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Compartmentalisation of the inflammatory response following aneurysmal subarachnoid haemorrhage

Yahia Z. Al-Tamimi[a,b,*], Deepti Bhargava[b], Nicolas M. Orsi[c], Anmar Teraifi[d], Michele Cummings[c], Uma V. Ekbote[c], Audrey C. Quinn[e], Shervanthi Homer-Vanniasinkam[f], Stuart Ross[b]

[a] Department of Neurosurgery, Sheffield Teaching Hospital NHS Foundation Trust, Glossop Road, Sheffield S10 2JF, United Kingdom
[b] Department of Neurosurgery, Level G Jubilee Wing, Leeds General Infirmary, Great George Street, Leeds LS1 3EX, United Kingdom
[c] Leeds Institute of Cancer & Pathology, Wellcome Trust Brenner Building, St James's University Hospital, Beckett Street, Leeds LS9 7TF, United Kingdom
[d] University of Liverpool School of Medicine, Cedar House, Ashton Street, Liverpool L69 3GE, United Kingdom
[e] Department of Anaesthesia, Level C Brotherton Wing, Leeds General Infirmary, Great George Street, Leeds LS1 3EX, United Kingdom
[f] Department of Vascular Surgery, Level B Brotherton Wing, Leeds General Infirmary, Great George Street, Leeds LS1 3EX, United Kingdom

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ABSTRACT

Introduction: There is some evidence to suggest that a systemic and central nervous system (CNS) inflammatory response occurs following aneurysmal subarachnoid haemorrhage (aSAH) which may be related to the pathophysiology of early brain injury and delayed ischaemic neurological deficit (DIND). The aim of this study was to measure inflammatory mediator levels in plasma and cerebrospinal fluid (CSF) in the days following aSAH and to determine their association with aSAH, DIND and clinical outcome.

Material and methods: Plasma and CSF samples were obtained prospectively from patients with aSAH on days 1–3, 5, 7 and 9 and profiled for interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-15, IL-17, IL-18, macrophage chemotactic protein (MCP)-1, vascular endothelial growth factor (VEGF) and tumour necrosis factor (TNF)-α. Plasma and CSF samples from non-aSAH patients undergoing spinal anaesthesia were used as controls.

Results: The CSF levels of all cytokines investigated except for IL-1α were significantly higher in aSAH compared to controls in the first seven days of ictus. CSF levels of IL-1α ($p = 0.014$), IL-18 ($p = 0.016$), IL-6 ($p = 0.0006$) and IL-8 ($p = 0.006$) showed significant increases in the days following aSAH. Conversely IL-17 demonstrated a decrease. In particular, IL-4 was higher in the CSF of patients who had DIND at all time-points ($p = 0.032$). Plasma IL-6 and IL-8 levels were higher, and IL-1α levels lower, than controls at most time-points. All mediators demonstrated persistent elevation in the CSF compared to plasma apart from IL-1α and IL-18 which followed the opposite trend. Day 3 plasma IL-6 levels predicted poor outcome at six months (Exp(B) 1.12 1.03-1.22, $P = 0.012$), although this association was lost in the second analysis incorporating Fisher grade, WFNS grade and age.

Conclusion: The post aSAH inflammatory response peaks on days 5–7 post ictus and remains largely compartmentalised within the CNS. IL-4 may have a particular association with DIND although its precise role in the pathophysiology of the disorder remains unclear. IL-6 predicted poor outcome but not independently of clinical grade, suggesting that it may be a surrogate marker of early brain injury.

1. Introduction

Recent studies have demonstrated that neuroinflammation plays a key role in early brain injury following aneurysmal subarachnoid haemorrhage (aSAH) and may be associated with delayed ischaemic neurological deficit (DIND) [1,2]. Inflammatory mediators may contribute to brain oedema, blood brain barrier (BBB) breakdown and neuronal apoptosis in addition to initiating a systemic inflammatory response due to the disruption of the BBB [3–18]. The initial haemorrhage can disrupt cerebral perfusion, increase intracranial pressure and activate Toll-like receptors and nucleotide-binding oligomerization domain-like receptors [2]. The activated inflammasomes activate procaspase-1, which in turn proteolytically cleaves the pro-cytokines pro IL-1β and pro IL-18 into their active forms. Cytokines are well recognised as low molecular weight pleiotropic mediators which both promote and control inflammatory processes [19]. The most widely investigated in the

* Corresponding author at: Department of Neurosurgery, Sheffield Teaching Hospital NHS Foundation Trust, Sheffield, United Kingdom.
E-mail address: y.al-tamimi@sheffield.ac.uk (Y.Z. Al-Tamimi).

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present setting include interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18 and tumour necrosis factor (TNF)-α.

The largely pro-inflammatory IL-1 family affects the expression of numerous genes encoding growth factors, adhesion molecules, matrix metalloproteinases (MMPs) and cytokines (e.g. IL-6) which may contribute to neurotoxicity and, paradoxically, also promote neuronal survival [20,21]. IL-1 has numerous effects on the endothelium that have implications for BBB integrity that could contribute to elevated intracranial pressure (ICP) and result in early brain injury. The overall effect depends on both the temporal and relative expression profile of these molecules. BBB function is tightly regulated by endothelial cells, with tight junctions at their lateral circumference and abluminal adherin junctions restricting molecular movement [22]. Inflammation-related compromise of this barrier is thought to take place in aSAH, with cytokines potentially originating from endothelial cells at sites of BBB disruption, brain parenchyma injury or as a result of blood within the CSF. These cytokines activate endothelial cells leading to blood-borne immune effector cell adhesion extracellular matrix and loss of endothelial tight junctions and adherin junctions [2,22]. Shortly after the onset of SAH, brain exposed to blood components activate the innate immune system leading to phagocyte and innate lymphocyte activation [23]. These adhere to brain vessel endothelium by binding with adhesion factors, produce cytokines and subsequently activate naïve T and B lymphocytes [24].

