Increased expression of NLRP3 associate with elevated levels of HMGB1 in children with febrile seizures: a case control study

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Abstract

Background

High mobility group box-1 (HMGB1) is an endogenous danger signal that mediates activation of the innate immune response including NLR pyrin domain containing 3 (NLRP3) inflammasome activation and pro-inflammatory cytokine release. Although HMGB1 and NLRP3 have been implicated in the pathophysiology of seizures, the correlation between HMGB1 and NLRP3 has not been determined in children with febrile seizures (FS). To explore the relationship between extra-cellular HMGB1 and NLRP3 in children with FS, we analyzed serum HMGB1, NLRP3, Capase-1, and pro-inflammatory cytokines of patients with FS.

Methods

Thirty FS children and thirty age-matched febrile controls were included in this study. Blood was obtained from the FS children within 1 hour of the time of the seizure; subsequently, the content of HMGB1, NLRP3, Capase-1, interleukin (IL)-1β, interleukin (IL)-6, and tumor necrosis factor-α (TNF-α) were determined by enzyme-linked immunosorbent assay. The Mann-Whitney *U* test was used to compare serum cytokine levels between FS patients and controls. The Spearman's rank correlation coefficient was calculated to detect significant correlations between cytokine levels.

Results

Serum levels of HMGB1, NLRP3, Capase-1, IL-1β, IL-6, and TNF-α were significantly higher in FS patients than febrile controls ($p < 0.05$). Serum levels of HMGB1 were significantly correlated with levels of NLRP3 and Capase-1 (both, $p < 0.05$). Serum levels of Capase-1 were significantly correlated with levels of IL-1β ($p < 0.05$). Serum levels of IL-1β were significantly correlated with levels of IL-6 and TNF-α ($p < 0.05$).

Conclusions

HMGB1 are up-regulated in peripheral serum of FS patients, what may be responsible, at least in part, for the increased expression of NLRP3 and Caspase-1. Increased expression of Capase-1 was significantly associated with elevated serum levels of and IL-1β. Given that activated Caspase-1 directly regulates the expression of mature IL-1β and positively correlates with activation of NLRP3 inflammasome, our data suggest that increased levels of peripheral HMGB1 possibly mediate IL-1β secretion through the activation of NLRP3 inflammasome in children with FS. Thus, both HMGB1 and NLRP3 might be the potential target for preventing or limiting FS.

Background

Febrile seizures (FS) are the most common type of convulsions in infants or children typically 6 months to 5 years of age in association with a fever more than 100.4°F (38°C), who have no evidence of any central nervous system infection or metabolic disturbance. Its overall prevalence in children is approximately at 2%-14% worldwide [1]. Though single short FS (generalized seizures lasting < 15 min) are generally benign, prolonged FS (pFS) (FS lasting > 15 min) are more likely to develop into temporal lobe epilepsy (TLE) later in life [2–6]. Retrospective studies have shown that 30%-60% of patients with TLE have a history of pFS [7]. Therefore, understanding the pathogenesis of FS is clinically important, because, if it associate with subsequent epilepsy, then predictive biomarkers and preventive therapies might be feasible.

The high mobility group box 1 (HMGB1) is a highly conserved, ubiquitously expressed non-histone DNA-binding protein presenting in eukaryotic cells, which functions in stabilizing nucleosome and regulating gene transcription [8]. Previous studies revealed increased expression levels of serum HMGB1 in FS patients [9–11]. Ito et al. found that HMGB1 enhances hypothermia induced seizures and contributes to FS pathogenesis and plays an important role in the acquired epileptogenesis of secondary epilepsy associated with pFS [12], indicating that HMGB1 network contribute to the generation of FS in children. Furthermore, Choi and colleagues found that increased expression of HMGB1 was associated with elevated serum levels of interleukin (IL)-1β in children who had FS [11]. Yang and colleagues found that increased expression levels of HMGB1 and toll-like receptor (TLR) 4 showed a positive correlation with the elevated serum levels of tumor necrosis factor-α (TNF-α) and IL-1β in a rat model and in children with TLE [13]. Taken together, the data above indicate a correlation between HMGB1 and IL-1β. However, the nature of the links between HMGB1 and IL-1β has not been clarified in FS children.

