

# Temporal patterns of inflammatory biomarkers measured in the cerebrospinal fluid of patients with aneurysmal subarachnoid hemorrhage using multiplex Proximity Extension Assay technology.

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## Research

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# Abstract

**Background** Neuroinflammation has been extensively studied in the context of subarachnoid hemorrhage (SAH) in recent years. A common approach is correlation of levels of single or few inflammatory biomarkers with clinical parameters such as cerebral vasospasm and outcome. However, the complexity of the inflammatory response post SAH may require a more comprehensive overall approach by analyzing multiple biomarkers simultaneously.

**Methods** Twenty-nine patients with SAH requiring insertion of external ventricular drainage were enrolled and ventricular cerebrospinal fluid was collected at days 1, 4 and 10 after hemorrhage. The levels of 92 inflammatory biomarkers were simultaneously measured in the samples using Proseek Multiplex Inflammation 1® assay (Olink Proteomics, Uppsala, Sweden) based on Proximity Extension Assay (PEA) technology. Thirty-eight proteins were excluded from further analysis due to lack of >10% of measurable values. Temporal patterns for each of the remaining 54 proteins were illustrated on graphs with medians and quartiles. Wilcoxon matched pairs test was used for comparison of the medians between time points.

**Results** Four different patterns were visually observed with an early peak and gradually decreasing trend (9 proteins), middle peak (14 proteins), late peak after a gradually increasing trend (27 proteins) and no specific pattern (4 proteins). Several biomarkers showed statistically significant increasing trends (defined as day 1 < day 4 < day 10 values) and late peaks (day 4 < day 10), such as chemokines CXCL6 and CCL23, or significant early peaks (that is, day 1 > day 4) such as leukemia inhibitor factor (LIF) and macrophage inflammatory factor-1 $\beta$  (MIP-1 $\beta$ ). No statistically significant decreasing trends (defined as day 1 > day 4 > day 10) were observed. Two proteins showed statistically significant middle peaks (chemokine CCL28 and Delta and Notch epidermal growth factor-related receptor-DNER).

**Conclusion** The comprehensive data set provided in this study may act as an illustration of an inflammatory profile of the acute phase of SAH showing groups of biomarkers with similar temporal patterns of activation. Further studies can be designed based on these data in order to explore potential implications on early and late clinical events in the disease course.

## Background:

Spontaneous subarachnoid hemorrhage (SAH) comprises approximately 5% of all strokes with an incidence of around 9 cases per 100.000 per year and aneurysm rupture being the cause in 85% of cases. [1] Brain injury in SAH occurs both at the time of the bleeding itself, termed primary injury, but also during the following days to weeks, a phenomenon known as secondary injury. Despite advances in the critical care of SAH patients and aneurysm treatment methods, mortality and morbidity rates among the survivors from the initial bleeding remain high, reflecting a lack of effective treatments targeting the pathophysiological mechanisms that underlie the secondary injury.

Delayed cerebral ischemia (DCI) develops in approximately 30–40% of SAH patients and is considered to be a major cause of unfavorable outcome.[2] For decades the condition was attributed to cerebral vasospasm (CV), i.e. the narrowing of basal cerebral arteries seen early on angiography and persisting for up to two weeks post SAH leading to decreased cerebral blood flow (CBF) and infarctions in the affected territories.[3] However, about 20% of SAH patients develop DCI without evidence of CV and only 30% of patients with CV actually suffer from DCI.[4] Moreover, the randomized multicenter CONSCIOUS-1 trial with the endothelin receptor antagonist Clazosentan failed to show any effect on functional outcome or incidence of cerebral ischemia despite significant decrease of CV.[5, 6] Thus, an uncoupling of angiographic vasospasm and DCI became apparent and new concepts emerged as potential underlying mechanisms of delayed brain injury.[7, 8]

Early brain injury (EBI) refers to the events occurring within the first 72 h from ictus and includes the primary injury and its direct consequences. Elevation of intracranial pressure (ICP), global ischemia, impairment of CBF autoregulation, cortical spreading depolarization (CSD), disruption of blood-brain barrier (BBB), cell death, oxidative stress and inflammatory processes are among the mechanisms that are activated shortly after aneurysm rupture and evolve during the following days.[9]

The processes triggered by the EBI are now believed to play an important role in the development of DCI. [9, 10] Neuroinflammation may be a mechanistic link between these two conditions and has been studied extensively in the past years.[11–13] Many clinical and experimental studies report levels of single or few inflammatory biomarkers (such as cytokines) in the peripheral blood, cerebrospinal fluid (CSF) and cerebral extracellular fluid through microdialysis (MD) in SAH patients or animal models and further correlate these biomarkers with clinical parameters and outcome.[14–22] Although this strategy provides useful insight in the interplay between inflammation and the disease course it fails to account for the complexity of the involved mechanisms that cannot be adequately described by a single (or few) parameter(s) alone.

The aim of the present study was to provide a comprehensive inflammatory profile of the acute phase of SAH by simultaneously measuring the levels of 92 inflammatory biomarkers in the CSF at days 1, 4 and 10 after admission using multiplex Proximity Extension Assay (PEA) technology. The biomarkers were then categorized into different groups according to their temporal expression.

## **Methods:**

### **Ethics**

The study was conducted in accordance with Declaration of Helsinki for human studies and approved by Uppsala University Ethics Committee. All participants or their next of kin gave written consent for participation in the study.

### **Patients**

Demographic data of the 29 patients included in the study are presented in Table 1. Inclusion period was between May 2013 and August 2014. Eligibility criteria was spontaneous SAH severe enough to require insertion of an external ventricular drain (EVD) within 24 h from ictus. Patients considered terminally ill from the bleeding were excluded. Brain CT scans were used to establish the diagnosis of SAH and were classified according to Fisher scale (median 4).[23] World Federation of Neurosurgical societies (WFNS) and Hunt & Hess scores were noted on admission (medians 4 and 3, respectively).[24, 25] CT angiography and catheter angiography were performed in all cases and aneurysms were identified in 28 patients. In 26 patients the aneurysms were endovascularly secured and the remaining two were surgically clipped. The patients were treated at the Neurointensive Care Unit of Uppsala University Hospital for at least 10 days after the bleeding. Standardized treatment protocols described in a previous publication were applied.[26] Functional outcome was assessed by a research nurse at 1 year using Glasgow Outcome Scale.[27]

Table 1  
Demographic data of the patient cohort.

