

Original article

**Diagnostic implications of urinary liver-type fatty acid-binding protein
and 8-hydroxy-2'-deoxyguanosine in forensic autopsy cases**

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Abstract:

Background/Aim: Liver-type fatty acid-binding protein (L-FABP) is a clinical biomarker of the progress of kidney disease. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is known as a biomarker of peroxidative DNA damage. We investigated both urinary L-FABP and 8-OHdG in forensic autopsy cases as biomarkers to elucidate the metabolic changes in survival periods after insults. **Methods:** In 196 urinary samples from forensic autopsy cases, we measured L-FABP and 8-OHdG by enzyme-linked immunosorbent assay (ELISA) and creatinine by enzymatic assay. Urinary L-FABP/Cr and 8-OHdG/Cr were obtained. **Results:** No significant correlation was observed between urinary L-FABP/Cr or 8-OHdG/Cr, and gender, age, or postmortem interval. Regarding urinary L-FABP/Cr or 8-OHdG/Cr, there were no significant differences among the causes of death. In the survival/agony period, urinary L-FABP/Cr under the cut-off value 31.3 might show that the survival/agony period was within 1 hour. Under the cut-off value of urinary 8-OHdG/Cr, 17.8, might indicate that it is within 24 hours. **Conclusion:** Urinary L-FABP/Cr may rise within a relatively short survival/agony period, and urinary 8-OHdG/Cr may increase when the damage continues longer. Measuring the urinary L-FABP/Cr and 8-OHdG/Cr might be useful in elucidating the survival/agony period.

Key Words: Liver-type fatty acid-binding protein; 8-Hydroxy-2'-deoxyguanosine; Urine; Antemortem reaction; Autopsy diagnosis

1. Introduction

In forensic medicine, the cause of death has a wider meaning of the causal relationship with not only the pathological cause of death, but also the passage from onset to death; however, metabolic changes in the survival period after insults are difficult to confirm in forensic autopsy cases. Comprehensive reviews of postmortem chemistry are available [1-4]. To elucidate the severity of the disease or trauma, several biomarkers in blood, such as c-reactive protein (CRP), neuron-specific enolase (NSE), and S100B for traumatic brain injury, have been reported [5, 6]. Postmortem chemistry can represent one of the most important ancillary procedures for the forensic pathologist in investigating the cause and the process of death, the contributing conditions and the predisposing disorders [3]. The purpose of this study was therefore to investigate the biomarkers useful for the autopsy diagnosis, especially elucidation of the death process [7].

Liver-type fatty acid-binding protein (L-FABP) is an isoform of FABPs. In the human kidney, proximal tubule cells express mRNA of L-FABP, and a certain amount is excreted into urine [8, 9]. Urinary L-FABP showed great potential for early and accurate detection of histological and functional decline in both nephrotoxin-induced and ischemia-reperfusion injury [10]. Urinary L-FABP was reported as a clinical biomarker that may be of use in monitoring and predicting the progression of chronic renal disease [11], and in the diagnosis of acute kidney injury (AKI) [12] or acute coronary syndrome [13].

8-hydroxy-2'-deoxyguanosine (8-OHdG) is produced by the oxidization of deoxyguanosine, a composition factor of DNA, by free radicals such as active and generated oxygen. 8-OHdG is an excellent biomarker that closely reflects living cell

damage caused by active oxygen, and the relationship between 8-OHdG and various stresses, including work, smoking [14-16], cancer, atherosclerosis and diabetes [17-22], has been clarified recently *in vivo*.

In postmortem chemistry, urine is not a practical alternative to blood; however, in forensic autopsy cases, blood samples are often not available. It is more stable for some markers to be analyzed in urine than in blood. For instance, in our preliminary study, it was revealed that urinary 8-OHdG was more stable than blood 8-OHdG [23]. Urine contains various biomarkers having biochemical information different from that of blood components, such as L-FABP. Previously, it was reported that increased urinary L-FABP levels represent an increase in the shedding of proximal tubule L-FABP, rather than just reflecting increased filtration of high serum L-FABP [24].

In this study, urinary L-FABP and 8-OHdG were investigated as potential supplementary biomarkers to elucidate the antemortem pathophysiological condition, especially the survival/ agony period.

