Characterization of NT-proBNP in Human Urine

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BACKGROUND: Urine amino-terminal probrain natriuretic peptide (NT-proBNP) concentrations may exclude the presence of heart failure and provide insight into renal clearance mechanisms for human NT-proBNP. We characterized the molecular forms of urine NT-proBNP detected by immunoassay.

METHODS: Urine from patients with heart failure was subjected to HPLC and analyzed using immunoassays specific toward different epitopes of NT-proBNP. We assessed urine NT-proBNP immunoreactivity in healthy subjects and patients with heart failure.

RESULTS: Size-exclusion chromatography of heart failure urine identified no NT-proBNP immunoreactivity coeluting with NT-proBNP(1–76); multiple immunoreactive NT-proBNP fragments were present. The absence of intact urinary NT-proBNP was supported by reversed-phase HPLC. Urine NT-proBNP immunoreactivity was higher in patients with acute [median 192 (interquartile range 108–1445) pg/mg creatinine] and chronic [52 (15–118) pg/mg creatinine] heart failure than in healthy subjects [4.2 (2.6–5.8) pg/mg creatinine] (P < 0.001). In 40 patients with heart failure, urine NT-proBNP immunoreactivity correlated with plasma NT-proBNP (r = 0.72, P < 0.001) and inversely with left ventricular ejection fraction (r = −0.35, P = 0.04).

CONCLUSIONS: Our findings clarify previous reported relationships of urine NT-proBNP–like immunoreactivity with plasma NT-proBNP concentrations and the diagnosis of heart failure. As urine NT-proBNP immunoreactivity is not intact NT-proBNP(1–76), but rather reflects assorted metabolites, the diagnostic performance of NT-proBNP assays in urine may be assay specific, necessitating validation of biomarker performance on an assay-by-assay basis.

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Characterization of Urinary NT-proBNP

NT-proBNP immunoreactivity in human urine compared with high plasma concentrations. Accordingly, we characterized the immunoreactive forms of NT-proBNP in human urine using HPLC coupled to 4 assays specific toward different epitopes of the NT-proBNP molecule.

Materials and Methods

PEPTIDES

We purchased human synthetic NT-proBNP(1–76) as 20 μg net peptide per vial from Phoenix Pharmaceuticals. Mass spectrometry conducted by our laboratory confirmed that the supplied peptide had a molecular weight identical to NT-proBNP(1–76). Synthetic human NT-proBNP(1–21), human NT-proBNP(1–13), and human NT-proBNP(1–13)-Tyr14 were synthesized by Mimotopes.

SAMPLE COLLECTION

All protocols were approved by the local regional ethics committee (Ministry of Health, New Zealand), and all patients gave informed consent. We collected specimens into chilled tubes containing 7.5 mg EDTA per 5 mL blood or 3.75 mg EDTA per 1 mL urine. Within 20 min of collection, specimens were centrifuged at 3000 g and 4 °C for 10 min; the supernatant was stored at −80 °C. Plasma (10–1 mL) and urine (60 mL) were extracted using Sep Pak C18 cartridges (Waters) and 4 °C for 10 min; the supernatant was stored at −80 °C. Plasma and urine were then filtered through 0.1% trifluoroacetic acid (TFA). The cartridge was further washed with 5 mL methanol and 5 mL of 0.1% trifluoroacetic acid (TFA). The cartridge was further washed with 5 mL of 0.1% TFA, and peptides were eluted with 2 mL 80% isopropanol in 0.1% TFA. After addition of 10 μL 1% Triton X-100, the eluates were dried under an air stream at 37 °C. We resuspended extract residues in either buffer before RIA or 20% CH3CN in 0.1% TFA before HPLC.

