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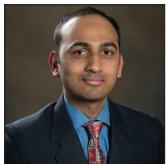
# Cytokines, brain proteins, and growth factors in acute stroke patients: A pilot study

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## ABSTRACT

**Background:** Immunomodulation and cell signaling involve several cytokines, proteins, and other mediators released in response to the trauma, inflammation, or other insults to the central nervous system. This pilot study is part of the registry designed to evaluate the temporal trends among these molecules after an acute ischemic stroke (AIS) in patients.

**Methods:** Twelve AIS patients were enrolled within 24 hours of the symptoms onset. Two sets of plasma samples were collected: First at admission and second at 24 hours after admission. Cytokines/chemokines and other inflammatory molecules were measured using multiplex assay kit.

**Results:** An increased trend in IL-6 (22 vs. 34 pg/ml), IL-8/CXCL8 (87 vs. 98 pg/ml), MMP-9 (16225 vs. 18450 pg/ml), and GMF-β (999 vs. 3739 pg/ml) levels was observed overtime after an AIS. Patients ≤60 years had lower levels of plasma MCP-1/CCL2 (50–647 vs. 150–1159 pg/ml), IL-6 (9–25 vs. 20–68 pg/ml), and IL-8 (30–143 vs. 72–630 pg/ml), when compared with patients >60 years old.

**Conclusion:** Cytokines/chemokines and other inflammatory mediators play an important role in the pathogenesis of stroke in addition to mediating poststroke inflammation. Further research is needed to evaluate and characterize the cumulative trends of these mediators for the clinical prognosis or as surrogate biomarkers.

**Keywords:** Acute ischemic brain injury, Cytokine, Interleukin, Poststroke inflammation, Stroke

## INTRODUCTION

In the United States (US), stroke is the fifth leading cause of mortality, affecting approximately 0.8 million people every year.<sup>[4]</sup> Ischemic stroke (IS) constitutes two-third of all strokes.<sup>[4]</sup> Ischemia to the brain precipitates cellular hypoxemia initiating oxidative damage, resulting in dysfunctional neurovascular unit and impair blood brain barrier (BBB). This inflammatory response is mediated by injured cells which produce interleukins (ILs)/cytokines/chemokines, interferons (INFs), and other growth stimulating factors, acting as both neuro- and immune-

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mediators.<sup>[27]</sup> Previous studies have observed the association of these molecules (both pro- and anti-inflammatory) with functional outcomes, infarct volume, stroke severity, and prognostication among IS patients.<sup>[15,24,26,27,29]</sup> However, there still exists a lack of consensus in the available data for a complete understanding of this intricate inflammatory response. Although, studies have observed these cytokines and cellular proteins dispersedly, the critical understanding is to elucidate the expression of these molecules focusing on the integrated synchronous trends and their association during the early phase. Glia maturation factor (GMF) is an intracellular, pro-inflammatory brain protein that has been implicated in neurodegenerative diseases. This is the first clinical study observing the role of GMF in acute stroke. This pilot study is part of the registry (<https://clinicaltrials.gov/ct2/show/NCT03297827>) to assess the collective temporal trends among these cytokines/chemokines and other mediators after acute ischemic stroke (AIS).<sup>[9]</sup>