The role of HMGB1 and IL-1β in generating and perpetuating seizures is well documented [14]. Physiologically, HMGB1 fundamentally resides in the nucleus translocates to the cytosol under stress conditions and is subsequently released into the extracellular [15]. Once released into the extracellular space, HMGB1 protein serves as a typical alarmin or damage associated molecular patterns (DAMPs) that binds to cell membrane pattern recognition receptors (PRRs), including TLR2, TLR4 and the receptor for advanced glycation end products (RAGE) predominantly expressed by activated monocytes, macrophages, T-lymphocytes in plasma, as well as microglia in the central nervous system [16]. Activation of TLR2 and TLR4 recruits MyD88 to activate several mitogen-activated protein kinases (MAPKs) that activate the downstream transcription factor nuclear factor kappa B (NF-κB). An activated NF-κB moves into the nucleus and promotes the formation of NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, thus enhancing the releasing of pro-inflammatory cytokine IL-1β [17–19]. Therefore, it was speculated that the extracellular HMGB1-activatied NLRP3 inflammasome possibly mediates IL-1β secretion in children with FS.

Given the correlation between HMGB1 and NLRP3 inflammasome, the aim of the current study was to investigate whether HMGB1-induced activation of NLRP3 inflammasome contributes to generation of FS by evaluating protein expression levels of HMGB1, NLRP3, Caspase-1, IL-1β, IL-6, and TNF-α in peripheral serum of FS patients.

Methods

Participants

A total of 30 FS patients (aged 6 months to 5 years) who visited to the department of pediatrics or emergency department of Affiliated Foshan Maternity and Child Healthcare Hospital, Southern Medical University from January 2019 and April 2020 were included in this study (Table 1). All individuals enrolled were unrelated ethnic Han Chinese who lived in southern China. None of the biological grandparents of the participants were from other ethnicities. Peripheral blood was obtained from patients within 1h of the time of seizure, and serum was immediately separated and frozen for subsequent cytokine assay. Patient inclusion criteria were age between 6 months and 5 years, body temperature ≥ 38.5°C, and patients with conditions known or suspected to cause seizures without fever were systematically excluded as our previous report [20].

Table 1
Clinical findings of febrile seizures and control children.

Variables	Febrile seizures (N = 30)	Febrile controls (N = 30)	*P* Value
Male/Female	20/10	16/14	0.292
Age (months)[a]	22.67 ± 11.08	28.33 ± 16.85	0.129
Severity of temperature (℃) [a]	39.16 ± 0.50	38.95 ± 0.61	0.216
C-reactive protein (mg/l)[a]	5.50 ± 8.85	6.57 ± 10.58	0.673
Leukocytes ($\times 10^9$/l)	10.65 ± 4.34	10.53 ± 6.57	0.936
Etiology of infection (viral/bacterial)	21/9	24/6	0.371
Duration of seizure			
<5min	22		
5–15min	8		
>15min	0		
[a] Mean ± Standard deviation.			

Clinical data for familial FS history, earlier FS attacks, as well as duration and semiology of FS were obtained from the patients' parents. Family history was regarded as positive when FS occurred in first-degree relatives. Laboratory findings, including complete blood counts, blood chemistry, and C-reactive protein, were checked at the time of seizure. Control samples were collected from children with febrile illness, but without convulsion. Control groups were matched for age and temperature criteria and had no

convulsions during the febrile illness and no known history of previous FS. Thirty controls were included in the final analysis. Control blood serum was collected and frozen as above. A diagnosis of FS was determined according to the International Classification of Diseases; Ninth Revision (ICD-9) codes (ICD-9 780.31, 780.32). All patients were followed up for more than 1 year.

