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The mechanism of serum cell-free DNA release in postmortem subjects and novel markers to estimate the postmortem intervals

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CRediT authorship contribution statement

Junko Fujihara: Conceptualization, Methodology, Visualization, Investigation, Writing - original draft, Funding acquisition, Supervision.

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Abstract

Cell-free DNA (cfDNA) is DNA released from dying cells into the serum. The aim of the present study is to elucidate the mechanism of cell-free DNA (cfDNA) release in postmortem subjects by assaying Cytokeratin 18 (M30 and M65), cyclophilin A (CyPA), and myeloperoxidase (MPO) levels and to evaluate whether these levels are useful as markers for estimating postmortem intervals. Serum (n = 54) was sampled from postmortem autopsied Japanese subjects. cfDNA was extracted and M30, M65, CyPA, and MPO in serum were assayed by enzyme-linked immunosorbent assay. Significantly higher serum levels of M30, M65, and MPO were observed in postmortem subjects than in living subjects. Although the difference was smaller, CyPA was also significantly higher in postmortem subjects than in living subjects. In addition, serum M30 and MPO levels were significantly correlated with cfDNA concentrations in postmortem subjects. Furthermore, M30 levels slightly increased according to the postmortem interval, and MPO levels at 2.5 days were significantly higher than those at <2 days. The result of regression analysis revealed a significant difference between M30/MPO levels and postmortem intervals. These findings suggested that elevated levels of cfDNA in postmortem subjects are released by apoptosis and neutrophils via NETosis and that M30 and MPO levels can be used as markers to estimate postmortem intervals.

Keywords: cfDNA, CyPA, M30, M65, MPO, postmortem subject

1. Introduction

Postmortem intervals (also known as time since death) indicate the time that has elapsed since a person's death. Estimating the postmortem interval is the most important factor in a forensic investigation because it helps to reveal the circumstances of death and to provide decisive evidence in criminal cases[1]. Today, postmortem intervals are usually estimated using conventional methods such as algor mortis (the state of body cooling), rigor mortis (postmortem muscle stiffness), and livor mortis (postmortem lividity)[2,3]. However, these methods can be used only for estimating relatively short postmortem intervals. Several studies have proposed the estimation of postmortem intervals by using postmortem biochemical markers. However, to date, no postmortem biochemical markers have been reliably utilized as scientific evidence[4].

Cell-free DNA (cfDNA) is DNA released from dying cells into the serum, plasma, and other body fluids[5,6]. We have previously reported elevated cfDNA concentrations in the plasma of patients with myocardial infarction and cardiac angina as well as DNA laddering in the plasma cfDNA of cardiac diseases patients; this ladder fragment pattern distribution of myocardial infarction patients differs from that of other cardiac disease patients[7]. We recently revealed greatly increased concentrations of cfDNA consisting of 150–200 bp fragments in postmortem samples compared with

living subjects and found cfDNA fragments (larger than 10,000 bp) derived from necrosis in only two postmortem subjects[8].

cfDNA is derived from apoptotic or necrotic processes[9–12]. Cytokeratin 18, a cytoskeletal protein of the epithelium, is broken down by caspases during apoptosis and non-apoptotic cell death. The caspase-cleaved cytokeratin 18 form M30 is used to detect apoptosis, while M65 is used to detect both apoptosis and necrosis[13]. Meanwhile, the cytosolic peptidyl-prolyl *cis*-trans isomerase cyclophilin A (CyPA) has been proposed as a biomarker of necroptosis[14]. NETosis is a cell-death pathway involving the release of neutrophil extracellular traps (NETs) that respond to sterile inflammation, infection, or hypoxia[15,16], and cfDNA is postulated to be released by NETosis. One neutrophil enzyme released by NETosis is myeloperoxidase (MPO)[12]. We recently suggested that plasma cfDNA from individuals with cardiac disease is released by neutrophils via NETosis, not just by apoptosis[17].

