, whether obtained from death certificates or hospital discharge data, contain major inaccuracies compared with autopsy diagnoses'.

Our university hospital has a catchment area of 500 000 inhabitants, and about 800 acute stroke patients are treated annually in our stroke centre. The ratio of thrombolysed patients is 19%, higher than the average of Western-European countries. In the everyday clinical practice at admission and during the course of the disease the various imaging modalities (CT, MRI) provide the main sources of information on structural/morphological changes in the brain. Although all of our acute stroke patients are immediately investigated by CT or MRI at admission (and repeated if required by the patient's deteriorating condition), neither ethical nor financial limitations allow performing daily CT/MRI during the agony phase for estimating the 'final' pathological findings of patients with poor outcome. Since the autopsy rate of patients who died at our neurology department was more than 90% over the previous years, we had access to the results of neuropathological evaluation which is a unique opportunity in the era of declining brain autopsy rates.

Ischaemic stroke has been in the focus of interest for several years due to its high frequency: cerebrovascular diseases are the most frequent diseases of the central nervous system; in 2010 the amount of ischaemic stroke cases was estimated 11.57

million. The one-third of the patients die, the rate of disability between survivors is high. Haemorrhagic infarction is a frequent complication of ischaemic stroke, although it is not always accompanied by clinical deterioration. The effect on clinical outcome is also unclear and most of the literature data regained from studies on thrombolysis for ischaemic stroke, mainly due to the fact that haemorrhagic transformation (HT) is a frequent complication of thrombolysis or anticoagulant therapy. Previous studies have focused on the possible aetiological role of the following parameters: age, systolic and diastolic arterial blood pressure, congestive heart failure, body temperature, serum glucose level, treatment with anticoagulants, pre-treatment with aspirin, early ischaemic signs on CT, mean infarct volume, plasma matrix metalloproteinase-9. The adequate time window for detection of HT is also disputed and autopsy is the method which may provide the most reliable data.

The urge for translational brain ischaemia research is derived from the clinical trials with neuroprotective regimens, which have been shown to be effective in experimental animal models but have failed in clinical trials of ischaemic stroke. The molecular mechanisms involved in the ischaemic human brain might differ from the controlled conditions of the animal experiments, which are performed with relatively young animals with no comorbidities or with selected, genetically determined morbidity (e.g. spontaneous hypertensive rats). The situation is very different in an aged stroke patient with multiple underlying risk factors and concomitant acute cardiovascular or respiratory diseases at the presentation of ischaemic stroke.

Apoptotic cell death is responsible only for a minority of the cells as compared to necrotic cell loss in ischaemic human brain, but is dominant in the penumbra, the target of neuroprotective regimens. The concomitant activation of the cell-death receptor (extrinsic) and mitochondrial (intrinsic) pathways of apoptosis has been investigated thoroughly in rats and mice after middle cerebral artery (MCA) occlusion. The role of mitochondria-dependent apoptosis in neurons has not been studied at all in ischaemic adult human brains. Only a handful of studies have described the cell death phenomena in ischaemic human brains, not to mention in tissues obtained with short post-mortem

delay after acute stroke. In experimental animal studies, neuroprotection has been suggested to ensue by inhibition of poly(ADP-ribosyl)ation. Furthermore, phase I and II clinical trials with PARP-1 inhibitors are underway as cardioprotecting agents or as an adjunct chemo- or radiosensitizer therapy. Now, clinical trials with PARP-1 inhibitors in ischaemic stroke are not yet found in the databases (e.g. clinicaltrials.gov). Still, stroke is suggested to be the prime indication for development of PARP-1 inhibitors.

Spontaneous intracerebral haemorrhage (ICH) affects 5 million people annually in the world, its incidence is 10-30/100 000/year and it takes 10-15% of all stroke cases. ICH has very high mortality; the 30-day mortality is 40%. The 50% of fatal outcome happen within the first two post-stroke days. The rate of disability between survivors is remarkable, 6 month later only 20% is able to live an independent life. There are much less medical or surgical treatment opportunities than in ischaemic stroke. Since no evidence-based effective treatment is known to decrease the primary brain injury caused by the haemorrhage, the treatment focuses mainly on the prevention of secondary injuries.

Early prognostication after ICH is riddled with uncertainties. Given this and the self-fulfilling pessimistic general opinion of poor outcome, reliable early prognostication after ICH can help to avoid a fatalistic approach by the wider care team including family members and enables evidence based DNR (do not resuscitate) orders. Thus, deliberated guideline-concordant therapy is essential for all ICH patients. Early prognostication helps clinicians to reach an objective opinion of predictable outcome.

2. OBJECTIVES OF THE STUDY

I. Clinicopathological analysis of patients who died in the Department of Neurology, University of Debrecen, and had brain autopsy in the Neuropathology Laboratory during the previous two calendar years.

Our specific goals were

1) to analyse the correlation between clinical and neuropathological diagnosis in cases of fatal ischaemic stroke, to assess the importance of the neuropathological evaluation in establishing the true frequency of HT,

2) to assess the clinically undisclosed findings revealed by the neuropathological analysis in a series of consecutive cases reflecting the routine practice of a stroke centre,

3) to assess the added value of autopsy and neuropathological analysis in the era of advanced imaging techniques.

II. Studying an autopsy cohort of 13 fatal ischaemic stroke cases treated in the Department of Neurology, Helsinki University Central Hospital.

Our specific aims were

1) to explore neuronal cell death pathways (necrosis, apoptosis) in ischaemic human stroke depending on both the post-stroke time and the examined region (core, penumbra, contralateral hemisphere) using immunohistochemical and immunofluorescent methods (caspase-3, PARP-1, p89 fragment, PAR, TUNEL)

III. Retrospectively analysing the data gained from a series of patients treated and died in the Department of Neurology, University of Debrecen during an 53-month long period due to primary intracerebral haemorrhage, combining these with classic, imaging-based data gained from CT volumetry in survivors and neuropathological ABC/2 volumetry in nonsurvivors.

Our specific goals were

1) to assess growth index of haematoma in nonsurvivors using the volumetric data assessed by the baseline CT and comparing these with the data obtained from ABC/2 volumetry by neuropathological examination,

2, to assess a relatively simple, reproducible, cost-effective scoring system suitable for the prediction of 30-day mortality of primary supratentorial ICH.

3. MATERIALS AND METHODS

3.1. Comparison Of Clinical And Neuropathological Diagnoses

3.1.1. Patient Selection and Data Collection

We retrospectively analysed the clinical records of patients who died in the Department of Neurology, University of Debrecen, and had general autopsy in the Department of Pathology and brain autopsy in the Neuropathology Laboratory during the previous two calendar years. All stroke patients were treated on specialized stroke units with multiparametric monitoring. All patients were older than 18 years of age, mean age was 62.66 years (SD 6.51). The following data were collected retrospectively from the patients' clinical notes: sex, age, survival time after stroke, suspected clinical diagnoses, administration of antiplatelets and anticoagulants over the hospitalisation, NIHSS (National Institutes of Health Stroke Scale) at admission, results of the performed CT, CTA (computed tomography angiography) or MRI examinations and the general autopsy findings.

Patients with ischaemic cerebral infarction had acute onset focal neurological deficit and brain imaging with or without ischaemic lesion.

Patients with haemorrhagic stroke had focal neurological deficit and brain imaging evidence of intraparenchymal haemorrhage.

