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Plasma High-Mobility Group Box-1 and Galectin-9 in Patients with Trauma and Their Prognostic Potentials

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Damage-associated molecular patterns (DAMPs) are endogenous molecules released from damaged tissues and elicit strong inflammatory responses of the host. Hence, they are supposed to play essential roles in the disrupted immune homeostasis after traumatic injuries. We examined plasma levels of galectin-9 (Gal-9), an immune checkpoint molecule as well as a DAMP, and a representative DAMP, high-mobility group box-1 (HMGB-1) in the trauma patient. Gal-9 was very high at admission, declined swiftly, and reached the normal level in 48 hours, while HMGB-1 was highest at admission, declined in 24 hours, then stagnated through the assessment period of 7 days with a level much higher than that of healthy subjects. The concentration of these DAMPs at admission correlated well with each other. HMGB-1 correlated with 6 prognostic parameters compared to only 2 for Gal-9, which reflects HMGB-1 but not Gal-9 could discriminate between survived and deceased patients. Receiver-operating characteristic (ROC) curve analysis demonstrated that plasma HMGB-1 possesses a moderate prognostic potential to discriminate deceased patients from survivors. Collectively, HMGB-1 has a potential to make a valuable blood biomarker for trauma, possibly in combination with other blood biomarkers.

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Introduction

Severe trauma is one of the leading causes of mortality worldwide. Patients who survive immediate or early death by severe injury develop systemic inflammation status resulting from injury, followed by disturbed immune homeostasis (Osuka et al. 2014). Damage-associated molecular patterns (DAMPs) are a vital mediator of inflammation released during traumatic injury and are supposed to play non-negligible roles in patients' immune alterations after trauma (Relja et al. 2018).

High-mobility group box-1 (HMGB-1) has been identified as a representative DAMP because of its atypical relocation to the extracellular milieu by infection or tissue dam-

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age and its prominent proinflammatory function. Hence, it has been a focus of research as a potential severity marker and therapeutic target for rescuing patients with inflammatory diseases (Tang et al. 2023). Galectin-9 (Gal-9) is an animal lectin that functions as an immune checkpoint molecule and was recently annotated as a DAMP (Dapat et al. 2017). Similar to HMGB-1, the blood concentration of Gal-9 has been demonstrated to increase in patients with various infectious or chronic inflammatory diseases (Iwasaki-Hozumi et al. 2021; Moar and Tandon 2021; Shete et al. 2024).

Interestingly both HMGB-1 and Gal-9 share many characteristics in common: ubiquitous expression, locating intracellularly but externalizing via non-conventional secretion pathway (Moar and Tandon 2021; Tang et al. 2023), having plural receptors of which T-cell mucin and immunoglobulin-3 (Tim-3) and Toll-like receptor 4 (TLR4) are common for both (Liang et al. 2021; Moar and Tandon 2021; Tang et al. 2023), activation of myeloid-derived suppressor cells (MDSCs) (Dardalhon et al. 2010; Tang et al. 2023), activation of bactericidal activity of neutrophils (Vega-Carrascal et al. 2014; Tang et al. 2023), promoting autophagy (Wiersma et al. 2015; Tang et al. 2023), stimulating coagulation (Zhi et al. 2022; Tang et al. 2023), etc. Conversely, there are several differences in their functions: Gal-9 activates dendritic cells (DCs) by binding to Tim-3 (Anderson et al. 2007), while the binding of HMGB-1 to Tim-3 abrogates HMGB-1/DNA complex from activating DCs (Chiba et al. 2012); Gal-9 activates regulatory T cells (Tregs) by binding to CD44 (Wu et al. 2014), while HMGB-1 suppresses Tregs by binding to either TLR4 or receptor of advanced glycation end product (RAGE) (Zhang et al. 2020; Zhou et al. 2021). However, contradicting reports exist as to the function of HMGB-1 on Tregs, suggesting context-dependent complex mechanisms (Wild et al. 2012; Hubert et al. 2021). Besides, HMGB-1 was reported to induce Gal-9 expression via the TLR4 pathway in the tumor microenvironment, which helps tumors escape from immune surveillance by employing the immunosuppressive function of Gal-9 (Teo Hansen Selnø et al. 2021), which adds further implications about the close relationships of both DAMPs in controlling immunity.