IL-6 has been implicated in endothelial cell dysfunction following experimental SAH [22]. Although their concentrations have been found to be elevated in cerebrospinal fluid (CSF) following aSAH, there is some evidence to suggest that increases in CSF IL-6 may be specifically associated with radiological vasospasm and DIND [10,11,16,25]. In addition, early CSF IL-1β, IL-18 and TNF-α levels are higher in aSAH exhibiting cerebral oedema and hydrocephalus [2]. Other cytokines have been associated with ischaemic stroke and traumatic brain injury (TBI), but remain to be investigated in the context of aSAH. These include IL-15, IL-17 and macrophage chemotactic protein (MCP)-1 which have been investigated in animal models of ischaemia/reperfusion and clinical ischaemic stroke studies. Anti-inflammatory cytokines such as IL-4 and IL-10 have also been associated with ischaemia but their involvement in aSAH is less clear [26,27].

Given that there is a paucity of information regarding the mechanisms and mediators involved in the putative inflammatory process following aSAH, this study aimed to measure cytokine profiles serially in both plasma and CSF of patients with aSAH in order to determine their potential associations with aSAH severity, their contribution to the development of DIND and overall clinical outcome.

2. Materials and methods

2.1. Patients

Informed consent was obtained from patients recruited to this exploratory study, which was approved by the regional ethics committee. Once aneurysmal pathology was confirmed radiologically, blood samples were collected from 43 patients with aSAH at set time-points following the presumed time of ictus (time = 0 h) (of whom 29 had a lumbar drain inserted and so CSF samples were also collected). The majority of patients were recruited to the LUMAS trial and so inclusion criteria was restricted to WFNS grade 1–3 and Fisher grade 2–4 patients only. Due to the variability associated with patients' presentation in relation to time of ictus, the first sample was taken at a time point defined as being < 96 h of ictus and < 12 h following intervention (endovascular or surgical). Fixed time points for sampling were on days 5 (n = 42 plasma, n = 25 CSF), 7 (n = 38 plasma, n = 18 CSF) and 9 (n = 13 plasma, n = 5 CSF) following ictus, as well as at the time of onset of DIND. Samples from patients with aSAH that developed clinical and biochemical signs of sepsis/infection were excluded from the analysis in order to remove the confounding effects of systemic inflammation. The first blood and CSF samples were collected at 9:00 am +/− 1 h in order to account for any potential circadian variations in cytokine production. Age-matched patients undergoing elective surgery requiring spinal anaesthesia for hip and knee replacements (excluding conditions featuring overt changes in systemic inflammatory profile such as rheumatoid arthritis) were used as controls (n = 11).

2.2. Sample collection, processing and analysis

Venous blood samples were collected from the antecubital fossa in EDTA tubes. CSF samples were collected aseptically from lumbar drains already *in situ*. All samples were immediately placed on ice and centrifuged at 3,000 rpm for 15 min (at 4 °C) to isolate plasma/remove CSF cell debris. CSF and plasma supernatants were stored at −80 °C until analysis.

Cytokine analysis was performed by multiplex fluid-phase immunoassay using commercial kits (Bio-Rad, Hemel Hempstead, UK) on a Luminex 100 cytometer (Luminex Corporation, Austin, Texas, USA) equipped with Bio-Plex Manager software (Bio-Rad), as previously described [28]. All samples were analysed in duplicate for IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-15, IL-17, IL-18, MCP-1, vascular endothelial growth factor (VEGF) and TNF-α. This choice of analytes was based on: (i) those previously investigated in the context of aSAH and shown to be associated with post haemorrhagic ictus inflammatory processes; and (ii) those mediators that demonstrated a strong association with other ischaemic conditions not yet investigated in aSAH.

2.3. Statistical analysis and data presentation

Initially, for data exploration, plots over time for each individual were created. In comparing patient observations with those of controls, Dunnett's *post hoc* tests were applied. Given the exploratory, hypothesis-generating nature of this study, no correction was applied for multiple testing and $P < 0.05$ was considered statistically significant. Where statistically significant results were found, the effect size and a 95% confidence interval were provided in order to determine potential clinical relevance. Comparisons of plasma and CSF mediator levels as well as those across groups were made with independent t-tests. In order to explore any changes over time and/or differences between DIND and non-DIND patients, ANOVA models were fitted for all cytokines. This included factors for both time point and DIND/non-DIND patients. A paired *t*-test was performed to compare values on the day of DIND with those on the previous day.

Binary logistic regression analysis was performed with a poor Modified Rankin Score (MRS 3–6) at day 10 and at 6 months post ictus as the outcome variable. All mediators were included in the model. Any mediators identified as significant predictors were placed into a second model as a second step following age, World Federation of Neurological Surgeons (WFNS) and Fisher grade placement. Box plots (with cytokine concentrations expressed as pg/ml) were utilised to present all datasets, with ordinate scale adjustments for clearer plot visualisation.

3. Results

Table 1 illustrates the clinical and demographic characteristics of aSAH patients and age-matched controls. The study consisted largely of WFNS grade 1–3 and Fisher grade 2–3 aSAH patients, where the majority underwent endovascular treatment for a ruptured cerebral aneurysm.

3.1. Comparison of plasma and CSF markers

When comparing plasma and CSF levels of each mediator at all time-points and across both groups, CSF concentrations were significantly higher in aSAH patients compared to controls for all mediators (except for IL-1α) at all time points, except day 9 where sample

Table 1
Characteristics of both groups of patients (aSAH patients and controls).