Patients with primary intracerebral tumours had histologically verified tumour, brain imaging evidence of the neoplasm; they were admitted due to disease progression (deteriorating hemiparesis, dysphagia or epileptic seizures). Patients with brain metastasis had clinically diagnosed or histologically verified extracerebral primary tumour, brain imaging evidence of the metastasis; they were admitted due to disease progression (increased intracranial pressure or epileptic seizures) or acute ischaemic stroke.

Patients with central nervous system infection had evidence of neurological symptoms or meningeal signs indicative of meningitis or meningo-encephalitis and cerebrospinal fluid analysis had evidence of elevated cell count and protein content with or without decreased sugar level depending on the infectious agent.

3.1.2. Neuropathological Analysis

Brains were immersed in 10% buffered formalin according to standard procedures to allow good fixation and avoid deformation. We measured the formalin fixed whole brain and brainstem&cerebellum weight, respectively, to have the weight ratio of the supra- and infratentorial part as an index of atrophy or weight gain (e.g. due to oedema). After detailed description of the general appearance coronal slices of 0.75 cm sickness were cut. This method is adequate to diagnose gross pathologies including haemorrhage, infarct, HT and other pathologies such as herniation, secondary brain stem haemorrhage, arachnoideal cyst, tumours. After the macroscopic evaluation approximately 2x2x0.5 cm tissue blocks were sampled (which fit into standard size cassettes used for histotechnical processing) from areas recommended by BrainNet Europe (frontal cortex, temporal cortex, cingulate gyrus, parietal cortex, pre- postcentral gyrus, occipital cortex, hippocampus anterior, hippocampus posterior, basal forebrain, striatum, thalamus, midbrain, pons, medulla, vermis, cerebellum). After histotechnical processing and embedding to paraffin wax, sections of 7 µm thickness were cut and stained with haematoxylin and eosin (H&E), and luxol fast blue and Nissl (LFB/Nissl) to assess basic pathological changes. Evaluation was performed by a neuropathologist aware of the sample localization and patient history. The clinical findings were compared with the neuropathological results.

3.2. Cell Death Mechanisms in Ischaemic Stroke

3.2.1. Autopsy Material

We studied autopsy specimens from 13 cases of fatal ischaemic stroke (symptom duration from 15 h to 18 days) treated at the Department of Neurology, Helsinki University Central Hospital. Autopsies were performed within a mean of 18.6 h after death (range 3.5–40 h). Two patients who died of a non-neurological cause served as controls. The study protocol was approved by the institutional review committee of the Helsinki University Central Hospital. Informed consent was given by relatives.

3.2.2. Neuropathology

Tissue sampling was based on the most recent CT scan to obtain samples from the infarct core and periinfarct area (ipsilateral to thrombosis) and from the corresponding areas of the contralateral hemisphere and from the control brains (n=2). On autopsy, infarction areas were identified macroscopically and approximately 1-cm^3 cortical samples including subcortical white matter were dissected and fixed with formalin prior to embedding in paraffin. Sections of 5 μm were cut and were stained with hematoxylin–eosin to confirm ischaemic neuronal changes. Evaluation was performed by a neuropathologist unaware of the patient history or the sample localization. Focusing on the integrity of the nucleus, scores for signs of ischaemic neuronal changes in each tissue section were given. In short, Score 0: normal neuronal morphology; Score 1: largely normal morphology but scattered neurons with nuclear abnormalities such as pyknosis, low nuclear cytoplasmic contrast and smearing of nuclear border; Score 2: large proportion of neurons with nuclear abnormalities, Score 3: large proportion of neurons with nuclear abnormalities, with scattered ones with irreversible signs such as shrunken cytoplasm with irregular borders and invisible nuclei; and Score 4: large proportion of neurons with irreversible changes. When comparing the neuropathological scoring with the CT scan and macroscopical sampling in autopsy, the sections with scores 0 or 1 were derived from contralateral hemisphere or control areas (ipsi- or contralaterally), with scores 2–3 from periinfarct area, and with score 4 from the infarct core.

3.2.3. Immunohistochemistry

The antibodies used were as follows: (1) MoAb rabbit anti cleaved Caspase-3 (ACA-3); 1:75; (2) MoAb anti poly(ADP-ribose) polymerase against full-length PARP-1; 1:100; (3) Mouse immunoglobulin G1 (m IgG1) as control antibody for Ab 2; 1:100; (4) Pc rabbit anti PARP-1 p85 fragment (Cleaved PARP-1); 1:100; (5) R IgG as control

for Ab 4; 1:5000; (6) MoAb anti-PAR; 1:400, (7) M IgG3 as control for Ab 6; 1:400. Abbreviations: Pc = polyclonal antibody (Ab). MoAb = monoclonal Ab.

Paraffin-embedded sections were deparaffinized with xylene and hydrated through graded alcohols. Antigen unmasking by microwave irradiation in 0.1 M citrate buffer (pH 6.0) twice at 750 W (5 min) and once at 650 W (5 min) was done prior to quenching in 0.3% H_2O_2 in methanol (3% peroxide in aqua for ACA-3 Ab) for 10 min, and blocking with 10% normal sera for 30 min (5% normal sera for 1 h for ACA-3 Ab) prior to incubation with the primary antibody at +4 °C overnight. Biotinylated secondary antibody (1:200; 30 min) was followed by avidin–biotin complex for 30 min. Incubation with diaminobenzidine (DAB) according to manufacturer's instructions was carried out prior to counterstaining with Mayer's hemalum. For cleaved PARP-1 (p85), NovoLink™ detection was carried out according to manufacturers' recommendation with the exception of washing buffer (0.05 M Tris–Hcl pH 7.6, 0.03 M NaCl, 0.1% Tween 20). Additional control experiments included omission of the primary or secondary antibody in the ABC-method, which resulted in loss of immunoreactivity. For PARP-1 and cleaved PARP-1 antibodies, Western blot testing was performed. Preabsorption of caspase-3 protein with ACA-3 antibody (1 h, RT) prior to immunohistochemical staining abolished immunoreactivity in duplicate experiments.

3.2.4. Western Blotting

Western blot analysis was used to assess the specificity of PARP-1 antibodies (from Serotec and Promega) in differentiating the full-length and cleaved fragment of PARP-1. Human recombinant caspase-3 protein was used to cleave purified full-length human PARP-1 protein in vitro. PARP-1 protein cleavage by ACA-3 was produced by mixing 750 ng of purified ACA-3 with 10 U of full length human PARP and incubating the reaction mixture at +37 ˚C for 30 min prior to applying 400 ng of protein/lane for separation under reducing conditions on 8% SDS-polyacrylamide gels followed by electroblotting to PVFD membranes. PARP-1 was detected by the antibody against full-lenght PARP-1 (Serotec Ab; dilution 1:1000) and cleaved PARP-1 by Promegas'

antibody against cleaved PARP-1 (p85) (1:1000). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG was used as secondary antibody. Protein bands were visualized using ECL Plus and Typhoon 9400 Variable Mode Imager. As a negative control the primary antibodies were omitted from the protocol resulting in abolishment of immunoreactive bands.