NT-proBNP ASSAYS

We measured NT-proBNP immunoreactivity using 4 NT-proBNP assays with different epitope specificities. All assays were conducted by technicians blinded to patient status. We used 2 established local assays for NT-proBNP. The first, referred to as NT-proBNP (1–13), used antisem raised against human NT-proBNP(1–13), radiolabeled NT-proBNP(1–13)-Tyr14, and synthetic NT-proBNP(1–21) standard, as described (19). Cross-reactivities using NT-proBNP(1–13) antisem, relative to NT-proBNP(1–21) (100%), were NT-proBNP(1–76) 58%, NT-proBNP(1–13) 77%, NT-proBNP(2–13) 15%, NT-proBNP(3–13) 3%, NT-proBNP(4–13) 0.03%, BNP(1–32) <0.0007%, atrial natriuretic peptide (ANP) <0.0005%, proANP(1–20) <0.0004%, and C-type natriuretic peptide (CNP)-22 < 0.004%. Intra- and interassay CVs for this NT-proBNP assay were 6.1% and 9.9% at 800 ng/L, 6.4% and 8.6% at 1500 ng/L, and 3.5% and 7.9% at 5000 ng/L. The assay limit of detection was 8.5 ng/L.

The second local NT-proBNP assay, referred to as NT-proBNP(62–76), used antisem raised against the carboxy-terminaI residues 62–76 of NT-proBNP, [125I]NT-proBNP(62–76), and synthetic NT-proBNP(1–76) standard (20). Cross-reactivities using NT-proBNP(62–76) antisem, relative to NT-proBNP(1–76) (100%), were NT-proBNP(58–76) 80%, proBNP(58–79) 0.07%, BNP(1–32) 0.14%, ANP 0.13%, and CNP-22 0.09%. The assay limit of detection was 135 ng/L. Intraassay CVs were 7.2% between 340 and 850 ng/L, 6.2% between 850 and 1700 ng/L, and 4.7% between 1700 and 6000 ng/L.

We also used our validated BNP(1–32) assay with antisem from Bachem (T-4021; Bachem) (21). Previous work has demonstrated that our NT-proBNP(1–13) assay detects proBNP(1–108) and NT-proBNP(1–76), our NT-proBNP(62–76) assay detects NT-proBNP(1–76) but does not cross-react with endogenous proBNP(1–108), and our BNP(1–32) assay detects both proBNP(1–108) and BNP(1–32) (20).

We also measured NT-proBNP immunoreactivity using first- and second-generation electrochemiluminescence immunoassays (Roche Diagnostics) on an Elecsys® 2010 analyzer. The first-generation assay (referred to as proBNP I) is based on 2 polyclonal antibodies recognizing epitopes within amino acids 1–21 and 39–50 of NT-proBNP(1–76) and proBNP(22, 23). The second-generation assay (referred to as proBNP II) is based on 2 monoclonal antibodies recognizing epitopes within amino acids 27–31 and 42–46 of NT-proBNP (Roche Diagnostics datasheet). The assay limit of detection was 5 ng/L for both assays. The interassay CV for both Roche NT-proBNP assays was ≤3.0% for concentrations up to 5500 ng/L.

SUBJECTS

Subjects were categorized into 3 groups: (1) 10 healthy individuals without a history of cardiovascular disease, (2) 20 unselected individuals with chronic heart failure (who had required hospitalization for acute heart failure within the previous 12 months), and (3) 20 unselected individuals within 8 h of hospitalization with acute heart failure. Acute heart failure was defined by Framingham criteria (24) and satisfied European Society of Cardiology guidelines (25) for heart failure. We measured plasma and urine NT-proBNP immunoreactivity in untimed samples without prior extraction using the second-generation Roche proBNP II assay. Urine NT-proBNP concentrations were adjusted for urine volume by indexing to urine creatinine measured by the standard colorimetric Jaffé reaction. We estimated glomerular filtration rate (eGFR) using the
Modification of Diet in Renal Disease (MDRD) equation (26). Fractional excretion of filtered NT-proBNP was calculated after normalization of urinary and plasma NT-proBNP concentrations to the respective creatinine concentrations as in the following equation:

\[
\text{Fractional excretion}_{\text{NT-proBNP}} \% = \frac{\text{urinary NT-proBNP}}{\text{plasma NT-proBNP}} \times \frac{\text{urine creatinine}}{\text{plasma creatinine}}
\]

In all heart failure subjects, a transthoracic echocardiogram was obtained using a Vivid 3 machine (General Electric) within 48 h of urine sampling. The standardized imaging protocol included apical 4- and 2-chamber views according to the American Society of Echocardiography (27). Parasternal short- and long-axis views (averaged over 4 cycles) were obtained for M-mode measurements of left ventricular dimensions. Left ventricular volumes were calculated from apical 4- and 2-chamber views using the method of discs formula. Biplane systolic and diastolic volumes and ejection fraction were calculated by planimetry according to Simpson’s method.

