Published in final edited form as:

Clin Chim Acta. 2010 December 14; 411(23-24): 1998–2003. doi:10.1016/j.cca.2010.08.021.

Effects of Anticoagulants on Human Plasma Soluble Corin Levels Measured by ELISA

Ningzheng Dong^{a,*}, Jing Dong^a, Peng Liu^a, Luyao Xu^a, Sengseng Shi^a, and Qingyu Wu^{a,b}

^a Cyrus Tang Hematology Center, Jiangsu Institute of Hematology, First Affiliated Hospital, Soochow University, Suzhou, China

^b Molecular Cardiology, Nephrology and Hypertension, Lerner Research Institute, Cleveland Clinic, Cleveland, OH

Abstract

Background—Recently, soluble corin was detected in human plasma. In patients with heart failure, plasma corin levels were lower than that of normal controls. In this study, we analyzed experimental conditions for measuring plasma or serum corin by an immunoassay.

Methods—Serum and plasma corin levels were measured by ELISA. Effects of different anticoagulants (EDTA, heparin and sodium citrate) on plasma corin levels were examined.

Results—Corin levels in serum were similar to that in plasma with heparin (950 ± 305 vs. 929 ± 301 pg/ml, $n=40$, $p=0.73$), but were significantly higher than those in plasma with sodium citrate (735 ± 237 pg/ml, $p<0.01$) or EDTA (716 ± 261 pg/ml, $p<0.001$). Native and recombinant human corin proteins were stable in human plasma with EDTA at 4°C or underwent freezing-and-thawing. In 348 healthy Chinese individuals, plasma corin levels ranged from 216 to 1663 pg/ml. The levels were higher in males than that in females (842 ± 283 vs. 569 ± 192 pg/ml, $p<0.001$).

Conclusion—Soluble corin was stable in plasma samples. Plasma soluble corin levels vary depending on anticoagulants used. Samples containing heparin had significantly higher levels of corin than that in samples with EDTA or sodium citrate.

Keywords

corin; membrane proteases; anticoagulants; heart failure

1. Introduction

Corin is a serine protease expressed primarily in cardiomyocytes [1–3], where it processes natriuretic peptides. Low levels of corin mRNA also were detected in other tissues such as the kidney, bone, brain and skin [4–6], but the biological function of corin in these tissues is not fully understood. In mouse models [7,8], corin deficiency leads to hypertension and cardiac hypertrophy, indicating the importance of corin in regulating blood pressure. In humans, corin gene variants have been associated with an increased risk for hypertension and heart disease in African Americans [9–12].

*Corresponding author: Ningzheng Dong, MD, PhD, Cyrus Tang Hematology Center, Jiangsu Institute of Hematology, First Affiliated Hospital, Soochow University, Suzhou, Jiangsu 215123, China. Tel: 86-512-65880987, Fax: 86-512-65880929, ningzheng_dong@yahoo.com.cn.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Structurally, corin is a member of the type II transmembrane serine protease (TTSP) family [13,14]. Corin is anchored on the cell surface by its N-terminal transmembrane domain [6,15]. Its protease domain is located at the C-terminus in the extracellular region. Like many membrane proteins, TTSPs can be cleaved proteolytically from the cell surface either in culture or *in vivo* [13,16,17]. More recently, soluble corin was detected in human plasma and serum samples by immunoassays [18]. We also found that plasma corin levels were significantly lower in patients with heart failure (HF) than that in normal controls [19]. More importantly, the reduction of the plasma corin levels appeared to correlate with the disease severity [19,20]. These results suggest that corin deficiency may play a role in the pathogenesis of HF and that plasma corin may be used as a biomarker for HF.

To better understand the value of plasma soluble corin as a potential biomarker and to assess experimental conditions that may influence the corin assay, we measured soluble corin levels in serum and plasma samples with different anticoagulants from healthy individuals. We also tested the stability of native and recombinant corin in pooled human plasma. Our results indicated that anticoagulants used in blood samples affected plasma corin levels when measured by ELISA assays.