The objective of this study is to examine the plasma levels of HMGB-1 and Gal-9 in patients with severe trauma and evaluate their predictive potential as a severity marker. Similar preceding reports on HMGB-1 exist (Cohen et al. 2009; Peltz et al. 2009; Polito et al. 2016; Sun and Xia 2019), but Gal-9 measurement in trauma has, to our knowledge, not yet been reported.

Materials and Methods

Patients

We performed a prospective, observational study of patients admitted to an academic, tertiary care emergency center from July 2011 to December 2013. Trauma patients with an abbreviated injury scale (AIS) score (Gennerelli and Wodzin 2008) of 3 or higher were enrolled in this study. Patients were excluded if they were younger than 20 years or were unable to give written informed consent. Hence, this study consisted of 78 patients including ten patients deceased in 28 days after admission. The ethics committee of our institution approved this study, and all study subjects provided written informed consent. The study is registered with the University Hospital Medical Information Network Clinical Trials Registry, UMIN-CTR ID UMIN00006714. The following scoring systems calculated patients' status and the severity of injury within 24 hours of admission: Glasgow coma scale (GCS), which composed of three parameters of responses of eye, verbal and motor (Zuercher et al. 2009); Injury severity score (ISS) (Baker et al. 1974); Revised trauma score (RTS) (Champion et al. 1989) and Probability of survival (PS) (Shackford et al. 1987).

Data collection

Patients' change of status over time was examined on days 1, 2, 3, 5, and 7 of admission by the following scoring systems; Sequential organ failure assessment (SOFA) score (Vincent et al. 1996); Acute physiology and chronic health evaluation II (APACHE-II) score (Knaus et al. 1985); SIRS positive number (SIRS+ number) (Rangel-Frausto et al. 1995); Japanese association for acute medicine disseminated intravascular coagulation (JAAM DIC) score (Gando et al. 2006) and International society on thrombosis and haemostasis DIC (ISTH overt DIC) score (Taylor et al. 2001). Bloods were drawn for cytological and biochemical studies on admission days 1, 2, 3, 5, and 7. A part of the blood samples was centrifuged at 3,000 rpm for 10 min, and the supernatants were stored as plasma samples at –80°C until use. According to the manufacturer's instructions, HMGB-1 concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit from Shino-Test (Sagamihara, Kanagawa, Japan). Gal-9 measurement by ELISA was described elsewhere (Chagan-Yasutan et al. 2009). Gal-9 ELISA used in this study recognizes an intact full-length form of the protein. The other clinical tests were outsourced to SRL Inc. (Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Microsoft Excel and Prism 8 (GraphPad software, version 8.4.3). Mann-Whitney U and Kruskal-Wallis tests with Dann's correction assessed the differences between the two groups and among multiple groups, respectively. The correlation between a set of data was examined using Spearman's rank correlation coefficient. Receiver operating characteristic (ROC) curve analysis was conducted to analyze the ability of DAMPs and other diagnostic tests to discriminate between the surviving and deceased groups of patients.

Results

Characteristics of patients

Seventy-eight patients with severe trauma were

enrolled in this study, including ten patients deceased in 28 days after admission. Table 1 summarizes the characteristics of patients in a form to compare between survived and deceased groups. The deceased group consists of a higher percentage of patients with head injuries that would reflect the injury and consciousness scores of the patients. Both groups differed significantly in well-established severity scores for trauma, i.e., ISS, RTS, and PS, and other severity scores, i.e., GCS, SOFA, and APACHE-II scores. ISTH overt DIC score, platelet count, and leucocyte elastase-generated cross-linked fibrin degradation products (E-XDP) related to coagulopathy, differed significantly. Lactate and base excess, metabolic signatures of severe trauma, were also different. Insulin-like growth factor 1 (IGF-1) is a versatile growth factor with the repair function of damaged tissues that were lower in the deceased group. Interleukin (IL)-6, IL-10, and interferon (IFN)-γ are known to increase in various inflammatory conditions but did not differ between the groups.