## MATERIALS AND METHODS

Twelve consecutive adult (>18 years) AIS patients presenting within 24 hours of the onset of symptoms were consented for this institutional (University of Iowa, Iowa City, IA) review board (IRB) approved study in accordance with the ethical standards of the Declaration of Helsinki. Blood samples were collected at the time of admission and after 24 hours. Exclusion criteria were; prior stroke or any other neurodegenerative or neuroinflammatory disease (Alzheimer's disease, Parkinson's disease [PD], multiple sclerosis [MS], etc.), acute infection, chronic inflammatory systemic illness, pregnancy, use of immunosuppressive medications or steroids, and a pre-morbid modified Rankin score  $\geq 5$ . Diagnosis of IS was based on clinical and radiological evidence at the discretion of the stroke neurologist. Patients clinical characteristics including stroke etiology and stroke severity were collected.<sup>[1,17]</sup> Detailed methodology, specimen collection, preparation, measurement, and additional laboratory processing are described previously.<sup>[9]</sup> Serum was separated from blood by centrifugation, aliquoted, and stored at  $-80^{\circ}\text{C}$ . Monocyte chemoattractant protein (MCP)/CCL2, ILs-8/CXCL8, IL-4, IL-10, IL-23, IL-36b/IF8, myeloperoxidase, tumor necrosis factor-alpha (TNF- $\alpha$ ), regulated and a normal T-cell expressed and secreted/CCL4, IL-1 $\beta$ , IL-6, IL-17A, IL-33, matrix metalloproteinase-9 (MMP-9), progranulin, and vascular endothelial growth factor were measured using human premixed multianalyte kit (R&D System, Minneapolis, MN); GMF- $\beta$  was measured by enzyme-linked immunosorbent assay (ELISA), Proteintech (Chicago, IL). All assays were performed as manufacturer's kit instructions, and the ELISA plate was read using a microplate reader (VERSAmax Microplate Reader, Molecular Devices, Sunnyvale, CA) and the multiplex assay plate was read using a BioRad BioPlex multiplexing system (Flow Cytometry Core

Facility, University of Iowa Carver College of Medicine, Iowa City, IA). Data will be made available on reasonable request from the corresponding author.

## RESULTS

Patient's demographics, stroke etiology, and severity are described. [Table 1] Majority (66.7%) were male, and the mean age of the cohort was 62.5 years. We observed an increasing trend in IL-6 (22 vs. 34 pg/ml), IL-8/CXCL8 (87 vs. 98 pg/ml), MMP-9 (16225 vs. 18450 pg/ml), and GMF- $\beta$  (999 vs. 3739 pg/ml) from baseline when compared with 24 hours, while other cytokines/chemokines and neural molecules remained stable overtime. The difference was insignificant [Table 2]. Moreover, patients presenting within 6 hours from their last known well had lower levels of MMP-9 (3287–20,215 vs. 16,435–20,936 pg/ml), IL-6 (9–39 vs. 17–68 pg/ml), IL-8/CXCL8 (34–143 vs. 31–630 pg/ml), and GMF- $\beta$  (300–2379 vs. 339–4209 pg/ml) as compare to patients presenting later [Figure 1]. We also observed that at admission, patients  $\leq 60$  years exhibit lower levels of plasma MCP-1/CCL2 (50–647 vs. 150–1159 pg/ml), IL-6 (9–25 vs. 20–68 pg/ml), and IL-8 (30–143 vs. 72–630 pg/ml) levels, when compared with patients >60 years old [Figure 2].

## DISCUSSION

Our pilot study observed an increasing trend overtime in IL-6, IL-8, MMP-9, and GMF- $\beta$  levels after an AIS. We

