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Temporal profile of serum mitochondrial DNA (mtDNA) in patients with aneurysmal Subarachnoid Hemorrhage (aSAH)

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Highlights

- Serum mitochondrial DNA contents, one of the potential mitochondrial DAMPs, were elevated after aSAH over two weeks
- Different mitochondrial genes (Cyt B, D-Loop and COX-1) showed association with post-SAH complications and clinical outcome
- Elevated serum mitochondrial DNA and their associations with post-SAH complications and clinical outcome implicate an important role of mtDNA in systemic inflammation after aSAH

Abstract

Aneurysmal subarachnoid hemorrhage (aSAH) is a highly complex disease. Majority of aSAH survivors confront post-SAH complications including cerebral vasospasm (CVS) and delayed cerebral ischemia (DCI) that mainly influence the clinical outcome. Tissue damage during early brain injury may lead to release of damage associated molecular pattern molecules (DAMPs) that may initiate and sustain inflammation during the course of aSAH through activation of pattern recognition receptors. Mitochondrial DNA (mtDNA) due to unmethylated CpG motifs acts as a DAMP via binding to toll-like receptor-9. The aim of this study was to investigate the cell free circulating mtDNA in the systemic circulation of aSAH patients and its association with post-SAH complications and clinical outcome.

The DNA was extracted from the serum of 80 aSAH patients at days 1, 3, 5, 7, 9, 11, 13 and from 18 healthy controls. Three representative mitochondrial gene fragments including Cytochrome B (CytB), D-Loop and Cytochrome c oxidase subunit-1 (COX-1) were quantified using a Taqman-probes based qPCR. Levels of mtDNA were quantified from standard curves generated using mtDNA extracted from HepG2 cell mitochondria. Clinical outcome of the patients was assessed by Glasgow outcome scale (GOS) and modified Rankin scale (mRS). Clinical data and post-SAH complications were recorded from patient's record file.

Serum D-Loop and COX-1 were significantly elevated early after aSAH and remained high over first 2 weeks. CytB levels were however, initially unchanged but elevated later at day 7 as compared to healthy controls. Cumulative levels measured over two weeks showed significant correlations with post-SAH complications including a negative correlation of D-Loop with pneumonia infection, hydrocephalus and occurrence of epilepsy, a positive correlation of Cyt B with occurrence of CVS and a negative correlation of COX-1 with occurrence of systemic infections and seizures. Cumulative D-Loop values negatively correlated with clinical outcome. Our data suggest that mtDNA may directly or indirectly influence post-SAH complications and clinical outcome.

Keywords

Mitochondrial DNA, DAMPs, aneurysmal subarachnoid hemorrhage, Cytochrome B, D-Loop, Cytochrome c oxidase.

Abbreviations

aSAH: Aneurysmal subarachnoid hemorrhage; CVS: cerebral vasospasm; DCI: delayed cerebral ischemia; DAMPs: damage associated molecular pattern molecules; mtDNA: Mitochondrial DNA; CytB: Cytochrome B; COX-1: Cytochrome c oxidase subunit-1; GOS: Glasgow outcome scale; mRS: modified Rankin scale; PRRs: pattern recognition receptors;

TFAM: mitochondrial transcription factor A; CT: computed tomography; CT-A: CT angiography; DSA: digital subtraction angiography; NICU: neuro-intensive care unit; TCD: transcranial Doppler; CT-P: CT perfusion; MTT: mean transient time; MAP: mean arterial blood pressure; IVH: intraventricular hemorrhage; ICB: intracerebral bleeding; CI: cerebral ischemia; VP-Shunt: ventriculoperitoneal shunt; H&H: Hunt and Hess grade; DIND: delayed ischemic neurological deficits

1. Introduction

Subarachnoid hemorrhage (SAH) is a subtype of hemorrhagic stroke accounting for only 5% of all stroke events. The mortality afflicted by subarachnoid hemorrhage is around 50% (van Gijn and Rinkel, 2001) and incidence, although varies geographically, is around 10 per 100,000 persons per year (Monstrey, 1998). Approximately 85% of SAH are due to rupture of intracranial aneurysms (Macdonald and Schweizer, 2017; van Gijn and Rinkel, 2001). Aneurysmal SAH (aSAH) affects at relatively younger age, around the mean age of 55 years and renders one third of the survivors dependent due to significant disability (van Gijn and Rinkel, 2001).

In the pathophysiology of aSAH, which is highly complex, two phases of brain injury have been described. The early brain injury occurs within 72 hours of aSAH due to transient global cerebral ischemia as a consequence of increased intracranial pressure and due to the toxicity of the extravasated blood and its catabolic products. The delayed brain injury phase exists after 72 hours after aSAH with delayed cerebral ischemia (DCI) as the prominent deteriorating complication during this phase (Macdonald and Schweizer, 2017). Evidence suggests that inflammation plays a key role from aneurysm formation to its rupture and in post-SAH complications (Aoki et al., 2017; Lucke-Wold et al., 2016; Miller et al., 2014; Provencio, 2013).

Mitochondria, due to their endosymbiotic nature, share many features to their ancestral bacteria (Galluzzi et al., 2012; Krysko et al., 2011) and are an important source of damage associated molecular pattern molecules (DAMPs). DAMPs released upon cellular injury from various cell compartments can be recognized by pattern recognition receptors (PRRs) on immune cells, leading to their activation and thus, upregulation of inflammation. In recent years, mitochondria have been recognized as a host of different DAMPs including TFAM (mitochondrial transcription factor A), N-formyl peptides, cardiolipin and hypomethylated/ non-methylated mitochondrial DNA, which are released upon cell stress, injury and necrosis (Galluzzi et al., 2012). Mitochondrial DNA (mtDNA) has been identified to induce TNF secretion from splenocytes and cause arthritis in mice joints on injection (Collins et al., 2004). In 2010, (Zhang et al. (2010b)) showed that circulating mtDNA can cause inflammation owing to its resemblance with bacterial CpG motifs and binding to toll like receptor 9 (TLR-9) on innate immune cells. There is now increasing evidence that mtDNA can upregulate innate immune response through several PRRs, most importantly TLR-9, NLRP3-, NLRC4-, AIM2-inflammasome complex and cGAS-STING (Boyapati et al., 2017; West and Shadel, 2017). The systemic levels of mtDNA has been shown to be raised in different diseased conditions such as sepsis (Kung et al., 2012), trauma (Lam et al., 2004; Mohamed et al., 2016), meningitis (Lu et al., 2010), HIV infection (Perez-Santiago et al., 2016), acute myocardial

infarction (Wang et al., 2015), autism (Zhang et al., 2010a), hepatic transplantation (Hu et al., 2015) and hemodialysis (Eleftheriadis et al., 2014). Multiple studies have shown that mtDNA as a potential biomarker of different neoplastic conditions such as prostate cancer (Ellinger et al., 2008), germ cell cancer (Ellinger et al., 2009) and breast cancer (Xia et al., 2014). A few studies have shown elevated circulating cell free mtDNA and its biomarker and prognostic potential in connection to diseases involving CNS pathologies (Lu et al., 2010; Mathew et al., 2012; Perez-Santiago et al., 2016; Podlesniy et al., 2013; Podlesniy et al., 2016a; Podlesniy et al., 2016b; Sondheimer et al., 2014; Varhaug et al., 2016). Role of mtDNA in the context of aSAH has not been investigated in detail.

Tissue damage during early brain injury may lead to release of damage associated molecular pattern molecules (DAMPs) that may initiate and sustain inflammation during the course of aSAH. The primary aim of this study was to investigate the systemic levels of mtDNA after aSAH. The secondary aim was to determine the association of mtDNA with post-SAH complications and clinical outcome of the patients.

2. Methodology

2.1. Patient Population

This study consists of 80 consecutive aSAH patients, which were recruited in the neurosurgery unit of the University Hospital Bonn during 2012 to 2016. Patients underwent standard diagnostic and treatment protocols as per institutional guidelines and were destined to either neurosurgical clipping or endovascular coiling of bleeding aneurysms based on an interdisciplinary decision. The inclusion criteria were aSAH patients presenting within 24 hours of onset of signs and symptoms. The exclusion criteria were age equal to or less than 18 years, presenting after 24 hours after the onset of symptoms, traumatic brain injury, ischemic stroke, SAH due to arteriovenous malformations or other reasons, signs of imminent death, not providing informed consent and pregnancy. Peripheral blood samples were collected in serum Monovette gel tubes (Sarstedt, Germany) from aSAH patients at day 1, 3, 5, 7, 9, 11, 13 and centrifuged at 3000 rpm for 10 minutes (Sigma, Germany).Control peripheral blood samples were obtained from 18 healthy volunteers. The serum was stored at -80 °C until analysis. The leucocyte counts and CRP values were retrieved from patients' records. This study was performed according to the guidelines of the Helsinki declaration and was approved by the local ethical committee of the medical faculty of the University of Bonn (Reference Number: LfD 138/ 2011 and 258/15).