<b>Number of patients</b>	<b>29</b>
Male vs Female	12 vs 17
Mean age (range)	57 (37–81)
Median Hunt & Hess score	3
Median World Federation of Neurosurgical Societies score	4
Median Fisher grade	4
Anterior vs posterior circulation aneurysm	23 vs 5 (1 patient with no aneurysm)
Embolization vs surgery	26 vs 2
Favorable vs unfavorable outcome (Glasgow Outcome Scale)	9 vs 20

## CSF samples and analysis

Ventricular CSF samples were collected through the EVD within 24 h, on day 4 and on day 9–11 after the bleeding, centrifuged directly after collection and frozen in -70 °C. The samples were then analyzed using Proseek Multiplex Inflammation 1®, a multiplex assay panel manufactured by Olink Proteomics AB, Uppsala, Sweden where 92 inflammation-related biomarkers are simultaneously measured using Proximity Extension Assay (PEA) technology (available online at <https://www.olink.com/products/inflammation>). The analytical method was previously described in detail.[28] Values are provided in output unit Normalized Protein Expression (NPX) on log<sub>2</sub> scale. NPX values express relative quantification between samples but is not an absolute quantification. Limit of detection (LOD) is determined for each biomarker based on the negative controls analyzed in each run.

## Biomarkers

A list of the 92 biomarkers included in the panel as well as their families is provided in Table 2. In summary, the panel included 31 cytokines, 20 chemokines, 9 growth factors, 8 tumor necrosis factor (TNF)-family members, 6 membrane glycoproteins, 6 neurotrophic factors, 5 proteases and 7 miscellaneous proteins. A total of 87 NPX values (29 patients x 3 time points) corresponding to protein concentrations were collected for each biomarker. Variations in the detectability of the biomarkers in the samples were noticed; for example, in 44 proteins all samples were successfully analyzed while in 6 proteins all values were below limit of detection. An inclusion criterion was therefore defined that the values needed to be above level of detection (LOD) in at least 90% of the samples for each protein (that is < 9 missing values), which lead to the exclusion of 38 proteins, thus leaving 54 proteins for further analysis.

Table 2  
List of biomarkers included in the panel

Nr	PROTEIN	FULL NAME	FAMILY
1	4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1	Translation factor
2	ADA	Adenosine deaminase	Deaminase
3	ARTN	Artemin	Neurotrophic factor
4	AXIN1	Axin-1	G-protein regulator
5	BDNF	Brain-derived neurotrophic factor	Neurotrophic factor
6	beta-NGF	Beta-nerve growth factor	Neurotrophic factor
7	CASP-8	Caspase-8	Cysteine protease
8	CCL11	C-C motif chemokine ligand 11 (Eotaxin)	Chemokine
9	CCL13 (MCP-4)	C-C motif chemokine ligand 13 (monocyte chemotactic protein 4)	Chemokine
10	CCL19	C-C motif chemokine ligand 19	Chemokine
11	CCL2 (MCP-1)	C-C motif chemokine ligand 2 (monocyte chemotactic protein 1)	Chemokine
12	CCL20	C-C motif chemokine ligand 20	Chemokine
13	CCL23 (MIP-3)	C-C motif chemokine ligand 23 (macrophage inflammatory protein-3)	Chemokine
14	CCL25	C-C motif chemokine ligand 25	Chemokine
15	CCL28	C-C motif chemokine ligand 28	Chemokine
16	CCL3 (MIP-1 $\alpha$ )	C-C motif chemokine ligand 3 (macrophage inflammatory protein-1 $\alpha$ )	Chemokine
17	CCL4 (MIP-1 $\beta$ )	C-C motif chemokine ligand 4 (macrophage inflammatory protein-1 $\beta$ )	Chemokine
18	CCL7 (MCP-3)	C-C motif chemokine ligand 7 (monocyte chemotactic protein 3)	Chemokine
19	CCL8 (MCP-2)	C-C motif chemokine ligand 8 (monocyte chemotactic protein 2)	Chemokine

Alphabetic list of the 92 biomarkers included in Proseek Multiplex Inflammation 1® panel (abbreviations and full names) together with their families. The 54 biomarkers that met the criterion of being above level of detection in at least 90% of the samples (that is < 9 missing values for each protein) to be included for further analysis are marked in bold.

Nr	PROTEIN	FULL NAME	FAMILY
20	CD244	Natural killer cell receptor 2B4	Membrane glycoprotein
21	CD40	Tumor necrosis factor receptor superfamily member 5	TNF receptor superfamily
22	CD5	T-cell surface glycoprotein CD5	Membrane glycoprotein
23	CD6	T-cell differentiation antigen CD6	Membrane glycoprotein
24	CDCP-1	CUB domain-containing protein 1	Membrane glycoprotein
25	CSF-1	Macrophage colony-stimulating factor 1	Cytokine
26	CST5	Cystatin-D	Cysteine proteinase inhibitor
27	CX3CL1	C-X3-C motif chemokine ligand 1 (Fractalkine)	Chemokine
28	CXCL1	C-X-C motif chemokine ligand 1	Chemokine
29	CXCL10	C-X-C motif chemokine ligand 10	Chemokine
30	CXCL11	C-X-C motif chemokine ligand 11	Chemokine
31	CXCL5	C-X-C motif chemokine ligand 5	Chemokine
32	CXCL6	C-X-C motif chemokine ligand 6	Chemokine
33	CXCL9	C-X-C motif chemokine ligand 9	Chemokine
34	DNER	Delta and Notch-like epidermal growth factor-related receptor	Growth factor
35	EN-RAGE	Protein S100-A12	Calcium-binding protein
36	FGF19	Fibroblast growth factor 19	Growth factor
37	FGF-21	Fibroblast growth factor 21	Growth factor
38	FGF-23	Fibroblast growth factor 23	Growth factor
39	FGF-5	Fibroblast growth factor 5	Growth factor
40	Flt3L	Fms-related tyrosine kinase 3 ligand	Cytokine

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<b>Nr</b>	<b>PROTEIN</b>	<b>FULL NAME</b>	<b>FAMILY</b>
41	GDNF	Glial cell line-derived neurotrophic factor	Neurotrophic factor
42	HGF	Hepatocyte growth factor	Growth factor
43	IFN-gamma	Interferon gamma	Cytokine
44	IL-10	Interleukin-10	Cytokine
45	IL-10RA	Interleukin-10 receptor subunit alpha	Cytokine
46	IL-10RB	Interleukin-10 receptor subunit beta	Cytokine
47	IL-12B	Interleukin-12 subunit beta	Cytokine
48	IL-13	Interleukin-13	Cytokine
49	IL-15RA	Interleukin-15 receptor subunit alpha	Cytokine
50	IL-17A	Interleukin-17A	Cytokine
51	IL-17C	Interleukin-17C	Cytokine
52	IL-18	Interleukin-18	Cytokine
53	IL-18R1	Interleukin-18 receptor 1	Cytokine
54	IL-1 $\alpha$	Interleukin-1 alpha	Cytokine
55	IL-2	Interleukin-2	Cytokine
56	IL-2RB	Interleukin-2 receptor subunit beta	Cytokine
57	IL-20	Interleukin-20	Cytokine
58	IL-20RA	Interleukin-20 receptor subunit alpha	Cytokine
59	IL-22 RA1	Interleukin-20 receptor subunit alpha-1	Cytokine
60	IL-24	Interleukin-24	Cytokine
61	IL-33	Interleukin-33	Cytokine
62	IL-4	Interleukin-4	Cytokine
63	IL-5	Interleukin-5	Cytokine
64	IL-6	Interleukin-6	Cytokine
65	IL-7	Interleukin-7	Cytokine

Alphabetic list of the 92 biomarkers included in Proseek Multiplex Inflammation 1® panel (abbreviations and full names) together with their families. The 54 biomarkers that met the criterion of being above level of detection in at least 90% of the samples (that is < 9 missing values for each protein) to be included for further analysis are marked in bold.