2. Materials and Methods

2.1. Anticoagulants in blood samples

Non-gel plastic vacuum serum collection tubes and blood collection tubes containing spray-coated K₂EDTA or heparin or 3.2% of sodium citrate were from BD Diagn., Franklin Lakes, NJ.

2.2. Serum and plasma samples

Human blood samples were obtained with the informed consent and the permission from local ethical committees. Venous blood was obtained from 348 healthy donors (aged 17–91 y). There were 40 individuals (22 females; 33.3 ± 11.4 y and 18 males; 36.6 ± 10.9 y) provided blood samples drawn into 3 tubes with different anticoagulants and 1 tube without anticoagulant. Plasma or serum samples were prepared by centrifugation at 3000 g for 10 min, aliquoted, and either used immediately or stored at -80°C for further use within 6 months.

2.3. Recombinant human corin

Recombinant human corin was expressed in transfected human embryonic kidney (HEK) 293 cells. The expression plasmid for the full-length human corin was described previously [21–23]. Recombinant corin contained a V5 tag at the C-terminus, which allowed protein detection by an anti-V5 antibody (Invitrogen, Carlsbad, CA) in Western analysis [21–23]. The transfected cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. All cells were cultured at 37°C in a humidified incubator with 5% CO₂ and 95% air.

2.4. Measurement of Corin by ELISA

An ELISA kit for human corin was purchased from R&D Systems (Minneapolis, MN). The experiment was performed according to manufacturer's instructions. Briefly, microtiter plates were coated with an anti-corin antibody. Plasma or serum samples or recombinant human corin protein standards were added and incubated at room temperature for 2 hours. After washing with a buffer, a biotinylated anti-human corin antibody was added and incubated for 2 h. After washing, peroxidase-conjugated streptavidin was added and incubated at room temperature for 20 min. After washing, a horseradish peroxidase substrate (3,3',5,5'-tetramethylbenzidine, TMB) was added and incubated in dark for 20 min. The

reaction was stopped by adding 2N-H₂SO₄ and the optical density was monitored with a spectrometer at wavelength of 450 nm.

2.5. Stability of native plasma corin

Experiments to test the stability of native corin were performed using pooled human plasma with EDTA as an anticoagulant. Fresh blood was drawn from 20 individuals. Pooled fresh plasma samples were prepared and assayed within 2 h. Plasma samples with or without a cocktail of protease inhibitors, which inhibit serine, cysteine and metalloproteases (Roche Diagnostics, #04693124001, 1:100 dilution), were stored at room temperature or 4°C. Corin levels were determined at different time points. We also tested corin protein stability with pooled plasma samples after several cycles of freezing-and-thawing.

2.6. Stability of recombinant human corin in pooled plasma

Recombinant human corin was expressed in a stable HEK 293 cell line. The cells were lysed in a buffer containing 50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, and 1% Triton X-100 (vol/vol) [21–23]. Cell lysate was added to pooled plasma with or without protease inhibitors. The samples were kept at room temperature, 4°C or –80°C. Corin levels in these samples were assayed by ELISA, as described above. In this set of experiments, similarly prepared cell lysate from parental HEK 293 cells was used as a negative control.

2.7. Statistical analysis

All data are presented as the mean $\pm$ SD. Difference between groups was analyzed by Student's *t*-test or ANOVA if groups were >2 . Analysis was done using the SPSS 12 software (SPSS, Chicago, IL). A $p < 0.05$ is considered to be statistically significant.

3. Result

3.1. Corin levels in human serum and plasma

Figure 1 shows corin levels in serum and plasma samples with three types of anticoagulants (heparin, sodium citrate and EDTA). Corin levels were similar in serum samples and plasma samples with heparin (950 ± 305 vs. 929 ± 301 pg/ml, $n=40$, $p=0.73$), but were significantly higher than that in plasma samples with sodium citrate (735 ± 237 pg/ml, $n=40$, $p<0.01$) or EDTA (716 ± 261 pg/ml, $n=40$, $p<0.01$). In linear regression analysis, corin levels in serum samples correlated with that in plasma samples with heparin ($r^2=0.66$, Fig. 2A). Similarly, corin levels in plasma samples with EDTA correlated with that in plasma samples with sodium citrate ($r^2=0.86$, Fig. 2B).