		Patients with aSAH	Control patients
Total number of patients (with CSF samples)		43 (29)	11 (10)
Median age/years (interquartile range)		58 (48–63)	65 (52–68)
Male : Female		11:32	4:7
WFNS grade	**1**	32	N/A
	2	10	N/A
	3	1	N/A
Fisher grade	**2**	12	N/A
	3	23	N/A
	4	3	N/A
	3 + 4	5	N/A
Aneurysm treatment	**Coiling**	27	N/A
	Clipping	11	N/A
	Both	5	N/A
DIND	**No**	31	N/A
	Yes	12	N/A
MRS at 10 days	**0–2**	18	N/A
	3–6	25	N/A
MRS at 6 months	**0–2**	31	N/A
	3–6	12	N/A

sizes were small and confidence intervals were much larger (Fig. 1). Plasma IL-1α levels in the aSAH group were significantly lower than in controls on days 3, 5 and 7 (Fig. 2). Plasma IL-6 and IL-8 levels were significantly higher on days 5 and 7. Cytokine concentrations were consistently higher in CSF apart from IL to 1α and IL-18 (which were higher in plasma) (Fig. 3), a significant finding for most mediators at most time points, notably for IL-6, IL-8, IL-15 and MCP-1 ($P < 0.0001$). Multivariate models to look for patterns between the plasma and CSF markers were fitted, but no patterns were observed.

3.2. Time analysis and DIND patients

Twelve patients experienced DIND with five providing CSF (all provided plasma). Levels in patients who developed DIND at some point in their time-course were compared with patients who never developed DIND (Fig. 4). ANOVA models were fitted for each cytokine to include factors for time-points and for DIND/non-DIND No statistically significant results were seen in plasma cytokine levels. Within CSF, statistically significant results were seen in IL-1α ($p = 0.014$), IL-18 ($p = 0.016$), IL-6 ($p = 0.0006$) and IL-17 ($p = 0.001$) for the effect of time (Fig. 1). When comparing DIND and non-DIND patients, only IL-4 was statistically significantly higher in the DIND group (Fig. 4). For those models which showed a statistically significant result, these were further examined to compare the different time points. For IL-1α, IL-18, IL-6 and IL-8, day 3 samples were significantly lower than on days 5 and 7. Only IL-18 showed this difference between days 3 and 9. For IL-17, day 7 values were significantly lower than on day 5 (Fig. 1). No successful models were found in a further single model analysis of all cytokines using DIND vs. no DIND as the outcome.

3.3. MRS analysis

Fig. 5 demonstrates CSF mediator levels according to six-month outcome. Day 3 plasma IL-6 levels predicted poor outcome at six months (Exp(B) 1.12 1.03–1.22, $P = 0.012$), although this association was lost in the second analysis incorporating Fisher grade, WFNS grade and age. No other significant findings were noted across all mediators, time-points and compartments.

4. Discussion

The present study demonstrated that CSF levels of all cytokines investigated except for IL-1α were higher in aSAH compared to controls in the first seven days of ictus. CSF levels of IL-1α ($p = 0.014$), IL-18 ($p = 0.016$), IL-6 ($p = 0.0006$) and IL-8 ($p = 0.006$) showed significant increases in the days following aSAH. Conversely IL-17 demonstrated a non-significant decrease. The novel finding of this study is that IL-4 levels were significantly elevated in the CSF of patients who developed DIND at all time-points ($p = 0.032$). Plasma IL-6 and IL-8 levels were higher, and IL-1α levels lower, than controls at most time-points. All mediators demonstrated persistent elevation in CSF compared to plasma, apart from IL-1α and IL-18 which followed the opposite trend.

The early elevation of CSF proinflammatory cytokines such as IL-1β, IL-6, IL-8, IL-18 and TNF-α noted in the current study and their subsequent rise in the days following aSAH is in keeping with published studies [2,3,6,8,9]. However, the IL-1β peak on day 5 post ictus followed by a steady decline to lower levels by day 10 that we report is not consistently demonstrated in all studies [3,9]. Trends for higher CSF IL-1β levels were noted in patients with DIND at all time points, a difference which was particularly pronounced on day 3. This is in agreement with other studies reporting that CSF IL-1β levels measured on admission in patients with aSAH were three-fold higher in those who subsequently developed DIND while later samples taken between days 8 and 15 also had higher IL-1β profiles in patients with DIND [6,11]. By contrast, there is no evidence in the current study to suggest that CSF and plasma IL-1α is associated with DIND, a finding consistent with other reports [5].

TNF-α concentration has been shown to be 5–30 fold higher in the CSF of aSAH patients when compared to controls (with levels peaking around day 7), which suggests intrathecal production [3,6,8]. CSF TNF-α levels have been shown to be higher in patients with increased flow velocities on transcranial doppler (TCD) (a surrogate measure of impending DIND), peaking at day 7 and subsiding at a slower rate in these patients [3]. Moreover, CSF TNF-α concentrations measured at the time of hospital admission have been recorded to be twice as elevated in patients subsequently developing DIND [6]. These findings are reflected by trends towards higher CSF TNF-α levels in DIND patients at all time points and a peak at day 5, which is sustained until day 9 in those with DIND in the present study. TNF-α appears to have different effects based on both its absolute concentration and the relative contribution of other synergistic/antagonistic cytokines. Although data from TNF-α knockout mice support a neurotrophic role which confers neuroprotection by inducing nerve growth factor (NGF) and IL-10, it has been largely associated with ICAM-1 activation, BBB dysfunction and intrathecal infiltration of activated leukocytes following TBI [29]. In the later stages following aSAH (days 4–7), this may be a significant contributor to secondary brain injury, further elevations in ICP and impaired cerebral perfusion. Reports suggest that CSF TNF-α increases relate to outcome and peak at day 9 in those with an unfavourable outcome, although this wasn't seen in the current study [8].