<b>Nr</b>	<b>PROTEIN</b>	<b>FULL NAME</b>	<b>FAMILY</b>
66	IL-8	Interleukin-8	Cytokine
67	LIF	Leukemia inhibitory factor	Cytokine
68	LIF-R	Leukemia inhibitory factor receptor	Cytokine
69	MMP-1	Matrix metalloproteinase-1	Protease
70	MMP-10	Matrix metalloproteinase-10	Protease
71	NRTN	Neurturin	Neurotrophic factor
72	NT-3	Neurotrophin-3	Neurotrophic factor
73	OPG	Osteoprotegerin	TNF receptor superfamily
74	OSM	Oncostatin-M	Cytokine
75	PD-L1	Programmed cell death ligand 1	Membrane glycoprotein
76	SCF	Stem cell factor	Cytokine
77	SIRT2	SIR2-like protein 2	NAD dependent deacetylase
78	SLAMF1	Signaling lymphocytic activation molecule	Membrane glycoprotein
79	ST1A1	Sulfotransferase 1A1	Transferase
80	STAMPB	STAM-binding protein	Protease
81	TGFA	Transforming growth factor alpha	Growth factor
82	TGF- $\beta$ 1	Transforming growth factor beta-1	Growth factor
83	TNF	Tumor necrosis factor	TNF family
84	TNFB	Tumor necrosis factor beta (Lymphotoxin-alpha)	Cytokine
85	TNFRSF9	Tumor necrosis factor receptor superfamily member 9	TNF receptor superfamily
86	TNFSF14	Tumor necrosis factor superfamily member 14	TNF receptor superfamily
87	TRAIL (TNFSF10)	TNF-related apoptosis-inducing ligand (TNF-superfamily member 10)	TNF receptor superfamily

Alphabetic list of the 92 biomarkers included in Proseek Multiplex Inflammation 1® panel (abbreviations and full names) together with their families. The 54 biomarkers that met the criterion of being above level of detection in at least 90% of the samples (that is < 9 missing values for each protein) to be included for further analysis are marked in bold.

Nr	PROTEIN	FULL NAME	FAMILY
88	TRANCE (TNFSF11)	Tumor necrosis factor superfamily member 11	TNF receptor superfamily
89	TSLP	Thymic stromal lymphopoietin	Cytokine
90	TWEAK (TNFSF12)	Tumor necrosis factor superfamily member 12	TNF receptor superfamily
91	uPA	Urokinase-type plasminogen activator	Protease
92	VEGF-A	Vascular endothelial growth factor A	Growth factor

Alphabetic list of the 92 biomarkers included in Proseek Multiplex Inflammation 1® panel (abbreviations and full names) together with their families. The 54 biomarkers that met the criterion of being above level of detection in at least 90% of the samples (that is < 9 missing values for each protein) to be included for further analysis are marked in bold.

## Statistics

The NPX values of the remaining 54 proteins were converted into linear scale using the formula  $2^{NPX} =$  linear NPX in order to better visualize the trends of the biomarkers during the observation period. Median values for each day and protein were then calculated from the linear NPX values and their development over time was illustrated in graphs with upper and lower quartiles. Statistical analysis was done on the linear NPX data that were visually not normally distributed. Therefore non-parametric statistical methods were chosen. Wilcoxon matched pairs test was used for the comparison of day 1 vs day 4 vs day 10 median values for each protein. Protein expression levels in graphs were illustrated with medians and upper and lower quartiles. The results were considered statistically significant at the  $p < 0.05$  level. All statistical analyses and graphical presentations were performed using the Statistica® software (Stat Soft, Inc., Tulsa, OK, USA).

## Results:

Visual inspection of the linear NPX graphs revealed four different temporal patterns (Table 3): an early peak at day 1 followed by a decreasing trend (Group A: 9 proteins), a middle peak at day 4 (Group B: 14 proteins), an increasing trend with a late peak at day 10 (Group C: 27 proteins) and finally no specific pattern (Group D: 4 proteins). Figures 1–4 illustrate typical graphs of median protein expression levels (linear NPX) and upper and lower quartiles over time for each group. Medians were then compared between time points for each protein using Wilcoxon matched pairs test. Table 4 shows the results (p-value considered significant at  $< 0.05$ ).

Table 3

Groups of biomarkers by their temporal pattern of protein expression

<b>GROUP A (n = 9) EARLY PEAK</b>	<b>GROUP B (n = 14) MIDDLE PEAK</b>	<b>GROUP C (n = 27) LATE PEAK</b>	<b>GROUP D (n = 4) NO PATTERN</b>
CCL11	CCL28	4E-BP1	CCL25
CCL20	CSF-1	ADA	MCP-1
FGF-19	CST-5	CASP8	TNFRSF9
LIF	DNER	CCL19	VEGF-A
MIP-1 $\alpha$ (CCL3)	FGF-5	CCL23	
MIP-1 $\beta$ (CCL4)	Flt3L	CD40	
MMP10	IL-10	CD5	
TGFA	IL-10RB	CDCP1	
TRAIL	IL-18R1	CX3CL1	
	IL-6	CXCL1	
	LIF-R	CXCL10	
	MCP-3	CXCL11	
	OSM	CXCL5	
	TGF- $\beta$ 1	CXCL6	
		CXCL9	
		EN-RAGE	
		HGF	
		IL-18	
		IL-7	
		IL-8	
		MCP-2	
		OPG	
		SCF	

Four different groups were identified based on visual inspection of the graphs, that is an early peak followed by a decreasing trend (Group A), a middle peak (Group B), a late peak after an increasing trend (Group C) and finally no specific pattern (Group D).

GROUP A (n = 9) EARLY PEAK	GROUP B (n = 14) MIDDLE PEAK	GROUP C (n = 27) LATE PEAK	GROUP D (n = 4) NO PATTERN
		SIRT2	
		STAMPB	
		TWEAK	
		uPA	
<p>Four different groups were identified based on visual inspection of the graphs, that is an early peak followed by a decreasing trend (Group A), a middle peak (Group B), a late peak after an increasing trend (Group C) and finally no specific pattern (Group D).</p>			

Table 4  
Expression levels of all proteins and time points.