**STABILITY OF NT-proBNP IMMUNOREACTIVITY IN URINE**

To assess the effect of urinary degradation on NT-proBNP immunoreactivity, we collected urine from 3 patients with acute heart failure. The samples were supplemented, with and without prior addition of EDTA, to 2500 ng/L with synthetic human NT-proBNP(1–76). Samples were incubated for 0, 30, 60, and 120 min at 37 °C. Incubated aliquots were removed at each time point, snap-frozen on dry ice, and stored at −80 °C before immunoassay.

**HPLC ANALYSIS**

We performed size-exclusion HPLC analysis on urine extracts from 3 patients and a pooled plasma extract from 2 patients, all with acute heart failure. We separated extracts using a TSK-GEL G2000SW (7.5 by 600 mm; Toyo Soda) column equilibrated with 20% 

CH3CN (ChromAR; Malinckrodt Baker Inc.) in 0.1% TFA (Uvasol; Merck KGaA) at 0.5 mL/min. After addition of 10 μL 1% Triton X-100 (10 μL), 0.5-mL HPLC fractions were dried under an air stream at 37 °C and reconstituted in buffer. Because the reversed-phase HPLC runs were conducted some months apart with differing batches of solvent, there were minor differences in the elution position of synthetic NT-proBNP(1–76) standard.

**STATISTICAL ANALYSIS**

Clinical results were calculated as mean (SD) or median and interquartile range (IQR) for nonparametric data. Nonparametric data were log-transformed before analysis to stabilize variances. Where urine NT-proBNP immunoreactivity was undetectable, the urine concentration was assumed to be equal to the assay limit of detection (5 ng/L) for indexation to urine creatinine. We tested agreement between actual and expected endogenous NT-proBNP immunoreactivity after serial dilution of extracted samples as determined by the Roche proBNP II assay using the method of Bland and Altman (28). The ratio between actual and expected NT-proBNP values was plotted against their mean.

Correlation coefficients were calculated using Pearson product–moment correlation. Independent *t*-tests and *χ*² tests were used to compare continuous or categorical variables, respectively, between groups of subjects. *P* values <0.05 were taken to indicate statistical significance. All data analyses were prepared using SPSS version 13 (SPSS Inc.).

**Results**

Serial 2-fold dilutions of urine extracts from patients with heart failure were parallel to standard curves of the NT-proBNP(1–13) and NT-proBNP(62–76) immunoassays (Fig. 1). Using the Bland–Altman method, we observed agreement between actual and expected concentrations of endogenous NT-proBNP immunoreactivity detected by the second-generation Roche assay for serial dilutions of urine extracts. The mean ratio between actual and expected NT-proBNP values was 1.17 (0.27) across a wide range of NT-proBNP immunoreactivity (8.5–200 000 ng/L).

**IN VITRO DEGRADATION OF NT-proBNP IN URINE**

NT-proBNP(1–13) immunoreactivity was stable in urine during 2 h of incubation at 37 °C. By contrast, NT-proBNP(62–76) immunoreactivity in urine decreased by approximately 50% in 2 h. Loss of NT-
proBNP(62–76) immunoreactivity was prevented by prior addition of EDTA (Fig. 2).