3.2. Stability of native and recombinant corin in plasma

We tested the effect of samples storage, either at room temperature or 4°C, on native plasma soluble corin levels. As shown in Figure 3A, plasma corin levels decreased after 24 hours at room temperature. In contrast, corin levels did not change significantly up to 72 h when samples were kept at 4°C (Fig. 3B). Similar results were obtained when the samples were stored with or without protease inhibitors (Figs. 3A and B). We also performed similar experiments using recombinant human corin that was added to pooled human plasma. In these experiments, corin levels decreased after 48 h when samples were kept at room temperature (Fig. 4A). No significant decrease in corin levels was observed if samples were kept at 4°C for up to 72 hours (Fig. 4B). Similar results were obtained in samples with or without protease inhibitors (Figs. 4A and B).

We then tested the effect of freezing-and-thawing on native and recombinant corin levels in plasma samples with EDTA. We did not detect any significant decrease in native (Fig. 5A)

or recombinant (Fig. 5B) corin levels when samples underwent up to three cycles of freezing-and-thawing ($p>0.05$, vs. cycle 0).

We did similar studies with serum samples or plasma samples with sodium citrate or heparin. Levels of native corin in serum samples (Fig. 6A) or plasma samples with sodium citrate (Fig. 6B) did not decrease significantly after three cycles of freezing-and-thawing. In contrast, the level was decreased in plasma samples with heparin after 2 cycles of freezing-and-thawing (Fig. 6C) ($p<0.05$ vs. cycle 0).

3.3. Normal range of plasma corin

We determined the normal range of plasma soluble corin in 348 healthy Chinese (182 males and 166 females of 17–91 years old) using samples with EDTA as an anticoagulant. In these individuals, corin levels ranged from 216 to 1663 pg/ml. The levels were higher in males than that in females (842 ± 283 vs. 569 ± 192 pg/ml, $p<0.001$). In both males and females, corin levels appeared to be similar in different age groups. When the samples were divided into 4 age groups, *i.e.* 16–30, 31–45, 46–60, and >60 y, there were no significant differences among these age groups within the same gender group (Figs. 7A and B). We also analyzed the data of the female group by dividing them according to the fertility age, *i.e.* younger or older than 55 y. There were no significant differences between these two groups (564 ± 193 pg/ml in females <55 y, $n=143$ vs. 601 ± 180 pg/ml in females >55 y, $n=23$, $p=0.39$).

4. Discussion

Natriuretic peptides play an important role in regulating blood pressure and cardiac function [3,24]. Corin is a newly identified membrane protease that processes natriuretic peptides in the heart [2,3,7,15]. Recent studies have detected soluble corin in human plasma by ELISA [18], indicating that corin is shed from the cell surface and that plasma corin levels may reflect cellular homeostasis in the heart. Indeed, plasma corin levels were progressively lower in patients with more severe HF [19,20]. These results suggest that soluble corin may be used as a biomarker for HF or other cardiovascular disease.

In this study, we measured soluble corin levels in human serum and plasma samples that were prepared with three types of anticoagulants (heparin, sodium citrate and EDTA). As measured by ELISA, soluble corin levels in serum and heparin-containing plasma samples were similar but significantly higher than that in plasma samples prepared from blood with EDTA or sodium citrate as an anticoagulant. This information is significant, highlighting the importance of using the same type of anticoagulant when comparing soluble corin levels in blood samples from different study groups.

Many trypsin-like serine proteases such as clotting factors are degraded quickly in test tubes. Surprisingly, soluble corin appeared to be stable in either serum or plasma [18]. When samples were stored at -35°C , serum corin exhibited no significant degradation for up to 1 yr. We confirmed this finding using an ELISA method based on similar antibodies. Our data showed that native and recombinant human corin proteins were stable in human plasma stored at either room temperature (up to 1 day) or 4°C (up to 3 days). The presence of cocktail protease inhibitors did not appear to increase its stability. The result was consistent with a recent proteomic study, showing that plasma protein levels were similar in samples with or without protease inhibitors [25]. We also found that corin levels in serum samples and plasma samples with EDTA or sodium citrate were stable after cycles of freezing and thawing. In contrast, plasma corin in samples with heparin was less stable after 2 cycles of freezing-and-thawing, suggesting that EDTA and sodium citrate are preferred anticoagulants when preparing samples to measure plasma corin. Given the remarkable protein stability, soluble corin may have an advantage over other peptides such as atrial and brain natriuretic

peptides (ANP and BNP) when used as a biomarker for HF [26,27]. Both ANP and BNP have short plasma half-lives, which may contribute to their assay variability in hospital settings [28–32].