2. Materials and Methods

2.1. Forensic autopsy cases

One hundred ninety-six urine specimens were collected from forensic autopsies performed in the Department of Forensic Medicine, Faculty of Medicine, Fukuoka University. All of the samples were collected within one-week postmortem interval (PMI) and centrifuged at 5 °C to remove sediments. The supernatant was stored at -30 °C until the assays. Obvious cases of renal disease were excluded.

The cause of death, the survival period, and the PMI had been diagnosed

based on the autopsy, pathological, toxicological and other examination findings. Further, the clinical records and the police investigated records were referenced for these diagnoses.

A summary of the cases, including age, gender, PMI, and survival/agony period in each cause of death, is shown in Table 1. The possible error in estimating PMI was within 1 h in witnessed death cases (n = 138), ranged from several hours to about 12 h (n = 48) and within 24 h (n = 10) in other cases, in accordance with circumstantial evidence and postmortem findings of their cases.

The examined cases, for which the survival period was known, were divided into 3 groups according to the suspected survival/agony period: within 1 hour (group A), within 24 hours (group B), and over 24 hours (group C). One hundred eighteen cases were included in group A, 29 in group B and 23 in group C. Another 26 cases had an unknown survival duration.

2.2. Quantitative analysis of L-FABP, 8-OHdG, cortisol, creatinine, and urea

L-FABP was measured using an L-FABP ELISA kit (Human L-FABP ELISA Kit; CMIC Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions [11]. Using an 8-OHdG-ELISA kit (New 8-OHdG Check; NIKKEN SEIL Co. Ltd, Shizuoka, Japan), 8-OHdG levels were measured according to the manufacturer's instructions [25, 26]. Creatinine (Cr) was measured by enzymatic assay involving a peroxidase-coupled reaction [27]. Urea was measured by urease and leucine dehydrogenase methods [28].

This study was approved by the Fukuoka University School of Medicine Ethics Review Board (No. 389).

2.3. Statistical analyses

Histograms of the overall distributions of urinary L-FABP/Cr and 8-OHdG/Cr were significantly right-skewed and indicated the need for nonparametric testing. We used the Mann-Whitney *U* test to analyze differences in measurements between male and female cases, and between natural and traumatic causes of death. Correlation analysis was performed by the Spearman rank correlation. The Kruskal-Wallis test was used to test for overall group differences and the Steel-Dwass test, a post-hoc nonparametric multiple comparisons procedure, was used to test for between-group differences in the causes of death and the survival/agony period. These statistical analyses were performed with the JMP 9 software program (SAS Institute, Inc., Cary, NC). $P < 0.01$ was considered significant.

2.4. Cut-off point of urinary L-FABP/Cr and 8-OHdG/Cr on the ROC curve

In order to clarify the standard value of urinary L-FABP/Cr and 8-OHdG/Cr for diagnostic implications, we examined the cut-off point of the value using the receiver operating characteristic (ROC) curve [29-31].

3. Results

Urinary L-FABP and 8-OHdG levels were corrected by urinary Cr levels, yielding urinary L-FABP/Cr and 8-OHdG/Cr. The median of urinary L-FABP/Cr ($\mu\text{g/g Cr}$) was 11.8 (interquartile range (IQR): 0–161.1). The median of urinary 8-OHdG/Cr ($\mu\text{g/g Cr}$) was 14.9 (IQR: 10.9–23.6).

Both urinary L-FABP/Cr ($P = 0.1258$) and 8-OHdG/Cr ($P = 0.1177$) showed no

significant difference between males and females. Furthermore, there were no statistically significant correlations using the Spearman rank correlation between urinary L-FABP/Cr and age or PMI, or urinary 8-OHdG/Cr and age or PMI.

There was also no significant correlation between urinary L-FABP/Cr and 8-OHdG/Cr. In cases in which alcohol was detected from blood or urine (>0.01 mg/ml), no significant differences were observed between blood alcohol and urinary L-FABP/Cr, blood alcohol and urinary 8-OHdG/Cr, urinary alcohol and urinary L-FABP/Cr, or urinary alcohol and urinary 8-OHdG/Cr.