2.2. Clinical monitoring and treatment

We followed our standardized diagnostic and treatment regimen. SAH was confirmed by computed tomography (CT) scan. CT angiography (CT-A) and digital subtraction angiography (DSA) were performed for further evaluation of the aneurysm. The treatment decision (coiling/ clipping) was based on an interdisciplinary approach. We followed an early treatment strategy (within 24 hours of admission). Our treatment protocol at the neuro-intensive care unit (NICU) included hourly neurological monitoring, continuous invasive blood pressure and body temperature measurements, daily transcranial Doppler (TCD) and the application of nimodipine for 21 days starting from the day of admission. Generally, patients who were not clinically assessable were screened for vasospasm by daily TCD and DSA on day 7 after ictus. CT-A and CT perfusion (CT-P) were performed upon suspicion of CVS to confirm the presence of the latter. The patients having significant perfusion deficits with mean transient time (MTT) above 6 seconds in CT-P were considered to be treated. CVS patients were treated with induced hypertension using catecholamines, maintaining a target mean arterial blood pressure (MAP) at around 110 mm Hg until resolution of CVS. Hypertensive treatment was stopped after final CT-A and CT-P showed no further evidence of CVS with perfusion deficits.

2.3. Isolation of serum DNA

Cell free circulating DNA in the serum of aSAH patients and controls was isolated using QIAMP DNA mini kit (QIAGEN, Germany) by following the manufacturer's instructions with slight modifications. Briefly, the serum samples were centrifuged for 3 mins at 6000 rpm (Eppendorf, Germany) to get rid of any contaminating cellular debris and obtain cell free DNA. A 200 µl aliquot of serum was applied to 20 µl proteinase K and 200 µl lysis buffer and incubated for 20 minutes at 56 °C. Afterwards, 230 µl of absolute alcohol was added and the mixture was applied to the provided columns after a brief spin down. The columns were washed twice sequentially with the provided washing buffers and finally, DNA was eluted from the column using 50 µl of elution buffer. The extracted DNA was stored at -80 °C until qPCR quantification.

2.4. Generation of mtDNA for standard curves

The mtDNA was extracted from the mitochondria isolated from HepG2 cells as described previously (Schafer et al., 2016). This mtDNA was then used as template to amplify different mt gene fragments i.e., mt Cytochrome B (mt CytB), mt D-Loop (mt D-Loop) and mt Cytochrome c oxidase subunit I (mt COX-1) by using following primers: mtCytB Fwd: 5'- CCT CCA AAT CAC CAC AGG A -3', Rev: 5'- TGA GTA GAG AAA TGA TCC GTA ATA -3'

(Eurogentec, Belgium); mtD-Loop Fwd: 5'- ATC AAC CCT CAA CTA TCA -3', Rev: 5'- ACT GTA ATG TGC TAT GTA -3'; and mtCOX-1 Fwd: 5'- TCA TCT GTA GGC TCA TTC -3', Rev: 5'- GGC ATC CAT ATA GTC ACT -3' (Invitrogen, Germany). A 2 µL of mtDNA template was applied to a 50 µL PCR reaction volume containing 40 nM concentrations of the above mentioned primers. The PCR profile was initial denaturation at 95 °C for 5 mins followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min. The PCR products were validated by gel electrophoresis and were then purified by High Pure PCR Product Purification Kit (Roche, Germany). Purified mitochondrial gene fragments were then quantified by using nanodrop (Thermoscientific, Germany) and finally serial dilutions were prepared using sterilized TE buffer (pH 7.4) ranging from 100 ng/ml to 1 pg/ml.

2.5. Real time PCR quantification of mtDNA

A real time PCR approach based on Taqman probes labelled with 6-carboxyflourescein (6-FAM) on their 5' end and a non-fluorescent minor groove binder (MGB) on their 3' end was established to quantify serum mtDNA levels. The following primers were employed for qPCR: mt CytB Fwd: 5'- AACCGCCTTTTCATCAATCG -3', Rev: 5'- TAGCGGATGATTCAGCCATAATT -3'; mt D-Loop Fwd: 5'- TCAACTATCACACATCAACTGCAACT 3', Rev: 5'- GGGTAGGTTTGTTGGTATCCTAGTG -3', and mt COX-1 Fwd: 5'- TCATCTGTAGGCTCATTCATTTCTCT -3', Rev: 5'- TCTACTATTAGGACTTTTCGCTTCGA -3'. The sequences of Taqman Probes used were as follows: mt CytB 5'-6-FAM-CCACATCACTCGAGACGT-MGB-Eclipse-3', mt D-Loop: 5'- 6-FAM-CAAAGCCACCCCTCA-MGB-Eclipse-3' and mt COX-1: 5'-6-FAM-TTTTCATGATTTGAGAAGCC-MGB-Eclipse-3'. Both the primers and probes were purchased from Eurogentec, Belgium. A qPCR was carried out using a reaction volume of 12.5 µL consisting of Taqman Universal qPCR mastermix (Life Technologies, Germany), 900 nM of each primer and 100 nM of the respective probe. The qPCR conditions were initial heating at 50 °C for 2 mins, then at 95 °C for 10 mins to activate the Taq Polymerase and finally 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. The data was acquired at the end of each cycle. The serum mtDNA levels were then computed from the respective standard curves for each mitochondrial gene fragment.

2.6. Statistical analysis

The categorical variables were expressed as percentages and the continuous variables as mean ± SEM unless otherwise stated. The mtDNA levels were log transformed before analysis. Two tailed student's t test was performed to compare two groups. The P value <0.05 was considered as a significant difference. Spearman's rank correlations were assessed for any association of different post-SAH complications and clinical outcome with

different mtDNA genes. The data was analysed using Graphpad Prism version 5.00 for Windows (CA, USA).

3. Results

3.1. Patient characteristics

The characteristics of the aSAH patient population has been summarized and represented in Table 1.

3.2. Serum levels of mitochondrial Cytochrome B, D-Loop and Cytochrome C oxidase subunit-1 were elevated after aSAH

The mt CytB levels were non-significantly elevated at day 1, 3, and 5 after aSAH as compared to healthy controls (Fig. 1A). However, at day 7 mt CytB levels were significantly raised in aSAH patients as compared to HC and remained significantly high till day 13. The mt CytB levels appeared to be slowly released into systemic circulation, peaking at day 9 and then started to decline (Fig. 1A). The levels of another gene fragment mt D-Loop were significantly higher early at day 1 and remained high till day 13 in serum of patients with aSAH as compared to healthy controls (Fig. 1B). Mitochondrial gene fragment of 136 bp was quantified from mtDNA encoding mt COX-1. Similar to mt D-Loop, mt COX-1 levels were significantly increased very early after aSAH as compared to HC at day 1 and remained elevated till day 13 (Fig. 1C). The release pattern of mt COX-1 in systemic circulation after aSAH was almost identical to mt D-Loop, however, all the quantified gene fragments reached peak levels at day 9 and then, started to decline. Interestingly, this represents an important time period during which post SAH complications likely occur and lead to secondary deterioration of patients.

3.2.1. Serum mt CytB and post SAH complications

For further analysis whether mt CytB levels influence the post SAH complications, we dichotomized the patients into two groups and compared the values of mtDNA in patients with or without a specific complication. Analysis was performed for severity of aSAH, treatment modality, location of aneurysms, development of different complications including cerebral vasospasm, delayed cerebral ischemia, CNS or systemic infections, seizures, hydrocephalus and clinical outcome. Serum levels of mt CytB tended to be higher in male patients with significant differences seen on day1, day 3 and day 9 (Fig. 2A). A significant difference existed only on day 1 between the patients who had intracerebral bleeding (ICB) as compared to the patients without intraventricular hemorrhage (IVH) and intracerebral bleeding (ICB). There was no impact of infections on mt CytB levels (Fig. 2C). Subgroup analysis based on the development of pneumonia, meningitis, and other infections (presence

of other infections such as UTI or in combination with pneumonia or meningitis) revealed significant lower mt CytB levels at day 9 among aSAH patients with pneumonia (Fig. 3D). Similarly, patients who developed cerebral infarction, here referred to as cerebral ischemia (CI), did not display any significant changes in mt CytB levels (Fig. 2E). However, further subgroup analysis between intervention related CI (aSAH patients who developed CI during to aneurysm treatment) detected in 24 hour cranial CT or DCI (aSAH patients where CI cause was not attributed to intervention) showed significantly reduced mt CytB levels at day 3, day 9 and day 13 in patients with interventional CI compared to without CI. On the other hand the patients with delayed cerebral ischemia (DCI) showed elevated mt CytB levels at day 9, day 11 (p=0.05) and day 13, compared to interventional CI group (data not shown). Our data suggests that mt CytB levels are sensitive to CI resulting from aneurysmal treatment with neurosurgical clipping or endovascular coiling.