As noted herein, CSF IL-6 and IL-8 levels have been shown to increase and peak around day 5 post ictus with a subsequent decline to lower, albeit pathological levels by day 14 [7,9,10]. These patients exhibited early CSF elevations followed by a more systemic inflammatory response after day 6 (based on elevated C-reactive protein levels and further increases in plasma, CSF and ECF IL-6 levels). Elevations have been shown to range from 1.5 to 10,000-fold higher compared to non-aSAH controls across a range of studies [3–6,9,10,12,25]. Whilst such variation in the reported level rise maybe related to methodological issues such as differences in analytical platforms, such marked increases are in agreement with our current findings of 680-fold and 50-fold higher CSF IL-6 and IL-8 levels, respectively, in aSAH patients. The results from this present study suggest that whilst IL-6 production is stimulated following aSAH (which may be a consequence of elevated ICP), this response is likely to resolve naturally within 6–21 days.

A number of associations between both IL-6 and IL-8 levels and DIND/poor outcome following aSAH have previously been described in several compartments (CSF, brain ECF and plasma) [3,4,6,9–11,15,16]. In the current study, IL-6 predicted poor outcome but not

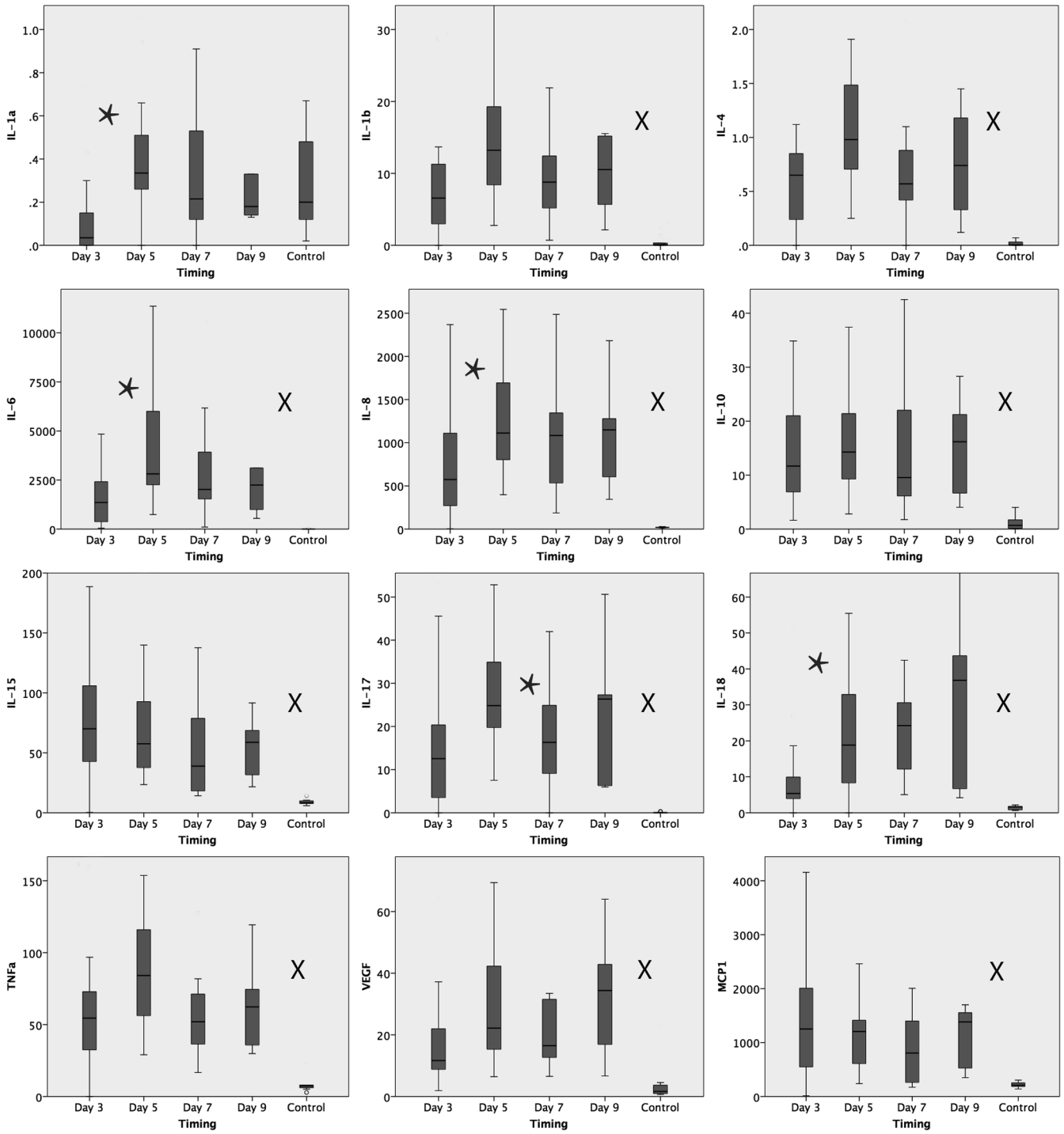

Fig. 1. Box plots demonstrating median CSF mediator concentrations. Interquartile range is highlighted. Units are pg/ml. Values are given at days 3, 5, 7 and 9 post-haemorrhage. * denotes $p < 0.05$ regarding the effect of time in the ANOVA analysis. X denotes significant ($p < 0.05$) difference between aSAH patient and controls at all time-points except day 9.

independently of clinical grade, suggesting that it may be a surrogate marker of early brain injury given that elevated serum IL-6 levels have been reported in aSAH patients with global cerebral oedema (a marker of early brain injury) [30]. Day 4 and 5 CSF levels were shown to be predictors of DIND, with a cut-off point of 2,000 pg/ml on day 4 conferring an 11-fold increase in the relative risk of DIND [10]. Day 7 CSF IL-6 levels were also associated with DIND. Although half of our patients with DIND and half without DIND had CSF IL-6 levels of > 2,000 pg/ml on day 3, elevations in CSF IL-6 and IL-8 levels were nonetheless noted at the time of onset of DIND. An explanation for this discrepancy may relate to differences in the aSAH cohort and DIND diagnostic criteria. In the cited study by Schoch and co-workers, there was a disproportionate weighting towards poor grade patients, in contrast to the current study which was limited to good grade aSAH patients.