Urea, examined in 54 cases, yielded a median of 139.3 mg/dL and an IQR of 97.9-331.0 mg/dL.

3.1. Urinary L-FABP/Cr and 8-OHdG/Cr levels for each cause of death

A summary of urinary L-FABP/Cr and 8-OHdG/Cr for each cause of death is shown in Table 2. Both urinary L-FABP/Cr ($P = 0.0002$) and 8-OHdG/Cr ($P = 0.001$) in the natural death cases were significantly higher than in trauma.

3.1.1. L-FABP/Cr

Each cause of death with more than 5 cases is summarized in Table 2. The overall Kruskal-Wallis test demonstrated important differences among the causes of death ($P = 0.0015$); however, in between-group comparisons, adjusted for multiple testing by the Steel-Dwass method, there was no statistically significant difference among the causes of death.

3.1.2. 8-OHdG/Cr

For each cause of death, urinary 8-OHdG/Cr is summarized in Table 2. The overall Kruskal-Wallis test demonstrated no significant difference among the causes

of death ($P = 0.169$).

3.2. Relationship between urinary L-FABP/Cr or 8-OHdG/Cr and the survival/agony period

Urinary L-FABP/Cr and 8-OHdG/Cr according to the survival/agony period were as follows: the median urinary L-FABP/Cr ($\mu\text{g/g Cr}$) was 1.0 (IQR: 0–21.3) in group A; 104.9 (43.2–396.7) in group B; and 335.9 (185.6–703.5) in group C. The median urinary 8-OHdG/Cr ($\mu\text{g/g Cr}$) was 13.2 (10.7–18.5) in group A; 16.6 (11.0–25.3) in group B; and 30.9 (19.7–57.3) in group C. The overall Kruskal-Wallis test demonstrated important differences among groups in both urinary L-FABP/Cr ($P < 0.0001$) and 8-OHdG/Cr ($P < 0.0001$).

In between-group comparisons, it was shown that both group B (within 24 hours) ($P < 0.0001$) and group C (over 24 hours) ($P < 0.0001$) were significantly higher than group A (within 1 hour) in urinary L-FABP/Cr (Fig. 1a). In urinary 8-OHdG/Cr, group C was significantly higher than both group A ($P < 0.0001$) and B ($P = 0.0028$) (Fig. 1b).

3.3. Cut-off point of urinary L-FABP/ Cr and 8-OHdG/Cr on the ROC curve

The ROC curve was obtained for sensitivity and specificity when assessing the survival/agony period. The optimal cut-off points for urinary L-FABP/Cr and 8-OHdG/Cr were calculated from the ROC curve. On the ROC curve of urinary L-FABP/Cr obtained from 170 cases with a known survival period, the optimal sensitivity and specificity were 0.78 and 0.88, respectively, at a cut-off value of 31.3 in discriminating whether the survival/agony period was within 1 hour (group A). On

the ROC curve of urinary 8-OHdG/Cr, they were 0.91 and 0.70 at a cut-off value of 17.8 in discriminating whether the survival/agony period was over 24hours (group C).

4. Discussion

In our preliminary study, urinary L-FABP/Cr and 8-OHdG/Cr in the urine of healthy volunteers showed no significant differences when keeping the samples at -30 °C until the assays, at least up to 6 months. To investigate the effects of urea on L-FABP and 8-OHdG in urine, urinary urea was measured in the specimens. In the relationship between urea and L-FABP or 8-OHdG, there was no tendency for high urea with low L-FABP. Both urea and Cr are indices of renal function. Urea/Cr was 5.64, 4.15–7.38 (median, IQR). It was considered that since the examined urea concentration had changed similarly to the concentration of Cr, urinary L-FABP and 8-OHdG could be used as an index if they were corrected by urinary Cr. Therefore, in this study, both urinary L-FABP/Cr and 8-OHdG/Cr were considered as suitable and stable biomarkers in postmortem examinations, when specimens were obtained within one-week postmortem.

From the results, it was considered that urinary L-FABP/Cr and 8-OHdG/Cr were not affected by gender, age, or PMI, and that blood and urinary ethanol contents did not correlate significantly with urinary L-FABP/Cr and 8-OHdG/Cr levels.