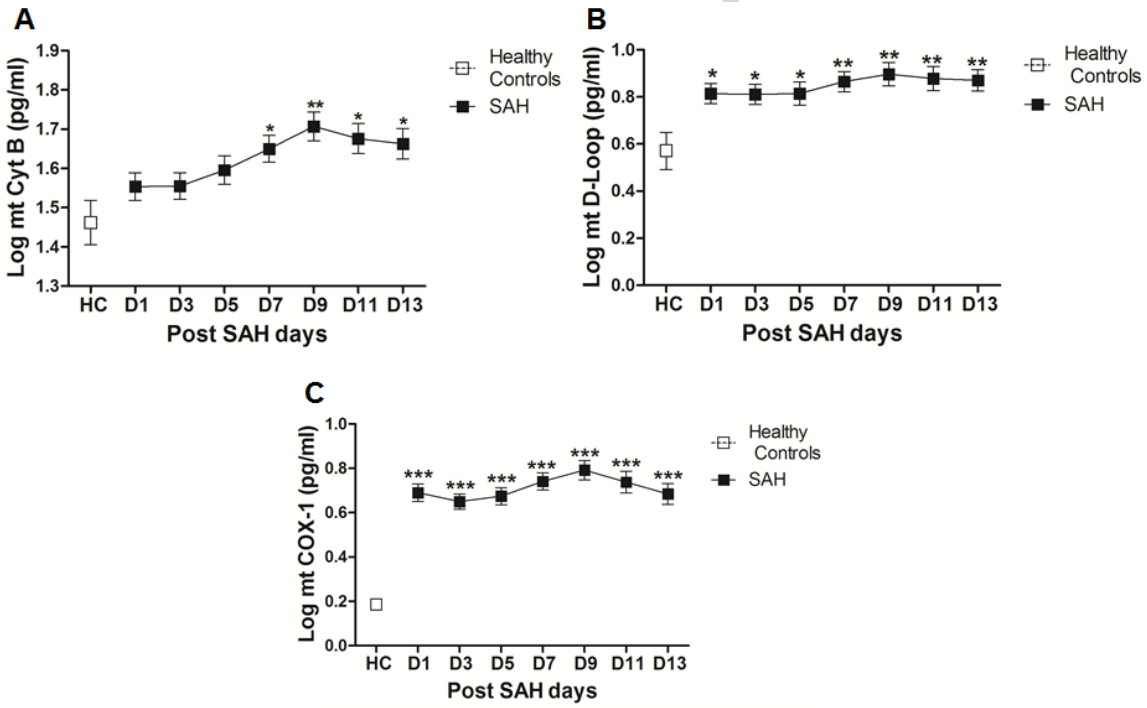

Figure 1: Comparison of serum mtDNA between healthy controls (HC, n =18) and aSAH patients (n = 80). **A.** Serum mt Cyt B levels between healthy controls and aSAH patients. **B.** Serum mt D-Loop levels between healthy controls and aSAH patients. **C.** Serum mt COX-1 levels between healthy controls and aSAH patients. Student's t test, $p < 0.05$.

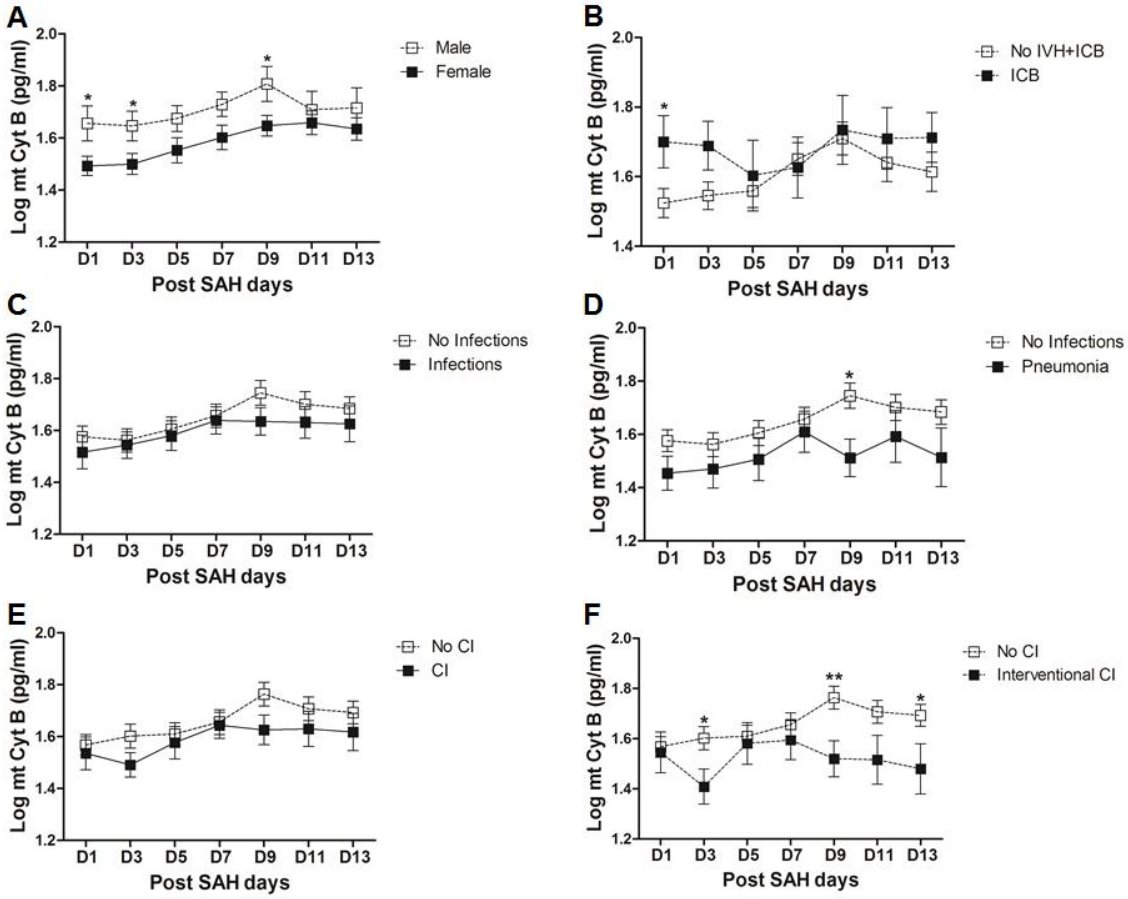

Figure 2: Comparison of mt Cyt B levels between different aSAH subgroups. **A.** Comparison of mt Cyt B levels between male (n = 30) and female (n = 50) aSAH patients. **B.** Comparison of mt Cyt B levels in patients showing No intraventricular hemorrhage and intracerebral bleeding (IVH+ICB) (n = 43) and only ICB (n = 16). **C.** Comparison of mt Cyt B levels between patients developing infections (n = 29) and no infections (n = 51). **D.** Comparison of mt Cyt B levels between patients with no infections (n = 51) and Pneumonia (n = 15). **E.** Comparison of mt Cyt B levels in aSAH patients showing cerebral ischemia (CI) (n = 33). **F.** Comparison of mt Cyt B levels between patients who have no CI (n = 47) and interventional CI (n = 17). Unpaired t test, $p < 0.05$ was considered as a significant difference.

3.2.2. Serum mt D-Loop and post SAH complications

An analysis analogous to mt CytB was performed with serum mt D-Loop levels. Serum mt D-Loop levels were only significantly high on day 9 in males and on day 13 in patients with severe aSAH (H&H III-V) (Fig. 3A, B). Like mt CytB, serum mt D-Loop levels were only downregulated in patients with pneumonia on day 9 compared to patients without infections (Fig. 3C). Patients who experienced post-SAH seizures have lower levels of mt D-Loop DNA levels with significant difference on day 3 and day 7 (Fig. 3D). A similar trend was seen in

patients who required ventriculoperitoneal shunt (VP-Shunt) placement due to development of chronic hydrocephalus on day 1 and day 9 (Fig. 3E). Interestingly, mt D-Loop DNA levels seem to be higher with significance difference on day 9 in patients with good clinical outcome (mRS 0-2) (Fig. 3F).

