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*Published in:*  
Clinical chemistry and laboratory medicine

*DOI:*  
[10.1515/cclm-2019-0152](https://doi.org/10.1515/cclm-2019-0152)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2019

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Harskamp, L. R., Meijer, E., van Goor, H., Engels, G. E., & Gansevoort, R. T. (2019). Stability of tubular damage markers epidermal growth factor and heparin-binding EGF-like growth factor in urine. *Clinical chemistry and laboratory medicine*, 57(10), E265-E268. <https://doi.org/10.1515/cclm-2019-0152>

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## Letter to the Editor

Laura R. Harskamp\*, Esther Meijer, Harry van Goor, Gerwin E. Engels and Ron T. Gansevoort

# Stability of tubular damage markers epidermal growth factor and heparin-binding EGF-like growth factor in urine

<https://doi.org/10.1515/cclm-2019-0152>

Received February 8, 2019; accepted February 11, 2019

**Keywords:** epidermal growth factor (EGF); heparin-binding EGF-like growth factor; pre-analytical conditions; sample storage; stability; tubular damage markers.

To the Editor,

There is an unmet need for biomarkers that predict accelerated kidney function decline caused by the relative insensitivity of the established biomarkers estimated glomerular filtration rate (GFR) and albuminuria [1, 2]. Recently, a kidney biopsy transcriptome-driven approach showed that urinary excretion of heparin-binding-epidermal growth factor (EGF)-like growth factor (uHB-EGF) and especially epidermal growth factor (uEGF) may fulfill the role of prognostic biomarker for chronic kidney disease (CKD) progression [3, 4]. In subsequent observational prospective cohort studies, uEGF was indeed identified as independent predictor of kidney function decline [3, 5, 6]. In these epidemiological studies urine samples often remain in the refrigerator for several hours prior to processing, and biomarker levels are in general measured in urine samples that have been stored for prolonged periods instead of immediately upon collection.

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Variations in handling and storage conditions of urine samples have been identified as a major sources of variation in epidemiological studies investigating the predictive value of urinary biomarkers [7–9]. So far, this has not been investigated for uEGF and uHB-EGF. In this present study, we investigated the effect of several storage conditions on the stability of urinary EGF and HB-EGF levels to provide evidence based instructions for optimal pre-analytical sample handling.

Ten volunteers, including five healthy subjects and five patients with kidney disease, were enrolled in this study in June 2018. These groups were chosen to represent a broad range of urinary EGF and HB-EGF levels. Subjects were invited to our out-patient clinic at the same day and instructed to collect an early morning void urine sample. These urine samples were divided over various aliquots immediately upon voiding. The aliquots that were used for studying the effect of short-term storage remained in the refrigerator at 4 °C until processing, while aliquots for studying the effect of freezing were immediately placed on ice. Thereafter, several pre-analytic sample handling procedures were evaluated. First, the effect of short-term storage at 4 °C on uEGF and uHB-EGF levels was evaluated at 6, 24 and 48 h. Second, the effect of long-term storage at –80 °C was assessed at 1 month, 2 months and 6 months. Finally, the effect of repeated freeze-thaw cycles on the uEGF and uHB-EGF levels was investigated after 6 months of frozen storage. During each cycle, a sample was frozen to –80 °C and thereafter thawed to 4 °C.

uEGF and uHB-EGF were measured with solid phase sandwich enzyme-linked immunoassays (R&D systems, USA). These assays were optimized to allow measurement in the lower range as described in a previous paper [10]. The lower limits of detection were 7.8 pg/mL and 6.3 pg/mL for uEGF and uHB-EGF, respectively. Values of uEGF below these limits were considered as equal to the detection limit in the analyses. No values below the detection limit were found for uHB-EGF.

For both EGF and HB-EGF two quality controls (one in the lower and one in the higher range), were analyzed

in duplicate in each assay run to investigate reproducibility. These quality control samples (QCs) were used from a batch of aliquots that had been stored frozen and never thawed during more than 5 years. These QCs were used for each study sample to correct for day-to-day variation using the following equation:

$$\left( \frac{\text{average of two study samples in one run/} \\ \text{([low QC + high QC in that same run]/2)}} \right)$$

To determine intra-assay coefficients of variation (CV) the results of duplicate urine samples were used that were analyzed in one immunoassay run, whereas for inter-assay coefficients of variation the results of quality control samples were used that were measured on the different measurement days during the study ( $CV = [\text{mean SD}/\text{mean concentration}] \times 100\%$ ).

Variables are presented as mean and standard deviation (SD). Student's t-tests were performed to compare values from two groups. p-Values of <0.05 were considered to indicate statistical significance. All analyses were performed using SPSS (IBM Statistics version 23) and Graphpad Prism (version 7.02).