4.1. Urinary L-FABP/Cr and 8-OHdG/Cr for each cause of death

4.1.1. Natural diseases

In a previous study, higher urinary L-FABP could differentiate patients with septic shock from those with severe sepsis, AKI and healthy controls [32]. In our results, urinary L-FABP/Cr in sepsis, observed pathologically by a general infection with lienitis, tended to be high, but statistically not significant. We assumed that since there were few cases of sepsis, a significant difference could not be proven. Urinary L-FABP/Cr might be useful information to diagnose septic shock as the cause of death. In a previous clinical study, patients treated for hypertension, diabetes mellitus, or chronic hepatitis showed significantly greater urinary L-FABP excretion than healthy subjects; however, urinary L-FABP in patients treated for hyperlipidemia, coronary artery disease, arrhythmia, hyperuricemia, thyroid diseases, chronic obstructive pulmonary diseases, prostate diseases, or gastroduodenal ulcer was not significantly different from that in healthy subjects [33].

Urinary 8-OHdG/Cr in natural deaths was also significantly higher than in traumatic deaths. Urinary 8-OHdG/Cr tended to be high in sepsis; however, our results could not confirm significant differences among the causes of death.

As for why both urinary L-FABP/Cr and 8-OHdG/Cr were high in sepsis, it was considered that sepsis induces a systemic disorder and prolongs the survival/agonny period. In addition, as for why urinary L-FABP/Cr in cardiac disease was low compared to natural deaths, it was thought that progress from the outbreak of the disorder to death was generally immediate, and the disordered organ was limited to the heart or the cardiovascular system.

4.1.2. Trauma

Regarding the level of urinary L-FABP/Cr, there was no significant difference

among the causes of death. In trauma, urinary L-FABP/Cr was relatively low in fire fatalities, which might reflect the short survival/agony period from fire to death.

Regarding urinary 8-OHdG/Cr, there was no significant difference among the causes of death. If there had been more cases, it might have been possible to confirm a significant difference.

The investigation of urinary L-FABP/Cr and 8-OHdG/Cr for each cause of death revealed that levels were affected by the survival/agony period rather than the cause of death.

4.2. Relationship of the survival/agony period and urinary L-FABP/Cr or 8-OHdG/Cr

Urinary L-FABP/Cr and 8-OHdG/Cr were investigated for the duration of pathological and traumatic damage.

It was revealed that both urinary L-FABP/Cr and 8-OHdG/Cr might increase with the survival/agony period. Moreover, from the results of the significant difference examination, urinary L-FABP/Cr may rise during a relatively short survival/agony period. In our cases, urinary L-FABP/Cr in fire fatalities showed a tendency to be low. All cases of fire fatalities were limited to within a one-hour survival/agony period (group A). In a previous study, urinary L-FABP showed promise as an early, accurate biomarker of AKI (pediatric bypass surgery, 4 h). The predictive ability of L-FABP for AKI requires further clinical confirmation in different patient populations [24]. It was therefore considered that L-FABP may be an immediate response marker antemortem.

Urinary 8-OHdG/Cr may increase when the survival/agony period is longer. In our cases, urinary 8-OHdG/Cr in infection cases showed a tendency to be high.

All cases of infection were limited to a >24-hour survival/agony period (group C). It is believed that L-FABP is secreted into the urine by a decrease in renal blood flow and causes damage to the proximal tubule. In a peroxidatively damaged DNA lesion, 8-OHdG is cut as a restoration mechanism to blood flow from the damaged target organs, and then 8-OHdG is excreted into the urine. It was therefore considered that an increase in urinary 8-OHdG/Cr occurs after an initial increase in urinary L-FABP/Cr.

4.3. Cut-off points of L-FABP/Cr and 8-OHdG/Cr to elucidate the survival/agony periods

The diagnostic performance of a test or the accuracy of a test to discriminate diseased from normal cases is evaluated using receiver operating characteristic (ROC) curve analysis [29-31]. On the ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate (1-specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC plot that passes through the upper left corner (100% sensitivity, 100% specificity); therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test [30, 31].