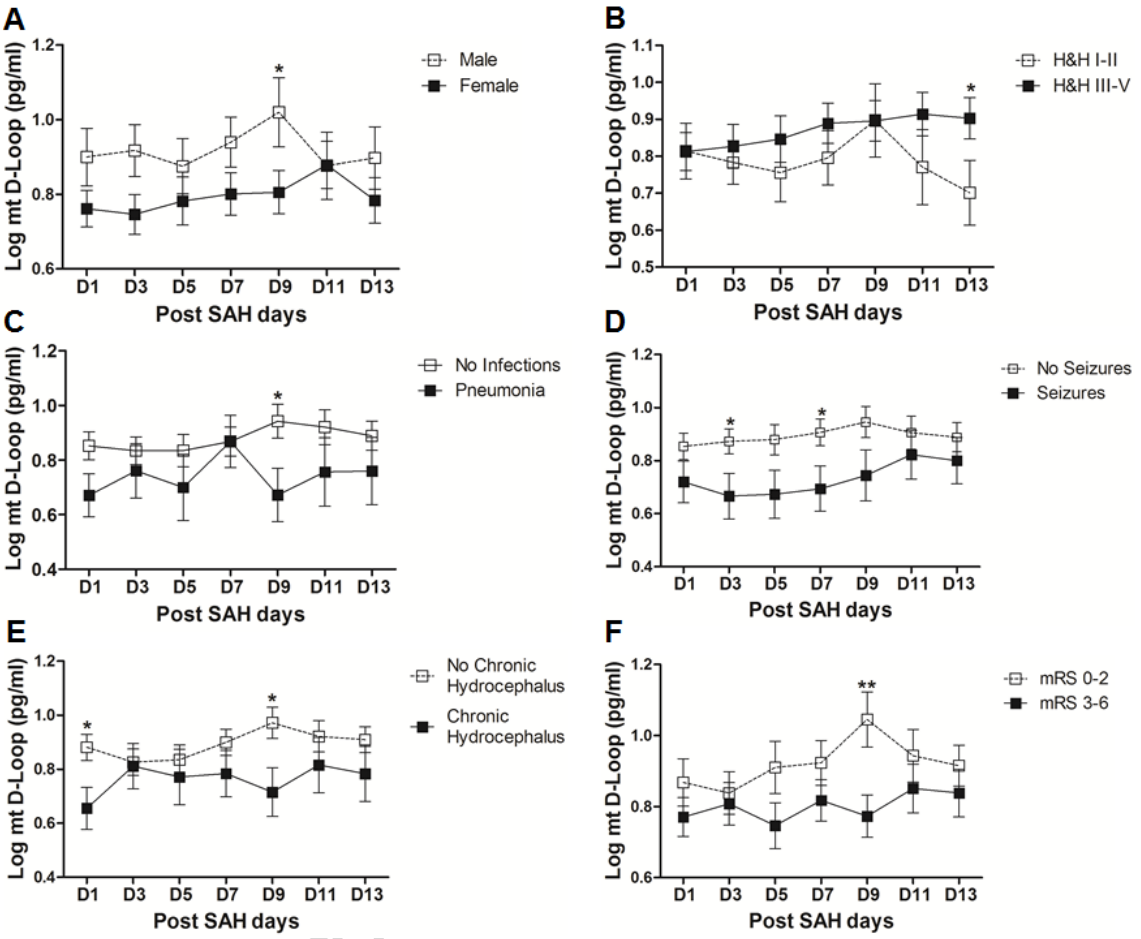

Figure 3: Comparison of mt D-Loop levels between different aSAH subgroups. **A.** Comparison of mt D-Loop levels between male (n = 30) and female (n = 50) aSAH patients. **B.** Comparison of mt D-Loop levels between patients with severe aSAH (Hunt & Hess grade (H&H) III-V, n = 51) and less severe aSAH (H&H I-II, n = 29). **C.** Comparison of mt D-Loop levels between patients with no infections (n = 51) and Pneumonia (n = 15). **D.** Comparison of mt D-Loop levels between patients who developed seizures (n = 24) and no seizures (n = 56). **E.** Comparison of mt D-Loop levels between patients who developed chronic hydrocephalus (n = 25) and who do not (n = 55). **F.** Comparison of mt D-Loop levels between patients with good clinical outcome (modified Rankin scale (mRS) 0-2, n = 35) and poor outcome (mRS 3-6, n = 45). Unpaired t test, $p < 0.05$ was considered as a significant difference.

3.2.3. Serum mt COX-1 and post SAH complications

Patients with ICB and the patients who have developed DIND (delayed ischemic neurological deficits) showed significantly higher mt COX-1 levels on day 1 and day 11, respectively (Fig. 4A & B). Group of patients who developed seizure showed significantly lower mt COX-1 levels on day 7 (Fig. 4C). Levels of mt COX-1 were significantly lower at day 3 and day 9 in patients who developed cerebral ischemia. Further dichotomy of patients who developed cerebral ischemia revealed significant lower mt COX-1 levels in patients with intervention related ischemia compared to the patients without ischemia at day 3, 9, 11 and13 (Fig. 4D, E).

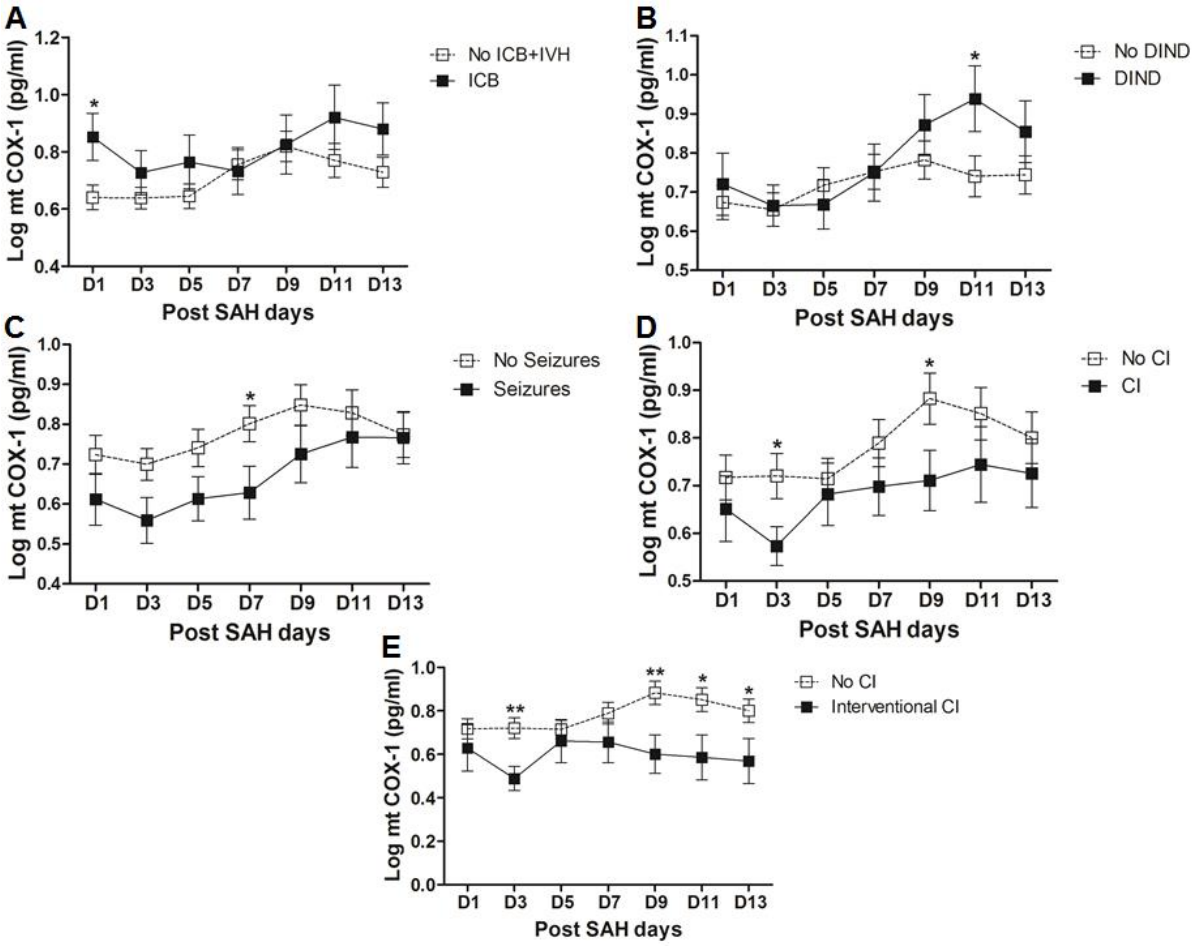

Figure 4: Comparison of COX-1 levels between different aSAH subgroups. **A.** Comparison of mt COX-1 levels in aSAH patients with No intraventricular hemorrhage and intracerebral bleeding (IVH+ICB) (n = 43) and only intracerebral bleeding (ICB) (n = 16). **B.** Comparison of mt COX-1 levels between patients with No delayed ischemic neurological deficits (DIND) (n = 52) and with DIND (n = 28). **C.** Comparison of mt COX-1 levels between aSAH patients developing seizures (n = 24) and no seizures (n = 56). **D.** Comparison of mt COX-1 levels in aSAH patients showing cerebral ischemia (CI) (n = 33) and no CI (n = 47). **E.** Comparison of

mt COX-1 levels between patients who have no CI (n = 47) and interventional CI (n = 17). Unpaired t test, $p < 0.05$ was considered as a significant difference.