**Table 1:** Demographics and clinical characteristics of the study participants.

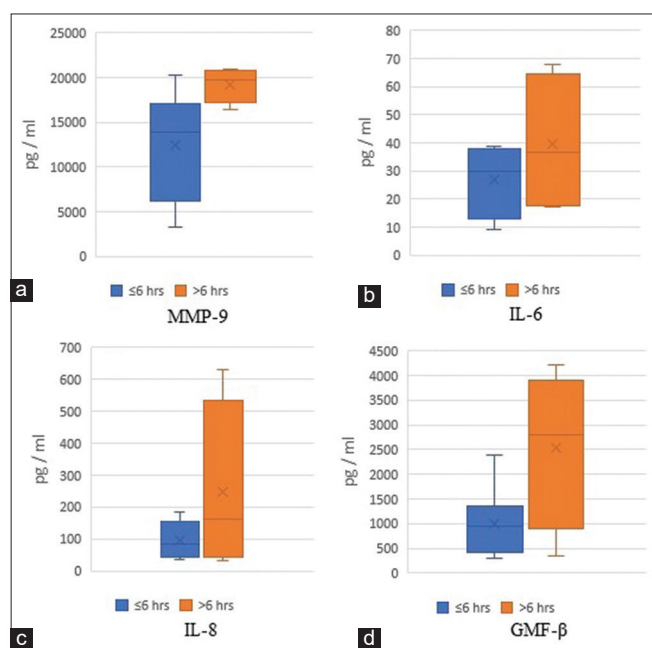
Number of participants	12
Age (mean $\pm$ SD)	62.5 $\pm$ 13.1
Gender	
Male	8 (66.7%)
Female	4 (33.3%)
Risk factors	
Diabetes mellitus	2 (16.6%)
Smoking	2 (16.6%)
History of coronary heart disease	4 (33.3%)
Hypertension	7 (58%)
Hypercholesterolemia	3 (25%)
NIH stroke scale	
Mild (0–7)	10 (83.3%)
Moderate (8–14)	1 (8.3%)
Severe ( $\geq 15$ )	1 (8.3%)
Stroke etiology	
LAA	3 (25%)
Cardioembolism	2 (16.6%)
Small vessel disease	3 (25%)
Stroke of other determined etiology	1 (8.3%)
Stroke of an undetermined etiology	3 (25%)
LKW to presentation (median mins, IQR)	360 (165–513)

LAA: Large artery atherosclerosis, LWK: Last known well

**Table 2:** Levels of cytokines/chemokines and other molecules at admission and after 24 hours among ischemic stroke patients admitted within 24 hours from last known well.

Cytokines/chemokines and other mediators	Baseline levels (pg/ml)	24 h levels (pg/ml)	P-value
MCP/CCL2	190 (52–518)	68 (26–470)	0.68
IL-8/CXCL8	87 (46–183)	98 (67–119)	0.8
IL-4	6 (6–7)	7 (6–7)	0.47
IL-10	13 (12–14)	14 (13–15)	0.52
IL-23	4744 (4511–5056)	4779 (4736–4924)	0.89
IL-36b/IF8	55 (40–67)	57 (32–76)	0.98
MPO	4104 (3631–4855)	4038 (3794–5835)	0.67
TNF- $\alpha$	14 (12–15)	15 (13–21)	0.16
RANTES/CCL4	16,599 (16,148–17,230)	16,781 (16,058–17,470)	0.32
IL-1 $\beta$	28 (18–50)	25 (23–45)	0.68
IL-6	22 (16–39)	34 (21–68)	0.32
IL-17a	8 (8–9)	8 (7–8)	0.6
IL-33	20 (18–22)	20 (18–23)	0.5
MMP-9	16,225 (12,867–20,145)	18,450 (11,845–19,124)	0.9
Progranulin	722 (567–1134)	876 (776–944)	0.71
GMF- $\beta$	999 (449–2589)	3739 (2829–4209)	0.16
VEGF	204 (163–270)	201 (194–422)	0.9

MCP: Monocyte chemoattractant protein, MPO: Myeloperoxidase, TNF- $\alpha$ : Tumor necrosis factor-alpha, RANTES: Regulated and a normal T-cell expressed and secreted, IL: Interleukin, MMP-9: Metalloproteinase-9, GMF- $\beta$ : Glia maturation factor beta, VEGF: Vascular endothelial growth factor