Participating subjects had a mean age of  $34.5 \pm 11.6$  years, and a mean baseline uEGF and uHB-EGF concentration of  $33.1 \pm 36.3$  ng/mL and  $147.1 \pm 52.6$  pg/mL, respectively. The overall intra- and inter-assay coefficients of variation were for uEGF 3.6% and 6.4%, respectively (in the QCs 3.2% and 14.1%, respectively), and for HB-EGF 4.6% and 6.2%, respectively (in the QCs 7.5% and 17.2%, respectively).

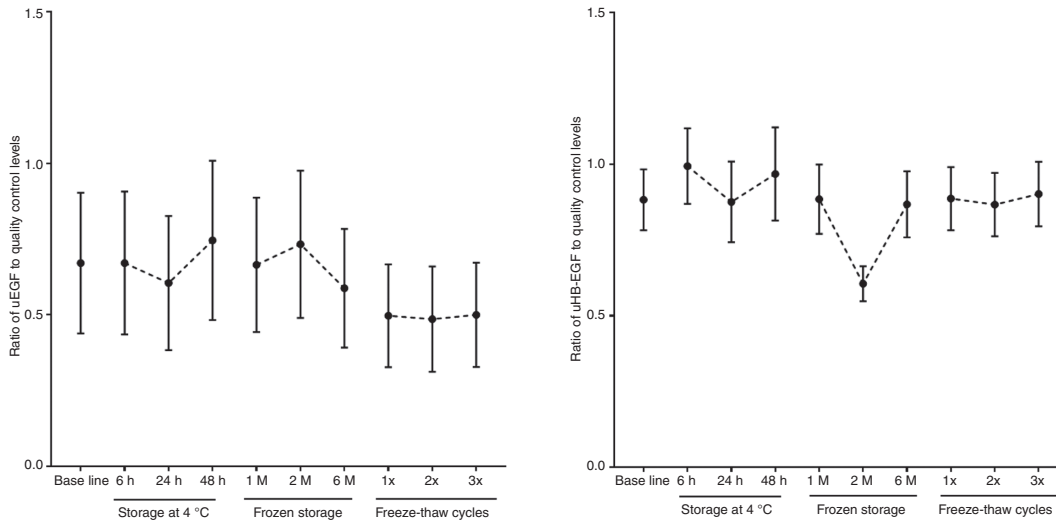
During storage at 4 °C, the uEGF and uHB-EGF levels remained unchanged, i.e. at 48 h the mean uEGF level in subjects and QCs was similar compared to baseline (+11.9%,  $p=0.2$ , and +2.0%,  $p=0.7$ , respectively) (Table 1). In line the ratio of uEGF to QC levels was unaffected during refrigerator storage (+25.0%,  $p=0.2$ ) as shown in Figure 1. The uHB-EGF levels decreased by -49.1% in the study subjects compared to baseline ( $p < 0.001$ ), but decreased also in the QC samples (-63.4%,  $p=0.06$ , Table 1). Figure 1 shows that as a result the uHB-EGF levels in subjects corrected for QC levels were unaffected (+4.2%,  $p=0.2$ ). Also variability was not affected by storage at 4 °C, as the SD of the mean uEGF and uHB-EGF levels, as well as the SD of uEGF and uHB-EGF adjusted for QC samples remained comparable over time (Table 1, Figure 1).

During frozen storage the uEGF and HB-EGF levels also remained similar. An increase in uEGF concentration was observed after 6 months of storage at -80 °C in subjects as well as in QCs (+13.6%,  $p=0.02$ , and +24.3%,  $p=0.07$ ), respectively. The ratio of uEGF in subjects to QCs was therefore not different compared to baseline (-11.3%,  $p=0.1$ ). uHB-EGF levels were lower after frozen storage,

**Table 1:** Effect of various storage conditions on urinary EGF and HB-EGF levels (mean  $\pm$  SD).

	Baseline	Storage at 4 °C				Frozen storage			Freeze-thaw cycles			
		6 h	24 h	48 h	1 M	2 M	6 M	1 FT	2 FT	3 FT		
uEGF												
Subjects	33.1 $\pm$ 36.3	34.4 $\pm$ 38.2	33.5 $\pm$ 38.8	37.6 $\pm$ 41.8	40.5 $\pm$ 42.8	38.2 $\pm$ 39.9	38.3 $\pm$ 40.3	32.4 $\pm$ 34.9	31.7 $\pm$ 35.9	32.6 $\pm$ 35.6		
Change	Reference	+3.9%	+1.3%	+11.9%	+18.3%	+13.3%	+13.6%	-2.2%	-4.4%	-1.6%		
QCs	49.3 $\pm$ 33.0	51.4 $\pm$ 31.8	55.4 $\pm$ 31.1	50.3 $\pm$ 36.0	61.0 $\pm$ 34.7	51.9 $\pm$ 26.5	65.1 $\pm$ 30.6	65.1 $\pm$ 30.6	65.1 $\pm$ 30.6	65.1 $\pm$ 30.6		
Change	Reference	+4.0%	+11.0%	+2.0%	+19.2%	+5.0%	+24.3%	N/A	N/A	N/A		
uHB-EGF												
Subjects	147.1 $\pm$ 52.6	165.3 $\pm$ 65.5	128.7 $\pm$ 61.7	98.6 $\pm$ 49.6	130.8 $\pm$ 53.6	93.5 $\pm$ 28.3	122.5 $\pm$ 48.6	125.0 $\pm$ 46.8	122.5 $\pm$ 46.8	127.5 $\pm$ 47.8		
Change	Reference	+11.1%	-14.3%	-49.1%	-12.4%	-57.3%	-20.0%	-17.6%	-20.1%	-15.3%		
QCs	166.5 $\pm$ 68.8	166.2 $\pm$ 59.9	146.8 $\pm$ 76.2	101.9 $\pm$ 59.6	147.7 $\pm$ 71.5	154.2 $\pm$ 62.2	141.2 $\pm$ 82.3	141.2 $\pm$ 82.3	141.2 $\pm$ 82.3	141.2 $\pm$ 82.3		
Change	Reference	-0.2%	-13.4%	-63.4%	-12.7%	-8.0%	-17.9%	N/A	N/A	N/A		