3.3. Correlations of total serum mtDNA with different aSAH characters

Serum mtDNA for different mitochondrial gene fragments revealed significant correlations with different aSAH parameters as represented in Table 2. Cumulative values of all these gene fragments measured over two weeks duration also showed significant correlations with different aSAH associated characters including a negative correlation of D-Loop with pneumonia, hydrocephalus and occurrence of epilepsy, a positive correlation of Cyt B with occurrence of CVS and a negative correlation of COX-1 with occurrence of systemic infections and seizures. Cumulative D-Loop values negatively correlated with clinical outcome (Supplementary Table 1).

4. Discussion

Aneurysmal subarachnoid hemorrhage, a morbid and lethal subtype of hemorrhagic stroke, is characterized by an intracranial bleed due to rupture of an intracranial aneurysm usually located at bifurcation of cerebral arteries (Macdonald, 2014; Suarez et al., 2006). The consequent transient global cerebral ischemia and extravasated blood toxicity leads to early brain injury that comprise all the events occurring within 72 hours of aSAH (Cahill and Zhang, 2009; Munoz-Guillen et al., 2013). Since the bleeding aneurysm can be obliterated from circulation by microsurgical clipping or endovascular coiling, but majority of the aSAH patients still experience a delayed deterioration phase after surviving the initial ictus (Macdonald, 2014). This delayed deterioration is the result of post-SAH complications such as cerebral vasospasm (CVS), hydrocephalus, rebleeding from the ruptured aneurysm, seizures, cortical spreading depression (CSD), and most importantly delayed cerebral ischemia (DCI) (Iadecola, 2009; Macdonald, 2014; Suarez et al., 2006). The failure of the past aSAH research focused solely on the reversal of CVS (a major contributor to DCI) to improve clinical outcome, led to explore additional mechanisms of brain injury. Increasing evidence suggests that aSAH is characterized by a systemic inflammatory response (Chaudhry et al., 2017; McMahon et al., 2013; Savarraj et al., 2017; Yoshimoto et al., 2001).

Sterile inflammation is the consequence of recognition of danger signal molecules or alarmins by the pattern recognition receptors (PRRs) on the immune cells. Such danger signal molecules are referred to as damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) depending on whether they are derived from stressed, injured and necrotic cells or from invading pathogens, respectively(Chen and Nuñez, 2010; Takeuchi and Akira, 2010). Over the past, mitochondria have gained value as a potential host of different DAMPs. A great body of evidence supports the role of mtDNA as

a DAMP mediating inflammation via different PRRs such as TLR-9, NLRP3-, NLRC4-, AIM2-inflammasome complex and cGAS-STING (Boyapati et al., 2017; West and Shadel, 2017). The current study aimed to investigate the temporal profile of systemic release of mtDNA after aSAH and assess its association with post-SAH complications and clinical outcome.

The systemic levels of mtDNA were elevated in aSAH patients compared to healthy controls. Interestingly, mtDNA for D-Loop and COX-1 were significantly higher from day 1 until day 13 in aSAH patients, however, mt CytB levels were significantly elevated at day 7 (Fig. 1). This suggests that different mtDNA gene fragments are differentially released into systemic circulation after aSAH. Interestingly, all of investigated mtDNA gene fragments showed a secondary delayed increase with a peak at day 9, which coincides with the delayed deterioration phase. Our results are in agreement with the findings of Wang and coauthors who observed elevated mitochondrial ND2 levels in a small group of aSAH patients (Wang et al., 2013).

Further subgroup analysis based on dichotomization of aSAH patients into two groups for different base line characters, development of post SAH complications and clinical outcome showed differentially elevated mtDNA gene products in some of base line characters and post SAH complications. Serum mtDNA levels of CytB and D-Loop were higher in males in comparison to female aSAH patients (Fig. 2A, 3A and Table 2). CytB and COX-1 mtDNA levels were sensitive to intracerebral bleeding on admission to hospital (Fig. 2B and 4A). There was non-significant difference in serum mtDNA levels in patients who developed infections. However, further subgrouping based on the type of infections revealed that mtDNA levels followed different trends and a significantly lower mt CytB and mt D-Loop levels were observed at day 9 in patients with pneumonia. Another interesting trend was seen in CytB and COX-1 mtDNA levels where mtDNA levels were down regulated in patients who developed intervention related cerebral ischemia as opposed to patients developing DCI or without cerebral ischemia. Whether this difference has some implications from immune paralysis may require further thorough investigations as immune depression is observed after aSAH. It is also known that mtDNA leads to immunosuppression via TLR-9 dependent mechanisms in cytotoxic T cells and deletion of cross presenting dendritic cells (Sarrafzadeh et al., 2011; Schafer et al., 2016). The aSAH patients experiencing convulsive seizures tended to have lower levels of both mt D-Loop and mt COX-1 levels, whereas in chronic hydrocephalus only mt D-Loop levels were downregulated. Another interesting difference, although significant at day 9, was seen in mt D-Loop levels where patients with poor clinical outcome (mRS 3-6) tended to have lower mt D-Loop levels compared to those with good outcome (mRS 0-2). However, this was in contrast to the findings of Wang et al. (2013) who found significant elevation of mtDNA at day 8 in poor outcome patients, but this difference

does not exist anymore after controlling for the aneurysm treatment effects (Wang et al., 2013). Furthermore, the study of Wang et al., (2013) was comprised of a small population of patients (n = 21) with more patients in the poor outcome group (n = 15), which might be the reason for divergence from their findings.

The increasing levels of mtDNA in peripheral circulation may likely involve mtDNA release from necrotic cells or probably the impairment of the DNA clearance mechanisms owing to systemic inflammation mediated organ damage in critically ill patients (Tsai et al., 2011). Inflammation can lead to increased leucocyte counts (Neil-Dwyer and Cruickshank, 1974) and interestingly mtDNA levels measured at different days were correlated with leucocytes counts (Table 2). However, cumulative levels of CytB and D-Loop mtDNA were positively correlated with leucocyte count (Supplementary Table 1), which is in agreement with previous findings (Tsai et al., 2011). This suggests that mtDNA may probably lead to leukocytosis owing to its DAMP nature. A negative correlation was found at different days between mtDNA and CRP levels. Systemic DNA levels has been reported to correlate with cerebral hematoma; however, we found a weak, but significant correlation among Fischer grade, cumulative mtDNA levels of CytB and COX-1 (Supplementary Table 2) (Rainer et al., 2003). It has already been mentioned that mtDNA levels are upregulated after CNS insult and TLR-9 is the major receptor mediating inflammatory effects of mtDNA (Boyapati et al., 2017). Therefore, TLR-9 represents an important target and different strategies based on oligodeoxynucleotides (ODN) aimed at antagonizing the inflammatory effects of TLR-9 activation are under development through preclinical or early clinical studies (Hennessy et al., 2010; Hoque et al., 2013; Savva and Roger, 2013). Another approach based on molecular scavenging of the free nucleic acids by nuclear acid binding polymers has been shown to limit the inflammation in preclinical studies (Holl et al., 2016; Holl et al., 2013). Therefore, further dissection of the inflammation associated with mtDNA and TLR-9 axis in animal models of SAH is warranted to unveil the biomarker and therapeutic potential of this axis.