GROUP A	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
CCL11	8,5 (5,2–12)	< 0,01	5,2 (3,4–7,8)	0,16	5,1 (4,3–7,2)	< 0,01
CCL20	57 (17–176)	0,32	51 (23–108)	0,08	43 (27–97)	0,25
FGF-19	17 (12–21)	0,07	16 (11–20)	0,62	13 (9,2–21)	0,16
LIF	52 (21–148)	< 0,01	13 (7,8–22)	0,93	14 (6,1–34)	< 0,01
MIP-1 $\alpha$ (CCL3)	9,6 (5,2–25)	0,13	8 (5,1–11)	0,99	7,7 (6,6–12)	0,17
MIP-1 $\beta$ (CCL4)	80 (47–404)	< 0,01	36 (22–64)	0,44	35 (28–59)	< 0,01
MMP-10	17 (9,9–35)	0,20	14 (12–19)	0,06	12 (8,8–18)	0,01
TGFA	7,7 (5,7–11)	0,23	6,9 (5,4–8,5)	0,03	6,5 (5,3–7,3)	0,01
TRAIL	9,4 (6,8–16)	0,01	7,3 (5,2–8,9)	0,99	6,7 (5,3–9,6)	0,04
GROUP B	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
CCL28	1,3 (1,2 – 1,5)	< 0,01	1,6 (1,5 – 1,7)	0,02	1,5 (1,3 – 1,6)	0,07
CSF-1	87 (48–126)	0,18	102 (74–131)	0,06	80 (50–111)	0,61
CST5	45 (38–61)	< 0,01	60 (54–65)	0,20	57 (48–65)	0,02
DNER	138 (112–216)	< 0,01	327 (250–381)	0,01	259 (170–360)	< 0,01
FGF-5	3,2 (2,5 – 4,9)	< 0,01	6,7 (5,5–11)	0,15	6 (4,2–9,7)	0,01
FIt3L	156 (129–231)	< 0,01	267 (185–374)	0,82	285 (166–367)	< 0,01
IL-10	5,1 (3,6–8)	0,31	6,3 (4,5–7,9)	0,31	4,9 (3,3–9,5)	0,92
IL-10RB	3,4 (2,9 – 6,2)	< 0,01	7,1 (4,7–9,6)	0,26	7,9 (4,3–12)	< 0,01

Comparison of median protein expression levels (linear NPX) with upper and lower quartiles within parentheses between time points for each protein (classified in groups according to Table 3); Wilcoxon matched pairs test was used for comparison; p-values were considered significant at < 0.05 level.

GROUP A	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
IL-18R1	3,9 (2,3–6,9)	0,12	5,7 (3,3–7,4)	0,34	5,3 (2,8 – 7,5)	0,34
IL-6	927 (267–2037)	< 0,01	2715 (537–5589)	0,64	2119 (611–6838)	< 0,01
LIF-R	4 (3,3–5,6)	< 0,01	5,2 (4,4–7,4)	0,26	5 (4,4–6,4)	0,03
MCP-3	38 (15–78)	0,02	95 (29–172)	0,31	98 (31–290)	< 0,01
OSM	11 (8,1–30)	0,02	30 (9,4–53)	0,41	31 (13–61)	0,03
TGF-β1	13 (9–25)	< 0,01	30 (19–36)	0,15	29 (17–54)	< 0,01
GROUP C	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
4E-BP1	2,3 (1,7 – 3,3)	< 0,01	21 (3,5–71)	< 0,01	111 (21–102)	< 0,01
ADA	16 (10–23)	< 0,01	32 (22–64)	0,055	53 (29–107)	< 0,01
CASP-8	2,9 (1,7 – 3,6)	0,26	2,8 (2,2–3,9)	< 0,01	5,6 (2,9–11)	< 0,01
CCL19	177 (115–388)	< 0,01	1772 (748–3642)	0,01	2875 (1196–5501)	< 0,01
CCL23	12 (7,1–25)	< 0,01	63 (31–104)	< 0,01	207 (83–331)	< 0,01
CD40	64 (43–101)	< 0,01	109 (75–189)	< 0,01	250 (137–395)	< 0,01
CD5	2 (1,5 – 3,5)	0,055	3,2 (2,2–4,2)	0,23	4 (2,2–5,3)	0,01
CDCP1	13 (9–15)	< 0,01	17 (15–26)	< 0,01	28 (17–42)	< 0,01
CX3CL1	6,5 (5,7–8,1)	0,10	9,5 (6,7–11)	0,19	11,7 (6,9–17)	0,07
CXCL1	183 (82–356)	< 0,01	830 (127–1066)	< 0,01	1037 (437–1973)	< 0,01
CXCL10	179 (76–417)	< 0,01	1573 (1024–4301)	0,25	2530 (1181–3539)	< 0,01

Comparison of median protein expression levels (linear NPX) with upper and lower quartiles within parentheses between time points for each protein (classified in groups according to Table 3); Wilcoxon matched pairs test was used for comparison; p-values were considered significant at < 0.05 level.

GROUP A	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
CXCL11	5,7 (3–8)	< 0,01	11,8 (8–22)	0,68	19,5 (10–39)	< 0,01
CXCL5	252 (144–572)	0,68	271 (160–753)	0,73	437 (185–737)	0,56
CXCL6	21 (8,8–44)	< 0,01	204 (22–408)	< 0,01	642 (172–959)	< 0,01
CXCL9	7,1 (6,2–19)	< 0,01	25,9 (15–42)	< 0,01	59 (18–116)	< 0,01
EN-RAGE	2,9 (2,5 – 4,2)	< 0,01	9,8 (5,8–29)	0,11	16 (5,9–39)	< 0,01
HGF	36 (27–70)	< 0,01	86 (67–116)	0,08	114 (64–188)	< 0,01
IL-18	6,3 (4,3–10)	0,33	9,8 (3,5–20)	< 0,01	24 (8,3–37)	< 0,01
IL-7	2,3 (2–2,5)	0,01	2,4 (2,2–3,4)	0,31	2,8 (2,5 – 3,4)	< 0,01
IL-8	6671 (4906–12663)	0,29	10867 (3084–16185)	0,03	15540 (6006–21024)	0,06
MCP-2	107 (53–188)	< 0,01	404 (126–644)	0,01	527 (198–1082)	< 0,01
OPG	638 (356–1629)	0,24	1076 (646–1584)	0,03	1381 (741–2481)	0,03
SCF	7,2 (5,2–11)	0,19	12 (7,9–15)	0,39	13 (6,1–19)	0,13
SIRT2	4,7 (3,3–8,2)	0,08	6,3 (3,9–10)	0,06	10 (5,2–22)	< 0,01
STAMPB	3,6 (2,4–4,2)	0,40	3,5 (2,9 – 6)	0,04	6,3 (3,2–8,9)	0,01
TWEAK	179 (123–246)	< 0,01	271 (202–438)	0,16	326 (214–497)	< 0,01
uPA	156 (120–227)	< 0,01	385 (244–565)	< 0,01	1230 (525–2239)	< 0,01
GROUP D	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
CCL25	2,5 (2,2–3,6)	0,04	2,2 (2–2,5)	0,04	2,5 (2–3,9)	0,58

Comparison of median protein expression levels (linear NPX) with upper and lower quartiles within parentheses between time points for each protein (classified in groups according to Table 3); Wilcoxon matched pairs test was used for comparison; p-values were considered significant at < 0.05 level.