3.2.5. Double-Labeling Immunofluorescent Staining of PARP-1 and PAR

The antibodies were applied in dilutions similar to immunohistochemistry. Paraffin-embedded sections were deparaffinized with xylene and hydrated through graded alcohols. Antigen unmasking by microwave irradiation in 0.1 M citrate buffer (pH 6.0) once at 750 W (5 min) and twice at 650 W (5 min) was done prior to blocking with 0.1 M Tris–Hcl/3% BSA and 20% FBS, pH 7.5 for 30 min. First primary antibody was incubated for 1 h (RT) followed by rabbit anti-mouse FITC 1:20 for 1 h (RT). Endogenous biotin blocking was performed according to manufacturers' recommendations prior to the second primary antibody incubation at +4C° overnight. Anti-mouse or anti-rabbit biotinylated secondary antibody (1:200) was followed by NeutrAvidin Rhodamine Red 1:200 for 30 min (RT). The slides were mounted on cover slips with Vectashield-DAPI fluorescence mounting media containing 4',6-diamino-2-phenylindole. Slides were washed twice in PBS for 5 min between all steps. All steps were performed in a humid chamber in dark.

3.2.6. Triple-Labeling Immunofluorescent Staining of in Situ Cell Death Detection by TUNEL with ACA-3 and DAPI

Microwave irradiation in 0.1 M citrate buffer (pH 6.0) at 370 W (5 min) was followed by blocking with 3% bovine serum albumine (BSA) in 20% normal bovine serum/0.1 M Tris–HCl (pH 7.5) for 30 min. Overnight incubation with the primary antibody (ACA-3) was succeeded by secondary biotinylated antibody at 1:200 dilution for 30 min and subsequent detection with RhodamineRed®-conjugated Neutralite™

avidin at 1:200 dilution (30 min). Subsequently, the TUNEL reaction (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) was carried out according to manufacturer's instructions with a FITC-tag. The sections were mounted on cover slips with Vectashield-DAPI fluorescence mounting media containing 4',6-diamino-2-phenylindole and then investigated with an epifluorescence microscope.

3.2.7. Acquisition of Microscopical Data and Statistical Analyses

The present investigational microscopical data were collected from tissue blocks from the infarct and periinfarct region and from homologous regions of the contralateral hemisphere. In each section, immunoreactivity was evaluated in five consecutive fields of 0.3125 mm^2 resulting in a mean (SE) of immunopositive cell structures/mm^2.

Although there is experimental evidence for apoptotic cell death of other cell types, including astrocytes, apoptotic cell morphology and TUNEL-labeling have been described most prominently as neuronal phenomena, which is in line with results in experimental animal model of permanent MCA occlusion. In the present study, we concentrated the systematic evaluation of the immunoreactivities on neuronal expression of cell death mediators, namely ACA-3 and PARP-1.

We confirmed the severity of ischaemic neuronal changes from an adjacent section (increasing damage scores from 0 to 4 as described in 'Neuropathology 3.2.2.'). This also made sure that the ischaemic neuronal changes associated with secondary ischaemia, e.g. due to bilaterally increased intracranial pressure, were represented as early ischaemic changes (i.e. score 1) in the results. The descriptive data are given as mean (SE). Statistical analyses were performed as described previously using the SPSS for Windows-program (version 13.0). In short, the difference between the mean numbers of immunoreactive cell structures/mm^2 in each brain location was evaluated by one-way ANOVA followed by LSD post hoc test. Jonckheere–Terpstra trend test was applied to determine the strength of associations between the continuously distributed variables, i.e. median immunoreactivities of ACA-3 and PARP-1 and the class-ordered ischaemic

neuronal score. Spearman correlation coefficient (*rS*) was used to assess the relation between PARP-1 and the percentage of TUNEL positive neurons. In the box plots, the median line is shown, the bar represents the upper 75% and lower 25% quartile, and the error bar depicts high and low values. *P* values <0.05 were considered statistically significant.

3.3. The 30-Day Outcome in Spontaneous Supratentorial Intracranial Haemorrhages

3.3.1. Patient Selection and Data Collection

We retrospectively reviewed the data of 156 Caucasian patients (71 nonsurvivors and 85 survivors) with primary supratentorial ICH admitted to our Intensive Care Unit (ICU) in a 53-month period. All patients were older than 18 years of age and were transported to our ICU within 24 hours of stroke onset. All patients were older than 18 years of age and were transported to our ICU, which is a specialized stroke unit with multiparametric monitoring, within 24 hours of stroke onset. If this point of time could not be ascertained, we used the last time when the patient was known to be well. All patients routinely underwent a baseline nonenhanced CT within 30 minutes of arrival, and the CT confirmed the ICH. A second CT was obtained on average on the 10^{th} day after admission or when symptoms deteriorated. Exclusion criteria were traumatic ICH, subarachnoid haemorrhage (SAH), vascular malformation, tumour, HT of ischaemic stroke (on admission or any CT performed later), postthrombolytic haemorrhage in ischaemic stroke, infratentorial ICH and primary intraventricular bleeding. We did not include subjects who had undergone neurosurgical evacuation or drainage. All patients were treated on specialized stroke units with multiparametric monitoring.

The following data were collected retrospectively from patients' clinical notes partly based on the Debrecen Stroke Database: sex, age, current smoking, excessive alcohol consumption, systolic and diastolic arterial blood pressure, and pulse rate at

arrival. Laboratory parameters were collected from the initial blood sampling: serum sodium, potassium, glucose levels, sedimentation rate, haemoglobin, white blood cell (WBC) and platelet counts, liver and kidney function tests and coagulation parameters.

3.3.2. CT Analysis

Image analysis was carried out retrospectively by two consultant neuroradiologists of our Department of Biomedical Laboratory and Imaging Science, who were blinded to the outcome. CT scans were performed on two 16-slice MDCT (multidetector computed tomography) scanners. Slice thickness was 5 to 10 mms for supratentorial and 2.5 to 4 mms for infratentorial regions. Images were transferred to an offline image processing workstation as DICOM (Digital Imaging and Communications in Medicine) files. Haemorrhage segmentation was carried out using the 3D Slicer software package developed by Brigham and Women's Hospital Surgical Planning Laboratory and MIT. This procedure allowed separation of the intracranial space from the skull and nonbrain structures; however, this method required verification and manual detachment of incorrectly labelled areas before performing volumetry with the built-in "Measurevol" module. The following variables were constructed: total intracranial volume; total haematoma volume; intraparenchymal haematoma volume; and intraventricular haematoma volume, each expressed as cm^3. Additionally, relative volumes were defined as the ratio of total, intraparenchymal and intraventricular haematoma volumes to intracranial volume yielding variables (without unit).

3.3.3. Neuropathological Analysis

Autopsies were performed within 48 hours after death. In our Neuropathology Laboratory, brains fixed in 10% formalin were cut into coronal slices. During the examination we verified the clinical diagnosis. Similarly to the in vivo diagnostic, the ABC/2 method was used to estimate haematoma volumes based on the assumption that the volume of an intracranial haematoma can be approximated by an ellipsoid unless the

haematoma is very irregular in shape. Ellipsoids can be described in terms of Cartesian coordinates using their three largest perpendicular axes. Consecutive coronal slices were laid down with the posterior surface up, and maximal diameters of the haematoma on every slice were measured in mms: one in the horizontal direction and another perpendicular to that. These two largest diameters were used as A and B in the ABC/2 formula and were not necessarily on the same brain slice; each slice has been photo documented. Although slices were originally 10 mms thick, we measured the thickness of each slice at four different locations assuming that the brains may have shrunk during fixation. The precise thickness of a slice was obtained as the average of these measurements. The resulting numerical data were then summed to obtain the maximum diameter of the haematoma perpendicular to the previous ones, which was used as C in the ABC/2 formula. In cases when the haematoma did not fully penetrate a slice, that is, the first and last brain slice containing the bleeding, we measured the depth of the portion of haematoma by sticking a probe into it. Due to fixation in formalin and brain slicing in cases of ventricular extension it was not always exactly discernible which part of the haematoma was in the parenchyma and in the ventricles, respectively. Therefore the haematoma volume calculated by the ABC/2 method was equal to the sum of the intraparenchymal and intraventricular parts. Volumes of haemorrhages were converted from mm^3 to cm^3. In these cases the relative haematoma volume was calculated using the initial CT intracranial volumetry data (for methods see above).