Our study with human population has interesting findings, but with some limitations. First of all, our patient population is very heterogeneous with wide age range, inclusion of both sexes and diverse grade of severity of subarachnoid hemorrhage with Hunt and Hess grade I-V. All these factors may lead to increase the variation as reflected by our data showing that male patients had higher serum mtDNA D-Loop and Cyt B levels. Hence, due to the heterogeneity of aSAH population the data should be interpreted carefully for any implications.

Moreover, the assessment of clinical outcome with common test batteries including GOS and mRS are not sensitive and roughly reflects the neurological status and hence, the discrete changes in neurological status may be overlooked.

Although with certain limitations, our data clearly demonstrates the elevated mitochondrial damage associated molecular patterns that might be implicated in an upregulated systemic inflammatory response after subarachnoid hemorrhage.

5. Conclusion

Systemic mtDNA levels were elevated after aSAH and display differential correlations with different aSAH associated characters, complications and clinical outcome.

6. Acknowledgements

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7. Declaration of interest

The authors declare no conflicts of interest.

Table 1: Characteristics of aSAH patients

aSAH (n)	80
Age (years) (mean±SD)	56.97 (±12.00)
Females (%)	62.5%
Treatment modality	
Neurosurgical clipping (%)	48.8%
Endovascular coiling (%)	51.3%
Intraventricular hemorrhage: IVH (%)	12.5%
Intracerebral bleeding: ICB (%)	20.0%
ICB and IVH (%)	13.8%
Hunt and Hess grade (median)	3
1 (%)	6.3%
2 (%)	30.0%
3 (%)	28.8%
4 (%)	16.3%
5 (%)	18.8%
Fischer grade (median)	3
1 (%)	1.3%
2 (%)	2.5%
3 (%)	85.0%
4 (%)	12.5%
Cerebral Vasospasm; CVS (%)	55.0%
Cerebral Ischemia (%)	41.3%

Intervention related CI (%)	21.3%
DCI (%)	20.0%
Seizures (%)	30.0%
VP-Shunt dependent hydrocephalus (%)	31.3%
Infections (%)	36.3%
Pneumonia (%)	18.8%
Meningitis (%)	8.8%
Others (%)	8.8%
Pneumonia+Meningitis (%)	2.5%
Pneumonia+UTI (%)	2.5%
Meningitis+UTI (%)	1.3%
Misc. (Osteomyelitis, sepsis) (%)	2.5%
Delayed Ischemic Neurological Deficits; DIND (%)	35.0%
Aneurysm location	
Anterior circulation (%)	86.3%
Posterior circulation (%)	13.8%
Glasgow Outcome Scale; GOS (median)	3
1 (%)	8.8%
2 (%)	12.5%
3 (%)	30.0%
4 (%)	7.5%
5 (%)	41.3%
Modified Rankin Scale; mRS (median)	3
0 (%)	2.5%
1 (%)	31.3%
2 (%)	10.0%
3 (%)	8.8%
4 (%)	21.3%
5 (%)	17.5%
6 (%)	8.8%

Table 2: Correlations of different mtDNA genes (mt CytB, mt D-Loop and mt COX-1) with different aSAH associated parameters

Log mtDNA	aSAH characters	No. of XY pairs	Spearman rho	P value	95% CI
CytB D1	Female	80	-0.254	0.023	-0.454 to 0.030
CytB D1	Leucocytes D13	61	-0.315	0.014	-0.531 to -0.061
CytB D3	Female	79	-0.221	0.051	-0.427 to 0.007
CytB D3	Leucocytes D5	76	0.323	0.005	0.098 to 0.516
CytB D3	Interventional CI	79	-0.243	0.031	-0.446 to -0.017
CytB D5	Female	77	-0.243	0.033	-0.448 to -0.014
CytB D5	CRP D13	32	-0.381	0.031	-0.650 to -0.027
CytB D7	Female	79	-0.225	0.046	-0.431 to 0.002
CytB D9	Female	78	-0.2316	0.041	-0.437 to -0.003
CytB D9	Infarcts	78	-0.239	0.035	-0.444 to -0.011
CytB D9	Interventional CI	78	-0.326	0.004	-0.517 to -0.105
CytB D9	Leucocytes D11	57	0.306	0.020	0.042 to 0.531
CytB D11	Leucocytes D11	55	0.375	0.005	0.114 to 0.588
CytB D13	Interventional CI	70	-0.284	0.017	-0.492 to -0.045
CytB D13	CRP D1	36	-0.392	0.018	-0.645 to -0.063
CytB D13	CRP D13	28	-0.426	0.024	-0.696 to -0.052
D-Loop D1	Chronic Hydrocephalus	80	-0.280	0.012	-0.476 to -0.058
D-Loop D1	CRP D1	43	-0.309	0.044	-0.564 to 0.000
D-Loop D1	Leucocytes D13	61	-0.278	0.030	-0.501 to -0.020
D-Loop D5	CRP D9	38	-0.337	0.038	-0.599 to -0.010
D-Loop D7	CRP D11	33	0.360	0.040	0.008 to 0.632
D-Loop D9	Chronic Hydrocephalus	78	-0.289	0.010	-0.486 to -0.065
D-Loop D9	GOS	78	0.288	0.011	0.063 to 0.485
D-Loop D9	mRS	78	-0.274	0.015	-0.473 to -0.048
D-Loop D9	Leucocytes D1	77	0.230	0.045	-0.001 to 0.437
D-Loop D13	CRP D1	35	-0.405	0.016	-0.656 to -0.073
COX1 D1	Leucocytes D13	61	-0.276	0.032	-0.499 to -0.018
COX1 D3	CRP D3	41	-0.328	0.036	-0.584 to -0.013
COX1 D3	Seizures	79	-0.224	0.047	-0.430 to 0.003
COX1 D3	Interventional CI	79	-0.297	0.008	-0.491 to -0.075
COX1 D5	CRP D1	41	-0.319	0.042	-0.577 to -0.003
COX1 D7	Seizures	79	-0.246	0.029	-0.448 to -0.019
COX1 D9	Infarcts	78	-0.234	0.039	-0.439 to -0.005
COX1 D9	Interventional CI	78	-0.323	0.004	-0.514 to -0.101
COX1 D11	Interventional CI	73	-0.336	0.004	-0.531 to -0.108
COX1 D11	DIND	73	0.242	0.039	0.006 to 0.453
COX1 D11	Leucocytes D11	55	0.305	0.024	0.0353 to 0.534
COX1 D13	Interventional CI	70	-0.335	0.005	-0.534 to -0.102
COX1 D13	CRP D1	36	-0.351	0.036	-0.616 to -0.016

Supplementary Table 1: Correlations of cumulative mtDNA levels of mt CytB, D-Loop and COX-1 measured over two weeks with different aSAH patient associated characters, complications and clinical outcome

Log mt DNA	Complications & Clinical Outcome	Spearman rho	P value	95% CI
D-Loop	GOS	0.113	0.010	0.024 to 0.200
D-Loop	mRS	-0.104	0.019	-0.191 to -0.015
D-Loop	Gender	-0.152	0.001	-0.238 to -0.064
D-Loop	Pneumonia	-0.1330	0.003	-0.219 to -0.044
D-Loop	Chronic hydrocephalus	-0.128	0.004	-0.215 to -0.039
D-Loop	Seizures	-0.152	0.001	-0.238 to -0.064
D-Loop	Leucocytes	0.120	0.0110	0.025 to 0.212
Cyt B	CVS	0.101	0.019	0.014 to 0.187
Cyt B	CI	-0.105	0.020	-0.190 to -0.018
Cyt B	Interventional CI	-0.179	<0.000	-0.262 to -0.094
Cyt B	Pneumonia	-0.149	0.001	-0.233 to -0.062
Cyt B	Others	0.1013	0.019	0.014 to 0.186
Cyt B	Fischer	0.113	0.009	0.026 to 0.198
Cyt B	Gender	-0.2012	<0.000	-0.283 to -0.116
Cyt B	Leucocytes	0.123	0.008	0.030 to 0.213
COX-1	CI	-0.150	0.000	-0.232 to -0.066
COX-1	Interventional CI	-0.208	<0.000	-0.288 to -0.125
COX-1	Fischer	0.100	0.018	0.015 to 0.183
COX-1	Infections	-0.117	0.005	-0.201 to -0.033
COX-1	Pneumonia	-0.107	0.011	-0.191 to -0.022
COX-1	Meningitis	-0.0834	0.049	-0.168 to 0.002
COX-1	Seizures	-0.094	0.026	-0.178 to -0.009