Plasma levels of HMGB-1 and Gal-9

Fig. 1 shows the kinetics of plasma levels of HMGB-1 and Gal-9. HMGB-1 was highest at admission with a median of 10.9 ng/mL (IQR 7.7-18.5) and rapidly decreased to 5.6 ng/mL (IQR 4.0-7.2) at 24 hours and remained near this level until day 7, which was much higher than the 1.7 ng/mL or the less in normal subjects (Fukami et al. 2009). Whereas plasma Gal-9 was highest on day 1 with a median value of 315 pg/mL (IQR 158-750), declined to 73 pg/mL (IQR 1-183 pg/mL) on day 2, and then reached the normal level thereafter. Fig. 2 examined the correlation between HMGB-1 and Gal-9 at day 1. Spearman's rank correlation test showed a moderate level of correlation ($R = 0.5701$, $P < 0.001$). We further explored the correlation analysis of these DAMPs with other diagnostic tests or assessments. Table 2 summarizes the items that exhibited statistically significant correlation with either of the DAMPs. HMGB-1 included all the items for which Gal-9 showed correlations. Among these items, 6 items, namely ISS, GCS (motor), lactate, base excess, ISTH overt DIC score, and APACHE-II, distinguished survived and deceased patients (Table 1). Gal-9 correlated only two items among them.

Prognostic potential of HMGB-1 and Gal-9 in severe trauma

HMGB-1 level was lower in the survived group than

Table 1. Characteristics of patients with trauma.

Patient information	All patients (n = 78)	Survived (n = 68)	Deceased (n = 10)	*P* value
Age: Years, median (IQR)	44.0 (25.0-63.8)	41.5 (24.0-63.0)	65.5 (43.8-76.3)	0.0621
Male sex: no. (%)	56 (71.8%)	49 (72.1%)	7 (70.0%)	> 0.9999
Head injury (%)	42 (53.8%)	33 (48.5%)	9 (90.0%)	0.0351
ISS		25 (16-32)	44 (28-47)	0.0009
RTS		7.6 (6.6-7.8)	4.7 (4.1-5.5)	0.0001
PS		93.3 (81.6-97.8)	24.3 (13.6-40.9)	< 0.0001
GCS (Eye)		3 (3-4)	1 (1-1)	0.0068
GCS (Verbal)		4 (2-5)	1 (1-1)	0.0005
GCS (Motor)		5 (5-6)	2 (1-2)	0.0002
SOFA		3 (2-5)	7 (6-8)	0.0017
APACHE-II		12 (7-15)	24 (22-28)	< 0.0001
IGF-1 (ng/mL)		108 (77-139)	74 (62-86)	0.0186
IL-6 (pg/mL)		114.0 (59.5 -269.5)	145.0 (52.2-422.5)	0.7423
IL-10 (pg/mL)		9.5 (1.0-46.5)	15.0 (5.8-69.3)	0.4026
IFN-γ (IU/mL)		0.05 (0.05-0.05)	0.05 (0.05-0.05)	0.8930
SIRS+ number		3 (2-3)	2 (1-4)	0.7650
Lactate (mmol/L)		1.8 (1.4-2.9)	4.4 (3.3-6.1)	0.0003
Base Excess (mmol/L)		–1.4 (–4.3-0.7)	–6.8 (–9.3- –2.4)	0.0022
Platelet (x 10^3/μL)		204 (130-246)	128 (111-192)	0.0494
E-XDP (U/mL)		4.6 (3.2-7.0)	8.5 (6.1-17.2)	0.0182
JAAM DIC		4 (3-4)	4 (3-5)	0.3203
ISTH overt DIC		3 (3-3)	4 (3-5)	0.0332

IQR, Interquartile range; ISS, Injury severity score; RTS, Revised trauma score; PS, Probability of survival; GCS, Glasgow coma scale; SOFA, Sequential organ failure assessment score; APACHE-II, Acute physiology and chronic health evaluation II score; IGF-1, Insulin-like growth factor 1; SIRS, Systemic inflammatory response syndrome; SIRS+ number, SIRS criteria positive number; E-XDP, leucocyte elastase-generated cross linked fibrin degradation products; JAAM DIC, Japanese association for acute medicine disseminated intravascular coagulation score; ISTH overt DIC, International society on thrombosis and haemostasis DIC score.