A possible mechanism for this association with DIND may be indirect i.e. related to elevated ICP, reduced cerebral perfusion and anaerobic parenchymal metabolism, all of which are associated with DIND and have been linked to elevated IL-6 [25]. IL-8 participates in neutrophil activation and chemotaxis as well as adhesion molecule

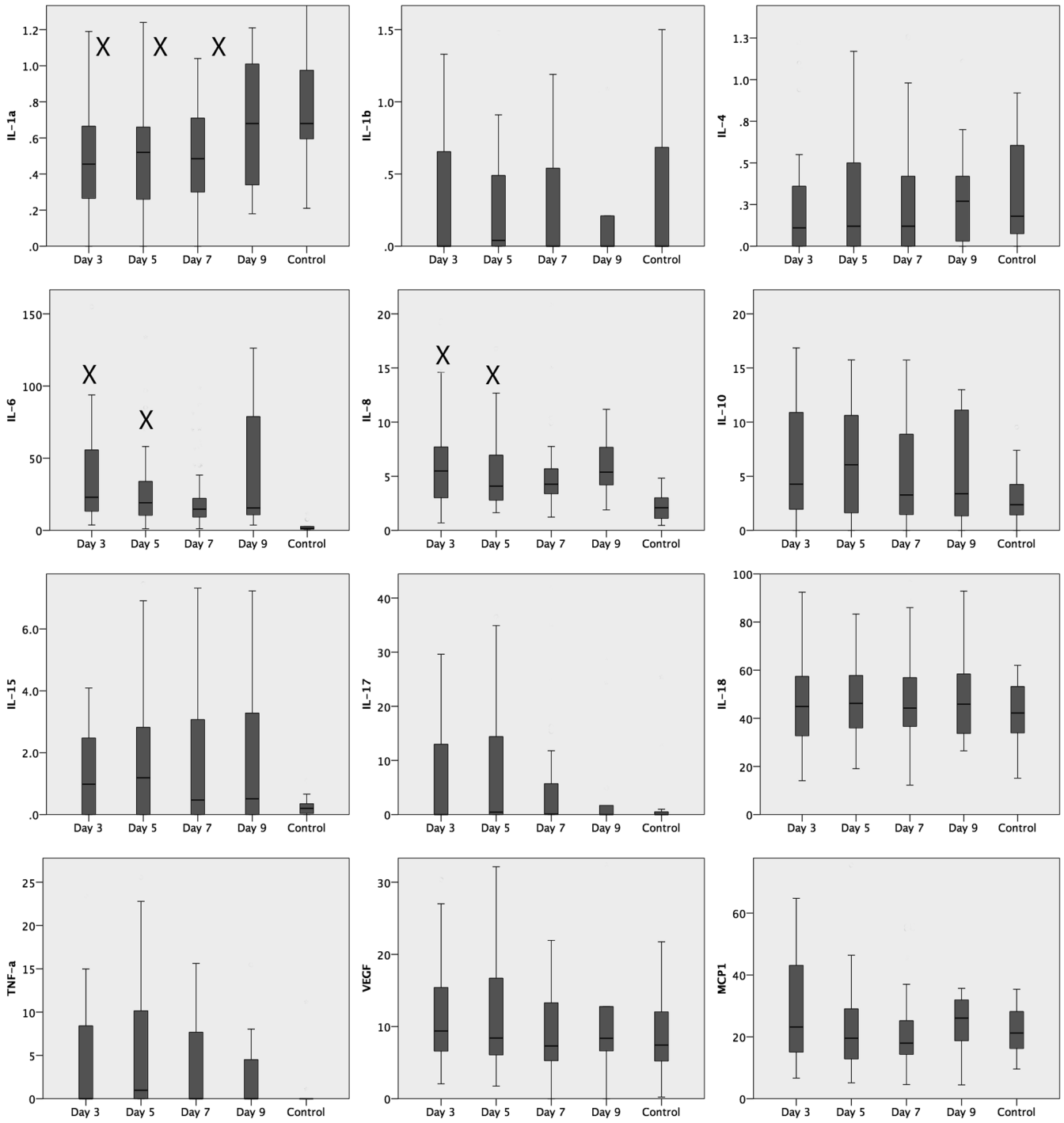

Fig. 2. Box plots demonstrating median plasma mediator concentrations on days 3, 5, 7 and 9 post haemorrhage. Interquartile range is highlighted. Units are pg/ml. X denotes significant ($p < 0.05$) difference between aSAH patients and controls at the time points specified by the symbol.

production and angiogenesis. Activated neutrophils may contribute to brain injury by causing microvascular occlusion and the production of further cytokines, reactive oxygen/nitrogen species and lipid mediators, such as bioactive eicosanoids [31], thereby perpetuating the inflammatory insult. This notion is supported by the fact that anti-neutrophil antibodies and neutrophil depletion have been shown to reduce neutrophil infiltration and infarct size in ischaemic rat brain models [32].