3.3.4. Statistical Analysis

The growth index of haematomas was calculated in two steps according to the following logistic formulas (log indicates natural logarithm): L=log(hpb2/(1-hpb2))-log(hpb1/(1-hpb1)), and *growth index*=exp(L)/(1+exp(L))-0.5, where *hpb* is haematoma per brain; *hpb2* is ratio of total haematoma volume obtained by the second volumetry (follow-up CT scan in survivors, neuropathological ABC/2 method in nonsurvivors) to intracranial volume based on the follow-up CT (survivors) or the first CT (nonsurvivors); *hpb1* is ratio of total haematoma volume to intracranial volume based on the first CT

volumetry (survivors and nonsurvivors). Negative growth indices denote reduction of haematoma; positive growth indices denote haematoma expansion.

Variables were described using mean and SD (continuous variables) or category percentages (categorical variables) stratified for survivors and nonsurvivors. Associations between explanatory factors and the outcome of death within 30 days were evaluated using logistic regression. Curvatures in relationships were assessed and allowed for by transformation or by adding a squared term if this substantially improved model fit. Variables showing remarkable associations in unadjusted models were selected for multiple regression modeling unless they are collinear with each other, in which case elimination was carried out on grounds of clinical practicability. Left-out variables were assessed for their potential contribution by adding to the prefinal multiple model one by one and left in if found clinically remarkable or statistically significant. The final multiple logistic regression model was checked for goodness of fit using the Hosmer-Lemeshow test. The level of significance was set at $\alpha=0.05$. To create the scoring system, model-predicted probabilities of outcome were generated for all subjects, and statistics for the minimum, the 10^{th}, 20^{th},…, 90^{th} percentage points, and the maximum were derived from the resulting set of values. The coefficients of the final model were built into a spreadsheet-based calculation interface which places any patient, with given input data, in terms of probability-of-death percentage range, given the percentage points observed on our sample of patients. All analyses were performed using Stata Statistical Software (StataCorp. 2009).

4. RESULTS

4.1. Comparison of Clinical And Neuropathological Diagnoses

All patients (n=100) had brain CT at admission. In possible thrombolysis candidates, computed tomography angiography (CTA) was also performed at arrival and all thrombolysed patients had a second control CT within 24 hours after thrombolysis. Repeat CT were done if the patient's condition deteriorated (loss of consciousness,

paresis, new clinical symptoms, etc.) to exclude any treatable cause of deterioration (e.g. haemorrhage, HT, secondary brainstem haemorrhage, oedema, etc.).

Clinically 64 patients (62.74%, female n=40, male n=24, mean age 62.6 years, SD 6.51) were diagnosed with acute ischaemic stroke during hospitalisation, regardless of CT signs of ischaemic infarct or absence of it at admission. In 10 of these patients HT of the infarct was diagnosed already by the clinicians. At autopsy we found territorial ischaemia in 59 patients and lacunar infarct(s) in 5 patients. Brain autopsy revealed HT in 34 cases (16 thrombolysed and 18 non-thrombolysed patients). We classified haemorrhagic infarcts as previously reported: HI1: small petechiae, HI2: more confluent petechiae, PH1: ≤30% of the infarcted area with some mild space-occupying effect, PH2: > 30% of the infarcted area with significant space-occupying effect, or clot remote from infarcted area. The distribution of HT in our material was the following: HI1 26.5%, HI2 29.4%, PH1 29.4% and PH2 14.7%.

None of the patients with HT received oral anticoagulant over their hospitalisation, although 4 of them were on coumarin or warfarin therapy at admission with ineffective INR (<1.7) level. Thirty-one patients received prophylactic low molecular weight heparin (LMWH) therapy and 17 patients received antiplatelet therapy.

According to CT, brain haemorrhage was the clinical diagnosis in 22 patients (21.56%, female n=6, male n=16, mean age 75 years, SD 19.7). The neuropathological findings were confirmatory in all clinically diagnosed haemorrhagic cases. According to the clinical records in one case trauma, in another case anticoagulant side effect was the aetiological factor. Underlying neuropathology in the background of cerebral bleeding was revealed in two cases (cerebral amyloid angiopathy and arteriovenous malformation, respectively). Brain autopsy revealed one clinically unknown cerebral haemorrhage; the male patient admitted with symptoms of mild vertebrobasilar insufficiency died unexpectedly, neuropathological evaluation described severe subarachnoideal haemorrhage as a consequence of ruptured basilar arterial aneurysm.

Neuropathological evaluation confirmed all primary brain tumours (n=3) and metastases (n=4) cases. The primary brain tumours were anaplastic astrocytoma in 2

patients and gliosarcoma in 1 patient. The metastases originated from pulmonary adenocarcinoma (n=1), invasive ductal breast cancer (n=1), sigillocellular gastric cancer (n=1) and carcinoma of the uvula in the oral cavity (n=1), respectively.

Infections of the central nervous system (CNS) were diagnosed clinically in 2 patients. The brain autopsy confirmed the meningitis in both patients.

In four cases with not life-threatening neurological conditions (e.g. neuronal heterotopy and microgyrification; arachnoideal cyst; silent aneurysm; opalescent meninges with mild mononuclear infiltration as a late consequence of a previous meningitis) the neuropathological evaluation confirmed the clinical diagnoses.

4.2. Cell Death Mechanisms in Ischaemic Stroke

4.2.1. Western Blotting

In Western blotting with purified PARP-1 protein, the PARP-1 antibody against full-length PARP-1 (Serotec) detected both a band of 116 kDa (equivalent to full-length PARP-1) and a less intense band of the size 89 kDa. PARP-1 protein cleavage by ACA-3 in vitro prior to Western blotting resulted in complete loss of signal for full-length PARP-1. Antibody against cleaved PARP-1 (Promega) detected only a band corresponding to cleaved PARP-1 (89 kDa).

Later in the text, PARP-1 refers to full-length PARP-1 (not totally excluding minor detection of cleaved PARP-1 p89 fragment) and cleaved PARP-1 to detection of p85 fragment using the specific antibody (Promega) in immunohistochemistry.

4.2.2. PARP-1 Immunoreactivity

Only scattered neurons showed PARP-1 immunoreactivity in control brains (n=2). Post-ischaemic PARP-1 immunoreactivity was evident both in neuronal nuclei and in cytoplasm. Interestingly, postischaemic PARP-1 immunoreactivity was also evident in the nucleolus. The maximum number of PARP-1 positive neuronal nuclei was evident in the infarct core during the first post-ischaemic day and this shifted to the

periinfarct area during the second day (highest at 23 h vs. at 39 h, respectively). The mean number of PARP-1 immunoreactive neuronal nuclei was the highest in the periinfarct area. Interestingly, the nuclear PARP-1 positivity correlated with increasing neuronal ischaemic (necrosis) score with the exception of most dense ischaemic damage (Score 4; only 2 brain sections). The induction was evident up to 2 weeks.

