assessed in human blood left at room temperature or at 4 °C. Ketamine, NK, and DHNK at a concentration of 500 μg/L were added to blood samples from volunteers. The samples were divided into two aliquots: one was stored at room temperature (~20 °C) and the other at 4 °C. At t₀ and at 30, 60, and 120 min, 8 mL of each aliquot was removed and immediately centrifuged at 2000g for 10 min at 20 °C. The plasma was decanted and stored immediately at ~20 °C until analysis. The assay was performed within 15 days of storage, and five replicates were used in each case. Results were compared using the nonparametric Mann–Whitney test. Two-tailed values <0.05 were considered significant. Statistical analysis was performed using Graphpad Instat software.

Ketamine, NK, DHNK, and nortilidine were stable in aqueous solutions at ~80 °C for at least 6 months. These solutions were used for the addition experiments in biological samples. No significant difference was observed in the concentrations of ketamine and its metabolites in human serum left at 4 °C for 2 days. Storage of ketamine, NK, and DHNK in human serum at ~20 °C did not produce significant changes in concentrations over a period of 10 weeks. These data are in accordance with those reported previously (7). As shown in Table 1, plasma concentrations of ketamine and NK remained constant when the centrifugation of blood was delayed for 2 h, and the stability of the two compounds was not affected by the change in storage temperature from 4 to 20 °C. On the other hand, a significant decrease in the plasma DHNK concentration was observed when blood samples were kept at 4 °C, whereas surprisingly, the DHNK concentration did not change significantly when blood samples were left for 2 h at room temperature.

After 30 min of storage at 4 °C, the plasma concentration of DHNK was, on average, 68% of the initial concentration. Furthermore, we also observed that the plasma concentrations of DHNK measured at t₀ represented, on average, 75% of the concentration added to blood and stored at 4 °C or at room temperature. These results could suggest that DHNK is unequally distributed between plasma and blood cells and that rapid permeation of this compound into blood cells may occur. Chemical degradation is not likely because the increase in temperature from 4 to 20 °C slowed the decrease in DHNK concentrations. Further investigations should be done to study the effect of various temperatures on the plasma concentration of DHNK. Moreover, the determination of DHNK concentrations in blood cells to estimate the partitioning behavior of this compound may be warranted.

In conclusion, the present study shows the necessity of observing rigorous conditions for the accurate estimation of ketamine metabolite concentrations in blood samples. The collected blood should be centrifuged without delay at ambient temperature to avoid the decrease in DHNK concentrations, which is most likely attributable to the permeation of this compound into the blood cells. The plasma samples can be transported at 4 °C within 2 days and can be stored at ~20 °C for 10 weeks without any change in the concentrations of ketamine, NK, and DHNK.

References

Diurnal Variation of Human Chorionic Gonadotropin β-Core Fragment Concentrations in Urine during Second Trimester of Pregnancy, Sigi Ratmensch,1 Claudia Celentano,1 Nelli Elliger,1 Oscar Sadan,1 Dan Lehman,2 Abraham Golan,1 and Marek Glezerman3 (Departments of 1 Obstetrics and Gynecology and 2 Clinical Immunology, The Edith Wolfson Medical Center, Holon, Holon 58100, and Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel; 3 Department of Obstetrics and Gynecology, Chieti University, 66031 Chieti, Italy; * address correspondence to this author at: Division of Obstetrics and Gynecology, The Edith Wolfson Medical Center, Holon 58100, Israel; fax 9723-502-8503, e-mail sigi-ro@zahav.net.il)

β-Core fragment (βcf) is the main metabolic breakdown product of human chorionic gonadotropin (hCG) (1). This analyte appears in maternal urine during pregnancy, but is undetectable in serum. Recent studies (2–4) have shown that concentrations of hCGβcf are substantially increased in pregnancies affected by Down syndrome, suggesting the use of this analyte for midtrimester antenatal screening. However, various groups of investigators have reported large differences in the efficacy of screening by means of hCGβcf (5–7). Some of these inconsistencies have been attributed to conditions used for storage of urine specimens (8), as well as chance, assay method, and study design (7).