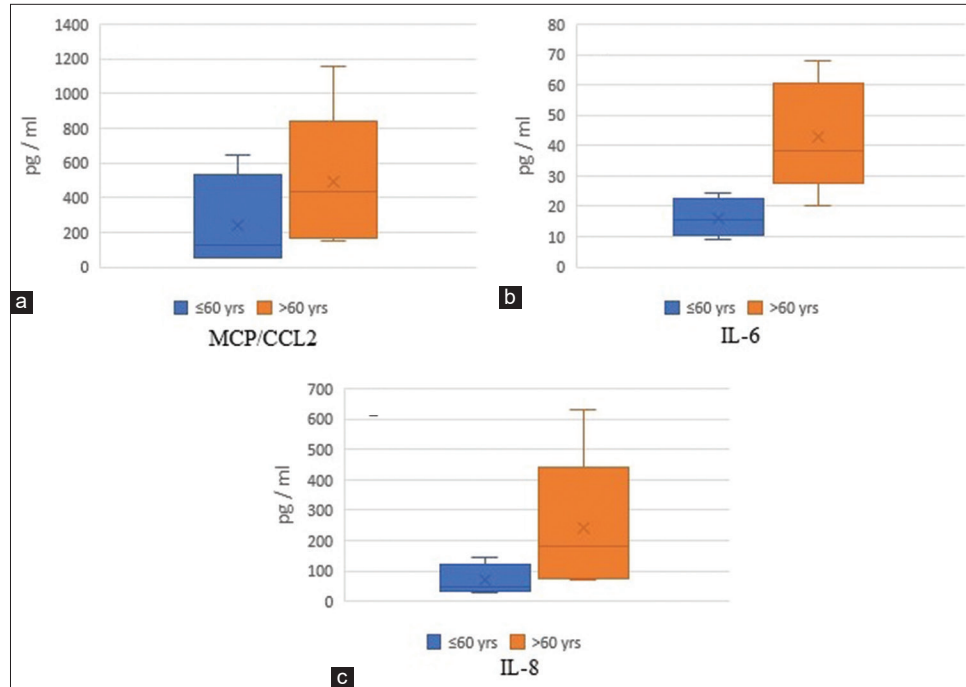
**Figure 1:** (a) Serum MMP-9 (b) IL-6, (c) IL-8, and (d) GMF- $\beta$  levels among patients presenting <6 h versus later after symptoms onset. MMP-9: Metalloproteinase-9, IL: Interleukin, GMF- $\beta$ : Glia maturation factor beta.

also observed that MCP/CCL2, IL-6, and IL-8 levels were increased among older AIS patients (>60 years).

This is the first report characterizing GMF- $\beta$  levels in AIS patients. GMF- $\beta$  is a pro-inflammatory protein expressed in the brain, especially in neurons, microglia,

and astrocytes.<sup>[16,28]</sup> Our study observed increasing levels of GMF- $\beta$  after AIS. GMF- $\beta$  is implicated in the activation and proliferation of microglial cells, consequently upregulating pro-inflammatory cytokines including TNF- $\alpha$ , IL-1  $\beta$ , and IL-6. We have recently shown that the absence of GMF- $\beta$  reduces inflammation and improves behavioral impairments in traumatic brain injury mice models.<sup>[2,25]</sup> In addition, we have also shown that GMF increases glial cell activation neuronal damage and that the absence of GMF decreased oxidative stress-associated neuroinflammation in the cell culture model of TBI.<sup>[3]</sup> Evidence indicate the role of GMF- $\beta$  as a pro-inflammatory trigger in the pathogenesis and progression of neurodegenerative and neuroinflammatory diseases including central nervous system injury, Alzheimer's, PD, and MS.<sup>[8,13,30]</sup> However, the exact mechanism involving the role of GMF- $\beta$  in AIS remains to be characterized.

We also observed increased levels of acute-phase pro-inflammatory cytokines, IL-6, IL-8, and MCP-1/CCL2. IL-6 is an endogenous pyrogen induced by IL-2, IL-4, prostaglandins, and interferon-gamma, while IL-8 is induced by macrophages and MCP-1/CCL2 by microglia/macrophages, endothelial cells, astrocytes, and neurons; acting as a chemoattractant for neutrophils, helping recruit inflammatory cells to the site of injury during the early phase inflammatory response. Studies have reported increased IL-6 and IL-8 levels as early as 1–4 hours after ischemic insult and remain elevated for the next 24–72 hours.<sup>[10,14]</sup> The REGARDS case-cohort study estimated an increased risk of IS incident with an incremental IL-6 levels being highest (HR: 2.4) among patients within