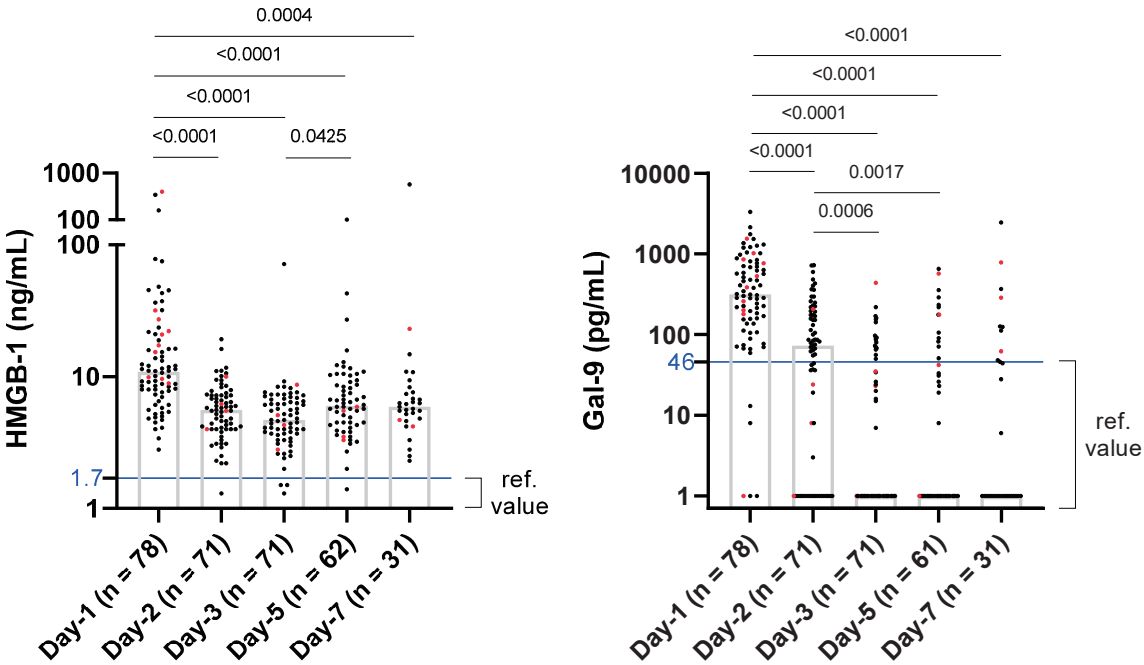

Fig. 1. Changes in the plasma concentration of HMGB-1 and Gal-9 after admission.
The top of the bar chart shows median value. Reference value (ref. value) indicates the concentration in which 95% of healthy subjects fall in from preceding literatures in which the same ELISA kits were used (Chagan-Yasutan et al. 2009; Fukami et al. 2009). Red dots express deceased patients. Statistical significance was assessed using Kruskal-Wallis test with Dann's correction and *P* values less than 0.05 were shown.

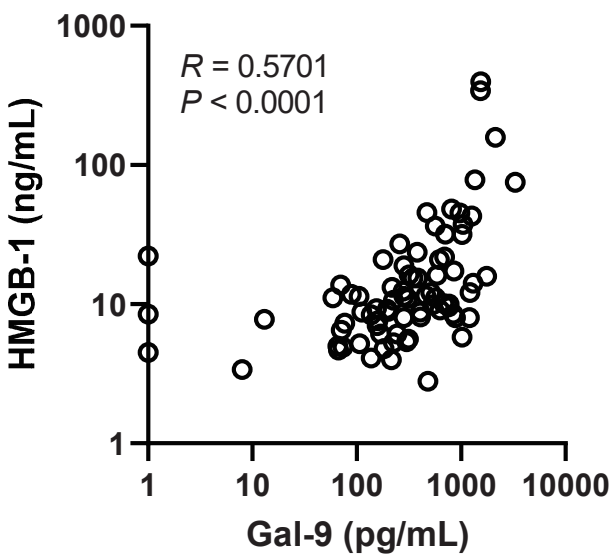

Fig. 2. The correlation between plasma HMGB-1 and Gal-9 at day 1 was examined using Spearman's rank correlation coefficient.
The result indicated a moderate correlation, suggesting a meaningful relationship between the two DAMPs.