IL-4 is a Th_2-derived anti-inflammatory cytokine [26,27] which downregulates pro-inflammatory cytokine production in activated monocytes. How IL-4 is involved in the regulation of inflammatory responses in acute cerebrovascular disease is unknown, although a negative feedback role to attenuate pro-inflammatory cytokines in ischaemic stroke has been demonstrated [27]. Its role in aSAH may be analogous. Results from the current study point to a strong CSF IL-4 response which peaks on day 5. This response was greater in those with DIND at all time points, suggesting that patients with DIND and a putatively larger inflammatory response mount an even greater compensatory anti-inflammatory response. Similar findings have been noted for IL-4 levels measured in the internal jugular vein [33]. M2a microglia/macrophage alternative activation by IL-4 supports growth and repair and CNS injury [34]. Impaired functional recovery following spinal

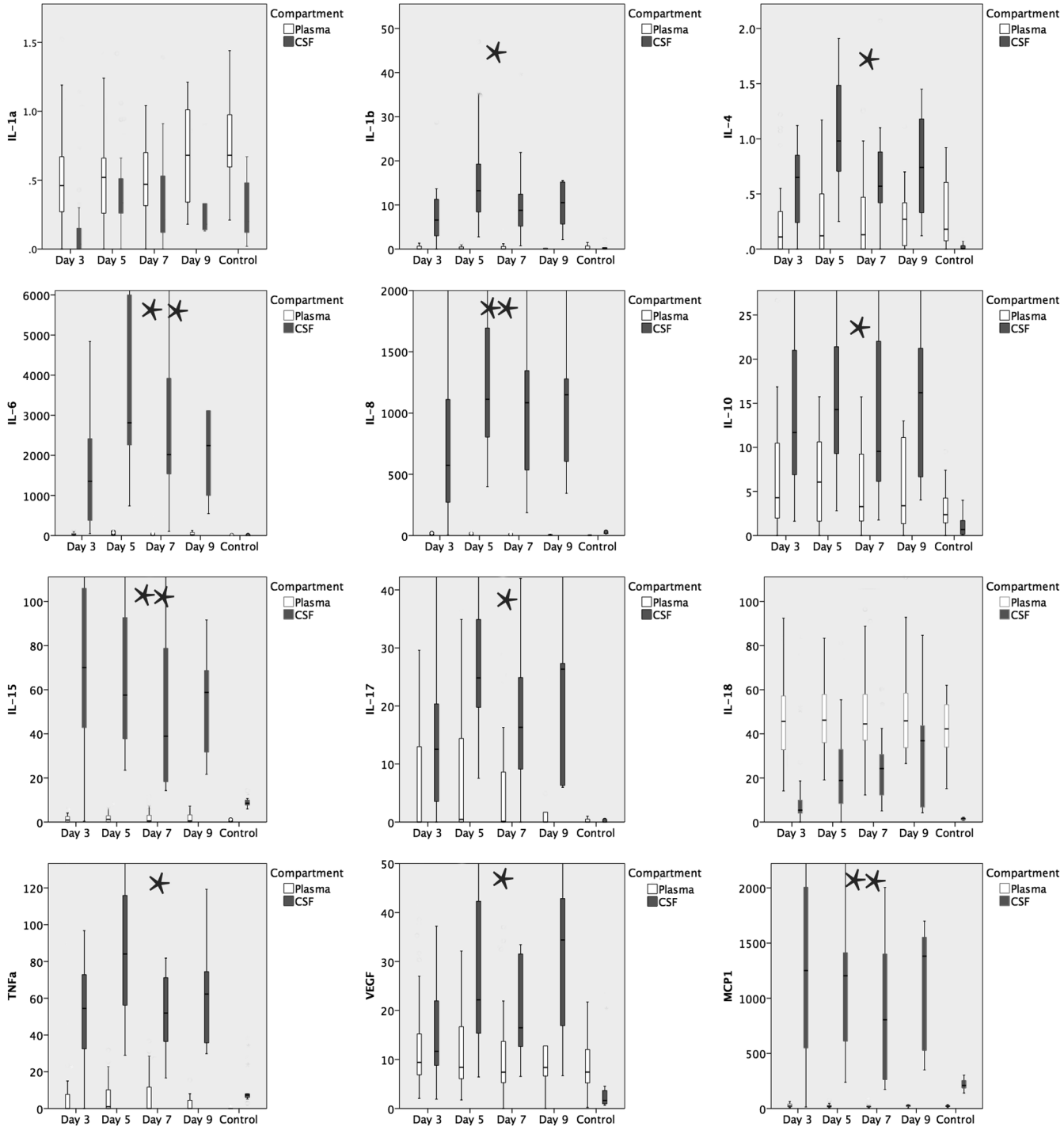

Fig. 3. Box plots demonstrating median plasma and CSF mediator concentrations. Interquartile range is highlighted. Units are pg/ml. Values are given at days 3, 5, 7 and 9 post-haemorrhage. * indicated significance ($p < 0.05$) at all time-points. ** indicates significance ($p < 0.001$) at all time-points.

cord injury in aged mice was associated with impaired induction of IL-4 receptor on microglia. This in turn was associated with attenuated arginase, IL-1β and chemokine ligand 2 (CCL2) expression and reduced recruitment of IL-4Rα^{+} macrophages to the injured cord. However, in the current study, the IL-4 response may be a larger and earlier inflammatory response to being a compensatory anti-inflammatory response and so needs further evaluation in the context of DIND.