Biochemical processes in the postmortem period have not been fully elucidated[17]. However, it has been reported that extensive biochemical changes due to the supply of oxygen being cut off lead to apoptosis, altered enzymatic reactions, and degradation of cells[18, 19]. Nevertheless, the origin and mechanism of cfDNA in postmortem subjects remains unclear. Therefore, in this study, we investigated the origin and mechanism of

cfDNA release in postmortem subjects by assaying cytokeratin 18 (M30 and M65), CyPA, and MPO levels. Moreover, we evaluated whether these levels are useful as markers for estimating postmortem intervals.

2. Materials and methods

2.1. Study subjects

Cardiac blood (n = 54) was collected in 15-mL polypropylene centrifugal tubes from postmortem subjects during autopsies performed at Shimane University from 2016 to 2020 (Table 1). After investigation by the police, the bodies of postmortem subjects were stored in the mortuary refrigerator at 4°C for 12 to 48 h before autopsy was performed. The post-mortem intervals of all subjects were within 4 days. To compare cytokeratin 18 (M30 and M65), CyPA, and MPO levels, blood samples were also collected from control subjects, and we used data on cardiac disease patients from our previous study[17]. The control venous blood samples were collected from the forearm cutaneous vein of healthy Japanese volunteers (n = 24) in EDTA tubes. The serum of autopsy subjects and the plasma of control subjects were prepared by whole blood centrifugation at 500 × g for 10 min, and the obtained serum and plasma were collected in 1.5-mL polypropylene microtubes and stored at −80°C until analysis. Data from our

previous study obtained from cardiac disease patients (n = 59) who had presented at the emergency department at Shimane University Hospital (Shimane, Japan) between 2016 and 2018 [17]. Information on the control subjects and cardiac disease patients is shown in Table 1. No subjects in this study were cancer patients. In both the present study and our previous study[17], informed consent was acquired from all healthy volunteers and cardiac disease patients. The study protocol and the use of samples from healthy volunteers, cardiac disease patients, and autopsy cases were approved by the Human Ethics Committee of Shimane University School of Medicine. The data collection methods were in accordance with approved guidelines.

2.2. Isolation of serum cfDNA

cfDNA was extracted from serum (1 mL) using a Maxwell® RSC cfDNA Serum Kit (Promega Corp., Madison, WI) as in our previous studies[6,7]. Using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), spectrophotometric absorbances at 260 nm (A_{260}), 280 nm (A_{280}), and 320 nm (A_{320}) were measured. A_{260}/A_{280} were used to evaluate purity. cfDNA concentrations were calculated according to the following formula: $(A_{260} - A_{320}) * 50 * (10/0.51)$.

To assess the quality and integrity of the isolated cfDNA as well as the efficiency of

the Maxwell® RSC cfDNA kit, all extracted cfDNA was visualized using MCE-202 MultiNA automated microchip-based electrophoresis using MultiNA Viewer software (Shimadzu Corp., Kyoto, Japan). Samples were run with the reagents (separation buffer and DNA marker reagent) from the DNA-1000 kit (Shimadzu Corp.). Fragments measuring 150–200 bp were observed in the control subjects; three fragments measuring 150–200, 300–400, and 500–600 bp, respectively, were observed in almost all cardiac disease patients; and fragments measuring 150–200 bp were frequently observed while those measuring 300–400 bp were sometimes observed in postmortem subjects.

2.3. Enzyme-linked immunosorbent assay

Serum M30 and M65 levels were assayed with the M30 Apoptosense and M65 Epideath enzyme-linked immunosorbent assay (ELISA) kits, respectively (Peviva, Bromma, Sweden). ELISA was used to measure serum CyPA levels (RayBiotech Life, Inc., Norcross, GA) and MPO levels (Proteintech Group, Inc., Rosemont, IL). Absorbance at 450 nm was measured using a Multiskan™ GO Microplate Spectrophotometer.