According to the results of the significant differences in the survival/agony period, we obtained the optimal cut-off point of urinary L-FABP/Cr and 8-OHdG/Cr using the ROC curve. That of urinary L-FABP/Cr was 31.3 in discriminating whether the survival/agony period was within 1 hour (group A), and urinary 8-OHdG/Cr was

17.8 in discriminating whether the survival/agony period was over 24 hours (group C).

The relationship between urinary L-FABP/Cr or 8-OHdG/Cr and the survival/agony period was investigated and it was considered that the survival/agony period might be elucidated by the cut-off point of urinary L-FABP/Cr and 8-OHdG/Cr.

The area under the ROC curve (AUC) is a measure of the overall diagnostic accuracy of the test [34]. AUC for urinary L-FABP/Cr and that of urinary 8-OHdG/Cr was 0.88 and 0.85, respectively. As a rule of thumb, an AUC >0.9 is considered highly accurate, while 0.7–0.9 indicates moderate accuracy, 0.5–0.7 low accuracy, and 0.5 a chance result [35, 36]; therefore, it was considered that both of our tests had moderate accuracy.

The sensitivity and specificity do not show the probability that the test will give the correct diagnosis [37]. Instead, positive and negative predictive values describe the probability of having an event, which is usually disease, once the test results are known [38]. Generally, the positive predictive value (PPV) of a test is defined as the proportion of people with a positive test result who actually have the disease, and the negative predictive value (NPV) is the proportion of people with a negative test result who do not have a disease. Regarding our data, the PPV of urinary L-FABP/Cr indicates the proportion of cases with urinary L-FABP/Cr <31.3 with a survival/agony period within 1 hour (group A). The NPV of urinary L-FABP/Cr indicates the proportion of cases with urinary L-FABP/Cr not <31.3 with a survival/agony period not within 1 hour (group A). The PPV of urinary 8-OHdG/Cr indicates the proportion of cases with urinary 8-OHdG/Cr >17.8, indicating that the

survival/agony period was >24 hours (group C). The NPV of urinary 8-OHdG/Cr indicates the proportion of cases with urinary 8-OHdG/Cr not >17.8, indicating that the survival/agony period was not >24 hours (group C). The PPV and NPV of urinary L-FABP/Cr were 0.94 and 0.63, respectively, and for urinary 8-OHdG/Cr were 0.32 and 0.98, respectively. So, urinary L-FABP/Cr under the cut-off value of 31.3 might show that the survival/agony period was within 1 hour (group A). Under the cut-off value of urinary 8-OHdG/Cr (17.8), might indicate that it was within 24 hours (groups A and B). Measuring urinary L-FABP/Cr and 8-OHdG/Cr might be useful to elucidate the survival/agony period.

5. Conclusion

- 1) Urinary L-FABP/Cr and 8-OHdG/Cr may be stable markers as they are not influenced by gender, age, or the postmortem interval.
- 2) With regards to the survival/agony period, lower urinary L-FABP/Cr may indicate a shorter period, and higher urinary 8-OHdG/Cr a longer period.
- 3) Urinary L-FABP/Cr and 8-OHdG/Cr may be used as indicators of the survival/agony period.

Disclosure statement

The authors have declared no conflicts of interest.

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Table legends

Table 1 Summary of cases

Table 2 Urinary L-FABP/Cr and 8-OHdG/Cr for each cause of death

Figure legends

Fig. 1 Box and whisker plots of urinary L-FABP/Cr and 8-OHdG/Cr for each group of survival/agony periods

a: L-FABP/Cr, b: 8-OHdG/Cr, * $P < 0.01$

The ends of the whiskers represent the lowest value still within 1.5 x interquartile range (IQR) of the lower quartile, and the highest value within 1.5 x IQR of the upper quartile.