We also determined plasma corin levels in EDTA-anticoagulated samples from healthy Chinese individuals. Our data showed that there was a significant gender difference in plasma corin levels, with the levels being higher in males than females. A similar gender difference also was observed in patients with HF [19]. At this time, it is not clear if this was due to higher corin expression levels or enhanced corin shedding in males than that in females. In contrast, there appeared to be no significant differences in plasma corin levels in different age groups. We noticed, however, that plasma corin levels in our samples from Han Chinese were lower than the reported values in groups of different ethnic backgrounds [18,20]. In a previous report, interestingly, plasma N-terminal (NT) pro-BNP levels also were found to be lower in a Chinese population than those in European and American populations [33]. It should be pointed out that in the reported studies of soluble corin, sample preparations differed. In one study, serum samples were used to measure soluble corin [18]. In the other 2, plasma samples containing EDTA were used [19,20]. Comparing the data from these two studies using plasma samples, soluble corin levels still were lower in Chinese individuals than that of Caucasians [19,20]. These results indicate the importance of establishing the normal range of plasma corin levels in different ethnic groups based on standardized experimental methods.

Acknowledgments

This work was supported in part by grants from Jiangsu Provincial Natural Science Foundation of China (BK2005036 to N.D.), the Ralph Wilson Medical Research Foundation and the National Institutes of Health (R01HL089298, R01HL089298-S1 to Q.W.).

Abbreviations

ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
HEK	human embryonic kidney
HF	heart failure
TTSP	type II transmembrane serine protease

References

1. Hooper JD, Scarman AL, Clarke BE, Normyle JF, Antalis TM. Localization of the mosaic transmembrane serine protease corin to heart myocytes. *Eur J Biochem.* 2000; 267:6931–6937. [PubMed: 11082206]
2. Wu Q. The serine protease corin in cardiovascular biology and disease. *Front Biosci.* 2007; 12:4179–4190. [PubMed: 17485366]
3. Wu Q, Xu-Cai YO, Chen S, Wang W. Corin: new insights into the natriuretic peptide system. *Kidney Int.* 2009; 75:142–146. [PubMed: 18716601]
4. Enshell-Seijffers D, Lindon C, Morgan BA. The serine protease Corin is a novel modifier of the Agouti pathway. *Development.* 2008; 135:217–225. [PubMed: 18057101]
5. Ono Y, Nakatani T, Sakamoto Y, Mizuhara E, Minaki Y, Kumai M, Hamaguchi A, Nishimura M, Inoue Y, Hayashi H, Takahashi J, Imai T. Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development.* 2007; 134:3213–3225. [PubMed: 17670789]