CHARACTERIZATION OF NT-proBNP MOLECULAR FORMS IN URINE USING HPLC

Separation of urine extracts by HPLC was conducted on samples from patients with acute decompensated heart failure; HPLC fractions of urine extracts from subjects with chronic heart failure were below the limits of detection for the NT-proBNP immunoassays used. Size-exclusion chromatography of heart failure plasma identified a single, well-defined peak of endogenous NT-proBNP immunoreactivity eluting at the position of synthetic human NT-proBNP(1–76) for both NT-proBNP(1–13) and NT-proBNP(62–76) assays (Fig. 3). In contrast, the HPLC profile of endogenous NT-proBNP immunoreactivity in heart failure urine was markedly heterogeneous (Fig. 3). In all 3 urine samples, the peak of highest MW NT-proBNP eluted at a lower MW than synthetic NT-proBNP(1–76) [fractions 28–29 (6.1 kDa)]. This material was immunoreactive in the NT-proBNP(1–13) assay but was not detected by the NT-proBNP(62–76) assay. Because the NT-proBNP(1–13) assay requires the first 3 residues of NT-proBNP for maximal immunoreactivity, this result suggests that a proportion of the highest MW peak observed in the urine chromatogram was NT-proBNP truncated by proteolytic degradation at the carboxy terminus. The loss of MW equivalent to about 2.5 kDa in this major chromatographic peak compared with the known NT-proBNP(1–76) MW of 8.5 kDa is suggestive of NT-proBNP cleavage near residues 55–58.

An HPLC peak was always detected at fractions 30–32 (Fig. 3) by the NT-proBNP(1–13) and both Roche NT-proBNP assays, consistent with a smaller NT-proBNP fragment of MW 2–3.5 kDa. A number of intermediate elution peaks of variable MWs were observed, representing numerous variably degraded NT-proBNP peptides. Minimal BNP immunoreactivity was identified in size-exclusion HPLC fractions excluding proBNP as an important contributor to the NT-proBNP immunoreactivity profile we observed (data not shown).

Separation of heart failure plasma by reversed-phase HPLC demonstrated elution of NT-proBNP...
Fig. 3. Size-exclusion HPLC analysis of immunoreactive NT-proBNP using antisera directed to NT-proBNP(1–13) (black circles) and NT-proBNP(62–76) (gray circles) and the first-generation (unfilled circles) and second-generation (black circles, dashed line) Roche NT-proBNP assays in Sep Pak C₁₈ extracts of urine from 3 patients with acute heart failure (A–C) and pooled extract of plasma from 2 patients with heart failure (D). The black triangle marks the elution position of synthetic human NT-proBNP(1–76) for the column. Arrows indicate approximate MW elution positions: 1, 8.5 kDa; 2, 6.1 kDa; 3, 2.1 kDa; and 4, 1.6 kDa. Note the different axis scales used for the local and Roche NT-proBNP assays for urine (A) and urine (C) owing to marked differences in concentrations measured by the different assays.
[fractions 64–71] earlier than synthetic nonglycosylated NT-proBNP(1–76) [fraction 73], consistent with its probable glycosylation. Using reversed-phase HPLC, we also identified a broad peak with NT-proBNP immunoreactivity in heart failure urine [fractions 60–64] (Fig. 4) eluting earlier relative to synthetic NT-proBNP [at fraction 76] compared with endogenous NT-proBNP in plasma.

**NT-proBNP IN HUMAN URINE**

*Healthy subjects.* Urine NT-proBNP was below the assay limit of detection (5 ng/L) in all healthy subjects and 3 (6%) patients with heart failure. In 10 healthy subjects (2 men, ages 22–53 years), the median urine NT-proBNP index was less than 4.2 (IQR 2.6–5.8) pg/mg creatinine.