in the deceased group, with median concentrations of 10.1 ng/mL (IQR 6.9-16.0) and 19.1 ng/mL (IQR 11.3-26.1), respectively ($P = 0.0231$). At the same time, Gal-9 did not differ between the groups (Fig. 3). To assess the diagnostic power of the DAMPs, receiver operating characteristic (ROC) curve analysis was performed (Fig. 4). The area under the curve (AUC) of HMGB-1 was 0.7213, which indicates the moderate diagnostic power of this DAMP to distinguish the deceased group of patients with trauma from the survivors. A similar level of AUC was seen in IGF-1, E-XDP, and ISTH overt DIC with the AUC values of 0.7316, 0.7324, and 0.7096, respectively, and they were higher than that of platelet counts with the AUC value of 0.6934 (Table 3).

Discussion

HMGB-1 and Gal-9 are multifunctional proteins categorized as DAMP and involved in complex immune modulation. They are located intracellularly but are released into the circulation in various diseases such as infection, cancer, and autoimmunity, and are assumed to play a role in the development of or recovery from the diseases. They may be involved in the pathological process of trauma as well, especially taking their functions of immune modulation (either pro- or anti-inflammatory) and coagulation into account. In this study, we examined plasma levels of HMGB-1 and Gal-9 for 7 days after the patient's admission. HMGB-1 was very high on day 1, promptly decreased in 24 hours, and then kept this level until the end of the study on day 7, the level being much higher than that of healthy subjects. We reported a similar kinetics of plasma HMGB-1 in post-cardiac arrest syndrome (PCAS) (Omura et al. 2016). Contrary to these observations, serum HMGB-1 in sepsis was reported to stay high during the study period of 1-6 days (Sundén-Cullberg et al. 2005). Therefore, in trauma

Table 2. Various parameters correlated with HMGB-1 or Gal-9 measurement.

	HMGB-1 (Day-1)		Gal-9 (Day-1)	
	R	*P* value	*R*	*P* value
ISS	0.3618	0.0011	0.1907	0.0945
GCS (Motor)	–0.3805	0.0006	–0.0151	0.8954
IL-6	0.3604	0.0012	0.4319	< 0.0001
IL-10	0.5165	< 0.0001	0.4845	< 0.0001
IFN-γ	–0.2671	0.0181	–0.2693	0.0171
Lactate	0.3466	0.0019	0.2043	0.0728
Base Excess	–0.4145	0.0002	–0.3552	0.0014
JAAM DIC	0.3056	0.0065	0.4314	< 0.0001
ISTH overt DIC	0.4122	0.0002	0.4067	0.0002
APACHE-II	0.2680	0.0177	0.0807	0.4827

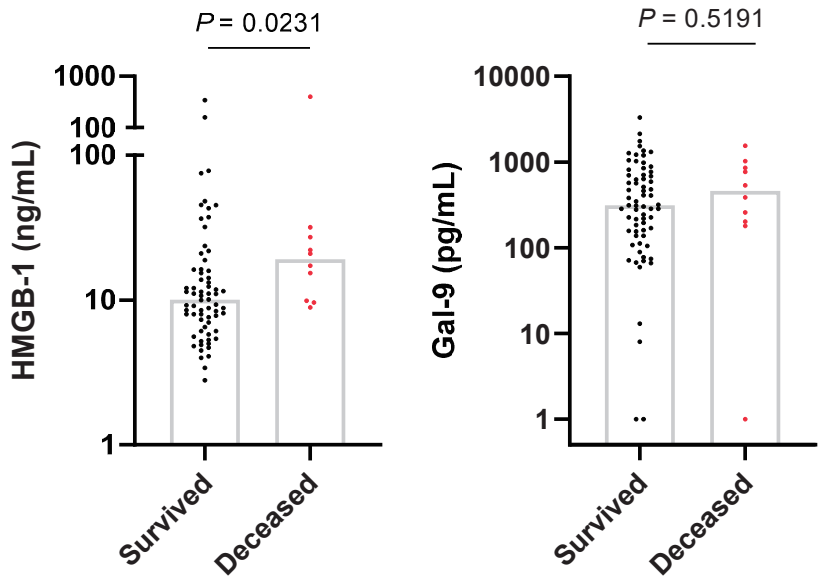

Fig. 3. Plasma concentration of HMGB-1 and Gal-9 in survived and deceased groups.
The measurements at day 1 were compared and assessed using Mann-Whitney U test. The top of the bar chart shows median value.