The number of neurons with cytoplasmic PARP-1 immunoreactivity was the highest in the periinfarct region compared to infarct core area (4.6 (SE 0.8) vs. 2.3 (SE 0.5); p=0.028). Cytoplasmic PARP-1 immunoreactivity followed a similar temporal course with maximum induction at 3 days like nuclear PARP-1. The cytoplasmic PARP-1 immunoreactivity correlated with increased percentage of TUNEL-labeled cells in the brain regions with neuronal ischaemic scores 2, 3 or 4, i.e. the periinfarct and infarct core regions (p=0.01) up to 6^{th} days survival time.

4.2.3. Post-Ischaemic Cleaved PARP-1 Immunoreactivity

The maximum number of neuronal nuclei immunoreactive for cleaved PARP-1 was observed between 3 days and 2 weeks compared with the peak in less than 3 days for PARP-1. The number of nuclei immunoreactive for cleaved PARP-1 correlated with increasing neuronal ischaemic score with the exception of most dense ischaemic damage (p=0.009). The maximum number of neuronal cytoplasm expressing cleaved PARP-1 was evident by less than 3 days. The cytoplasmic cleaved PARP-1 expression was in inverse correlation with increasing neuronal ischaemia score (p=0.001).

4.2.4. PAR-Polymer Immunoreactivity

PAR immunoreactivity was prominent in the cytoplasm of neurons in the control brains. PAR immunoreactivity was very intense both in the neuronal nuclei and cytoplasm in two brain sections from the periinfarct area with a neuronal ischaemia score 2 (Cases 1 and 6).

PAR immunoreactivity was mostly seen in the neuronal nuclei in two brain sections (Cases 12 and 13) with advanced ischaemic damage (Score 4; infarct core).

4.2.5. Double Labeling of PARP-1 and PAR

The amount of neuronal somas immunofluorescent for cleaved PARP-1 (p85) was bigger than for full-length PARP-1 (Case 2) in keeping with the results from immunohistochemical staining. PAR-fluorescence in neuronal nuclei colocalized both with the full-length and cleaved PARP-1 enzyme. Less PAR-immunofluorescent cells were detected in a section from the contralateral hemisphere (Case 2).

4.2.6. ACA-3 Immunoreactivity

ACA-3 immunoreactivity was seen only in scattered cells in the control brains (n=2). Post-ischaemic ACA-3 expression became evident in the cytoplasm of the gray matter neurons after 15 h of ICA occlusion. About a fourfold increase in the number of ACA-3 immunopositive neurons was detected in the infarct core very acutely and it subsided during the following days up to 1 week. In the periinfarct area, the magnitude of the increase in the number of ACA-3 immunoreactive neurons was roughly threefold, and it tended to be increased up to a week. A decrease in the number of ACA-3 positive neurons between the groups of acute ischaemia (<3 days of symptoms) and >2 weeks ischaemia duration was detected ($p=0.002$). In the contralateral hemisphere, neuronal ACA-3 immunoreactivity showed an increasing trend up to 3 days, after which the number of ACA-3-positive neurons was equivalent to controls (n=2). ACA-3 expression was seen in the neuronal processes in the infarct core on the 2nd day after MCA occlusion (Case 4). In double-labeling immunofluorescence, ACA-3 positive neuronal processes colocalized to some extent with the TUNEL-positive nuclei.

4.3. The 30-Day-Outcome in Spontaneous Supratentorial Intracranial Haemorrhages

Of the 81 survivor patients, eight were excluded because they did not have a second CT. Of the 75 nonsurvivor patients we excluded two based on brain autopsy results: ruptured aneurysm of the MCA and a metastatic tumour, respectively, had been confirmed. Twelve brains were unsuitable for volumetry due to fragmented, multilobar haematomas or having been damaged during removal from the skull, cases in which the ABC/2 method fails to accurately estimate haemorrhage volumes. In addition, medical records were not sufficiently detailed in a total of nine cases. Thus, we analyzed the complete dataset of 59 nonsurvivor and 66 survivor patients.

4.3.1. Predictors of 30-Day Mortality

The relationship between age and greater odds of death was of borderline significance. Higher systolic blood pressure at admission was a predictor of lethal outcome. Other variables, including sex, alcohol consumption, smoking and pulse rate at admission, were not significantly associated with mortality.

There was no statistical significance regarding sex, current smoking, excessive alcohol consumption, diastolic arterial blood pressure and pulse rate at arrival. Laboratory parameters were collected from the initial blood sampling: serum sodium, potassium, glucose levels, sedimentation rate, haemoglobin, white blood cell (WBC) and platelet counts, liver and kidney function tests, and coagulation parameters. Of these, serum potassium concentration was significantly associated with fatal outcome: high and low levels outside the normal range both represented elevated odds of death. We identified higher serum glucose concentration and lower platelet count as further predictors of fatal outcome.

Comparing CT characteristics and volumetric findings between survivors and nonsurvivors, the mean time from symptom onset to initial CT was strongly and inversely related to occurrence of death within 30 days. In the survivor group, the mean

time between baseline and second CT was 10.7 (SD 5.05) days; in the nonsurvivor group, the mean time between symptom onset and death was 7.7 (SD 6.4) days. Differences between the two groups in absolute volumes of total and intraparenchymal haematoma on baseline CT were strongly significant, similarly to relative haematoma volumes. The growth index of haematoma was found to be a very strong predictor of the outcome. The early presence of intraventricular haemorrhage and its volume was also strongly associated with fatal outcome. There was an approximate 43% decrease in the mean volume of total haemorrhage from baseline to follow-up CT in survivors and a 54% increase from initial CT to death in the other group and, consistently, there was a 42% decrease in survivors and a 56% increase in nonsurvivors in relative total haematoma volume. Intraventricular extension of the haematoma was more frequent in the nonsurvivor group both on initial CT and on follow-up examinations.

4.3.2. The SUSPEKT Scoring System

The final multiple logistic regression model showed statistically significant associations between 30-day case fatality and a number of variables: serum glucose (***Su***gar); total haematoma volume (***S***ize); systolic blood ***P***ressure; presence of intraventricular haemorrhage (***E***xtension to the ventricular system); and serum potassium level (***K***alium). Using these and adding age (life***T***ime) which was of borderline significance we developed the six-factor scoring system ***SUSPEKT***. The SUSPEKT scoring system is suitable for prediction of 30-day mortality after primary supratentorial ICH. Probability (*pr*) can be derived by summing all *bx* (product of means observed in the present study and coefficients) values (*sbx*), and using the formula *esbx/(1 + esbx)*, where *e* denotes Euler's number. Probability (*pr*) may be referred by a table of percentiles.

5. DISCUSSION

5.1. Comparison of Clinical And Neuropathological Diagnoses

The Department of Neurology, University of Debrecen is a regional stroke centre explaining the predominance of stroke patients in our autopsy series.