GROUP A	DAY 1	1 VS 4	DAY 4	4 VS 10	DAY 10	1 VS 10
MCP-1	9428 (7822–10942)	0,50	9491 (7628–10487)	0,85	8998 (7870–10266)	0,18
TNFRSF9	7 (3,5–10)	0,17	7,2 (5–9,8)	0,96	8,1 (3,7–11)	0,31
VEGF-A	1039 (520–1414)	0,04	689 (493–1007)	0,10	996 (535–1247)	0,38
Comparison of median protein expression levels (linear NPX) with upper and lower quartiles within parentheses between time points for each protein (classified in groups according to Table 3); Wilcoxon matched pairs test was used for comparison; p-values were considered significant at < 0.05 level.						

Group A biomarkers with statistically significant early peak, defined as significantly higher day 1 vs day 4 values, were CCL11, LIF, MIP-1 $\beta$  (CCL4) and TRAIL; no biomarkers in this group showed statistically significant decreasing trend (that is, significant differences between day 1 vs day 4 and day 4 vs day 10 values).

Several Group B biomarkers (Table 4) showed significantly higher values between day 4 vs day 1 (i.e. CCL28, CST5, DNER, FGF-5, Flt3L, IL-10RB, IL-6, LIF-R, MCP-3, OSM and TGF- $\beta$ 1). Half of these biomarkers showed decreasing NPX values towards day 10 and the rest remained stable. The only biomarkers with statistically significant middle peak (that is, significant difference between day 4 vs day 1 and day 4 vs day 10 values) were CCL28 and DNER.

Fifteen of the 27 biomarkers in Group C (Table 4) showed statistically significant late peak defined as significantly higher day 10 vs day 4 values (4E-BP1, CASP-8, CCL19, CCL23, CD40, CDCP1, CXCL1, CXCL6, CXCL9, IL-18, IL-8, MCP-2, OPG, STAMPB and uPA). Ten of them also showed statistically significant increasing trend during the entire observation period, that is significantly higher day 10 vs day 4 and day 4 vs day 1 values (i.e. 4E-BP1, CCL19, CCL23, CD40, CDCP1, CXCL1, CXCL6, CXCL9, MCP-2 and uPA).

## Discussion:

In this study we present a concise data set of the sequential production of multiple biomarkers of inflammation in the CSF of patients during the first 10 days post SAH. To our knowledge, this is the largest data set to describe the inflammatory profile of SAH ever reported with 92 inflammatory biomarkers included, many of which never studied in SAH patients previously. This is also the first report of Proximity Extension Assay (PEA) technology being used for measuring biomarker expression levels in the context of SAH. Similar studies were recently published in patients with traumatic brain injury (TBI) and trigeminal neuralgia, as well as numerous other non-neurosurgical conditions (such as neuropathic pain, cardiovascular diseases, gastric cancer, etc.).[29–31]

The analyses in the present study were performed in CSF compartment alone (not plasma or cerebral interstitial fluid) as this seems more suitable to describe the disease pathophysiology, given also that major early and late clinical complications (i.e. CV and chronic hydrocephalus) spatially correlate best with this compartment. Many of the included proteins have previously been associated to SAH inflammation, for example IL-1ra, IL-6, IL-8, TNF-a, LIF, MCP-1, and VEGF-A.[32] On the other hand, there is scarce or non-existing literature on many other proteins, some of which showed interesting temporal patterns and statistically significant peaks and trends, such as LIF, CCL11, CCL28, 4E-BP1, CD40, CXCL6, CXCL9, and IL-18.[20, 33–36]

## **Group A**

Nine biomarkers showed higher day 1 values and decreasing trends throughout the observation period (Tables 3 and 4). Four of them (CCL11, LIF, MIP-1 $\beta$  and TRAIL) showed statistically significant early peaks with day 1 > day 4 levels but no statistically significant decreasing trends were observed. MIP-1 $\beta$  (also known as CCL4) and CCL11 are members of the chemokine family (C-C motif) and are involved in chemotaxis of macrophages and activated T-cells, respectively, as well as other proinflammatory actions. Their early peak can possibly be associated to the recruitment of leukocytes at the site of the bleeding. LIF is a cytokine involved in activation of signaling pathways that regulate cell growth among other actions. A similar early peak in serum has been observed previously.[37] TRAIL, a member of the TNF superfamily (also known as TNFSF10) involved in apoptosis, has not been studied in the SAH literature.

## **Group B**

Among the 14 proteins included in this group only two (CCL28 and DNER) showed statistically significant middle peaks, that is significantly higher day 4 values than both day 1 and day 10. Production of the chemokine CCL28 is induced by other proinflammatory cytokines and its chemotactic actions are exerted on B and T cells and eosinophils. Delta and Notch-like Epidermal growth factor-Receptor (DNER) is an activator of NOTCH1 pathway. None of these biomarkers have been studied in a SAH context earlier and their potential involvement in the SAH complications, mainly CV that coincides temporally with the observed middle peaks, should be examined.

It should be noted that the majority of the biomarkers in this group showed a significant increase in their levels between day 1 and day 4 (11/14, Table 4). In almost all cases though, with the exception of the two biomarkers named above, these dynamics seemed to wear off between day 4 and day 10. Interestingly, IL-6 was recently studied by our group using a quantitative routine monoclonal antibody-based method in CSF samples from 44 patients with severe SAH. The results showed a very similar temporal IL-6 pattern as in the present study, with increasing values between day 1 and day 4 followed by decreasing values towards day 10, although remaining higher than day 1 values.[38]

## **Group C**

Half of the studied biomarkers (n = 27) showed higher levels towards the end of the observation period, reflecting a more prolonged activation post SAH that may indicate an involvement in the healing

processes or the development of late complications, such as late vasospasm, posthemorrhagic hydrocephalus, etc. Fifteen biomarkers (4E-BP1, CASP-8, CCL19, CCL23, DC40, CDCP1, CXCL1, CXCL6, CXCL9, IL18, IL8, MCP-2, OPG, STAMPB and uPA) showed statistically significant late peaks, meaning significantly higher day 10 than day 4 values. Apart from CASP-8, IL-18, IL-8, OPG and STAMPB all the remaining proteins in the list showed statistically significant increasing trends throughout the observation period with day 1 < day 4 < day 10 values.