6. Yan W, Sheng N, Seto M, Morser J, Wu Q. Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. *J Biol Chem.* 1999; 274:14926–14935. [PubMed: 10329693]
7. Chan JC, Knudson O, Wu F, Morser J, Dole WP, Wu Q. Hypertension in mice lacking the proatrial natriuretic peptide convertase corin. *Proc Natl Acad Sci U S A.* 2005; 102:785–790. [PubMed: 15637153]
8. Nigrovic PA, Gray DH, Jones T, Hallgren J, Kuo FC, Chaletzky B, Gurish M, Mathis D, Benoist C, Lee DM. Genetic inversion in mast cell-deficient (W(sh)) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. *Am J Pathol.* 2008; 173:1693–1701. [PubMed: 18988802]
9. Dries DL, Victor RG, Rame JE, Cooper RS, Wu X, Zhu X, Leonard D, Ho SI, Wu Q, Post W, Drazner MH. Corin gene minor allele defined by 2 missense mutations is common in blacks and associated with high blood pressure and hypertension. *Circulation.* 2005; 112:2403–2410. [PubMed: 16216958]
10. Rame JE, Drazner MH, Post W, Peshock R, Lima J, Cooper RS, Dries DL. Corin I555(P568) allele is associated with enhanced cardiac hypertrophic response to increased systemic afterload. *Hypertension.* 2007; 49:857–864. [PubMed: 17296875]
11. Rame JE, Tam SW, McNamara D, Worcel M, Sabolinski ML, Wu AH, Dries DL. Dysfunctional corin I555(P568) allele is associated with impaired brain natriuretic peptide processing and adverse outcomes in blacks with systolic heart failure: results from the Genetic Risk Assessment in Heart Failure substudy. *Circ Heart Fail.* 2009; 2:541–548. [PubMed: 19919978]
12. Wang W, Liao X, Fukuda K, Knappe S, Wu F, Dries DL, Qin J, Wu Q. Corin variant associated with hypertension and cardiac hypertrophy exhibits impaired zymogen activation and natriuretic peptide processing activity. *Circ Res.* 2008; 103:502–508. [PubMed: 18669922]
13. Bugge TH, Antalis TM, Wu Q. Type II transmembrane serine proteases. *J Biol Chem.* 2009; 284:23177–23181. [PubMed: 19487698]
14. Wu Q. Type II transmembrane serine proteases. *Curr Top Dev Biol.* 2003; 54:167–206. [PubMed: 12696750]
15. Yan W, Wu F, Morser J, Wu Q. Corin, a transmembrane cardiac serine protease, acts as a proatrial natriuretic peptide-converting enzyme. *Proc Natl Acad Sci USA.* 2000; 97:8525–8529. [PubMed: 10880574]
16. Lin CY, Anders J, Johnson M, Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *J Biol Chem.* 1999; 274:18237–18242. [PubMed: 10373425]
17. Wu Q. Gene targeting in hemostasis. *Hepsin Front Biosci.* 2001; 6:D192–200.
18. Peleg A, Jaffe AS, Hasin Y. Enzyme-linked immunoabsorbent assay for detection of human serine protease corin in blood. *Clin Chim Acta.* 2009; 409:85–89. [PubMed: 19751717]
19. Dong N, Chen S, Yang J, He L, Liu P, Zheng D, Li L, Zhou Y, Ruan C, Plow E, Wu Q. Plasma soluble corin in patients with heart failure. *Circ Heart Fail.* 2010; 3:207–211. [PubMed: 20061521]
20. Shrestha K, Troughton RW, Borowski AG, Yandle TG, Richards AM, Klein AL, Tang WH. Plasma Corin Levels Provide Minimal Prognostic Utility Incremental to Natriuretic Peptides in Chronic Systolic Heart Failure. *J Card Fail.* 2010; 16:621–627. [PubMed: 20670840]
21. Knappe S, Wu F, Madlansacay MR, Wu Q. Identification of domain structures in the propeptide of corin essential for the processing of proatrial natriuretic peptide. *J Biol Chem.* 2004; 279:34464–34471. [PubMed: 15192093]
22. Knappe S, Wu F, Masikat MR, Morser J, Wu Q. Functional analysis of the transmembrane domain and activation cleavage of human corin. *J Biol Chem.* 2003; 278:52363–52370. [PubMed: 14559895]
23. Liao X, Wang W, Chen S, Wu Q. Role of glycosylation in corin zymogen activation. *J Biol Chem.* 2007; 282:27728–27735. [PubMed: 17660514]
24. Levin ER, Gardner DG, Samson WK. Natriuretic peptides. *N Engl J Med.* 1998; 339:321–328. [PubMed: 9682046]
25. Ayache S, Panelli M, Marincola FM, Stroncek DF. Effects of storage time and exogenous protease inhibitors on plasma protein levels. *Am J Clin Pathol.* 2006; 126:174–184. [PubMed: 16891190]
26. Braunwald E. Biomarkers in heart failure. *N Engl J Med.* 2008; 358:2148–2159. [PubMed: 18480207]