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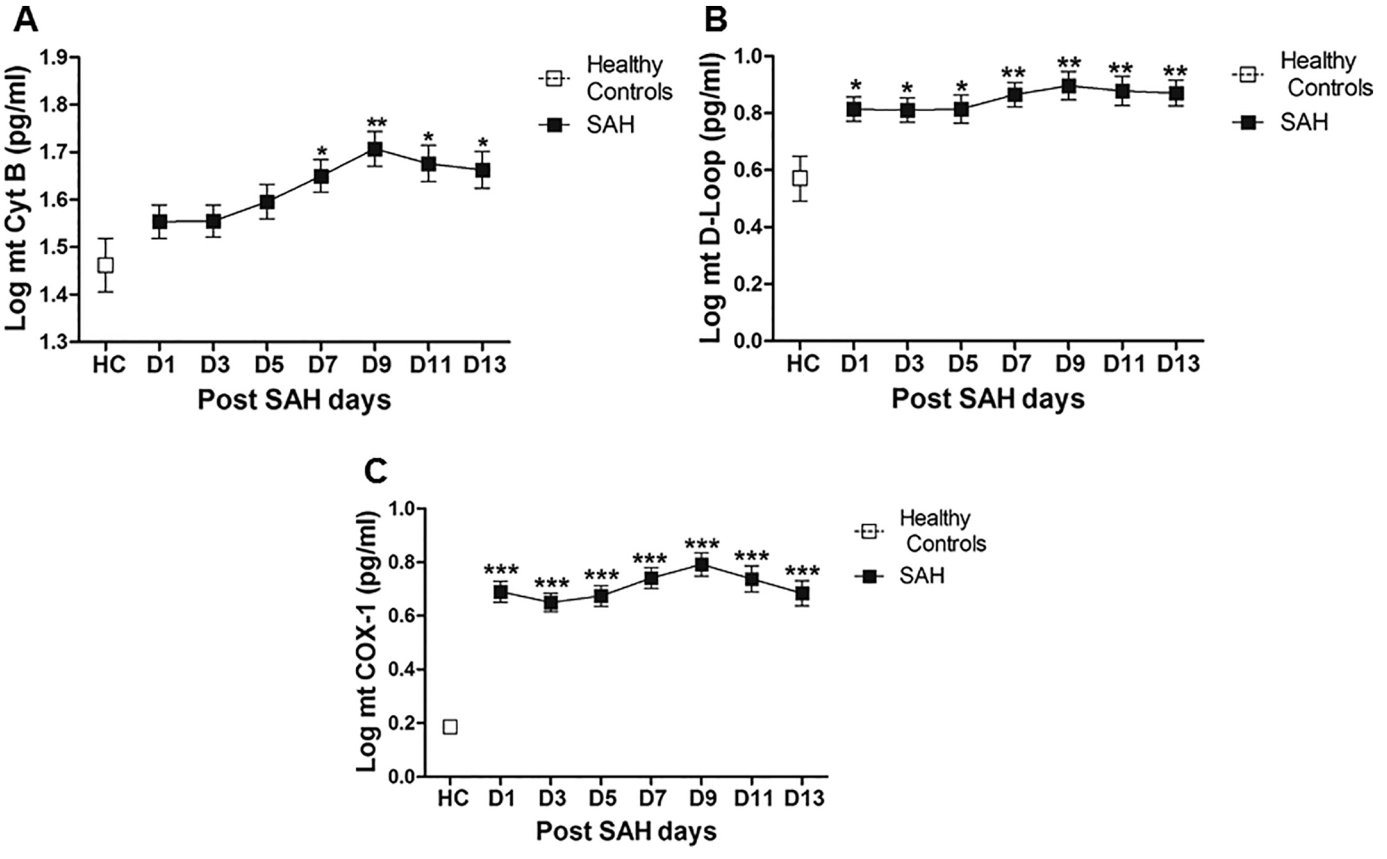
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Healthy Controls
SAH
B
Log mt D-Loop (pg/ml)
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Post SAH days
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Figure 1

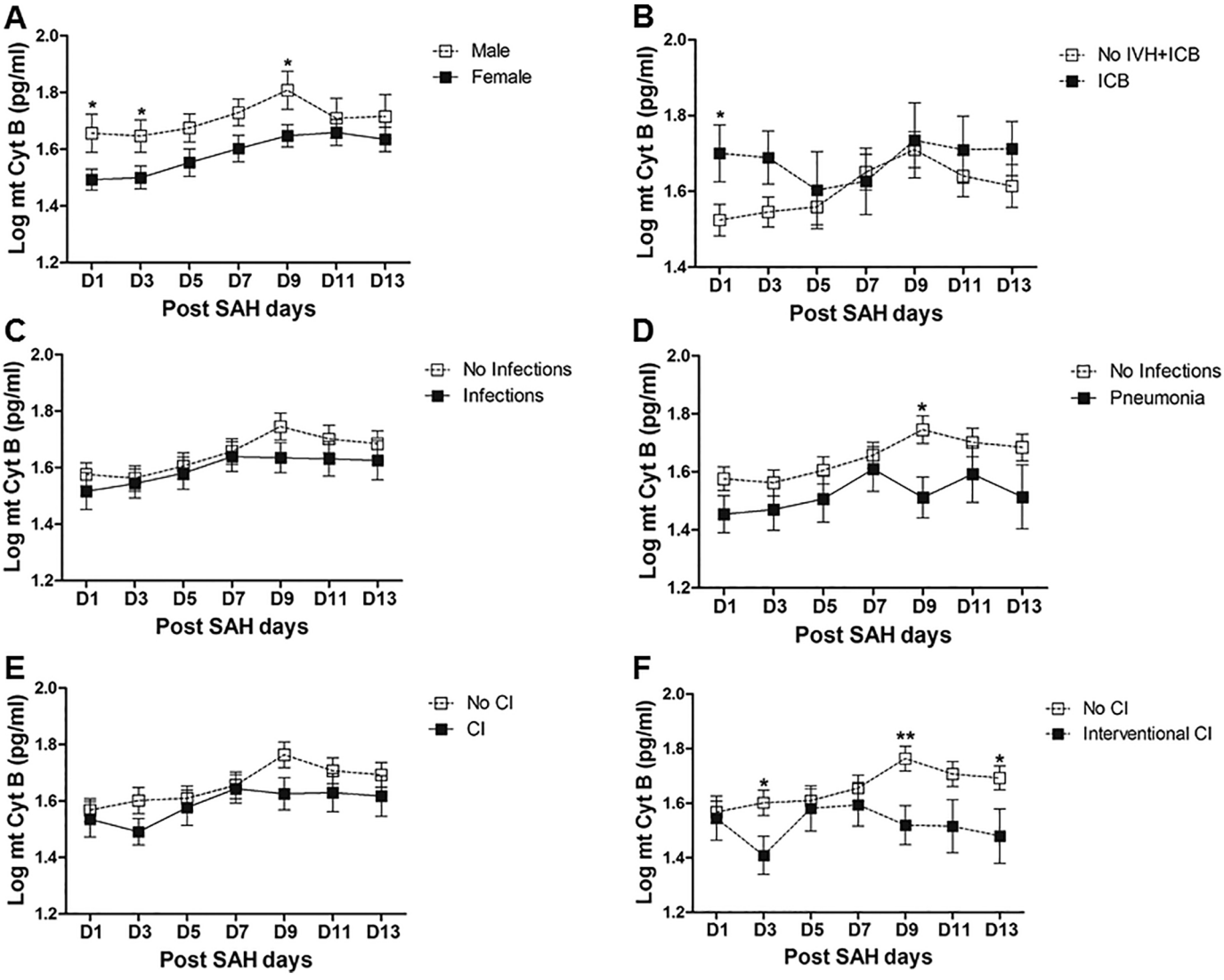
A
Log mt Cyt B (pg/ml)
2.0
1.8
1.6
1.4
1.2
D1 D3 D5 D7 D9 D11 D13
Post SAH days
Male
Female
B
No IVH+ICB
ICB
C
No Infections
Infections
D
No Infections
Pneumonia
E
No CI
CI
F
No CI
Interventional CI