**Figure 2:** (a) MCP/CCL2, (b) IL-6, and (c) IL-8 levels among patients with age  $\leq 60$  years versus  $>60$  years at baseline. MCP: Monocyte chemoattractant protein, IL: Interleukin.

the fourth quartile of IL-6 levels.<sup>[12]</sup> The study also reported increased IL-8 levels; however, it was not associated with an increased risk of stroke incidence. This contrasted with the studies from Kostulas *et al.* and Ormstad *et al.*, where they reported significant association of increased IL-8 levels among AIS patients.<sup>[14,21]</sup> Our study also corroborates these findings characterizing increase IL-6 levels during the earliest phase of the ischemic injury, thus, supporting the notion that IL-6 may be an early inflammatory predictive marker of acute ischemic brain injury.<sup>[21]</sup> Moreover, results demonstrated that IL-6, IL-8, and MCP-1/CCL2 are also increased among older AIS patients. Similar observations were noted in REGARDS study where they observed increasing IL-6 levels with an incremental increased age (3.6 vs. 4.1 vs. 4.7 ng/ml;  $P < 0.001$  for  $<64$ ,  $64-75$ , and  $>75$  years, respectively) and the Health ABC study, where the authors reported an increased risk of IS (RR: 3.7) among older patients within the highest IL-6 tertile levels.<sup>[7]</sup> Likewise, Ormstad *et al.* observed a significant association of IL-8 levels with an increasing age ( $r = 0.52$ ,  $P < 0.001$ ). Similar observations have been noted for MCP-1/CCL2, establishing the association of increased levels with stroke severity and functional outcome after an AIS.<sup>[5]</sup> Population studies have also observed that an increased circulating level of MCP-1/CCL2 is an independent predictor of IS.<sup>[11]</sup> However, age-related characterization of MCP-1/CCL2 among AIS patients still remains to be elucidated. We also observed an increasing trend in MMP-9 as demonstrated by the previous studies,<sup>[18,23]</sup> peaking during the early phase within 12–24 hours.<sup>[19]</sup> MMP-9 are zinc metalloproteins

involved in the breakdown of the extracellular matrix. The previous studies have shown that MMP-9 is involved in the inflammatory response and BBB breakdown after an ischemic insult.<sup>[22]</sup> Increased MMP-9 levels after AIS are also associated with poor functional outcome and hemorrhage after thrombolytic therapy among AIS patients.<sup>[6,20]</sup>

These observations corroborate the role of these pro- and anti-inflammatory cytokines and other mediators in AIS patients, however, prognostication and clinical utility of these cytokines in predicting functional outcome, stroke types, and mortality are still debatable. Cytokines/chemokines constitute part of a complex immune system. Disruption in the intricate pathway implies downstream modulation on the dynamic feedback circuitry. As we expand our understanding of these molecular interactions, there still remains a lack of knowledge concerning integrative molecular alterations and their impact. The proposed comprehensive database comprising clinical, imaging, and molecular data would help expand our understanding and elucidate molecules that are systemically altered in AIS patients. “Cytokine Registry In Stroke Patients,” NCT03297827 was established to study the role of these cytokines and molecules and their trend variations in stroke patients, eventually leading to develop new therapeutic and management strategies.

## CONCLUSION

This preliminary study reports the trends in GMF- $\beta$ , a pro-inflammatory protein in AIS patients. We also corroborated

findings of acute-phase inflammatory cytokines/chemokines and protein, including IL-6, IL-8, MCP-1/CCL2, and MMP-9 during the early phase after an acute ischemic injury.

### Clinical trial registration

URL: <http://www.clinicaltrials.gov> Unique identifier: NCT03297827.

Cytokine Registry In Stroke Patients (CRISP) trial.

### Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

### Financial support and sponsorship

Stoppelmoor – Adams Stroke Education and Research Fund.

### Conflicts of interest

There are no conflicts of interest.

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