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## Progressive DNA and RNA damage from oxidation after aneurysmal subarachnoid haemorrhage in humans

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### ABSTRACT

Free radical toxicity is considered as a key mechanism in the neuronal damage occurring after aneurysmal subarachnoid haemorrhage (SAH). We measured markers of DNA and RNA damage from oxidation (8-oxodG and 8-oxoGuo, respectively) in cerebrospinal fluid from 45 patients with SAH on day 1–14 after ictus and 45 age-matched healthy control subjects. At baseline, both markers were significantly increased in patients compared to controls ( $p$  values  $< .001$ ), and exhibited a progressive further increase (to  $>20$ -fold above control levels) from day 5–14. None of the markers predicted the occurrence of vasospasms or mortality, although there was a trend that the 8-oxoGuo marker was more strongly associated with mortality than the 8-oxodG marker. We conclude that SAH leads to a massive increase in damage to nucleic acids from oxidative stress, which is likely to play a role in neuronal dysfunction and death. As only patients in need of a ventriculostomy catheter were included in the study, the findings cannot necessarily be extrapolated to all patients with SAH.

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### KEYWORDS

Aneurysm; nucleic acids; oxidative stress; subarachnoid haemorrhage; vasospasm

## Introduction

Subarachnoid haemorrhage (SAH) is an acute and life-threatening disease caused by bleeding into the subarachnoid space. Eighty-five percent of spontaneous SAH cases are caused by a ruptured intracranial aneurysm, of which the case fatality is estimated to be around 50%. Most survivors experience severe sequelae that include focal neurological deficits, epilepsy, and cognitive and psychosocial dysfunction [1].

Free radical toxicity is considered as a key mechanism in the neuronal damage occurring after aneurysmal SAH, and is a possible target for neuroprotective interventions [2–5]. The extravascular release of haemoglobin after aneurysm rupture catalyses increased oxidative stress through the Fenton reaction, in which free iron reacts with hydrogen peroxide to form hydroxyl radicals, which are highly reactive and readily interfere with the integrity of macromolecules such as lipids, proteins, and nucleic acids (DNA/RNA) [2].

The oxidative damage to neuronal DNA, or the inability to enzymatically repair such damage, is a key

mechanism in disorders of chronic neurodegeneration [6,7]. More recently, a role of oxidative damage to RNA in neurodegenerative disorders has been established [8]. Markers of lipid peroxidation have previously been found to be increased in cerebrospinal fluid (CSF) after SAH [9–11], as well as in traumatic brain injuries [12]. Animal studies have indicated increased DNA damage from oxidation in the brain after experimental SAH [2,13]. However, to our knowledge, a determination of the amount of damage from oxidation to DNA/RNA after SAH in humans has never previously been performed.

## Materials and methods

### Subjects and investigations

The details of the study cohort are described elsewhere [14]. One hundred and eleven patients admitted to the neurointensive care unit at the Copenhagen University Hospital between February 2011 and December 2011 with spontaneous aneurysmal SAH, verified by

computed tomography (CT), were studied prospectively in the 14 days after ictus. A medical history was obtained from the patient or family members. Based on the diagnostic CT-scan, the extent of bleeding was rated by the Fisher scale [15]. The presence of an aneurysm was confirmed by CT angiography, digital subtraction angiography, or during surgery. Exclusion criteria were prior spontaneous SAH, age <18, or pending cerebral herniation. Of the full study population, 45 patients had sequential CSF samples obtained from a ventriculostomy catheter, which was inserted following standard clinical criteria (i.e. signs of increased cerebral pressure). These individuals constitute the patient sample of this study.

The occurrence of cerebral vasospasms was identified as previously described [16]. Briefly, transcranial Doppler measurements of the mean flow velocity in the middle cerebral artery (VMCA) were performed bilaterally in duplicate with colour-coded duplex ultrasound (Micromaxx, Sonosite Ltd., Hitchin, UK; P17/5–1MHz probe). Cerebral angiographic vasospasm was defined as a VMCA  $>200 \text{ cm s}^{-1}$  or a difference between sides of  $>50 \text{ cm s}^{-1}$  occurring between days 3 and 12 (both included) after SAH. Cerebral vasospasm was defined as clinical symptoms in combination with a positive angiogram. Patients were subjected to angiography only on indication by the treating clinician.

Lumbar CSF samples from 45 control subjects from the Danish Dementia Research Centre and the Copenhagen Memory Clinic, Rigshospitalet, Copenhagen, Denmark, were used as controls. Controls were either healthy control subjects recruited for research purposes or patients referred to the Copenhagen Memory Clinic. All controls were cognitively intact and had no neurological or psychiatric disease.