*Subjects with heart failure.* Table 1 shows the clinical characteristics of the patients with heart failure who participated in the study. The median urine NT-proBNP indexed to creatinine was higher in patients with acute and chronic heart failure than in healthy subjects ($P < 0.001$ for both comparisons). Urine NT-proBNP concentrations were also significantly higher in patients with acute compared with chronic heart failure ($P = 0.003$) (Fig. 5). Urine and plasma NT-proBNP concentrations correlated with each other ($n = 40$, $r = 0.72$, $P < 0.001$). Urine NT-proBNP index was inversely correlated with left ven-

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**Table 1.** Baseline characteristics for patients with acute and chronic heart failure.\(^a\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute heart failure</th>
<th>Chronic heart failure</th>
<th>$P^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>73.6 (13.5)</td>
<td>74.2 (8.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>12 (60)</td>
<td>11 (55)</td>
<td>NS</td>
</tr>
<tr>
<td>NYHA functional class, n (%)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I</td>
<td>2 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>11 (55)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8 (40)</td>
<td>7 (35)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12 (60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR, mL/min/1.73m²</td>
<td>51.7 (22.4)</td>
<td>58.2 (17.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>37.6 (15.5)</td>
<td>47.3 (16.7)</td>
<td>NS (0.06)</td>
</tr>
<tr>
<td>Plasma NT-proBNP, ng/L</td>
<td>4313 (2548–13752)</td>
<td>1381 (436–2677)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine NT-proBNP, ng/L</td>
<td>113 (45–683)</td>
<td>30 (14–102)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urine NT-proBNP index, pg/mg creatinine</td>
<td>191 (108–1445)</td>
<td>52 (15–118)</td>
<td>0.003</td>
</tr>
<tr>
<td>Fractional excretion NT-proBNP, %</td>
<td>8.5 (4.0–22.7)</td>
<td>4.6 (2.8–15.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^{a}\) Data are mean (SD) or median (IQR), unless noted otherwise.

\(^{b}\) $P$ values represent group comparisons between acute heart failure and chronic heart failure status using $\chi^2$ or independent $t$-tests. NS, not significant; NYHA, New York Heart Association.
tricular ejection fraction (n = 40, r = −0.33, P = 0.04) and eGFR (n = 40, r = −0.45, P = 0.004) across the eGFR range of 18–107 mL/min/1.73m² (Fig. 5). Plasma NT-proBNP was also inversely correlated with left ventricular ejection fraction (n = 40, r = −0.48, P < 0.01) and eGFR (n = 40, r = −0.38, P = 0.01). Median fractional excretion of filtered NT-proBNP was 6.3% (IQR 3.3%–19.9%) and was inversely correlated with eGFR (n = 40, r = −0.41, P = 0.009) but not with left ventricular ejection fraction (n = 35, r = −0.05, P = 0.77) or plasma NT-proBNP immunoreactivity (n = 40, r = 0.13, P = 0.45).

Discussion

We used HPLC, coupled to multiple RIAs incorporating antisera raised toward a range of epitopes of NT-proBNP(1–76), to characterize NT-proBNP forms in the urine of patients with acute heart failure. We validated a commercial assay for determination of urine NT-proBNP levels. Our data indicate that immunoreactive NT-proBNP is present in human urine as multiple fragments of the 76–amino acid peptide. We confirmed previous work showing that urine NT-proBNP immunoreactivity correlates with concurrent plasma concentrations (11, 12, 16) in patients with heart failure, is significantly increased in patients with heart failure compared with healthy controls (11, 12, 15, 16), and correlates inversely with left ventricular ejection fraction (13) and creatinine clearance (12).

The sources of NT-proBNP fragments in human urine are unknown. At a MW of 8.5 kDa (or even twice that if heavily glycosylated) and possessing a neutral charge at pH 7.4 (29), NT-proBNP is likely to be freely filtered at the glomerulus and may undergo subsequent proteolysis in urine. Additionally, NT-proBNP may undergo degradation by renal tubular cells. The lack of correlation between eGFR and fractional renal NT-proBNP extraction across a broad range of kidney function (30) favors a contribution from tubular metabolism and secretion rather than glomerular filtration alone as the major renal clearance mechanism for circulating NT-proBNP. The observation in the current study, that urinary NT-proBNP indexed to creatinine was more discriminating for acute heart failure than urinary NT-proBNP concentration alone, is consistent with this conclusion. Markedly reduced concentrations of NT-proBNP in urine compared with plasma are also consistent with a cellular proteolytic mechanism for renal NT-proBNP clearance. In the current study, the inverse relationship between both urine NT-proBNP immunoreactivity and fractional excretion of filtered NT-proBNP to eGFR suggests that renal NT-proBNP clearance is increased rather than decreased with decreasing eGFR, but does not define the mechanisms by which degraded immunoreactive NT-proBNP enters the urinary space. Although not definitive, these data are consistent with upregulated renal excretion of circulating NT-proBNP associated with reducing renal function.