2.4.Statistical analysis

M30, M65, CyPA, and MPO levels among the groups were compared by factorial measure analysis of variance (ANOVA). If ANOVA revealed an overall significant difference among the groups, then between-group differences (i.e., cfDNA concentration in healthy controls/cardiac disease patients/postmortem subjects; M30, M65, CyPA, and MPO levels by postmortem intervals; and M30, M65, CyPA, and MPO levels by cause of death) were analyzed with the Tukey–Kramer test. This test can be used in a wide range of applications to determine which pairs of means have statistically significant differences with high statistical power. Because this study aimed to evaluate a marker for estimating postmortem intervals, a Tukey–Kramer test with high statistical power was used. Spearman's correlation coefficient was used for correlation analysis. All analyses were performed using Bell Curve for Excel (Social Survey Research Information Co. Ltd., Tokyo, Japan).

3. Results

3.1. M30, M65, CyPA, and MPO levels in postmortem subjects

Figure 1 shows a comparison of M30, M65, CyPA, and MPO levels in postmortem subjects with those in controls subjects and cardiac disease patients, which was reported previously[17]. Serum M30 levels were 18-fold higher in postmortem subjects than in

control subjects and 12-fold higher than in cardiac disease patients ($p < 0.01$) (Fig. 1a); M65 levels were 33-fold and 18-fold higher in postmortem subjects than in control and cardiac disease patients, respectively ($p < 0.01$, Fig. 1b); and MPO levels were 30-fold higher in postmortem subjects than in control subjects and 8-fold higher than in cardiac disease patients ($p < 0.01$) (Fig. 1d). Although the difference was smaller than for M30, M65, and MPO, CyPA levels were still significantly higher in postmortem subjects than in healthy controls and cardiac disease patients ($p < 0.01$, 2-fold) (Fig. 1c).

3.2. Correlation analysis

Figure 2 shows the correlations between cfDNA levels and biomarker (M30, M65, CyPA, and MPO) levels in the plasma of control subjects and cardiac disease patients and the serum of postmortem subjects. In control subjects, a significant correlation was not observed between plasma cfDNA level and biomarker levels (Fig. 2a–d). However, a significant correlation ($p < 0.05$) was observed between plasma cfDNA and MPO levels in cardiac disease patients (Fig. 2h). In postmortem subjects, serum M30 and MPO levels were positively correlated with cfDNA concentrations ($p < 0.001$, Fig. 2i and 2l). Table 2 shows the results of correlation analysis among the five markers in postmortem subjects. A significant positive correlation was observed between serum M30 and M65 ($p < 0.01$)

levels in postmortem subjects, in addition to the above correlations.

3.3. M30, M65, CyPA, and MPO levels by postmortem interval

Figure 3 shows the associations of M30, M65 CyPA, and MPO levels with postmortem intervals. M30 and MPO levels showed a tendency to increase according to postmortem interval (Fig. 3a and 3d). MPO levels at 2.5 days were significantly higher than those at 0.5, 1, 1.5, and 2 days (Fig. 3d). The regression analysis was performed between M30/MPO levels and postmortem intervals, and the results were statistically significant for M30 levels vs. postmortem intervals ($p < 0.01$) and for MPO levels vs. postmortem intervals ($p < 0.05$). In contrast, M65 levels were constant regardless of the postmortem interval, except the 1.5-day postmortem interval (Fig. 3b). CyPA levels were also constant regardless of the postmortem interval (Fig. 3d).

3.4. Relationship of M30, M65, CyPA, and MPO levels with cause of death

Figure 4 shows the M30, M65, CyPA, and MPO levels by cause of death (data with <3 samples were excluded). Although no significant differences were observed, serum M30 levels in asphyxia and death from internal causes were higher than those for other causes of death. In contrast, serum M65 levels, CyPA levels, and MPO levels were

constant regardless of the cause of death.