27. Daniels LB, Maisel AS. Natriuretic peptides. *J Am Coll Cardiol.* 2007; 50:2357–2368. [PubMed: 18154959]
28. Luckenbill KN, Christenson RH, Jaffe AS, Mair J, Ordonez-Llanos J, Pagani F, Tate J, Wu AH, Ler R, Apple FS. Cross-reactivity of BNP, NT-proBNP, and proBNP in commercial BNP and NT-proBNP assays: preliminary observations from the IFCC Committee for Standardization of Markers of Cardiac Damage. *Clin Chem.* 2008; 54:619–621. [PubMed: 18310156]
29. Maisel AS, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, Duc P, Omland T, Storrow AB, Abraham WT, Wu AH, Clopton P, Steg PG, Westheim A, Knudsen CW, Perez A, Kazanegra R, Herrmann HC, McCullough PA. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. *N Engl J Med.* 2002; 347:161–167. [PubMed: 12124404]
30. Wu AH. Serial testing of B-type natriuretic peptide and NTpro-BNP for monitoring therapy of heart failure: the role of biologic variation in the interpretation of results. *Am Heart J.* 2006; 152:828–834. [PubMed: 17070141]
31. Wu AH, Smith A. Biological variation of the natriuretic peptides and their role in monitoring patients with heart failure. *Eur J Heart Fail.* 2004; 6:355–358. [PubMed: 14987588]
32. Xu-Cai YO, Wu Q. Molecular forms of natriuretic peptides in heart failure and their implications. *Heart.* 2010; 96:419–424. [PubMed: 19451138]
33. Shi X, Xu G, Xia T, Song Y, Lin Q. N-terminal-pro-B-type natriuretic peptide (NT-proBNP): reference range for Chinese apparently healthy people and clinical performance in Chinese elderly patients with heart failure. *Clin Chim Acta.* 2005; 360:122–127. [PubMed: 16002063]

Fig. 1. Corin concentrations in serum and plasma samples

Serum and plasma samples with heparin, sodium citrate or EDTA were from 40 individuals. Each box represents the median and interquartile range values. The outlines that are >1.5 times the interquartile range are indicated by filled circles. * $p<0.01$ and ** $p<0.001$ vs. indicated groups.

Fig. 2. A. Correlation between corin levels in serum and heparin plasma samples. B. Correlation between corin levels in EDTA and citrate plasma samples
Linear regression lines and 95% confidence limits are shown.

Fig. 3. Stability of native corin in plasma

Corin concentrations were measured in pooled plasma samples stored at room temperature (A) or 4°C (B) for up to 72 h with or without protease inhibitors. Data were from three independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. fresh samples.

Fig. 4. Stability of recombinant corin in pooled plasma

Corin concentrations were measured in pooled plasma samples that contained recombinant corin (~1000 pg/ml) and were stored at room temperature (**A**) or 4°C (**B**) for up to 72 h with or without protease inhibitors. The results were calculated by subtracting values for native corin in the plasma (~719 pg/ml). Data were from three independent experiments. * $p < 0.05$ vs. fresh samples.

Fig. 5. Levels of native and recombinant corin after freezing-and-thawing
Corin concentrations were measured in pooled plasma with EDTA (A) or cell lysate containing recombinant corin (B). The samples underwent up to three cycles of freezing-and-thawing. The results for recombinant corin were calculated by subtracting values for native corin in the plasma. Data were from three independent experiments.

Fig. 6. Levels of native corin in serum and plasma after freezing-and-thawing
Corin concentrations were measured in pooled serum samples (A) and pooled plasma samples with sodium citrate (B) or heparin (C). The samples underwent up to three cycles of freezing-and-thawing. Data were from three independent experiments. * $p < 0.05$ vs. cycle 0.

Fig. 7. Plasma corin levels in EDTA-anticoagulated samples from healthy individuals

Data were from 182 males (**A**) and 166 females (**B**). Each box represents the median and interquartile range values. The outlines that are >1.5 or >3 times the interquartile range are indicated by circles and asterisk, respectively.