Renal tubular epithelial cells possess numerous peptidases, including enzymes that are known to degrade BNP (31). These include the membrane-bound
metalloprotease neutral endopeptidase (EC 3.4.24.11) (32) and the transmembrane serine protease, dipeptidyl peptidase IV (DPP4) (EC 3.4.14.5) (33, 34) that degrades peptides where proline or alanine is the penultimate amino terminal residue (35). ProBNP(1–108) and its cleavage products NT-proBNP(1–76) and BNP(77–108) all have proline as the second amino-terminal residue and are known (23, 36) or potential substrates for DPP4 amino-terminal degradation. Substantially lower urine NT-proBNP concentrations in HPLC fractions identified by the NT-proBNP(1–13) (amino-terminal specific) assay compared with mid-peptide assays (Roche proBNP first- and second-generation assays) is consistent with DPP4-mediated degradation of NT-proBNP. DPP4 is present in both glomerular epithelial (37) and renal tubular (38) cells and may play a role in renal metabolism of NT-proBNP before urinary excretion.

In reversed-phase HPLC, all immunoreactive urine NT-proBNP eluted ahead of a synthetic (and nonglycosylated) NT-proBNP(1–76) standard and relatively earlier than NT-proBNP in plasma. Taken together with our size-exclusion results, the data suggest that NT-proBNP present in plasma has a greater molecular weight than the forms in urine of these subjects with heart failure. Glycosylation of NT-proBNP may account for its early elution on reversed-phase HPLC. In any case, it is clear that the urine and plasma NT-proBNP forms are different.

Analysis of patients with documented heart failure and healthy controls confirmed that urine NT-proBNP concentrations are increased in heart failure (11, 12, 15, 16). The inverse relationship between urine NT-proBNP and left ventricular ejection fraction, together with correlations between plasma and urine concentrations in heart failure, suggests that urine NT-proBNP immunoreactivity is a potential biomarker in heart failure, as previously suggested (11–17). Urine concentrations may reflect severity of cardiac decompensation. Accordingly, urine NT-proBNP values represent a potential screening tool for the presence of heart failure.

A single landmark study has evaluated the utility of urine NT-proBNP concentrations to exclude left ventricular systolic dysfunction (LVSD) assessed by echocardiography (n = 1308, prevalence of LVSD 2.1%) in a community population without antecedent heart failure (15). In the study, using a noncompetitive immunoluminometric assay (11), a urine NT-proBNP index of 0.16 pg/mg creatinine was comparable to plasma NT-proBNP (cutoff value of 222 ng/L) as a screening tool to exclude LVSD, with positive and negative predictive values for presence of LVSD of 5.7% and 99.8%, respectively. The utility of urine NT-proBNP concentrations to detect heart failure (as opposed to detection of LVSD) in an unselected symptomatic population, however, remains to be assessed and is necessary before the diagnostic test accuracy of urine NT-proBNP for diagnosis of heart failure is known.

In conclusion, degradation of circulating plasma NT-proBNP in renal tissue before excretion in urine is likely to contribute to renal clearance, although the mechanisms remain to be elucidated. Clinical studies of urine NT-proBNP values in dyspneic patients, as previously conducted for plasma NT-proBNP (39, 40), are now needed to determine the diagnostic test accuracy of urine NT-proBNP for the detection or exclusion of heart failure in symptomatic patients. There is little, if any, intact NT-proBNP(1–76) present in heart failure urine, and therefore immunoreactivity will vary according to the epitopes to which assorted antisera bind. The diagnostic performance of NT-proBNP assays applied to urine may be assay specific, necessitating analysis of biomarker performance on an assay-by-assay basis.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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