4. Discussion

Apoptosis is a controlled type of cell death that can be caused by mild injuries and antemortem changes[20]. In contrast, necrosis is difficult to prevent and can be caused by severe injuries and postmortem changes[21]. We previously reported that cfDNA concentrations were greatly increased in postmortem subjects compared with healthy controls (90-fold) and cardiac disease patients (9-fold)[8]. To our knowledge, no studies have examined M30, M65, CyPA, and MPO levels in the serum of postmortem subjects. In the present study, M30, M65, CyPA, and MPO levels in serum from autopsied samples were compared with those in the plasma cfDNA obtained from control subjects and cardiac disease patients in our previous study[17]. Elevated cfDNA concentrations have been reported in cancer patients, and thus cfDNA is considered to be a potential biomarker for cancer diagnosis and prognosis[5, 22]. In the present study, subjects with cancer were excluded to avoid the effect of cancer on cfDNA, M30, M65, CyPA, and MPO levels. Significantly higher serum M30 (18-fold), M65 (33-fold), MPO (30-fold), and CyPA (2-fold) levels were observed in postmortem subjects than in living subjects (Fig.1). These results suggest that apoptosis and NETosis are dominantly induced in

postmortem samples. Previously, we reported a significant relationship between MPO and cfDNA concentrations and that M30, M65, and CyPA were not correlated with cfDNA concentrations in cardiac disease patients[17]. In the present study, serum M30 and MPO levels were significantly correlated with cfDNA concentrations and M65 level was significantly correlated with M30 level in postmortem subjects (Fig. 2 and Table 2). These results demonstrated that elevated levels of cfDNA from postmortem subjects are released by apoptosis and neutrophils via NETosis.

Previous studies have attempted to identify biomarkers that could be used to estimate post-mortem intervals. Donaldson et al. reported that blood concentrations of hypoxanthine, ammonia, NADH, and formic acid increased with time and suggested that these may be potential markers for postmortem intervals[19]. Swain suggested that the potassium concentration in the vitreous humor is the single best parameter to estimate postmortem intervals[23]. Marrone et al. identified a total of nine potential postmortem interval biomarkers by using mass spectrometry[24]. Moreover, other studies have attempted to estimate postmortem intervals by evaluating the degradation pattern of the DNA[25-27], RNA[28-30], and proteins[31-33]. To our knowledge, no study has reported the postmortem changes in M30, M65, CyPA, and MPO levels. In the present study, M30, M65, CyPA, and MPO levels in serum from postmortem subjects were investigated

according to postmortem interval (Fig. 3). M30 levels showed a tendency to increase according to postmortem intervals (Fig.3a). A previous study reported that apoptosis was positive in autopsied subjects with postmortem intervals of <20 h, and negative in autopsied subjects with postmortem intervals of >20 h who died of acute myocardial infarction[34]. In the present study, it is speculated that apoptotic processes occur in postmortem subjects, plateauing at 2–3 days postmortem, and dropping to baseline after 4 days postmortem. MPO levels at 2.5 days were significantly higher than those at <2 days (Fig. 3d). To our knowledge, there are no previous studies on MPO levels in postmortem subjects. It is speculated that the NETosis process peaked at 2.5 days postmortem and dropped to baseline thereafter. The result of regression analysis revealed a significant difference between M30/MPO levels and postmortem intervals. In contrast, M65 and CyPA levels were relatively constant regardless of the postmortem interval. These results suggest that M30 and MPO levels might be useful as markers to estimate postmortem intervals. As mentioned above, previous studies have attempted to estimate postmortem intervals by using biomarkers and the degradation patterns of DNA, RNA, and proteins. These previous methods involved flow cytometry, reverse transcription PCR, mass spectrometry, and so on. Compared with these previous methods, M30 and MPO levels can be measured more easily, for example, by performing ELISA on blood

samples.