Chemokines CCL23, CXCL6 and CXCL9, all potent chemotactic agents for resting T-cells/monocytes, neutrophilic granulocytes and T-cells respectively, have not been studied in SAH patients earlier. Signaling pathway molecule 4E-BP1 has been implicated in the development of vasospasm in a canine SAH-model but no reports on human studied are available.[35] Protein CD40, a member of TNF family, is found on antigen-presenting cells and mediates multiple inflammatory responses. Elevated serum levels of CD40 have been associated with poor outcome and severity of neurological deficits in SAH patients.[36, 39] IL-18, a strong proinflammatory cytokine involved in the synthesis of inflammatory mediators, has recently been shown to be a predictor of early brain injury and clinical prognosis in SAH patients as elevated concentrations correlated to cerebral edema and acute hydrocephalus.[20] It should be noted that the observed temporal pattern of IL-18 in that study differed from our study as we demonstrated a late peak of this cytokine. Urokinase (or uPA) is a serum protease that activates plasminogen to plasmin which in turn leads to thrombolysis and tissue degradation. Plasma concentrations of its receptor (soluble uPA-receptor) was not shown to correlate with neurological outcome post SAH.[40]

## General considerations

The great variability of the expression levels and temporal patterns of the measured biomarkers is an indicator of the complexity of the inflammatory response after SAH. Many of the included proteins are well established in the SAH research both in humans and in preclinical animal models while others are novel in the SAH field. Their exact role as well as interplay with each other is not easy to establish, especially considering the fact that many of these substances are described to play both a detrimental and a beneficial role in the disease course depending on the time after bleeding.[41] This study may offer guidance for further research on groups of biomarkers based either on their families or their pattern of activation (e.g. early vs late peak) to identify potential underlying inflammatory mechanisms of SAH complications as well as novel targets of intervention in order to prevent/treat these conditions and improve outcome.

## Limitations

A limitation with the study is that the PEA analysis method in its presently available form does not give absolute protein concentrations. However, the protein expression levels illustrate the relative concentrations and how these change over time. They also illustrate the relations between the levels of the different biomarkers. The study may be limited by the relatively small number of patients included. Another drawback may be the fact that the study is limited to the CSF compartment. Similar analyses could be performed in the CSF, cerebral interstitial fluid and plasma, giving the opportunity to compare

protein levels in the different fluid compartments providing a more thorough inflammatory profile of the acute phase of the disease. Comparisons with healthy individuals could also serve as an indicator of the intensity of activation for each biomarker. Moreover, correlations of the biomarker expression levels with clinical parameters were not performed in this study, as the main goal was to provide general information of as many proteins as possible in order to look for patterns of expression for further investigation. Finally, measurements in more than three time points could possibly describe the temporal patterns of each protein more accurately.

## **Conclusion:**

The temporal patterns of expression of multiple inflammation-related proteins in the acute phase of SAH are reported in this study providing an inflammatory profile of the disease that facilitates further research in the field of biomarkers. Proximity Extension Assay technology enables the measurement of the expression levels of several biomarkers simultaneously in small amounts of sample, adding a useful tool in the quest of finding relevant biomarkers to better describe and understand the complex pathophysiology of SAH.

## **Abbreviations**

SAH  
subarachnoid haemorrhage  
PEA  
Proximity Extension Assay  
DCI  
delayed cerebral ischemia  
CV  
cerebral vasospasm  
CBF  
cerebral blood flow  
EBI  
early brain injury  
ICP  
intracranial pressure  
CSD  
cortical spreading depolarization  
BBB  
blood-brain barrier  
CSF  
cerebrospinal fluid  
MD

microdialysis  
EVD  
external ventricular drainage  
CT  
computerized tomography  
WFNS  
World Federation of Neurosurgical Societies  
GOS  
Glasgow outcome scale  
NPX  
normalized protein expression  
LOD  
limit of detection  
TNF  
tumor necrosis factor  
TBI  
traumatic brain injury

## Declarations

### *Ethics approval and consent to participate*

The Uppsala University Ethical review board for research on humans granted permission for the study. Informed consent for participation was obtained from the patients when feasible or their next of kin.

### *Consent for publication*

Not applicable.

### *Availability of data and material*

The datasets used in the current study are available from the corresponding author on reasonable request.

### *Competing interests*

None.

### *Funding*

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### *Authors' contributions*

The first and last author were responsible for the study design and data collection and analysis. The first author was responsible for writing the manuscript. All the other authors contributed with critically revising and improving the manuscript.

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### *Authors' information (optional)*