### **Determination of 8-oxodG and 8-oxoGuo in cerebrospinal fluid**

CSF content of the oxidatively modified guanine nucleosides was quantified using a modification of the previously described ultraperformance liquid chromatography/tandem mass spectrometry method [17]. The chromatographic methods for the detection of oxidized nucleosides are superior to ELISA-based methods in both sensitivity and precision, and have been optimized and applied by our group in several previous studies [18–22]. Briefly, the CSF samples were added internal standards filtered through prewashed filters (VectaSpin 3 centrifuge filters, 10-K molecular weight cut off, Whatman, Kent, UK) before injection. The chromatographic separation was performed on an Acquity UPLC

HSS T3 column (1.8  $\mu\text{m}$ ,  $2.1 \times 100 \text{ mm}$ ) protected with an HSS T3 precolumn (1.8  $\mu\text{m}$ ,  $2.1 \times 5 \text{ mm}$ ) both obtained from Waters. The analytes were separated by gradient elution using 0.5% acetic acid and acetonitrile. The column temperature was 1 °C. MS/MS detection was performed in the positive ionization mode. The MS/MS transition for detection of 8oxoGuo was  $m/z$  300 –  $>$  168, and  $m/z$  284 –  $>$  168 for 8oxodG.

### **Ethics**

Written informed consent was obtained from each participant or by the nearest relative or the patient's general practitioner. The study protocols were approved by the Regional Ethical Committees of the Copenhagen and Zealand Regions (H-3-2010-136, H-1-2013-121, and SJ-214). The study was performed in accordance with the Declaration of Helsinki.

### **Statistics**

Baseline patient vs control characteristics and levels of 8-oxodG and 8-oxoGuo were compared with chi-square tests and Mann–Whitney *U*-test. In patients, the baseline value was defined as the marker concentration in the first sample obtained after ictus (restricted to day 1–4). Patient levels of 8-oxodG and 8-oxoGuo at day 1–14 were analysed with a linear mixed model with time as fixed factor and 8-oxodG/8-oxoGuo as the dependent variables. *Post hoc* comparisons to day 1 levels, using the least significant difference option, were included. The predictive value of the oxidation markers vs outcome measures (mortality and vasospasms) was analysed with Cox regression, where CSF 8-oxodG/8-oxoGuo were dichotomized into “low” and “high” groups by the median of the datasets, which consisted of the first available measurement after ictus at any time point (in the vasospasm analyses, only observations from before the occurrence of vasospasms was included). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0 (IBM Corporation, Armonk, NY). Statistical significance was defined as  $p < .05$ . All statistical tests were two sided.

## **Results**

### **Clinical data of the participants**

Patients and controls did not differ with respect to age (mean age  $\pm$  SD =  $57 \pm 12$  vs  $60 \pm 9$ ,  $p = .09$ ), but there were slightly more females in the patient group (39/45 (86%)) vs the control group (30/45 (67%)) ( $p = .025$ ).

Among patients, 16/45 (36%) had a medical history of hypertension, and 34/45 (76%) were smokers. Based on the initial CT-scan, 2/45 (4%) were rated to be Fisher grade 2, 37/45 (82%) as grade 3, and 6/45 (13%) as grade 4. Vasospasms occurred in 14/45 (31%) patients during the 14-day follow-up. 10/45 (22%) patients died within the first 30 days after ictus.