5. Conclusions

In this study, cytokeratin 18 (M30 and M65), CyPA, and MPO levels were measured to elucidate the origin and mechanism of cfDNA release in postmortem subjects. Serum M30, M65, and MPO levels were greatly increased in postmortem subjects compared with living subjects. Serum M30 and MPO levels were significantly correlated with cfDNA from postmortem subjects, suggesting that cfDNA in postmortem subjects is released by apoptosis and neutrophils via NETosis. M30 levels exhibited a tendency to increase according to postmortem interval, and MPO levels were significantly elevated at a 2.5-day postmortem interval compared with those at <2 days. In addition, regression analysis revealed a significant difference between M30/MPO levels and postmortem intervals. These results suggest that M30 and MPO levels might be useful as markers to estimate postmortem intervals. This study is the first to report the origin and mechanism of cfDNA release in postmortem subjects by assaying M30, M65, CyPA, and MPO levels in serum from postmortem subjects and to suggest that both M30 and MPO might be useful as biomarkers to estimate postmortem intervals.

Declaration of ethics

The study protocol and the use of samples from healthy volunteers, cardiac disease patients, and autopsy cases were approved by the Human Ethics Committee of Shimane University School of Medicine (20110803-2; 20151214-2).

Declarations of Conflicts of interest

The authors declare that they have no conflicts of interest.

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Table 1. Information of autopsied subjects (cause of death, age, sex, and postmortem intervals), healthy control, and cardiac disease patients

Subjects	*n*	Age, years*	Male/female	Postmortem interval
Autopsied subjects				
Asphyxia	30	67 (8–98)	17/13	0.5–4 d
Traumatic shock	5	55 (20–85)	4/1	1.5–3 d
Head injury	4	65 (25–81)	3/1	0.5–2.5 d
Cerebral stroke	3	48 (32–53)	0/3	0.5–2.5 d
Poisoning	2	29, 84	2/0	1–2.5 d
Hypothermia	1	86	0/1	1–4 d
Sudden manhood death syndrome	1	29	1/0	0.5 d
Septic shock	1	38	1/0	1.5 h
Death from internal causes (unknown)	7	46 (44–69)	3/4	1.5–3.5 d
Healthy control subjects	24	46 (27–55)	23/1	-
Cardiac disease patients [13]	59	76 (44–93)	28/31	-

*Data are median (interquartile range) and each age when the number of samples is less than two. d, day(s).

Table 2. Correlation analysis among five markers in postmortem subjects

	Cytokeratin 18 (M30)	Cytokeratin 18 (M65)	Cyclophilin A	Myeloperoxidase
Cell-free DNA	r = 0.5397 ***P* < 0.001**	r = 0.1246 *P* = 0.9874	r = −0.0245 P = 0.6487	r = 0.3064 ***P* < 0.001**
Cytokeratin 18 (M30)	-	r = 0.1674 ***P* < 0.01**	r = 0.2449 *P* = 0.1210	r = 0.1947 *P* = 0.1230
Cytokeratin 18 (M65)	-	-	r = 0.2498 *P* = 0.0535	r = 0.0913 *P* = 0.1992
Cyclophilin A	-	-	-	r = 0.0875 *P* = 0.4293