Absolute values are provided as mean  $\pm$  SD in ng/mL for uEGF and in pg/mL for uHB-EGF. Percentages indicate mean percentage change compared to the reference value. uEGF, urinary epidermal growth factor; uHB-EGF, urinary heparin-binding EGF-like growth factor; h, hour(s); M, month(s); FT, freeze-thaw cycles; QC, quality control.



**Figure 1:** Effect of various storage conditions on urinary EGF and HB-EGF levels.

Data points are expressed as ratio of concentrations of biomarker in study subjects compared to quality control samples (mean  $\pm$  SEM). uEGF, urinary epidermal growth factor; uHB-EGF, urinary heparin-binding EGF-like growth factor; h, hour(s), M, months.

but again in subjects as well as in QCs ( $-20.0\%$ ,  $p=0.002$ , and  $-17.9\%$ ,  $p=0.2$ , respectively). The ratio of uHB-EGF levels in subjects to QCs was stable after 6 months of storage at  $-80^\circ\text{C}$  compared to baseline ( $-5.9\%$ ,  $p=0.7$ ).

Repeated freeze-thaw cycles did not affect mean uEGF or uHB-EGF levels. After three cycles, compared to baseline uEGF levels were  $-1.6\%$  ( $p=0.7$ ), and uHB-EGF levels  $-15.3\%$  ( $p=0.005$ ), with uHB-EGF adjusted for QC samples  $-1.0\%$  ( $p=0.6$ ) (Table 1 and Figure 1).

To the best of our knowledge, this is the first study to examine the effect of several pre-analytic sample handling and storage conditions on urinary EGF and HB-EGF levels. We show that EGF and HB-EGF levels remain stable in urine after storage at  $4^\circ\text{C}$  up to 48 h, after long-term storage at  $-80^\circ\text{C}$ , as well as after repeated freeze-thaw cycles.

For uHB-EGF levels it may seem that levels decreased after 48 h of storage at  $4^\circ\text{C}$ . However, uHB-EGF levels in QCs measured in the same run were also lower compared to baseline indicating that the change in uHB-EGF was actually the result of day-to-day variability in the uHB-EGF measurement. After adjusting the subjects' samples for QC levels, we observed that levels of uHB-EGF were unaffected by refrigerator storage (Figure 1). Similarly, we noticed decreased uHB-EGF levels after repeated freeze-thaw cycles, but adjusting for QCs showed its levels remained stable. The use of QCs as a measure for reproducibility, is therefore a significant strength of this study. We propose therefore that QCs are used in epidemiological research in which (urine) biomarkers are measured over time.

Other strengths of this study were the use of optimized assays for biomarker measurements, samples measured

in duplicate to minimize random variability, and including subjects with kidney disease and healthy subjects to guarantee a broad range of biomarker levels. Limitations are that for practicality reasons stability was examined after a frozen storage up to 6 months, while storage in clinical research studies can sometimes last several years.

There is great interest in measuring uEGF and uHB-EGF for both research and clinical care, as these growth factors have the potential to serve as surrogate biomarkers for renal tubular function. In patients with CKD and diabetes mellitus uEGF indeed added prognostic value to estimated glomerular filtration rate and albuminuria to predict CKD progression [5, 6]. Our present results highlight the importance of testing the influence of sample storage conditions on urinary biomarker levels prior to their use in research or clinical practice. The stability that we showed supports the use of uEGF and uHB-EGF in biomarker research.

In conclusion, measurement of uEGF and uHB-EGF levels is relatively reproducible, and not affected by pre-analytical conditions, suggesting no need for specific instructions for pre-analytical sample handling. The levels of these biomarkers can also be measured accurately after prolonged frozen storage, which is common in biomarker research.

**Author contributions:** All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

**Research funding:** None declared.

**Employment or leadership:** None declared.

**Honorarium:** None declared.

**Competing interests:** The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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