The study was approved by the Ethics Committee of Affiliated Foshan Maternity　Child Healthcare Hospital, Southern Medical University (Approved number: FSFY-MEC-2018-016). All experiments and methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from the patient's legal guardians.

Cytokines Measurement

Four milliliters of blood was taken from the peripheral vessels of children in all the groups, and serum was obtained by centrifugation at 4000 rpm for 5 min at 4℃. The serum was then poured into acid-washed tubes and stored in a refrigerator at -80℃ until assay. Serum levels of HMGB1, NLRP3, Capase-1, and pro-inflammatory cytokines, including, IL-1β, IL-6, and TNF-a, were examined for FS patients and febrile controls using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Cusabio Biotech, Wuhan, China).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 19.0 for Windows (SPSS Inc., Chicago, IL, USA) program. The chi-square test or t-test was used for the comparison of clinical characteristics between FS patients and the controls. The Mann-Whitney *U* test was used to compare serum cytokine levels and laboratory findings between FS patients and controls. The Spearman's rank correlation coefficient was calculated to detect significant correlations between cytokine levels. GraphPad Prism v.7.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform the above tests. Values are expressed as means. Statistical significance was defined as $P < 0.05$.

Results

Table 1 shows the comparison of the selected patient's clinical data. Thirty FS children and 30 age matched control children with febrile illness without convulsion were included in this study. The mean age was 22.67 ± 11.08 months in the FS group and 28.33 ± 16.85 months in the febrile control group. Boys were more prevalent than girls were (respectively, 66.7% vs. 33.3%). All patients had their first FS attack and 73.3% (22/30) patients have duration of seizure < 5 min. There were no statistically significant differences between the two groups with respect to sex, age, severity of temperature, C-reactive protein, leukocytes and type of febrile disease ($p > 0.05$).

When we compared the FS group with the febrile control group, the serum levels of HMGB1 (Fig. 1a, p = 0.023), NLRP3 (Fig. 1b, p = 0.016), Capase-1 (Fig. 1c, p = 0.001), IL-1β (Fig. 1d, p = 0.007), IL-6 (Fig. 1e, p =

0.023), and TNF-α (Fig. 1f, p = 0.026) were significantly higher in the FS group than those in the febrile controls group (Table 2). Additionally, HMGB1 serum levels were significantly correlated with NLRP3, Capase-1, and IL-1β (respectively: Fig. 2a, 2b, and 2c; r = 0.814, r = 0.652, and r = 0.675; all $p < 0.001$). Capase-1 serum levels were significantly correlated with IL-1β (Fig. 2D, r = 0.589; $p < 0.001$). Serum IL-1β levels were significantly correlated with IL-6 and TNF-α levels (respectively: Fig. 2E and 2F; r = 0.564 and r = 0.668, both $p < 0.001$).

Table 2
Comparison of HMGB1, NLRP3, Capase-1, and cytokine levels between the febrile seizures group and febrile control group.

Variables	FS group [a] (N = 30)	Control group [a] (N = 30)	*P*-Value
HMGB1 (pg/ml)	399.84 (329.69-626.52)	304.56 (240.86-495.16)	0.023*
NLRP3 (pg/ml)	2330.15 (1956.64-3179.77)	1666.14 (1302.69-3231.45)	0.016*
Capase-1 (pg/ml)	2550.69 (1845.07-3560.79)	1504.81 (1134.57-2909.78)	0.001*
IL-1β (pg/ml)	87.90 (75.58-139.83)	65.31 (45.66-142.17)	0.007*
IL-6 (pg/ml)	40.87 (27.15–53.46)	25.06 (16.94–42.56)	0.003*
TNF-α (pg/ml)	181.05 (146.58-239.76)	134.39 (87.23-213.34)	0.026*
FS, febrile seizure; HMGB1, high mobility group box-1; IL-1β, interleukin-1beta; N, number; TNF-α, tumor necrosis factor α. The *P*-value is for Mann-Whitney U-test.			
[a]. median (interquartile range);			
*indicates a significant difference.			