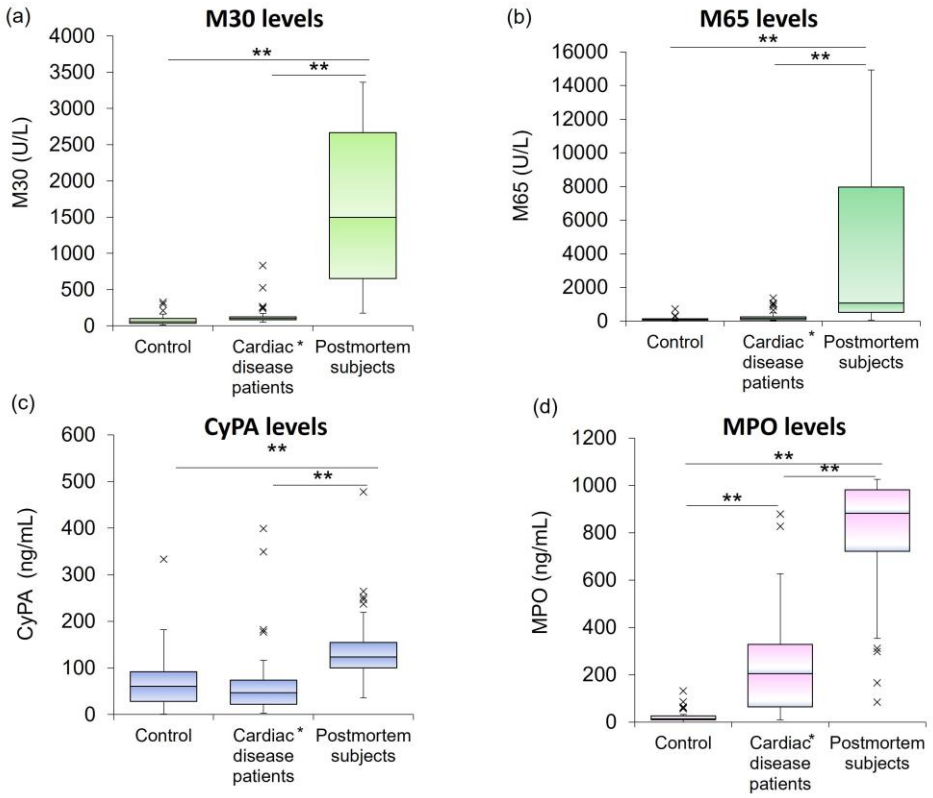

Fig. 1. Levels of (a) M30, (b) M65, (c) CyPA, and (d) MPO in plasma from cardiac disease patients and healthy control subjects and serum from postmortem subjects. $^{**}p < 0.01$ when compared using the Tukey–Kramer test. The top and bottom of each box show the 25th and 75th percentile, respectively. The line of the box is the median and the error bars indicate the 5th and 95th percentiles. *Data from our previous study [17].