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## Figures

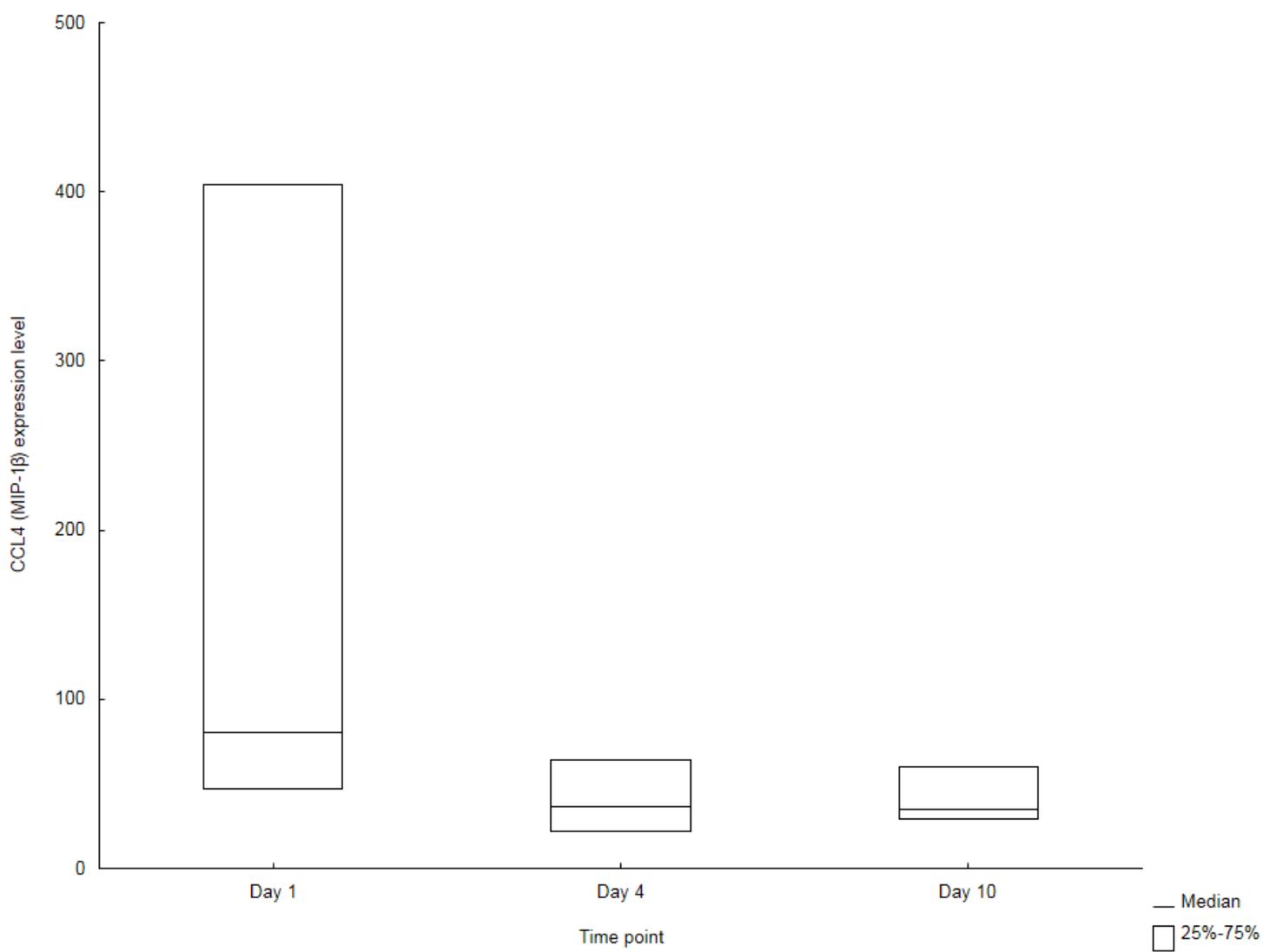
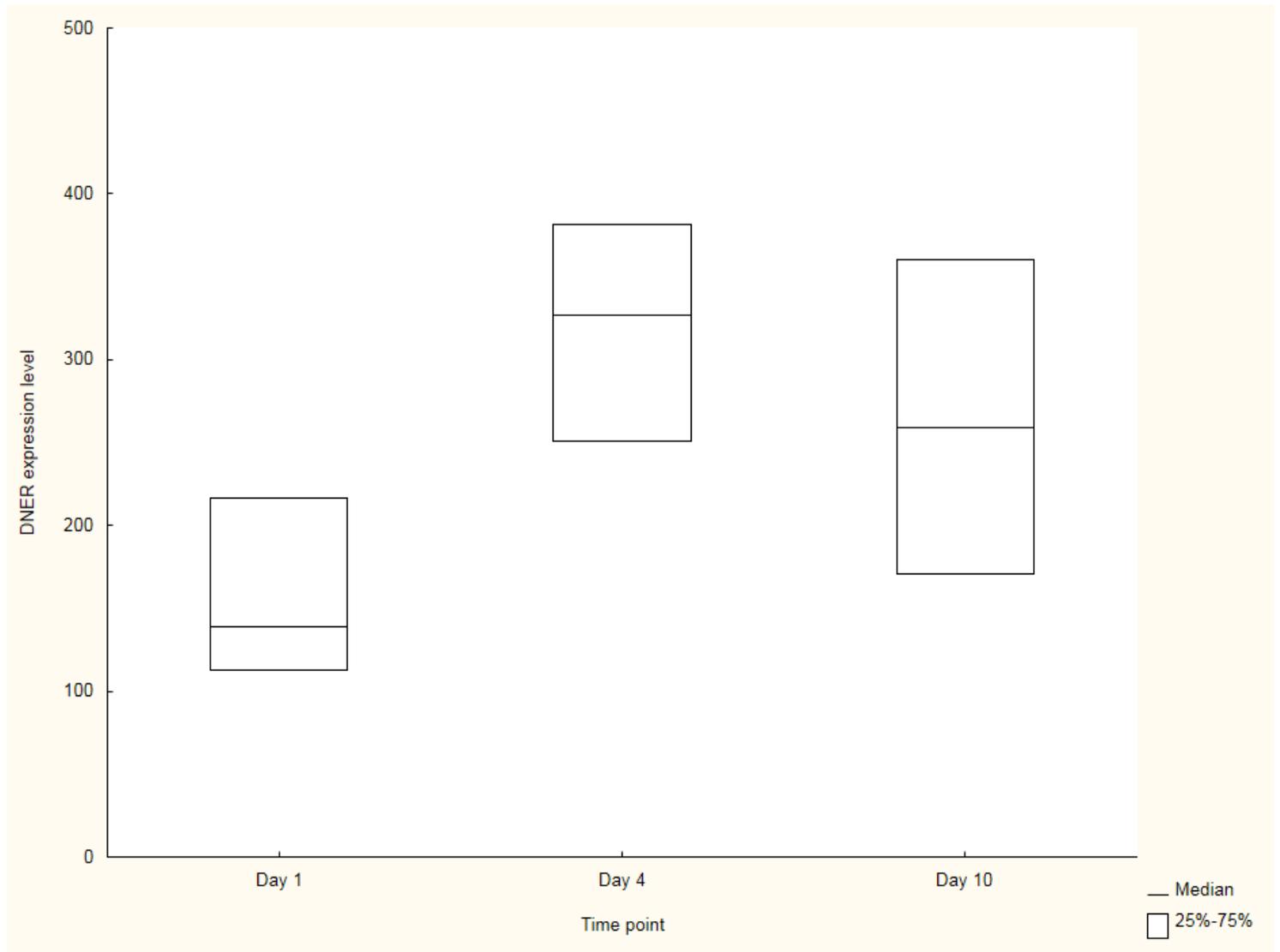