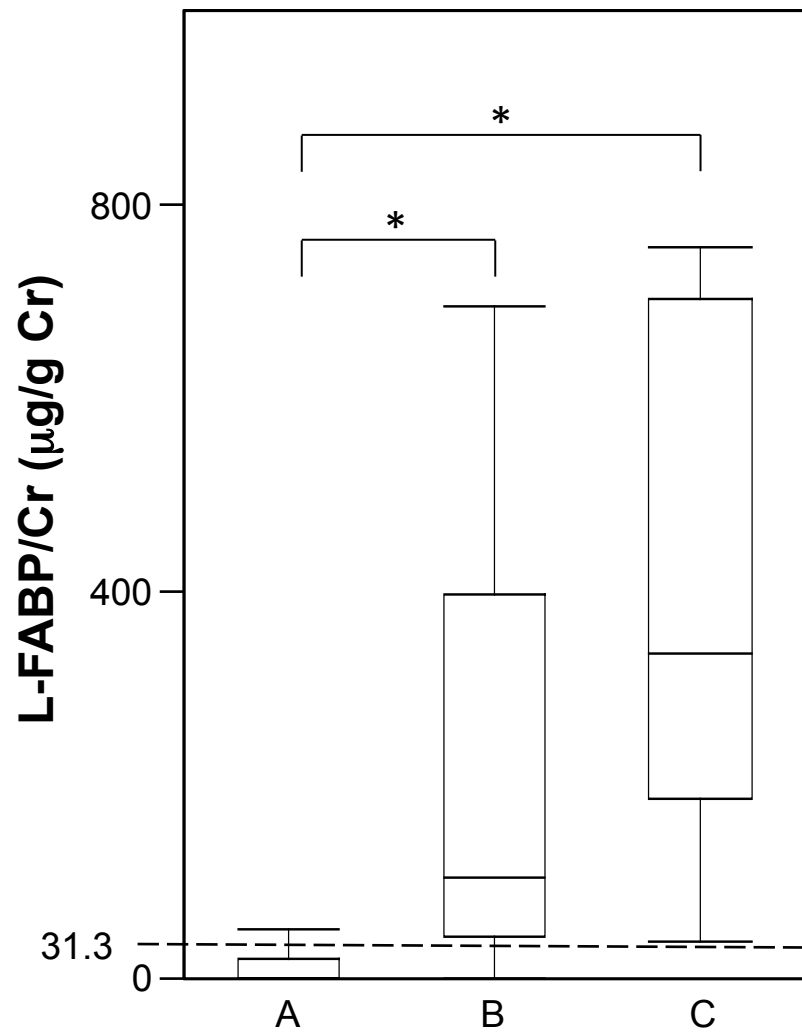
The cut-off point for urinary L-FABP/Cr in discriminating whether the survival/agony period was within 1 hour (group A): 31.3

The cut-off point for urinary 8-OHdG/Cr in discriminating whether the survival/agony period was over 24hours (group C): 17.8