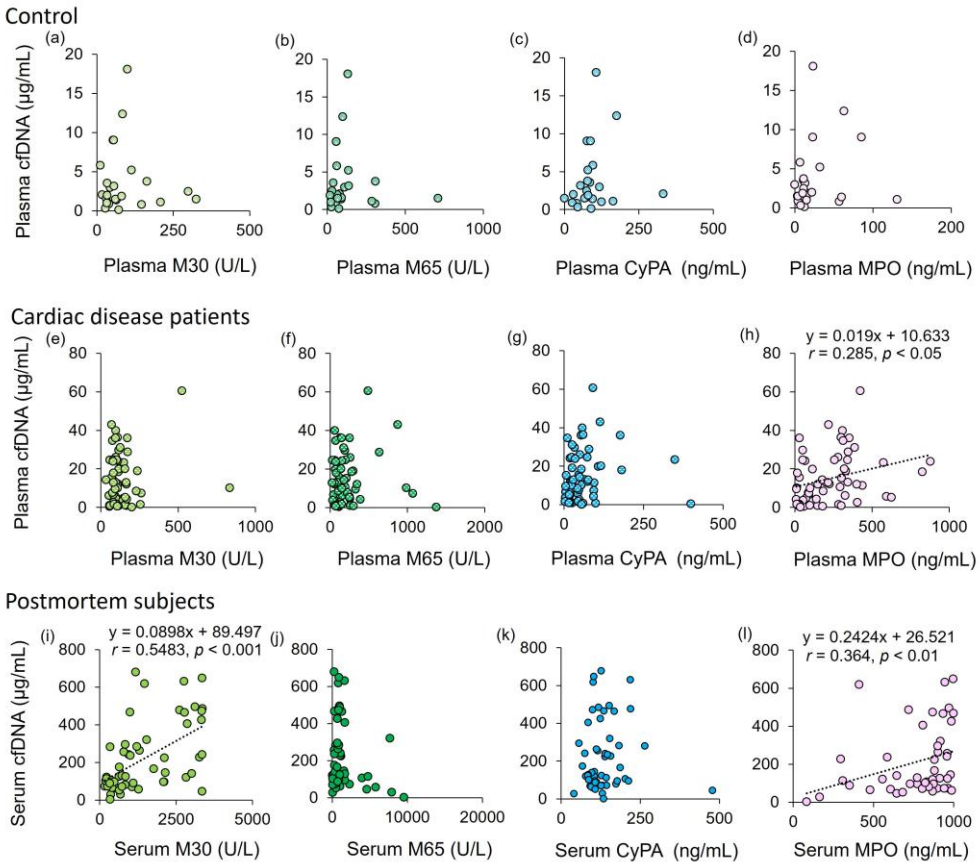

Fig. 2. Correlations between cfDNA concentration and four biomarkers (M30, M65, CyPA, and MPO) in the plasma of control subjects (a–d), plasma of cardiac disease patients (e–h), and postmortem subjects (i–l).

