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Prolonged Frozen Storage of Urine Reduces the Value of Albuminuria for Mortality Prediction

To the Editor:

Albuminuria is increasingly recognized as a cardiovascular risk factor in patients with diabetes and in the general population. Cardiovascular disease risk increases continuously with increasing urinary albumin excretion, starting at concentrations that once were considered healthy *(1)*. We recently reported that, after prolonged frozen storage of urine samples, albumin concentrations may decrease, particularly those within the reference and microalbuminuric intervals *(2)*. We also observed sample variation in the extent to which urinary albumin concentrations (UAC) decreased. In 2005, to investigate whether outcome predictions based on albuminuria were affected by assessment of UAC from frozen samples, we reassessed UAC in Prevention of Renal and Vascular ENdstage Disease (PREVEND) study baseline urine samples, collected and stored frozen at −20 °C from 1997 to 1998. The PREVEND study was approved by the local medical ethics committee and conducted in accordance with the guidelines of the Declaration of Helsinki *(3)*.

The PREVEND dataset was enriched for albuminuria. To create a representative sample of the general population, the enriched subset (UAC >10 mg/L) was reweighed by proportionally taking an SPSS-generated random subset. Study participants with missing data on UAC and/or with leucocyturia, erytrocyturia, and/or kidney disease were excluded. Of the remaining 3249 persons, 104 died during follow-up. Power calculations indicated that 330 cases would provide a power of 90% with $\alpha = 0.05$ for detection of a difference of 0.05 in area under the ROC analyses between fresh and frozen samples. In the total PREVEND cohort, from 1997 to 1998 until December 2004, 340 persons died during follow-up. The dataset was enriched, therefore, with samples from persons who died, leading to a final study population of 3485 persons.

Handling of fresh and frozen urine samples, including hand inversions and centrifuging before analysis by nephelometry, was performed as described previously *(2)*. The variability with calibration and reagent lot changes was 1.6% to 4.4% and 1.1% to 2.4%, respectively. The intra- and interassay CVs were 2.7% and 4.5%, respectively.

Participants in the PREVEND study gathered 2 urine samples on 2 consecutive days, allowing us to investigate whether prolonged frozen storage leads to additional error. We used the Pythagorean Theorem ($CV^2_{total} = CV^2_{analytical+biological} + CV^2_{freezing}$) to calculate $CV_{freezing}$ from the day-to-day variation in UAC for fresh and frozen samples. The median (interquartile range) UAC in fresh urine samples was 5.0 (3.3–8.6) mg/L. UACs were reassessed after a mean (SD) time period of 7 (0.4) y (range, 7.7–8.8 y) of frozen storage. After frozen storage, the mean (SD) percentage UAC concentration change was −27 (26)%, −51 (29)%, −43 (30)%, and −14 (17)% for samples with fresh UAC concentrations of <10, 10–20, 20–200, and >200 mg/L, respectively.

The between-day variations were 24%, 26%, 25%, and 16% for fresh samples ($CV_{analytical+biological}$), and 27%, 40%, 37%, and 23% for frozen samples (CV_{total}) for the <10, 10–20, 20–200, and >200 mg/L categories, respectively. Freezing, therefore, introduced an additional variation ($CV_{freezing}$) of 12%, 30%, 27%, and 17% in the respective categories. The overall additional measurement error ($CV_{freezing}$) from frozen storage was 18% ($CV_{analytical + biological}$ 25% and CV_{total} 31%). ROC analysis revealed a mean (SE) area under the curve of 0.80 (0.014; $P < 0.001$) for the prediction of mortality by UAC assessed from fresh samples and 0.74 (0.016; $P < 0.001$) from frozen samples ($P = 0.006$ for comparison with fresh samples) (Fig. 1).

Our study is the first prospective report on a decrease in predictive properties of albuminuria for mortality after prolonged frozen storage of urine samples at −20 °C. We also found that freezing introduced additional measurement error along with a decrease in UAC. Introduction of an additional measurement error by freezing was suggested previously *(2, 4)*, but to the best of our knowledge no previous studies actually compared frozen with fresh samples.

Based on the results of 2 previous studies, urine samples were more stable at −70 °C than at −20 °C storage *(4, 5)*. In the 1st study, which investigated samples stored at −20 °C and −70 °C for 22 weeks, neither a significant decrease in UAC nor a significant difference between frozen storage at −20 °C and −70 °C was encountered. In the 2nd study, which assessed UAC only after frozen storage at −20 °C and −70 °C and not in fresh samples, significantly higher concentrations were found in samples stored at −70 °C than at −20 °C. This study, however, did not investigate whether storage at −70 °C indeed prevented a decline.

Our study shows that prolonged frozen storage of urine samples at −20 °C adversely affects prediction of outcome by albuminuria. This effect is relevant for the interpretation and design of epidemiological stud-

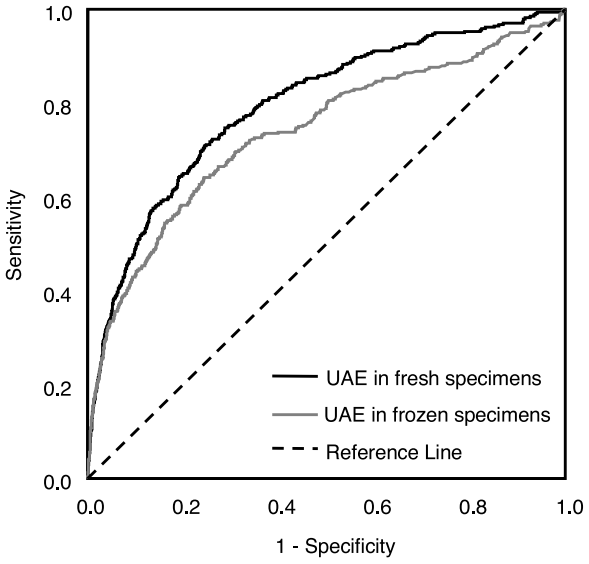

Fig. 1. ROC curves for albuminuria assessed in fresh and thawed samples as predictors of all-cause mortality. $P < 0.05$ between UAE in fresh and frozen urine.

ies, screening programs, and intervention trials.

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