### CSF 8-oxodG and 8-oxoGuo concentrations in healthy controls and in patients at day 1–14 after ictus

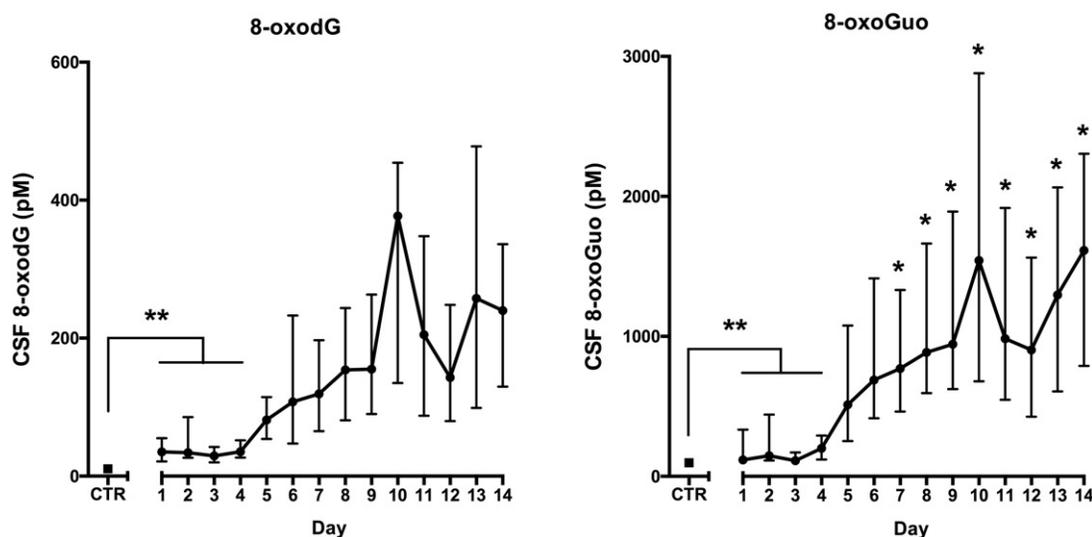
The average number of CSF samples available from each patient were 5.5 (range 1–10). A baseline value (i.e. from day 1–4) was available for 33/45 (73%) patients. Patient baseline CSF concentrations of both markers were increased compared to control subject levels (8-oxodG, patients (median (interquartile range)): 33.7 (23.7–72.8) pM, controls: 9.9 (7.2–11.5) pM, Mann–Whitney  $U = 70.0$ ,  $p < .001$ . 8-oxoGuo, patients: 125.8 (99.9–286.5) pM, controls: 80.6 (68.0–107.0) pM,  $U = 248.5$ ,  $p < .001$ ). In both markers, a significant effect of time was found in the linear mixed model (8-oxodG:  $F = 4.025$ ,  $p < .001$ . 8-oxoGuo:  $F = 6.282$ ,  $p < .001$ ), reflecting increasing levels from approximately day 5 after ictus (not significant in *post hoc* comparisons for 8-oxodG, significant from day 7–14 for 8-oxoGuo ( $p = .015$ – $0.001$ )) (Figure 1). Hence, at day 14, levels of both markers were increased >20-fold compared to healthy control levels (Figure 1). CSF 8-oxodG and 8-oxoGuo concentrations did not predict mortality or the

occurrence of vasospasms. Compared to 8-oxodG, high 8-oxoGuo showed a stronger association to mortality, but this was not significant in the Cox regression (hazard ratio (95%CI) = 1.84 (0.56–5.94),  $p = .26$ ) (Figure 2).