Discussion

In the current study, we evaluated the expression of HMGB1 and NLRP3 inflammasome alongside Caspase-1 and IL-1β in FS patients compared with febrile controls. Despite their role in triggering neuroinflammatory response, HMGB1 and NLRP3 inflammasome have been poorly studied in FS. We confirmed previous studies of increased HMGB1 and NLRP3 expression in FS [11, 20], reporting increased expression of NLRP3 was associated with elevated plasma levels of HMGB1 in FS for the first time. Moreover, serum levels of other pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6 were significantly higher among our patients with FS.

Over the past two decades, neuroinflammatory response and the released pro-inflammatory cytokines, including HMGB1, IL-1β, TNF-α, and IL-6, has been implicated in the pathophysiology of FS [11, 21–24]. Of these pro-inflammatory cytokines, HMGB1 and IL-1β are key initiators of neuroinflammation contributing not only to the generation of FS, but also to the epileptogenesis after prolonged FS [12, 13, 25–30]. Experimental studies have shown that increased levels of HMGB1 and IL-1β contribute to chronic

inflammation, neuronal excitotoxicity and reduction of seizure threshold [12, 14, 17, 27–29, 31–33]. Moreover, HMGB1 and IL-1β levels are increased in epileptogenic brain tissue [13, 28, 30]. Interestingly, the levels of HMGB1 was positively correlated with the serum levels of IL-1β in a rat model and in children with TLE, while HMGB1 treatment of hippocampal neurons induced a significant increase in the levels of IL-1β [13]. In this study, we showed that patients with FS also display higher circulating (i.e. plasma) levels of HMGB1 and IL-1β. We also found that increased expression of HMGB1 was associated with elevated serum levels of IL-1β in peripheral blood after FS in children, indicating that there is a correlation between HMGB1 and IL-1β in children with FS. However, it was unclear how HMGB1 induce IL-1β.

HMGB1 is a highly conserved, ubiquitously expressed protein and could serve as a representative DAMPs [33]. DAMPs are pivotal for activation of NLRP3 inflammasome pathways [34]. Under normal circumstances, microglia and astrocytes express insufficient amounts and NLRP3 inflammasome exist in an inactive form. When cells are subjected to specific stimuli, such as lipopolysaccharide (LPS), NLRP3 inflammasome can be activated [35]. Assembly and activation of the NLRP3 inflammasome requires two functionally distinct steps: ‘priming’ and ‘activation’ [36]. Recent studies have demonstrated that HMGB1 could stimulate increased expression of NLRP3 to a critical level necessary for inflammasome formation, thus cause priming process of the NLRP3 inflammasome via TLR4/NF-κB signaling pathway [37], and cause sustained activation the NLRP3 inflammasome [32, 38]. NLRP3 inflammasome-dependent Caspase-1 activation is an important pathway related to IL-1β release [39], and has been implicated in the pathophysiology of neurological diseases, including Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and epilepsy [40–42]. In this study, we demonstrated a significant increase expression of NLRP3 in peripheral blood after FS in children, and a significant correlation between Capase-1 expression and serum levels of IL-1β, as previously described in our previous study [20]. As expected, we also observed a positive correlation between HMGB1 and NLRP3, and a positive correlation between HMGB1 and Capase-1. Given that activated Caspase-1 directly regulates the expression of mature IL-1β and positively correlates with activation of NLRP3 inflammasome [20], our results suggest that increased levels of peripheral HMGB1 possibly mediate IL-1β secretion through the activation of NLRP3 inflammasome in children with FS, and HMGB1/NLRP3 inflammasome/Caspase-1/IL-1β network may contribute to the generation of FS in children. Further studies are needed to further verify the mechanism.