and PCAS, large amounts of HMGB-1 may be released predominantly from injured tissues by physical or ischemia/reperfusion insult, at least in the initial phase. On the other hand, active release may predominate in sepsis from the pathogen-stimulated tissues. Gal-9 was very high at admission but swiftly decreased to the normal level in 48 hours. This sharp decline may involve proteolytic clearance of Gal-9. The concentration of HMGB-1 and Gal-9 were correlated moderately, and they both correlated with several other diagnostic tests in common. These findings are consistent with the overlapping functions of these DAMPs. However, HMGB-1 correlated more diagnostic test items that dissociate between survived and deceased patients, which reflected that HMGB-1, but not Gal-9, showed a diagnostic power to distinguish the group of patients who will die of trauma.

Several reports examined the blood concentration of HMGB-1 in patients with trauma. However, the reported concentrations are varied, even within the studies where the same ELISA kit was utilized. This might be explained, at least to some extent, by the different timing of blood collection after the injury (Peltz et al. 2009), different severity of patients, and potential differences in handling of samples (Ottestad et al. 2019). As a blood biomarker, all the publications so far admit an association between HMGB-1 concentration and severity of trauma except one of the very initial publications (Peltz et al. 2009). Therefore, our study would add one more positive data to bolster the potential clinical utility of HMGB-1 measurement in trauma.

Blood biomarkers are generally useful because it is minimally invasive, and allows real-time objective evaluation of patients largely irrespective of the quality of assessors. If HMBG-1 measurement is to be used in a real clinical setting, it could be combined with other biomarkers to increase the performance. HMGB-1 alone demonstrated high sensitivity of 1.000, but the specificity was only

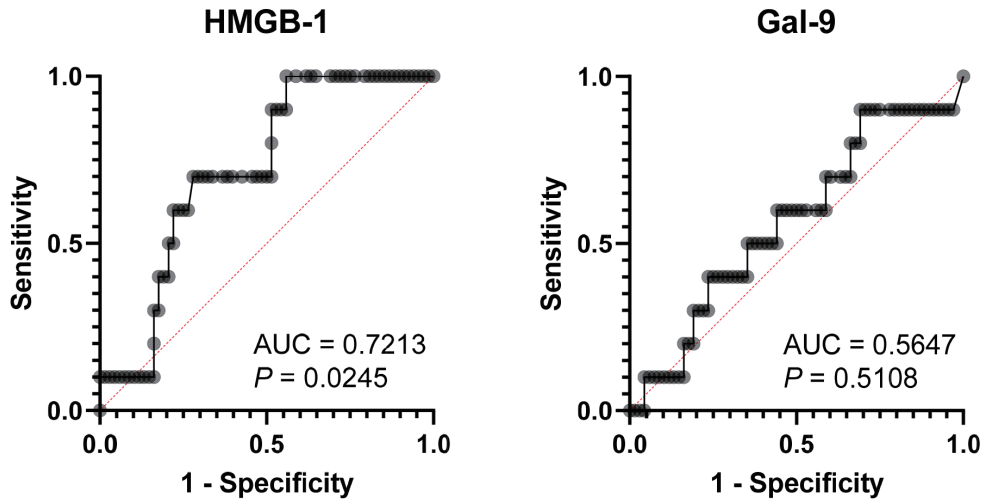

Fig. 4. Prognostic potential of plasma HMGB-1 and Gal-9.
Measurements at the admission of patients were separated between survived and deceased groups in 28 days and used for ROC curve analysis, and area under the curve (AUC) and *P* values were calculated.

Table 3. Prognostic accuracy of each parameter to distinguish the deceased group of patients with trauma by ROC curve analysis.