MCP-1 is a member of the cysteine-cysteine chemokine gene family which chemoattractant for monocytes, memory T-cells and natural killer cells *in vitro* [35]. MCP-1 is expressed in astrocytes in experimental models of ischaemia, with MCP-1 knockout mice sustaining smaller infarct volumes and reduced monocyte/neutrophil infiltration compared to their wild-type counterparts [36–38]. An early reduction in IL-1β is noted within ischaemic tissue [36]. In clinical studies, MCP-1 concentrations are elevated in both plasma and particularly CSF following aSAH [4,12]. In the current study, a comparable response was only noted in CSF. CSF MCP-1 levels rose and peaked on day 3 following early rises in CSF IL-6 in aSAH [39]. Levels of MCP-1 have previously been shown to reflect patient grade and the weaker plasma response is thought to represent a weaker systemic inflammatory response in good grade patients, in keeping with the similar plasma mediator levels recorded across aSAH patients in the present study.

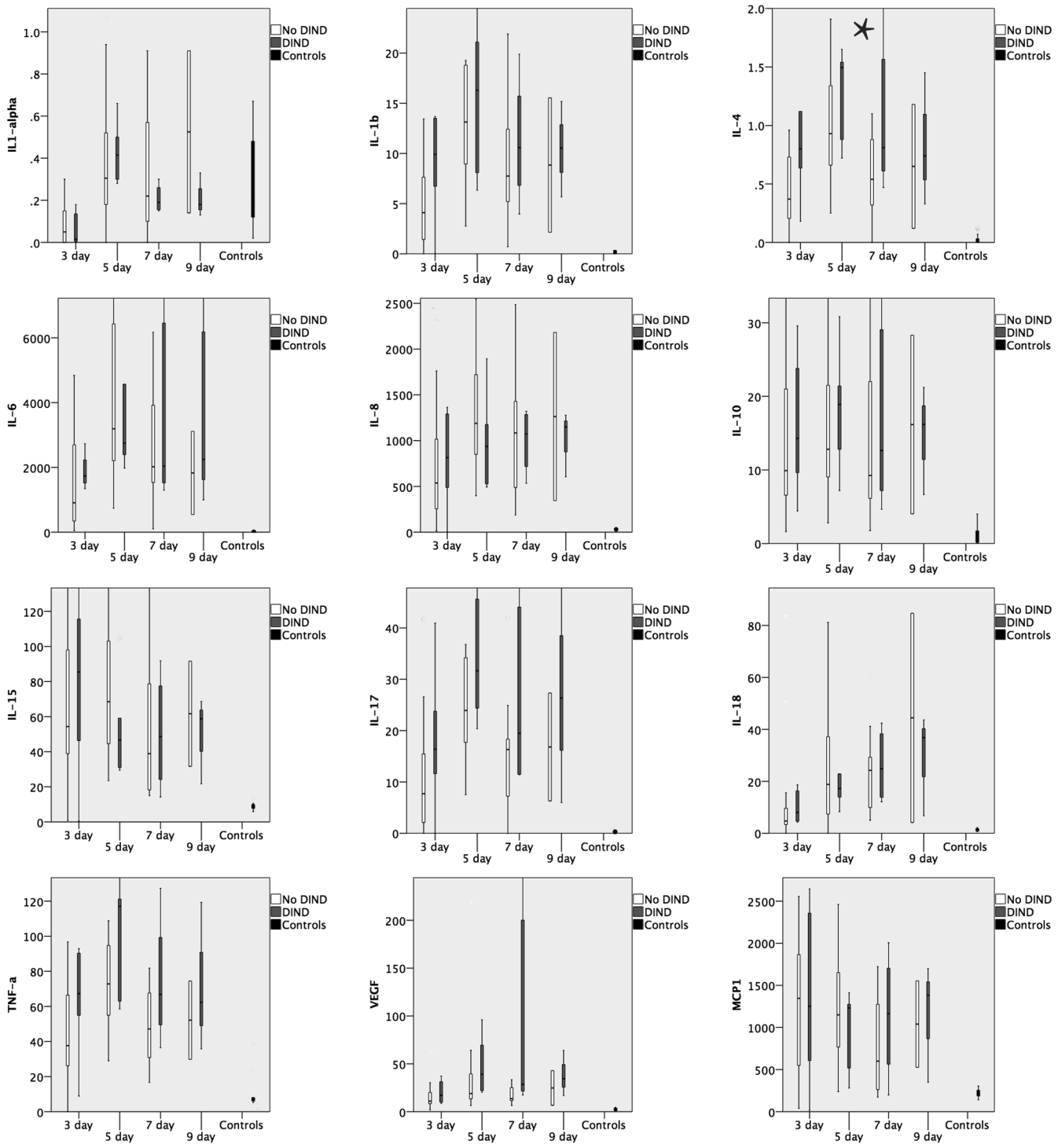

Fig. 4. Box plots demonstrating median CSF mediator concentrations for patients with and without DIND. Interquartile range is highlighted. * $P < 0.05$ regarding the effect of DIND in the ANOVA analysis.

Following aSAH, evidence suggests that pro-inflammatory mediators are produced by microglia, astrocytes and neurones [2,18]. This notion of intra-thecal, compartmentalised production is supported by the abundance of these mediators in the CSF following aSAH compared to plasma [3,9,13]. In agreement with our current findings, IL-1β, IL-6, IL-8 and TNF-α have been shown to be lower in plasma following aSAH compared to simultaneously sampled CSF [3,7,9,13]. Arterial to jugular venous concentration gradients have nevertheless been noted for various mediators, particularly IL-6, raising the question of whether brain-derived mediators can enter the systemic circulation due to a dysfunctional BBB and propagate to a post-aSAH systemic inflammatory response syndrome (SIRS; see below) [18]. The *caveat* underlying these interpretations lies with the iatrogenic haemodilutional influence of aSAH management subsequent to ictus which makes interpretation of absolute circulatory levels difficult.