In addition to IL-1β and HMGB1, inflammatory cytokines, including IL-6 and TNF-α, might also have facilitatory effect on the development of FS [43]. IL-1β can binds to IL-1 receptor type 1 (IL-1R1), a toll receptor family member, and induces the transcription of various genes that encode several downstream mediators of inflammation, including TNF-a and IL-6, via an NF-κB pathway [31, 44]. In this study, we found that IL-6 and TNF-α serum levels were significantly higher in our FS patients than in febrile children without seizures, and IL-6 and TNF-α positively correlated with the serum levels of IL-1β in children with FS. These observations, together with the experimental animal studies that transgenic mice over-expressing high amounts of IL-6 or TNF-α in astrocytes were reported to increased seizure susceptibility [45–48], support the possibility that IL-1β is a pluripotent pro-inflammatory cytokine and the key interleukin involved in FS pathogenesis.

Conclusion

In conclusion, our present study showed that HMGB1 are up-regulated in peripheral serum of FS patients, what may be responsible, at least in part, for the increased expression of NLRP3 and Caspase-1. Increased expression of Capase-1 was significantly associated with elevated serum levels of and IL-1β. Our data suggest that increased levels of peripheral HMGB1 possibly mediate IL-1β secretion through the activation of NLRP3 inflammasome in peripheral blood after FS. Thus, both HMGB1 and NLRP3 inflammasome might be the potential target for preventing or limiting FS.

Abbreviations

HMGB1	High mobility group box-1
NLRP3	NLR pyrin domain containing 3
FS	febrile seizures
IL-1β	interleukin-1β
IL-6	interleukin-6
TNF-α	tumor necrosis factor-α
TLE	temporal lobe epilepsy
TLR	toll-like receptor
DAMPs	damage associated molecular patterns
PRRs	pattern recognition receptors
RAGE	receptor for advanced glycation end products
MAPKs	mitogen-activated protein kinases
NF-κB	nuclear factor kappa B
LPS	lipopolysaccharide

Declarations

Ethics approval and consent to participate The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the ethics committee of Affiliated Foshan Maternity Child Healthcare Hospital, Southern Medical University (Approve number: FSFY-MEC-2018-016).

Written informed consent to participate in this study was provided by the participants' legal guardian.

Consent for publication This study adhered to the guidelines of the International Committee of Medical Journal Editors regarding patient consent for research or participation, and all the individuals or legal guardians undergoing testing consented to their data being used for research.

Data availability The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Competing interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions Zhi-Gang Liu contributed to the conception of the study. Xing-Guang Ye and Feng-Zhi She contributed to the interpretation of clinical data and drafting of the figures and the manuscript. Dong-Ni Yu, Li-Qian Wu, and Yan Tang examined the patient and participated in drafting of the manuscript. Ben-Ze Wu, Shi-Wei Dong, Jie-Min Dai, Xing Zhou, and Zhou-Lian Qin contributed to the collection and analysis of clinical data. Zhi-Gang Liu provided critical review and substantially revised the manuscript.