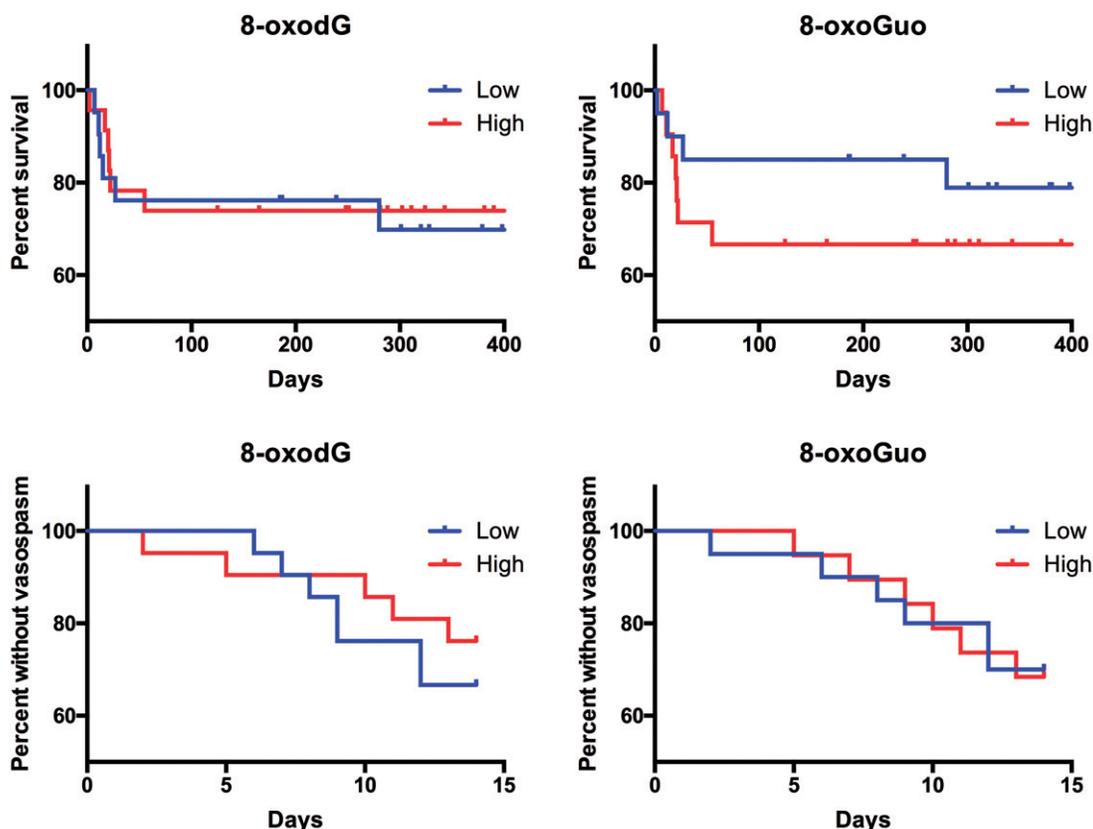
### Discussion

We measured markers of DNA and RNA damage from oxidation in cerebrospinal fluid from 45 patients with SAH on day 1–14 after ictus and 45 healthy control subjects. We found that SAH is associated with severely increased DNA and RNA damage due to oxidative stress. The observed increase in the marker concentrations was biphasic, with a stable increase compared to controls in the acute phase (day 1–4), and a progressive further increase at day 5–14. The further increase in CSF markers concentration from day 5 coincides with the time point at which delayed cerebral ischaemia occurs; a phenomenon thought to involve impaired microcirculation and – among other events – increased oxidative stress [1,4].

The massive increase in damage to nucleic acids from oxidation is likely to play a role in neuronal dysfunction and death after SAH. The predicted downstream consequence of DNA damage is activation of apoptotic pathways and subsequent cell death, and indeed, this has been established after SAH [23]. RNA damage may cause malformed or truncated proteins, which can cause cell dysfunction, endoplasmic reticulum stress, and activation of the unfolded protein



**Figure 1.** CSF markers of oxidatively generated DNA and RNA damage (8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively) in cerebrospinal fluid from healthy control subjects (CTR) and patients with aneurysmal SAH at day 1–14 after ictus. There is a significant difference in both markers between controls and patients at baseline (as defined as the first available sample from day 1–4). From approximately day 5 after ictus, there is an increase of both markers compared to day one levels (not significant in *post hoc* comparisons for 8-oxodG, significant from day 7 for 8-oxoGuo). Data are presented as medians (interquartile range). \* $p < .05$ , \*\* $p < .001$ .



**Figure 2.** Kaplan–Meier plots of CSF markers of oxidatively generated DNA and RNA damage (8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively) as predictors of mortality (top panel) and vasospasms (bottom panel) after aneurysmal SAH. 8-oxodG/8-oxoGuo was dichotomised into “low” and “high” groups, defined by the median of the datasets. None of the associations are significant. Data were analysed with Cox regression.

response (UPR), and these pathways have been observed to be activated after a range of acute brain injuries [24]. Collectively, it could be hypothesized that damage to nucleic acids from oxidation is an early and causal event in the molecular pathways leading to cell death after SAH. In the present cohort, however, biomarkers of DNA/RNA oxidative damage only showed a weak association with intracerebral vasospasms and in-hospital mortality. Other factors besides oxidative damage may influence outcomes in critically ill SAH patients, and the significance of the present findings has to be tested in larger studies.

In two previous studies, we have found the DNA/RNA oxidation markers to be strongly linked to systemic iron levels [25,26]. Animal studies have found that pharmacological blockade of brain iron accumulation after SAH reduced oxidative stress and early brain injury [27,28]. The above-mentioned study by Lee et al. [2] showed that treatment with the iron chelator deferoxamine reduced brain levels of the 8-oxodG DNA lesion and concomitantly reduced early cell death and mortality after experimental SAH in rats. Our demonstration that DNA, as well as RNA, damage from oxidation also

occurs in humans – and is severely further increased later in the disease course – adds support to the notion that iron chelation could be of beneficial effect in humans with SAH. It should be noted, however, that many other biochemical processes in the acute phase of SAH, such as e.g. inflammation and alterations in DNA repair, could influence levels of oxidative stress and the extracellular release of the nucleic acid markers.

The main limitations of the study are the relatively low sample size; the limited number of samples from each patient at each time point, and the selection bias that is potentially introduced by only including patients in need of a ventriculostomy catheter (i.e. the most severely ill). Hence, the findings cannot necessarily be extrapolated to all patients with SAH. Furthermore, we cannot completely rule out that the difference in anatomical site of sampling between patients and controls may have influenced the results. Finally, SAH patients had a high prevalence of smoking, which could potentially influence levels of oxidative stress.

We conclude that both early and delayed brain injury after aneurysmal SAH coincides with an increase in DNA/RNA damage from oxidation; a phenomenon

which could be of importance to the neuron loss occurring after SAH, and which could potentially be targeted for treatment.

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## Disclosure statement

None of the authors declare any conflicts of interest.

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