Brain autopsy confirmed the clinical diagnoses in all cases of cerebral haemorrhages, primer and metastatic tumours and central nervous system infections. Neuropathological evaluation revealed the aetiology in two cases with parenchymal bleeding; it described cerebral amyloid angiopathy in one and arteriovenous malformation in another case. Only brain autopsy could reveal a clinically unknown cerebral haemorrhage; the male patient was admitted with symptoms of mild vertebrobasilar insufficiency, while neuropathological evaluation described severe subarachnoideal haemorrhage in the background of unexpected death as a consequence of ruptured basilar arterial aneurysm.

Autopsy provided the most additional information in ischaemic stroke cases. There are not many clinico-pathological reports on consecutive autopsy series with predominantly stroke cases and only few with special emphasis on HT in ischaemic infarcts. The reported frequency of HT in previous studies depended on several factors, e.g. whether the study was based on imaging methods or on autopsy results, whether the patients had received anticoagulant, antithrombotic or fibrinolytic treatment, or on the post-stroke time of control imaging modality. Additionally the results of the clinical and neuropathological diagnostic tools are heterogenous, difficult to compare them, and the population examined can make it even more heterogenous due to the fact that autopsy results can be gained from fatal cases exclusively. Kerényi *et al*. analysed an autopsy series of 245 patients with ischaemic stroke with HT in 29%. Lodder *et al.* reported an autopsy series with 48 patients dying within 15 days following a supratentorial cerebral infarct. In their analysis 16 patients (33%) had HT. Toni *et al.* examined 150 consecutive patients with cerebral infarct in the anterior circulation. They performed CT or autopsy one week after the stroke and observed HT in 43%, mostly (89%) petechial

transformations and in 11% larger haematomas. Celik *et al.* evaluated a series of 86 MCA territorial infarction cases, who had received before neither antiplatelet nor anticoagulant therapy. By the follow up CT performed 72 hours after stroke they found HT in 8.5% of all examined patients, but in 40.6% of MCA territorial infarction cases. Okada *et al.* examined 160 acut ischaemic stroke patients. Their CT based results showed all together 40.6% HT, from which 15.4% could be seen in the first 1-4 days, 67.7% in the first 10 days and 100% within the first 30 days and they haven't detected any new HTs after the first month. Hornig *et al.* evaluated 65 patients' data. They detected HT with brain CT in 28 cases, 40% in the first week, and the rest 60% in the second post-stroke week. These earlier reports show how difficult is to define the most appropriate time for the diagnosis of HT. Furthermore, diagnostic possibilities are often different in the routine practice as compared to well-defined and sponsored studies which last for a particular period of time. Moreover, ischaemic stroke patients usually have days or weeks (in our sample 11.71 days, SD 13.16) before death with reduced level of consciousness and critical medical status. Therefore, the premortal diagnosis of HT could only be possible with daily repeat CT or MRI during the critically ill period, which is not possible from both the ethical and financial point of view. Repeat CT are done in all thrombolysed patients within 24 hours after thrombolysis, but not necessarily perfomed in non-thrombolysed patients, if the patient's condition does't require or allow it. However it is well established that HT is not necessarily accompanied by deterioration of neurological status.

The reported frequency of the different types of HT in the literature depends on the treatment used in the acute phase of ischaemic stroke, but also on the HT subtypes' definitions used in that particular source. The distribution of HT in our material was the following: HI1 26.5%, HI2 29.4%, PH1 29.4% and PH2 14.7%. Okada *et al.* defined four types of HT: spotty and scattered petechial haemorrhage, along the cortical margin of the ischaemic lesion (56.9%), diffuse haemorrhage (18.5%), small (<3 cm in diameter) (13.8%) and massive (>3 cm in diameter) (10.8%) haematoma. Fiorelli *et al.* examined the subtypes and frequency of HT after iv. rtPA treatment in the ECASS I. HT detection

was based on control brain CT performed 36 hours later. Due to the very different patient population from ours and the exclusively imaging based HT diagnosis, the comparison between their and our results is meaningless. However, their results support those previous findings that the frequency and even the severity of HT are higher after rtPA treatment. In our sample most of the PH1-2 transformations developed in thrombolysed patients. The high proportion of HT in our sample diagnosed only at brain autopsy can be explained partially by the fact that control CT is perfomed only 24 hours after thrombolysis. Although the half-life of t-PA is only few minutes, there is a prolonged effect on the coagulation cascade.

Fiorelli *et al.* and Berger *et al.* evaluated the ECASS I and ECASS II data and established that only parenchymal haematoma type 2 was associated with an increased risk of clinical deterioration; all other types of haematomas did not independently increase the risk of late deterioration. In the NINDS rtPA study 70% of the symptomatic HT corresponded to the parenchymal haematoma (PH1 and PH2) subtypes by ECASS classification. The vast majority (73,5%) of symptomatic HT in the ECASS were in the PH1 and PH2 subgroups. These data show that HTs accompanied by clinical worsening usually belong to parenchymal haemorrhages, appear as dens haematomas and they have pronounced space occupying effect. In our sample PH2 type transformations developed in thrombolysed patients and all of them were diagnosed already clinically. On the other hand relatively large proportion of HT1-2 and PH1 transformations were diagnosed only by brain autopsy even in a specialized stroke unit with multiparametric monitoring, despite the control CTs 24 hours later in thrombolysed patients and other control CTs perfomed due to clinical worsening (in 6 cases).

Although some prior studies demonstrated the negative impact of the asymptomatic HTs, even of the HI forms, on the outcome compared with those without HT of acute ischaemic stroke. According to a previous study, based on both imaging and autopsy results, the pathogenesis of haemorrhagic infarctions and parenchymal haematomas are different; while haemorrhagic infarctions are the consequences of multifocal extravasation of red blood cells, usually in the grey matter, parenchymal

haematomas probably represent haemorrhages from single damaged vessels, injured by ischaemia and following reperfusion. Since the transformation occurs in the already necrotic tissue, often no clinical worsening, reflecting the development of HT, could be detected. The general condition of acut stroke patients with fatal outcome is very poor, there are several additional extracerebral factors (ie. bronchopneumonia, acute myocardial infarction, pulmonary embolism) which may lead to the fatal outcome.

Instead of focusing on the reasons, clinical signs or treatment of HT, our aim was to emphasize the possibility and much higher than expected frequency of its occurrence even without clinical signs of the development and emphasize the need of clinical alertness even in asymptomatic cases. Since we evaluated only fatal cases, our results cannot be extrapolated to all ischaemic stroke cases. However keeping this in mind, in individual cases (e.g. in thrombolysed patients) clinicians may consider requesting brain CT immediately before discharging the ischaemic stroke patient.

Additionally we underline the importance of clinical autopsies which are still crucial in the correct diagnosis of HT of fatal stroke cases even in the era of advanced imaging techniques. Our study provided solid evidence that post-mortem neuropathological examination is important not only in confirming the clinical diagnosis but also reveals in a high proportion of patients undisclosed clinically relevant brain pathologies. Furthermore, post-mortem neuropathology is of educational value for trainees and specialists alike in clinical neurosciences, imaging and general pathology.

5.2. Cell Death Mechanisms in Ischaemic Stroke

In human brain ischaemia the critical decrease of blood supply results in neuronal damage in few minutes after stroke. Acute neuronal injury causes overactivation of PARP-1, which results in unregulated poly(ADP-ribose) (PAR) synthesis and due to the ATP and NAD^+ decrease, widespread neuronal cell death.