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Figures

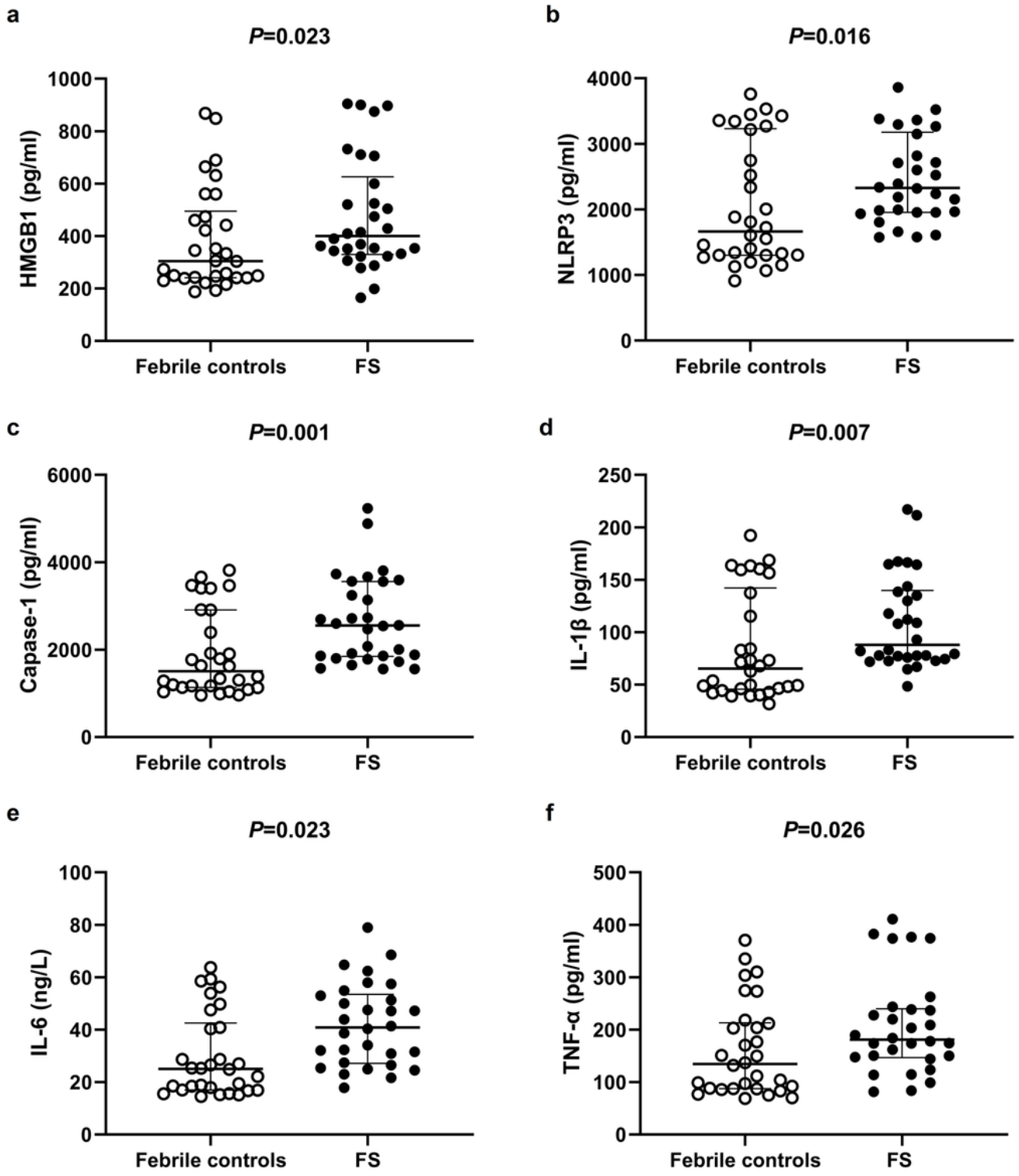

Figure 1

Comparison of serum levels of HMGB1 (**a**), NLRP3 (**b**), Capase-1 (**c**), IL-1β (**d**), IL-6 (**e**), and TNF-α (f) between febrile seizures group and control group. The median (interquartile range) values are indicated by three parallel lines. Analysis of serum cytokine levels between two groups was performed by Mann-Whitney U test. HMGB1, NLRP3, Capase-1, IL-1β, IL-6, and TNF-α levels are significantly high in febrile seizures group than control group ($p < 0.05$ indicates a significant difference).