hCG serum concentrations show distinct diurnal variation (9). We investigated whether urine concentrations of hCGβcf, the terminal breakdown product of serum hCG, also show diurnal variation. Alterations in the time of day when urine specimens were collected in previously reported studies could potentially explain the inconsistent findings on the screening efficacy of this analyte for Down syndrome. We prospectively and longitudinally examined hCGβcf concentrations in urine from women with uncomplicated midtrimester pregnancies.
Sampling times are reported in Table 1. Crude hCG concentrations was 211%. When hCG was stored at 2°C and then frozen within 4 h, and stored at −20°C until assayed for hCG within 7 days of collection. hCG concentrations were measured by use of a commercially available enzyme-immunoassay reagent set (Novamed Ltd.). The test was repeated at a different dilution if the measured concentration was outside the operating range. Measurements of hCG were indexed to urine creatinine concentrations, as measured by an enzymatic creatinine assay. Fetal karyotypes obtained by amniocentesis were available for 14 participants and were normal. Fetuses without available karyotypes showed distinct diurnal variation, with concentrations highest at 0600 and lowest at 1800 (Fig. 1); ANOVA showed that the difference was statistically significant (P < 0.001). Compared with baseline values at 0, concentrations were significantly different at 0600 (P < 0.005) and 1200 (P < 0.005).

The main finding of our study was that creatinine-normalized hCG concentrations in urine showed significant diurnal variation. The difference between peak and nadir concentrations was equivalent to 0.8 MoM. One would expect, therefore, that calculations of the risk of Down syndrome must be substantially affected by the timing of urine specimen collection. In turn, these findings could explain some of the reported inconsistencies in reported screening efficacies.

Urine hCG concentrations is the final metabolic breakdown product of the nicked hCG β subunit. Increased nicking of the β subunit in Down syndrome pregnancies was first demonstrated by Rotmensh and coworkers (10, 11), but the mechanism for this phenomenon remains unclear. Greater phagocytic activity against the placenta as a foreign semiallograft for the mother and increased nicking activity in the sera of pregnant women have been proposed. Early studies found urine concentrations of hCG between 1.3- and 6.1-fold higher than the unaffected median have been reported (12). Variability suggests that factors other than the biology of Down syndrome pregnancies affect the results. It is unclear, at present, whether dividing

| Table 1. Urine β-core and creatinine concentrations in relation to time of day. |
|------------------|------------------|------------------|------------------|------------------|
|                  | Median (range)   | Median (range)   | Median (range)   | Median (range)   |
|                  | at 0000          | at 0600          | at 1200          | at 1800          |
| β-core, MoM      | 1.74 (0.05–47.76) | 2.29 (0.23–37.68) | 1.94 (0.07–10.44) | 1.49 (0.04–8.06) |
| Creatinine, MoM  | 0.78 (0.18–3.58)  | 0.74 (0.37–3.64)  | 0.70 (0.19–2.38)  | 0.70 (0.29–2.98)  |
| β-core/creatinine, MoM | 1.58 (0.09–10.24) | 2.10 (0.29–12.64) | 1.66 (0.30–13.91) | 1.39 (0.12–6.82) |

We prospectively studied 46 healthy pregnant women at 15–19 weeks of gestation. Pregnancies were dated by menstrual history and first-trimester ultrasonographic examination and followed until delivery. A detailed history was obtained from all volunteers to exclude preexisting medical or obstetric complications.

Midstream urine samples were collected at 0600, 1200, 1800, and 0000. Specimens were frozen within 4 h and stored at −20°C until assayed for hCGβcf within 7 days of collection. hCGβcf concentrations were measured by use of a commercially available enzyme-immunoassay reagent set (Novamed Ltd.). The test was repeated at a different dilution if the measured concentration was outside the operating range. Measurements of hCGβcf were indexed to urine creatinine concentrations, as measured by an enzymatic creatinine assay. Fetal karyotypes obtained by amniocentesis were available for 14 participants and were normal. Fetuses without available karyotypes showed distinct diurnal variation, with concentrations highest at 0600 and lowest at 1800 (Fig. 1); ANOVA showed that the difference was statistically significant (P < 0.001). Compared with baseline values at 0, concentrations were significantly different at 0600 (P < 0.005) and 1200 (P < 0.005).

The main finding of our study was that creatinine-normalized hCGβcf concentrations in urine showed significant diurnal variation. The difference between peak and nadir concentrations was equivalent to 0.8 MoM. One would expect, therefore, that calculations of the risk of Down syndrome must be substantially affected by the timing of urine specimen collection. In turn, these findings could explain some of the reported inconsistencies in reported screening efficacies.