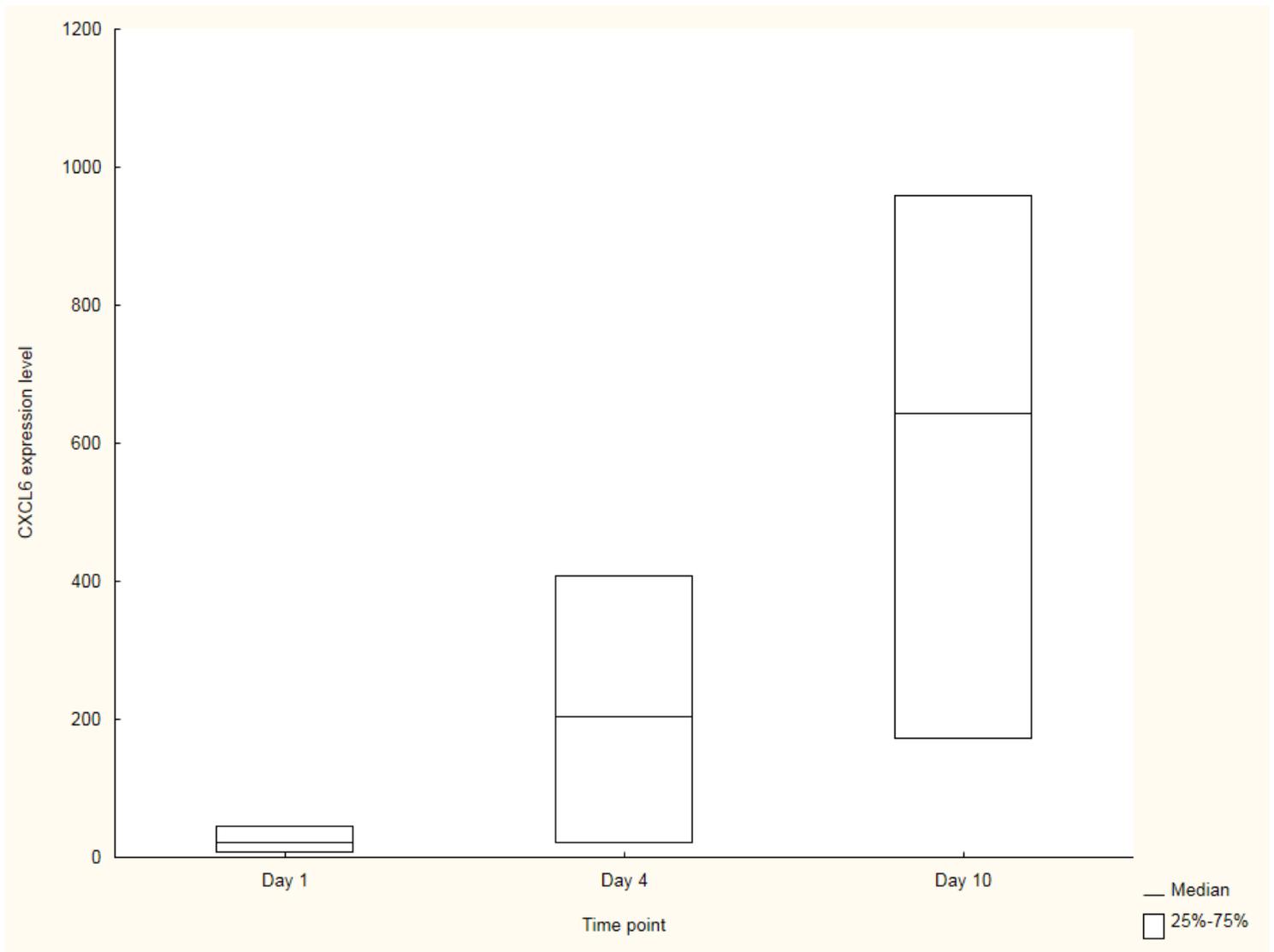
Figure 1

Group A example; CC4 (or MIP-1 $\beta$ ): temporal pattern of protein expression level (linear NPX) for C-C motif chemokine ligand 4 (CCL4) or macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ). Statistically significant early peak (median day 1 vs day 4 values=80.3 vs 36.4,  $p<0.01$ ) but no significantly decreasing trend since values seemed to stagnate between day 4 and 10 (36.4 vs 35.1,  $p=0.44$ ). Of note is the large spread of day 1 values compared to day 4 and day 10 values that were more concentrated.



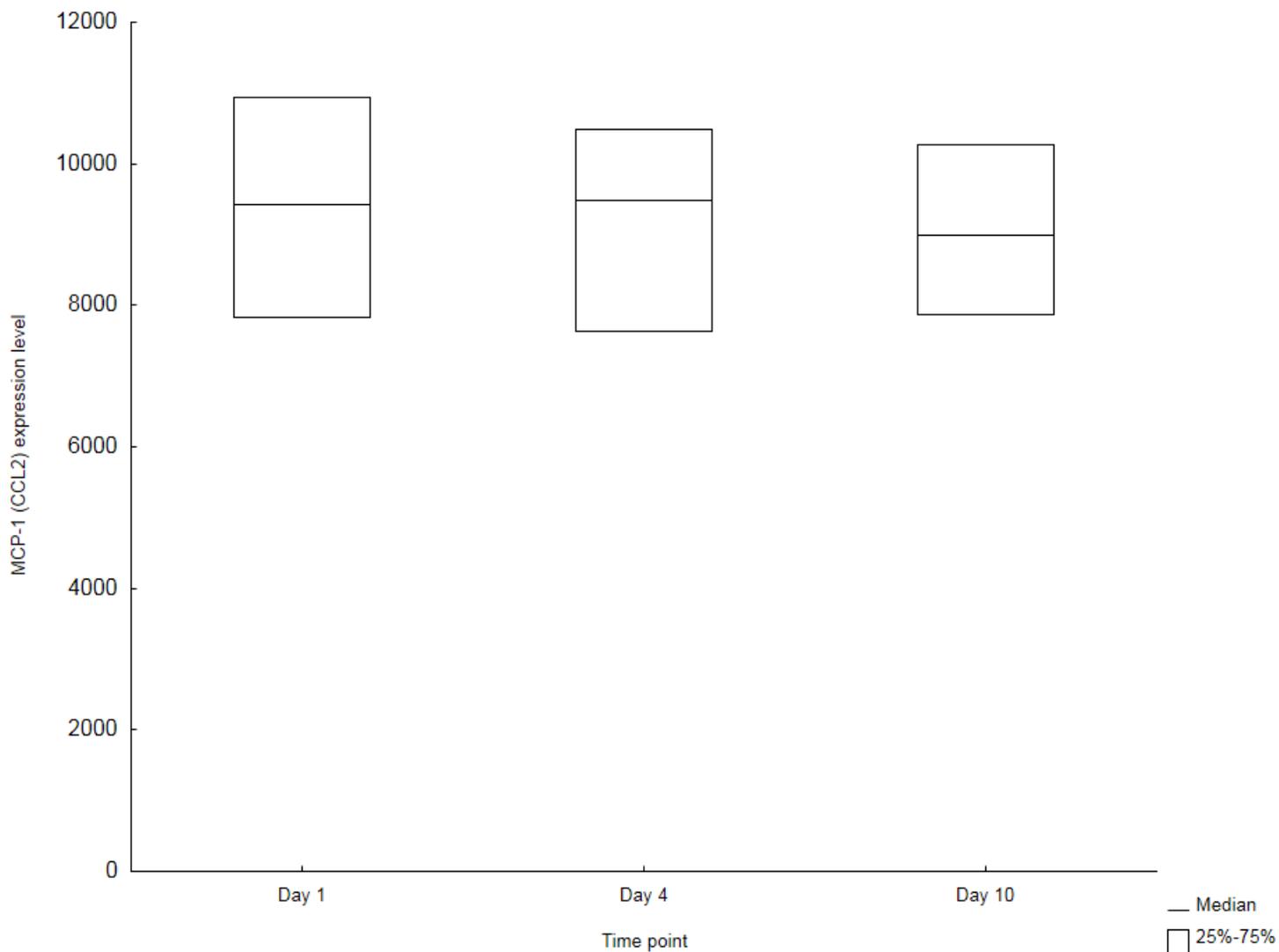
**Figure 2**

Group B example; DNER: temporal pattern of protein expression level (linear NPX) for Delta and Notch-like epidermal growth factor-related receptor (DNER). Statistically significant peak in the middle of the observation period defined by significantly higher day 4 vs day 1 (327.1 vs 138.8,  $p<0.01$ ) and day 4 vs day 10 median values (327.1 vs 259.2,  $p=0.01$ ).



**Figure 3**

Group C example; CXCL6: temporal pattern of protein expression level (linear NPX) for C-X-C motif chemokine ligand 6 (CXCL6) showing statistically significant late peak and increasing trend, that is day 1 < day 4 < day 10 median values (21.1 vs 204.3 vs 642.6 respectively,  $p < 0.01$ ). Of note the gradually increasing spread of values towards the end of the observation period.



**Figure 4**

Group D example; MCP-1: temporal pattern of protein expression level (linear NPX) for monocyte chemotactic protein 1 (MCP-1) or C-C motif chemokine ligand 2 (CCL2) showing essentially stable median values throughout the whole observation period (9428.7 vs 9491.3 vs 8998.3,  $p=0.50$  and  $p=0.85$  respectively).