The findings of the current study suggest that in response to the presence of blood within the subarachnoid and *peri*-adventitial space, an inflammatory response is triggered with early induction of pro-inflammatory mediators including IL-1β, TNF-α and MCP-1 as well as modulatory anti-inflammatory mediators which attract immune

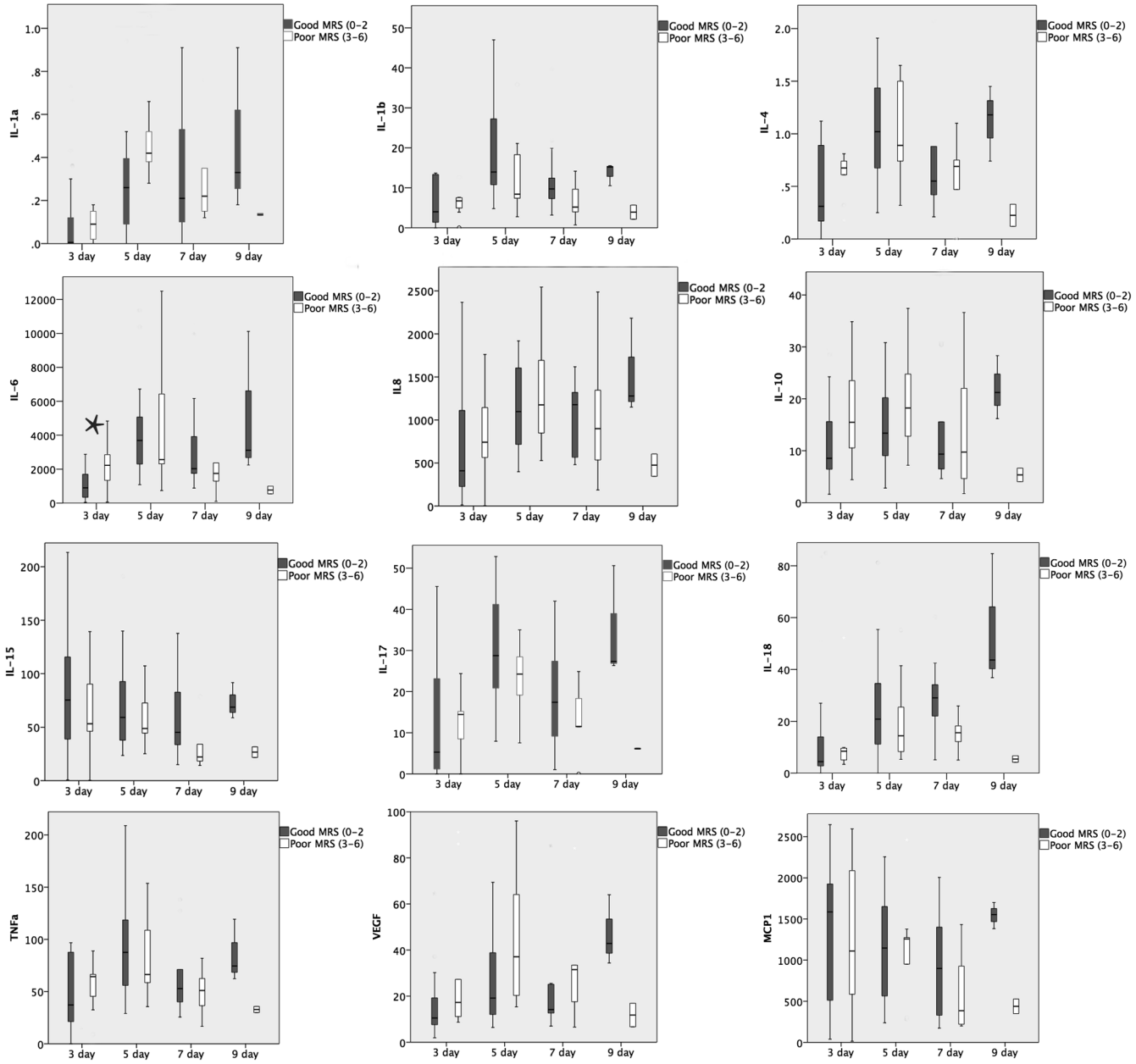

Fig. 5. Box plots demonstrating median CSF mediator concentrations for patients with a good (0–2) and poor (3–6) Modified Rankin Score at 6-months. Interquartile range is highlighted. * Indicates $p < 0.05$.

effector cells to the affected region. Simultaneous disruption of the BBB (which may be contributed to directly by mediators such as TNF-α) allows these mediators to leak into the circulation, resulting in a systemic response. Other factors, particularly relating to clinical aSAH grade and necrotic load, will also contribute to the severity of this SIRS (e.g. sepsis and organ failure). Moreover, after acute CNS injury, a temporary impairment of cellular immune function is believed to be a risk factor for systemic infections [24]. In those who will develop/have developed DIND, this response is more pronounced (probably proportional to the concentration/quantity of the original causative agent(s) within the initial haemorrhagic blood load). One of the unanswered questions is whether ischaemic deficits are caused by the inflammation within the CNS, or co-exist with it as surrogate markers, both chronologically and spatially.

In summary, aSAH triggers an inflammatory response which peaks on days 5–7 post ictus and remains largely compartmentalised within the CNS. Within this process, IL-4 has a particular association with DIND, although its precise role in the pathophysiology of the disorder remains unclear. IL-6 predicted poor outcome but not independently of clinical grade, suggesting that it may be a surrogate marker of early brain injury

Author contributions

Y.Z. Al-Tamimi is the principal investigator and author of manuscript.

D. Bhargava helped with sample collection.

A. Teraifi assisted with the literature review.

N. Orsi helped with the laboratory analysis and co-authored the manuscript.

M. Cummings and U. Ekbote helped with the laboratory analysis.

A. Quinn, S. Homer-Vanniasinkam and S. Ross helped with editing and review of the manuscript.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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