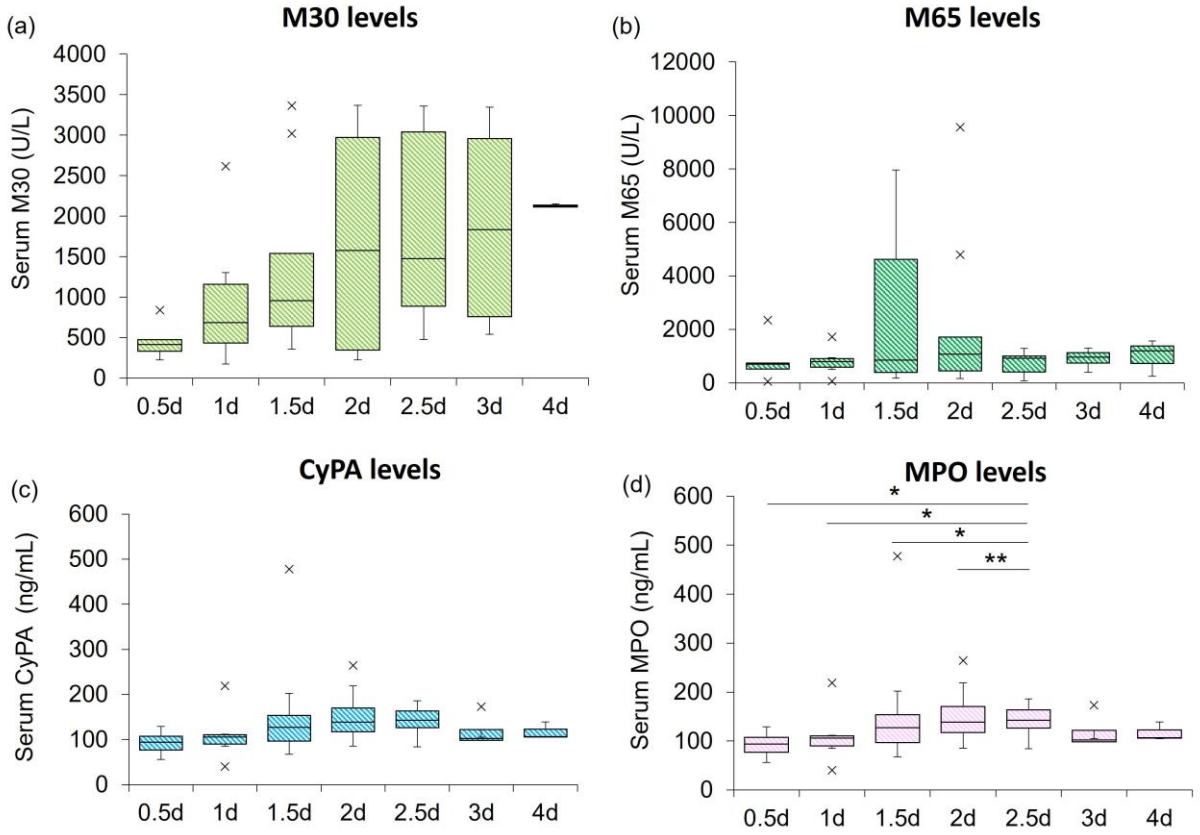

Fig. 3. Box plot of serum M30 levels (a), M65 levels (b), CyPA levels (c), and cfDNA concentrations (d) in postmortem subjects according to postmortem interval. *$p < 0.05$, **$p < 0.01$ when compared using Tukey–Kramer test. The top and bottom of each box represent the 25th and 75th percentile, respectively. The line of the box is the median and the error bars show the 5th and 95th percentiles. d, day(s).

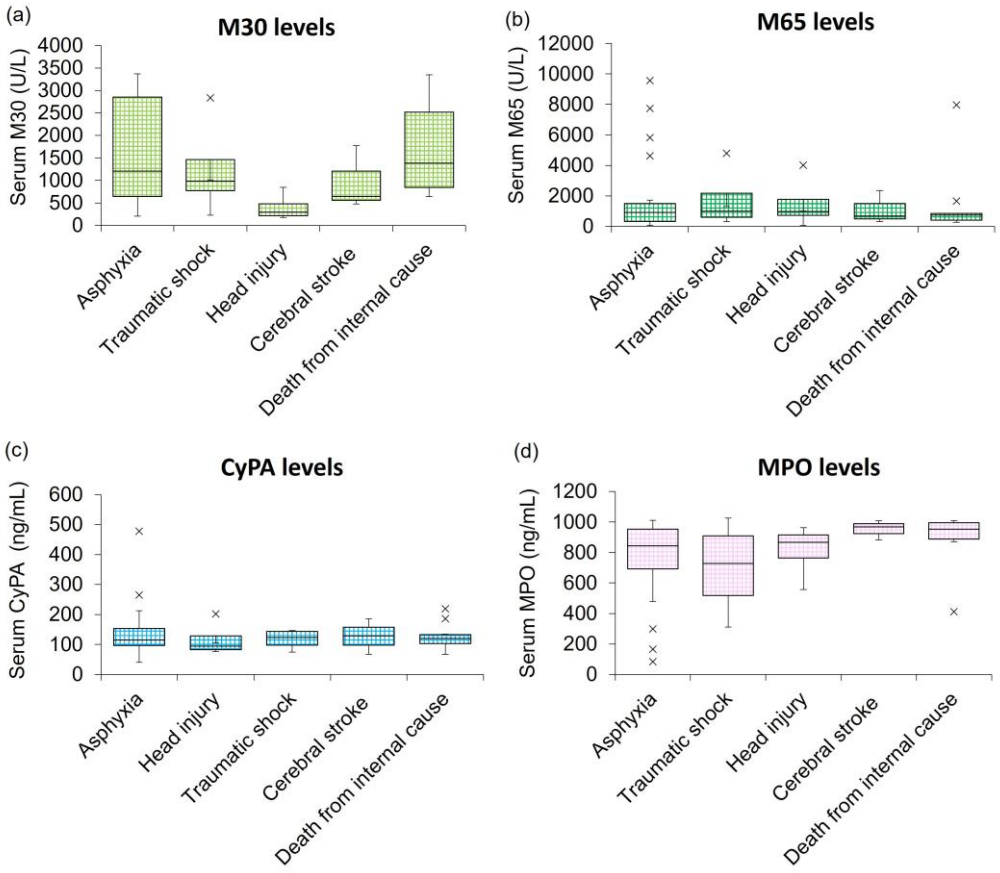

Fig. 4. Box plot of serum M30 levels (a), M65 levels (b), CyPA levels (c), and cfDNA concentrations (d) in postmortem subjects according to cause of death. The top and bottom of each box represent the 25th and 75th percentile, respectively. The line of the box is the median and the error bars show the 5th and 95th percentiles.