Figure 2

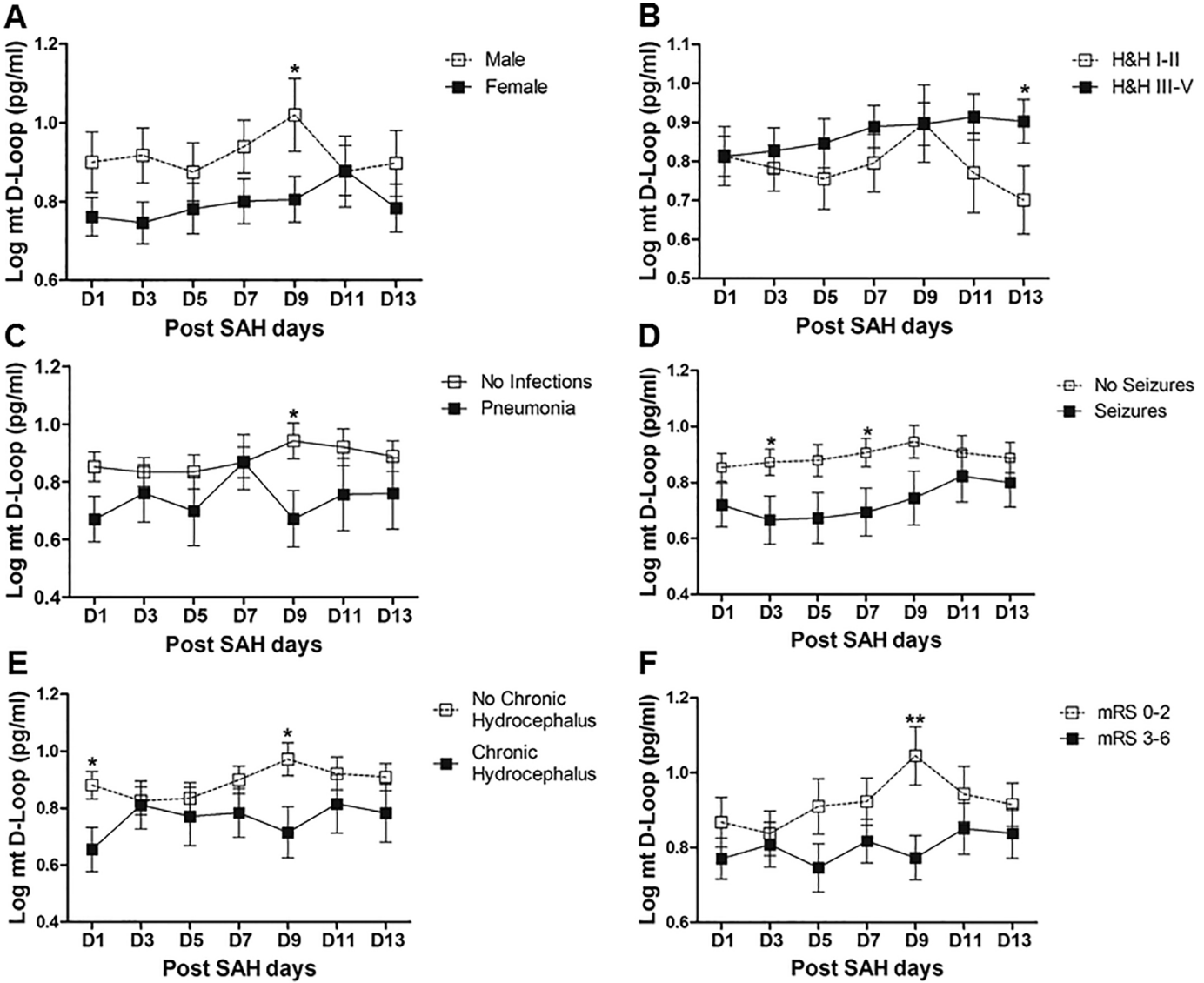
A
Log mt D-Loop (pg/ml)
1.2
1.0
0.8
0.6
*
D1 D3 D5 D7 D9 D11 D13
Post SAH days
Male
Female
B
Log mt D-Loop (pg/ml)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
D1 D3 D5 D7 D9 D11 D13
Post SAH days
H&H I-II
H&H III-V
C
Log mt D-Loop (pg/ml)
1.2
1.0
0.8
0.6
0.4
*
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No Infections
Pneumonia
D
Log mt D-Loop (pg/ml)
1.2
1.0
0.8
0.6
0.4
* *
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No Seizures
Seizures
E
Log mt D-Loop (pg/ml)
1.2
1.0
0.8
0.6
0.4
* *
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No Chronic Hydrocephalus
Chronic Hydrocephalus
F
Log mt D-Loop (pg/ml)
1.2
1.0
0.8
0.6
**
D1 D3 D5 D7 D9 D11 D13
Post SAH days
mRS 0-2
mRS 3-6

Figure 3

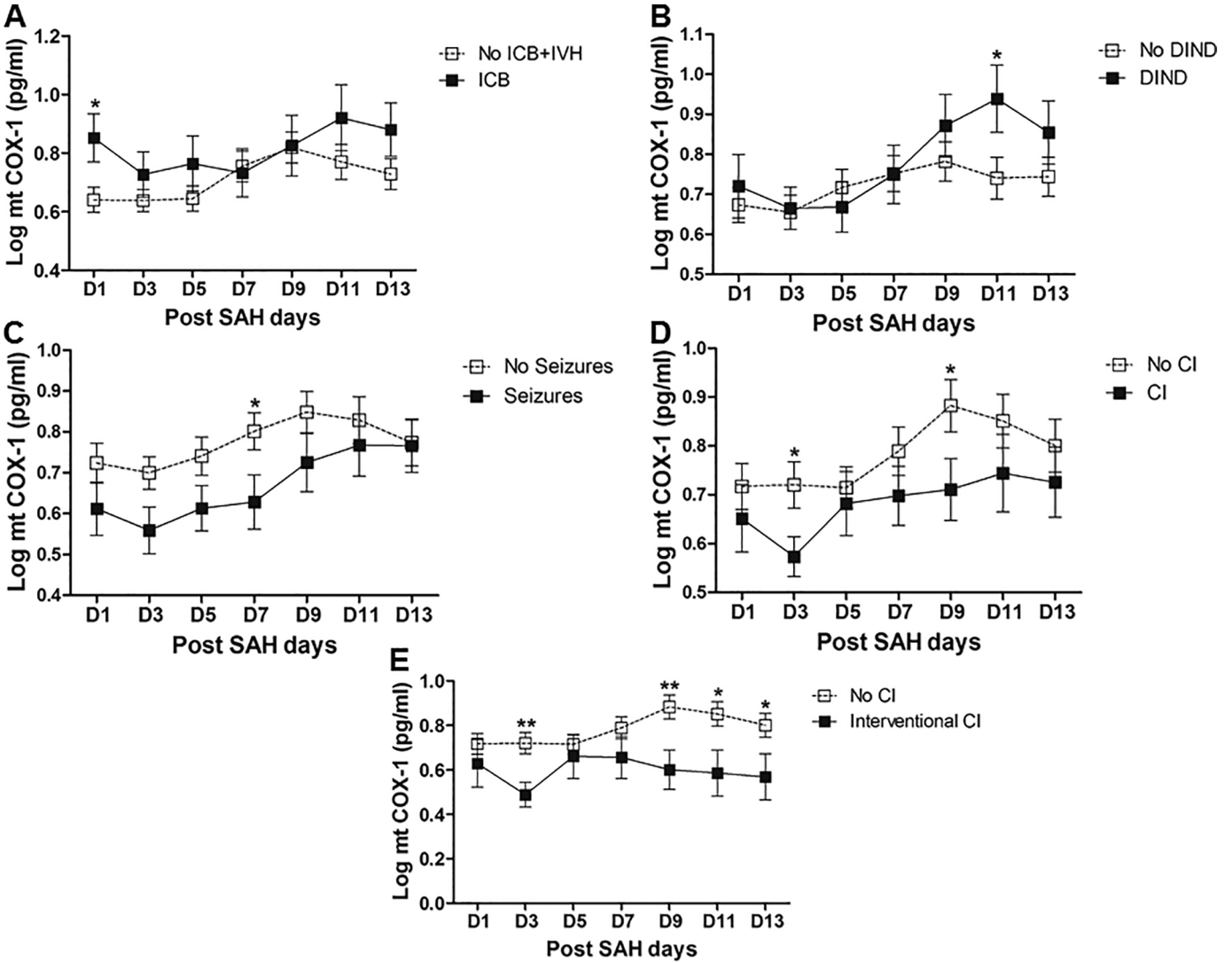
A
Log mt COX-1 (pg/ml)
1.2
1.0
0.8
0.6
0.4
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No ICB+IVH
ICB
*
B
Log mt COX-1 (pg/ml)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No DIND
DIND
*
C
Log mt COX-1 (pg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No Seizures
Seizures
*
D
Log mt COX-1 (pg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No CI
CI
*
*
E
Log mt COX-1 (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
D1 D3 D5 D7 D9 D11 D13
Post SAH days
No CI
Interventional CI
**
**
*
*

Figure 4