Urine hCGβcf is the final metabolic breakdown product of the nicked hCG β subunit. Increased nicking of the β subunit in Down syndrome pregnancies was first demonstrated by Rotmensh and coworkers (10, 11), but the mechanism for this phenomenon remains unclear. Greater phagocytic activity against the placenta as a foreign semiallograft for the mother and increased nicking activity in the sera of pregnant women have been proposed. Early studies found urine concentrations of hCGβcf to be five- to sixfold higher in Down syndrome than in normal pregnancies (12). However, as more studies have been published, the early results have been at the high end of the range. In fact, the variability of results has been unusually great; concentrations of hCGβcf between 1.3- and 6.1-fold higher than the unaffected median have been reported (12). This variability suggests that factors other than the biology of Down syndrome pregnancies affect the results. It is unclear, at present, whether dividing

![Fig. 1. Diurnal variation in log-transformed β-core/creatinine ratios. The box plots show medians, 25th and 75th percentiles, and the 10th and 90th percentiles (bars).](https://academic.oup.com/clinchem/article-abstract/47/9/1715/5639538/fig1)
hCGb fragment concentrations by creatinine concentrations properly normalizes for sample concentrations (13). It is conceivable that in very dilute urine, the relationship between creatinine and urine concentrations of hCGb fragment is altered. Cole et al. (8) showed that urine concentrations of hCGb fragment in Down syndrome pregnancies decrease over time in storage, possibly because of aggregation. This finding implied that the interval between sampling of urine and the assay could substantially affect hCGb fragment concentrations. Cuckle et al. (7) considered the possibility that hCGb fragment concentrations in urine may vary during the day, even after normalization with creatinine. As part of a large multicenter study on the screening efficacy of hCGb fragment for Down syndrome, cross-sectional information on the time of voiding was obtained toward the end of patient recruitment. The data showed an impressive increase in urine concentrations of hCGb fragment, from 0.7 MoM at 0400 to 1.4 MoM at 0800. These findings appear to be consistent with our own data, showing a similar increase between 0000 and 0600. However, the study by Cuckle et al. (7) reported cross-sectional data between 0400 and 1600, whereas in our study, participants were followed longitudinally for 24 h.

Our findings make it plausible that diurnal variation in urine concentrations of hCGb fragment could affect the efficacy of screening for Down syndrome. On the one hand, there is no reason to believe that women with Down syndrome pregnancies and women with normal pregnancies have systematically collected samples at different times of the day in the previously reported studies. However, the number of Down syndrome pregnancies has been very small in most published studies. Therefore, we cannot exclude differences in timing of sampling for these few specimens, compared with the women with normal pregnancies, as a cause of the reported inconsistencies. In summary, hCGb fragment displays substantial diurnal variation. Assessment of the value of hCGb fragment as a screening marker for Down syndrome should be reconsidered when data adjusted for time of sampling are available.

References
9. Pekonen F, Altfhan H, Stennanf NH, Ylikorkala O. Human chorionic gonado-

Protein Fragments in Urine Have Been Considerably Underestimated by Various Protein Assays, Kerryn A. Gretev, Nicholas D.H. Balataz, and Wayne D. Comper†
(1 Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia 3800; 2 Biochemistry Unit, S.H.C. Pathology Service, Monash Medical Centre, Clayton, Victoria, Australia 3168; † author for correspondence: fax 61-3-9876-5881, e-mail wayne.comper@med.monash.edu.au)

It has recently been discovered that filtered proteins in the kidney are not excreted in their intact forms (I, 2). During renal passage, high-molecular mass proteins, including albumin, transferrin, and IgG, undergo degradation. The products of this degradation are then excreted in urine as heavily degraded fragments. In rats, ~90% of the excreted albumin is heavily degraded to fragments with molecular masses <10 000 Da, and in humans this percentage is even higher (I, 2).

The excretion of filtered protein in the form of protein fragments that are not detected by conventional protein assays has not been recognized in the clinical chemistry literature. Previous studies have shown that several techniques used routinely to measure urinary total protein and more specific techniques used to measure urinary albumin concentrations are insensitive to albumin fragments (3). Such techniques include immunochemical albumin assays, the benzethonium chloride method, the sulfosalicylate assay, the pyrogallol red assay, and the Coomassie blue assay for proteins (I, 3). This means that analysis of the total amount of a particular protein (intact plus fragments) excreted has never been accomplished and that total urinary protein/peptide excretion has been severely underestimated. The problem is particularly evident for normal and microalbuminuric urine: Whereas normal human excretion of intact albumin (as measured by RIA) is <25 mg/day, the excretion of accompanying derived fragments can be >1300 mg/day (I).

The aim of the present study was to analyze urine samples from 20 diabetic patients with varying degrees of microalbuminuria for albumin by immunochromatographic immunoelometry (Beckman Array Protein Analysis System) and for total urinary protein by the Biuret assay (for peptide