Only scattered neurons expressed PARP-1 in normal brain tissue, in accordance with previous results. Post-ischaemic nuclear expression of PARP-1 peaked in the infarct core and in the periinfarct area inside 3 days, which is consistent with previous human

results. On the other hand, by 2 days only very sparse cells contained detectable PARP-1 within the infarcts, while in our material, the induction was evident for up to 2 weeks. The number of neurons with cytoplasmic PARP-1 immunoreactivity was highest in the periinfarct region and it correlated with increasing number of TUNEL-labeled neurons showing also apoptotic morphology. Regardless of the survival time, intense nuclear and cytoplasmic immunoreactivity were not detected in the same cells, supporting an ischaemia-induced subcellular translocation of PARP-1.

For nuclear immunoreactivity, both PARP-1 and cleaved PARP-1 correlated with increasing neuronal ischaemic damage score, with a delayed peak in the expression of cleaved PARP-1 after 3 days of ischaemia. The number of neurons with cytoplasmic expression of cleaved PARP-1 was in inverse correlation with increasing ischaemic damage. As a sign of PARP-1 enzymatic activity, PAR immunoreactivity was very intense both in the neuronal somas and in the cytoplasm of post-ischaemic neurons. It has been hypothesized that PAR is generated mainly in the nucleus and localizes to cytosol and interacts there with mitochondria to induce cell death. Cytoplasmic PARP-1 has been described in normal brains in a previous study, where subcellular localization of PARP-1 was studied in three non-neurological patients. In another study of human traumatic brain injury, PARP-1 was found either exclusively in the nucleus or in both nuclear and cytoplasmic compartments. The dominance of cytoplasmic neuronal staining was linked with a short time interval from trauma to surgery. All these post-mortem studies carry a hazard that the changes in PARP immunoreactivity may be due to cell death or partial cell death, during which the nuclear content becomes more accessible to immunohistochemical staining. Strikingly, in the present study, PARP-1 was detected in nucleoli either alone or with simultaneous nuclear expression between 15 and 39 h and only in the periinfarct and contralateral regions, which retain more physiological energy metabolism than the infarct core. Although nucleolar PARP-1 expression has not been reported in neurons before, it has been described in vitro in MDBK, HeLa and CHO cells and in murine fibroblasts fitting as a sign of active RNA synthesis in general.

The scattered expression of ACA-3 in controls is in line with the suggested roles of caspases in axon guidance, synaptic plasticity and preservation of normal brain integrity. Taken together, neuronal cytoplasmic ACA-3 immunoreactivity was highest in the periinfarct area - the area of most prevalent apoptosis. Interestingly, post-ischaemic ACA-3 immunoreactivity became evident in the cytoplasm of the gray matter neurons, although the well-established role of ACA-3 as an inactivator of the nuclear protein PARP-1 (as well as other nuclear substrates) would suggest a nuclear localization. In a previous study of human brain ischaemia ACA-3 was strongly expressed both in the cytoplasm and in the nucleus. Our results are in keeping with the results of Ferrer *et al.* that demonstrated the exclusive expression of ACA-3 in the cytoplasm in a Western blot analysis from penumbral samples of adult rats. Interestingly, post-ischaemic ACA-3 immunoreactivity became evident in the cytoplasm of the gray matter neurons, although the well-established role of ACA-3 as an inactivator of the nuclear protein PARP-1 (as well as other nuclear substrates) would suggest a nuclear localization.

The present and our previous observational findings generate a hypothesis of overexpressed nuclear full-length and cleaved PARP-1 as a kind of surrogate marker of necrosis while cytoplasmic PARP-1 might reflect concomitant ongoing apoptosis. After a delay of some days, PARP-1 cleavage could reflect an attempt to substitute necrosis with apoptosis in the periinfarct region, a process requiring ATP. A previous study describing PARP-1 cleavage during apoptosis in vitro demonstrated that the larger p89 PARP-1 fragment migrates from the nucleus to the cytoplasm in apoptotic cells.

Based on a series of human atherothrombotic infarcts, it has been hypothesized that apoptosis makes little contribution to neuronal death in focal brain infarcts in man, because many neurons showed obvious morphological changes of ischaemic neuronal death without immunolabeling for caspase-3 in the infarcted regions. In contrast to that, our results support a concomitant role for apoptosis and necrosis in neuronal ischaemic cell death in keeping with Sairanen's previous results demonstrating the decrease in TUNEL-labeling with increased necrosis.

There is a great interest in the potential clinical benefit of PARP-1 inhibition in human diseases, such as myocardial infarction, stroke and cancer based on the double-edged role of PARP-1 in the pathogenesis of cardiovascular and inflammatory diseases and on the other hand on its ability to repair injured DNA. As reviewed by Graziani and Szabo, a vast body of literature suggests that cells with mild, repairable DNA damage as well as cells with severely damaged DNA are diverted by PARP-1 inhibition to a common pathway, i.e. apoptotic cell elimination. Caspase-3 mediated PARP-1 inactivation by its cleavage suggests that blocking PARP-1 activation is crucial for the execution of apoptosis. This theory of protection against necrosis by inhibition or inactivation of excessive PARP-1 renders the necrotic process amenable to pharmacological intervention. Our results support inhibition of the pathological PARP-1 overactivation in neurons as a therapeutic strategy in human brain ischaemia. It might possibly prevent cell necrosis and ensue loss of cellular energy together with downregulation of various pro-inflammatory signal transduction pathways.

The major limitations of our study are that the data are descriptive (no possibility of interventions) and data are based only on immunohistochemical data. In our opinion, the autopsy brain cohort is best used for microscopical evaluation instead of homogenization for, e.g. Western blotting. In the latter approach, the knowledge on cellular localization would be lost. Due to the extensiveness of data collected by counting the number of positive cells (semiquantitative) under a microscope, our analysis lacks systematic evaluation of PARP-1, PAR, and ACA-3 immunoreactivities in other cells of the neurovascular unit besides neurons. Third, the autopsy cohort consists of serious, lethal (in many cases death due to herniation) brain infarct cases with main artery occlusion, which limits the interpretation of the results to a less severe stroke.

5.3. The 30-Day-Outcome in Spontaneous Supratentorial Intracranial Haemorrhages

Predictors of fatal outcome in primary ICH have been widely studied. However no reliable and widely used scoring system has been established as yet. Our aim was to

assess a relatively simple, reproducible, cost-effective predictive scoring system by analysis of a wide range of clinical and epidemiological data. Our results show that systolic blood pressure, serum potassium and glucose levels, and platelet count independently and statistically significantly predict the 30-day fatal outcome in primary supratentorial ICH. Therefore, we propose a predictive scoring system for clinical outcome in ICH using six parameters (serum glucose; total haematoma volume; systolic blood pressure; presence of intraventricular haemorrhage; serum potassium level; and age) with the acronym SUSPEKT.

Of the laboratory parameters, we found serum glucose (*SU*) level on admission to be significantly associated with lethal outcome and this result is also supported by previous studies, while others reported that, in ICH, hyperglycaemia is not a significant and independent outcome predictor. Stead *et al.* described the elevated serum glucose level at admission as a predictor of early (<7 days) mortality both in diabetic and in nondiabetic patients.

We also found that absolute and relative haematoma volumes ("*S*" for size), haematoma growth index, and presence and size of intraventricular haemorrhage are predictors of death within 30 days after ICH. We recommend the use of absolute haematoma volume since it is easier to calculate and its predictive value is similar to relative volume. Haematoma volume is a well-known essential predictor of mortality and poor outcome in patients with ICH.