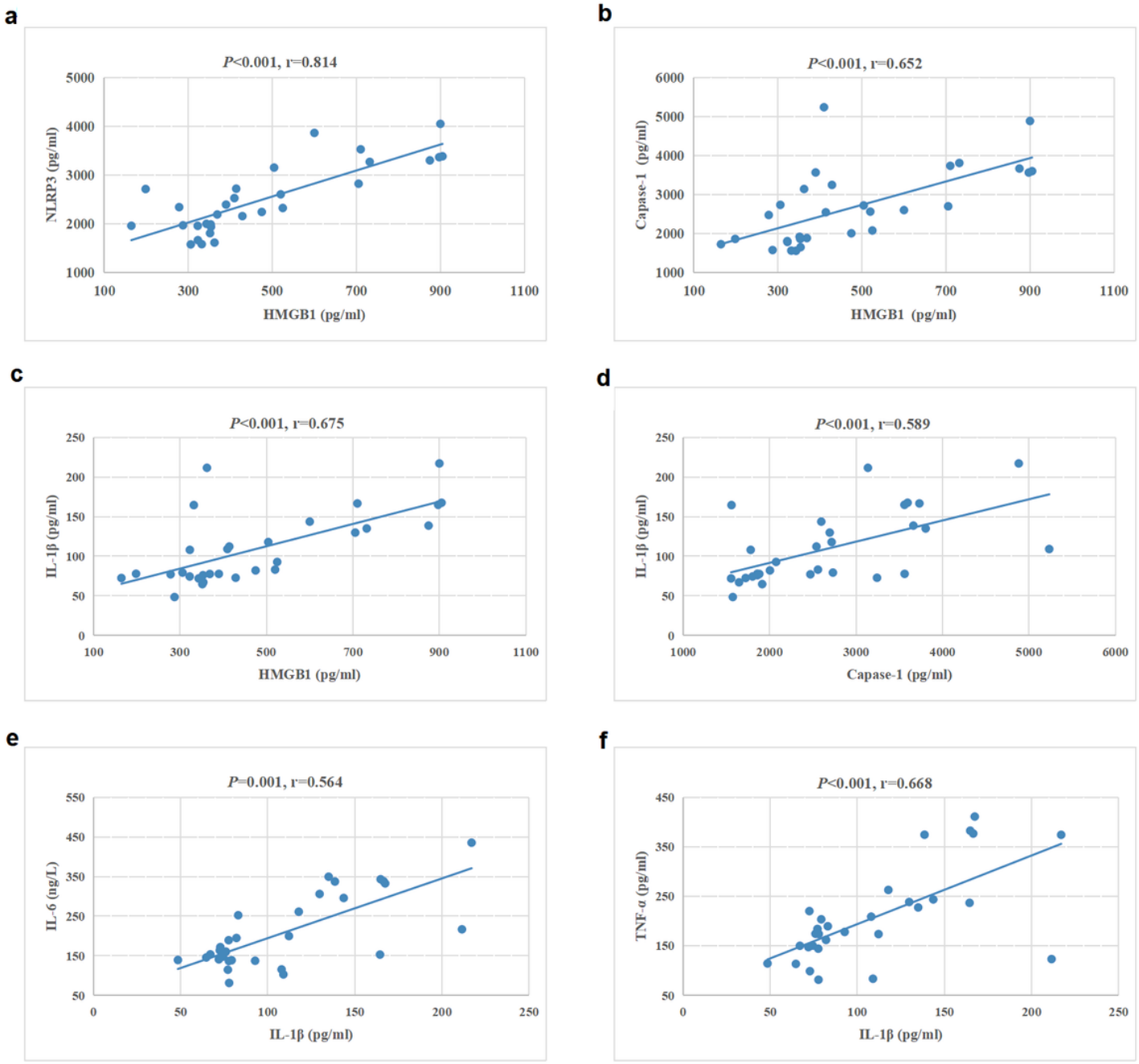

Figure 2

Correlation between serum cytokine levels in febrile seizuresgroup. (**a-f**) Correlation between serum levels of NLRP3 and HMGB1 (**a**), Capase-1 and HMGB1 (**b**), IL-1β and HMGB1 (**c**), and IL-1β and Capase-1 (**d**), IL-6 and IL-1β (**e**), TNF-a and IL-1β (**f**) in febrile seizures children. HMGB1 levels are significantly correlated with NLRP3, Capase-1, and IL-1β levels (all, $p < 0.05$, r = 0.814, r = 0.652, and r = 0.675, respectively). Capase-1 levels are significantly correlated with IL-1β levels ($p < 0.05$, r = 0.589). IL-1β levels are significantly correlated with IL-6 and TNF-a levels (both, $p < 0.05$, r = 0.564 and 0.668, respectively).