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Fig. 1

a



b

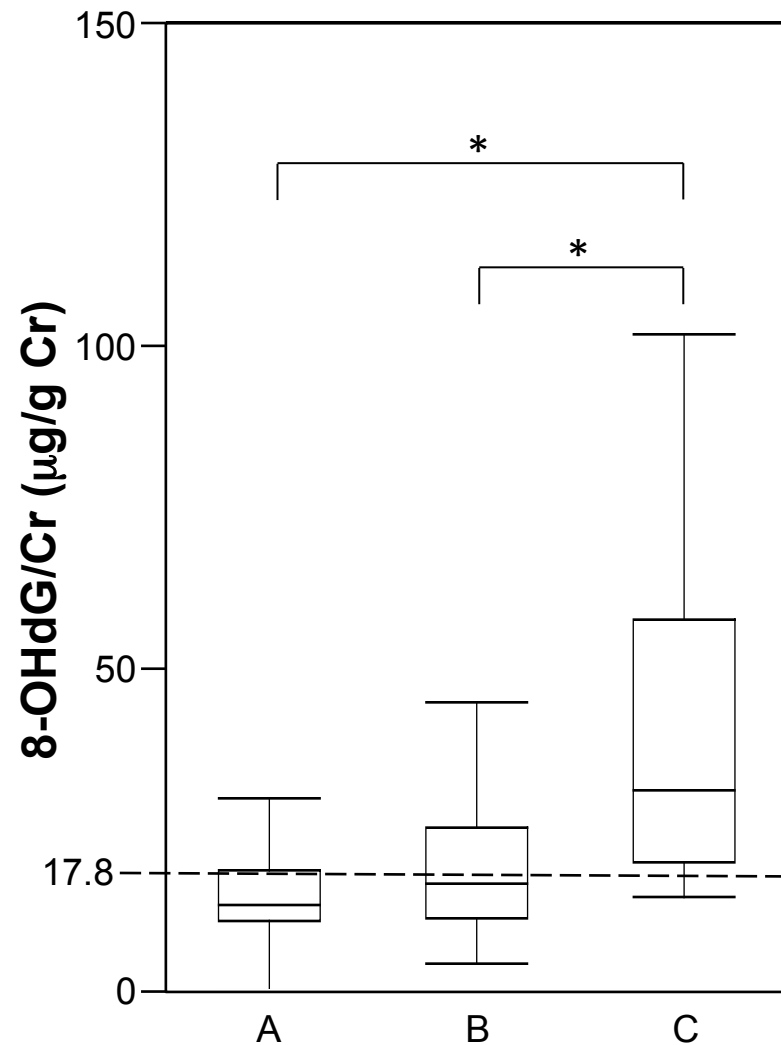


Table 1

Cause of death	n	Age Median (IQR)	Gender	PMI Median (IQR)	Survival Time ^b Group A/B/C
<i>Natural diseases</i>	41	61 (37.25-72)	32/9	24 (14.75-56.5)	5/2/15
Cardiac diseases	19	66.5 (46-75.5)	15/4	27 (20-60)	1/0/4
Cerebrovascular stroke	6	55.5 (44-64.25)	5/1	18 (11.5-114)	2/2/0
Infections	5	53 (0.42-67) ^a	5/0	20 (4.5-26) ^a	0/0/5
Others	11	44 (28-76)	7/4	19 (12-48)	2/0/6
<i>Trauma</i>	155	52 (36-64)	117/38	20 (12-36)	113/27/8
Injury	63	51.5 (34.75-61)	52/11	15.5 (11-27)	41/11/7
Drowning	28	53 (37-66)	22/6	60 (36-72)	26/2/0
Fire fatality	20	62 (50.5-67.5)	12/8	15 (10-23.5)	20/0/0
Mechanical asphyxiation					
Strangulation	15	48 (27-67)	11/4	30 (15-34)	14/1/0
Choking	6	43 (21.25-54.25)	4/2	12 (9.375-33.75)	5/1/0
Intoxication	9	41 (31.5-54)	5/4	30 (14.5-54)	2/5/1
Hypothermia	8	74 (51.25-80.5)	5/3	20.5 (11-60)	1/6/0
Others	6	43 (25.25-55.75)	6/0	25 (15.75-49.5)	4/1/0

IQR: interquartile range; gender: male/female

^a For causes of death that number five or less, the number in parentheses expresses the range.

^b The number of the cases does not include cases with an unknown survival/agonny period.

Table 2

Cause of death	L-FABP/Cr ($\mu\text{g/g Cr}$)		8-OHdG/Cr ($\mu\text{g/g Cr}$)	
	Median	(IQR)	Median	(IQR)
<i>Natural diseases</i>	159.2	(4.0–402.5)	22.0	(12.2–43.2)
Cardiac diseases	69.2	(0.9–248.6)	22.0	(10.5–31.2)
Cerebrovascular stroke	200.9	(27.7–448.2)	21.0	(8.4–42.8)
Infections	335.9	(193.2–8225) ^a	49.0	(17.8–505.9) ^a
Others	270.5	(4.2–1198.6)	19.7	(13.1–51.9)
<i>Trauma</i>	6.4	(0–91.2)	14.0	(10.9–20.4)
Injury	2.5	(0–78.1)	13.6	(10.4–18.6)
Drowning	37.7	(0–208.9)	14.7	(12.7–24.1)
Fire fatality	0.6	(0–28.6)	13.4	(9.7–16.5)
Mechanical asphyxiation				
Strangulation	6.7	(0–67.7)	17.0	(11.3–30.7)
Choking	3.5	(0–193.8)	10.1	(6.1–15.2)
Intoxication	104.9	(8.7–420.0)	16.0	(8.4–44.1)
Hypothermia	41.8	(7.6–519.5)	23.5	(18.2–31.7)
Others	4.5	(0.8–14.8)	12.3	(9.8–13.3)

IQR: interquartile range

^a For causes of death that number five or less, the number in parentheses expresses the range.