According to previous findings, high systolic and diastolic blood pressure are associated with larger ICH volume. High mean arterial blood pressure and diastolic pressure correlate significantly with mortality and poor outcome. Dandapani *et al.* demonstrated that higher than 145 Hgmm mean arterial blood pressure is unfavourable regarding the outcome. However, according to previous results, low blood pressure (below 120 Hgmm) is also associated with poor outcome. We found only systolic blood pressure („*P''*) to strongly predict 30-day fatal outcome.

Presence and size of intraventricular haemorrhage ("*E*" for expansion) are also strongly associated with outcome in both overall and supratentorial cerebral

haemorrhages. Haematoma expansion is a significant determinant of both mortality and poor functional outcome after ICH.

We observed a previously unreported, very strongly significant association between serum potassium ("*K*") level and 30-day lethal outcome, which was related to both high and low levels outside the normal range. According to a previous study based on 55 haemorrhagic stroke cases, abnormal potassium level was almost in 40% of the cases, mostly (34.55%) hypokalaemia and less (3.63%) hyperkalaemia, although the effect of these alterations on prognosis was not in focus. Another article analyses electrolit alterations among intracerebral haemorrhage cases. Patients with thalamus bleeding had significantly much frequently altered sodium, potassium and chlorid levels, than patients with nonthalamic bleeding. Those patients who showed electrolit abnormality had significantly higher mortality than those who didn't.

Our result could be explained also by the fact that high and low potassium levels are associated with morbidity and mortality also through cardiac effects: ventricular fibrillation and cardiac arrest. At extreme serum potassium levels death probably occurred because of cardiac dysfunction and not directly due to intracranial mass effect.

Age ("*T*" for lifetime) had a borderline predictive *p* value of 0.054 consistent with previous observations that the role of age in outcome after ICH is controversial. Some prior studies reported that age was significantly and independently correlated with fatal outcome, while others did not find this association or only for patients aged above either 65 or 80 years.

We identified low platelet count as a predictor of fatal outcome and it is also reported that low platelet count and platelet dysfunction significantly correlate with haematoma growth. Low haemoglobin showed no association with lethal outcome within 30 days. Although Kumar *et al.* reported that anaemia and WBC count were associated with larger haematomas, none of these variables were predictors of 30-day mortality in concert with our results.

Time from symptom onset to initial CT was inversely and strongly associated with fatal outcome. This paradoxical relationship could be explained by cases of rapidly

worsening complaints or severe signs caused by massive haemorrhages having presented to hospital sooner and, in contrast, slower response by patients experiencing mild impairment due to small haemorrhages.

There was no correlation between smoking and 30-day mortality. In a Chinese study smoking was an independent risk factor for death in both the ischaemic and haemorrhagic stroke, while other reports found no association. Ikehara *et al.* reported that heavy alcohol consumption is significantly associated with increased mortality from haemorrhagic stroke for men; however, in the present study in accordance with others' results we found no association between alcohol consumption and fatal outcome in ICH.

Several prognostic models have been developed to predict fatal outcome after ICH. Prognostic scores limited to supratentorial haemorrhages are also known. These studies focused on several factors: age, sex, volume or location of haemorrhage, presence of intraventricular haemorrhage, subarachnoidal extension of haemorrhage, presence of hydrocephalus on initial computed tomography scan, level of consciousness, focal neurological deficit on admission, high NIHSS total score, pulse pressure. Compared with the previously used prognostic score, undoubtable forcefulness of SUSPEKT score is that it includes serum potassium level which is a novelty and hyperglycaemia which can be corrected. Another advantage of our scoring system is that it consists of only 6 parameters and except haematoma volume these can be managed and influenced by conservative (i.e. nonsurgical) therapeutic interventions.

Our findings are limited by some shortcomings. Prehospital deaths, exclusion of large, multilobar haematomas, and missing second CT in the survivor group may have led to possible selection bias.

NEW DISCOVERIES

1) Analyzing the results of an autopsy series performed in the Neuropathology Laboratory Unversity of Debrecen over a two-year long period we provided solid evidence that post-mortem neuropathological examination is still important and essential

not only in confirming the clinical diagnosis but it also reveals in a high proportion of patients undisclosed clinically relevant brain pathologies.

2) According to the previous results the frequency of the HT of ischaemic stroke is relatively high and its occurence is often asymptomatic. The vast majority of literature on this topic is based on imaging techniques. Our study provides retrospective data about the proportion of HT diagnosed by imaging tools performed by the suggestions of actual guidelines and the proportion diagnosed by brain autopsies. We demonstrated that HT is still frequent and frequently undiscovered despite the fact that clinicians are aware of its possibility and take efforts to diagnose it. We also demonstrated that autopsy has a unique and essential role in diagnosing HT.

3) We reported nucleolar PARP-1 expression which is a novelty. It has been described in vitro in MDBK, HeLa and CHO cells and in murine fibroblasts before, but not in neurons. In our sample PARP-1 was detected in nucleoli either alone or with simultaneous nuclear expression between 15 and 39 h and only in the periinfarct and contralateral regions, which retain more physiological energy metabolism than the infarct core.

4) The present observational findings generate a hypothesis of overexpressed nuclear full-length and cleaved PARP-1 as a kind of surrogate marker of necrosis while cytoplasmic PARP-1 might reflect concomitant ongoing apoptosis.

5) Our present study supports the previously *in vitro* demonstrated subcellular translocation of PARP-1 from nucleus to the cytoplasm in human ischaemic stroke.

6) The present study on a unique set of human post-mortem brains demonstrates the concomitance of necrosis and apoptosis and their relation to the extent of ischaemic neuronal damage in human ischaemic stroke.

7) We demonstrate that serum potassium concentration is significantly associated with fatal outcome: high and low levels outside the normal range both represents elevated odds of death.

8) The final multiple logistic regression model showed statistically significant associations between 30-day case fatality and a number of variables: Using serum

glucose (*Su*gar); total haematoma volume (*S*ize); systolic blood *P*ressure; presence of intraventricular haemorrhage (*E*xtension to the ventricular system); serum potassium level (*K*alium) and adding age (life*T*ime) which was of borderline significance we developed a six-factor scoring system *SUSPEKT*. The SUSPEKT scoring system is suitable for prediction of 30-day mortality after primary supratentorial ICH. Compared with the previously used prognostic score, undoubtable forcefulness of SUSPEKT score is that it includes serum potassium level which is a novelty and hyperglycaemia which can be corrected. Another advantage of our scoring system is that it consists of only 6 parameters and except haematoma volume these can be managed and influenced by conservative (i.e. nonsurgical) therapeutic interventions.

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List of publications related to the dissertation

1. **Szepesi, R.**, Széll, I.K., Hortobágyi, T., Kardos, L., Nagy, K., Lánczi, L.I., Berényi, E., Bereczki, D., Csiba, L.: New prognostic score for the prediction of 30-day outcome in spontaneous supratentorial cerebral haemorrhage.
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**T. Sairanen and R. Szepesi contributed equally to this study.*
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3. Murnyák, B., Bodoki, L., Vincze, M., Griger, Z., Csonka, T., **Szepesi, R.**, Kurucz, A., Dankó, K., Hortobágyi, T.: Inclusion body myositis: Pathomechanism and lessons from genetics.
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Total IF of journals (all publications): 17,574
Total IF of journals (publications related to the dissertation): 7,976

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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