	AUC	*P* value	Cut off value	Sensitivity	Specificity
HMGB-1	0.7213	0.0245	8.85 ng/mL	1.0000	0.4412
Gal-9	0.5647	0.5108	174 pg/mL	0.9000	0.3088
ISS	0.8272	0.0009	37	0.7000	0.9118
RTS	0.8772	0.0001	6.3	0.9000	0.7647
PS	0.9529	< 0.0001	68.25	1.0000	0.8824
GCS (Eye)	0.7662	0.0068	1.5	0.8000	0.7941
GCS (Verbal)	0.8404	0.0005	1.5	0.9000	0.8382
GCS (Motor)	0.8669	0.0002	3.5	0.9000	0.9118
SOFA	0.8088	0.0017	6.5	0.7000	0.8676
APACHE-II	0.9265	< 0.0001	20.5	0.9000	0.9118
IGF-1	0.7316	0.0186	97.5 ng/mL	0.9000	0.5735
Lactate	0.8581	0.0003	3.15 mmol/L	0.8000	0.7794
Base excess	0.8015	0.0022	–1.65 mmol/L	1.0000	0.5735
Platelet	0.6934	0.0494	$216.5 \times 10^3/\mu L$	1.0000	0.4265
E-XDP	0.7324	0.0182	5.15 U/mL	0.9000	0.5735
ISTH overt DIC	0.7096	0.0332	3.5	0.5000	0.7941

0.4412 (Table 3). Combination with lactate improves the specificity at the slight cost in the sensitivity (Fig. 5A). Moreover, combination with base excess and platelet was as good as PS, the scoring system used in clinical practice with the highest performance in the current study (Fig. 5B,C). These results are only preliminary assessment but encouraging enough for the further studies with increased number of patients.

Recently, HMGB-1 was shown to change the function by oxidation (Ferrara et al. 2020). Reduced form works for tissue repair, while oxidized form works for pro-inflammation, and both forms are supposed to interconvert depending on the local redox status. So, different forms of HMGB-1 may be released at different stages of trauma. The ELISA system used in the current study cannot distinguish these two forms. Gal-9 measurement in this study utilized an ELISA system that recognizes only full-length Gal-9. Gal-9 is easily degraded by proteolysis and lose the activity, and hence we thought the active form should be focused. However, in conditions where inflammation and coagulopathy are being developed, proteolysis may be accelerated. In these situations, the measurement of degraded Gal-9 may provide a better diagnosis than that of active but fragile full-length Gal-9 (Padilla et al. 2020). Focusing on such molecules of HMGB-1 and Gal-9 may demonstrate different figures in terms of the diagnostic potentials.

The limitation of this study is that the setting is observational and the number of patients is small for stable esti-

A: HMGB-1 + Lactate

	Decease	Survive
Positive	8	10
Negative	2	58

$P < 0.0001$
Sensitivity = 0.8000
Specificity = 0.8529

B: HMGB-1 + Base Excess + Platelet

	Decease	Survive
Positive	10	9
Negative	0	59

$P < 0.0001$
Sensitivity = 1.0000
Specificity = 0.8676

C: PS

	Decease	Survive
Positive	10	8
Negative	0	60

$P < 0.0001$
Sensitivity = 1.0000
Specificity = 0.8824

Fig. 5. Prognostic accuracy of HMGB-1 in combination with other blood biomarkers.
The contingency tables were generated based on the following diagnosis: A) HMGB-1 and lactate, B) HMGB-1, base excess, and platelet, C) PS, the scoring methods used in clinical practice and demonstrated the highest performance in the current dataset (Table 3). The cutoff values of each biomarker or score were calculated by ROC curve analysis (Table 3) and the statistical significance was assessed by Fisher's exact test.

mation using a quasi-experimental analysis such as propensity score matching. Increasing the number of patients and measuring the aforementioned specific molecules of HMGB-1 and Gal-9 would warrant the future studies.

In conclusion, HMGB-1 demonstrated a potential to be a prognostic biomarker for traumatic death, especially in combination with other blood biomarkers for higher performance. Further study with an increased number of patients is recommended to clarify the utility. Meanwhile, Gal-9, at least the measurement of the full-length form, did not predict the outcome of traumatic patients.

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Conflict of Interest

The authors declare no conflict of interest.

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