

Fig. 1. Concentrations of serum creatinine (A) and cystatin C (B) before and after treatment of hypo- and hyperthyroidism.

hyperthyroidism, but the cystatin C values in hyperthyroidism are 46% higher than those in hypothyroidism. Existing literature suggests that it is creatinine that is providing the correct assessment of GFR changes (17), but because definitive work formally assessing GFR in thyroid disease (using a "gold standard" marker) has yet to be performed, this cannot be stated categorically.

Until now, one of the most appealing aspects of using cystatin C as a marker of GFR has been the apparent lack of influence of medical conditions on its clinical utility, with the only debate being whether cystatin C concentrations are influenced by some metastatic malignancies or after renal transplantation (18, 19). A likely explanation for our findings here is that hyperthyroidism is associated with a reversible increase in cystatin C production that is in excess of the expected increase in GFR, whereas in hypothyroidism, cystatin C production is reduced to a greater extent than is the GFR. The reasons for these changes in production remain speculative, but if similar to the cause suggested for β_2 -microglobulin, then it would seem that it is simply through a metabolic-rate-mediated mechanism (14).

In summary, this study has shown a clinically significant discrepancy between GFR assessed by serum creatinine and that found using cystatin C in untreated thyroid disease. This raises doubts as to the reliability of cystatin C measurement in these common conditions, but it also suggests that further work needs to be performed to confirm which marker is giving the true reflection of GFR.

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References

1. Star R, Hostetter T, Hortin GL. New markers for kidney disease. *Clin Chem* 2002;48:1375–6.
2. Deinum D, Derkx FHM. Cystatin for the estimation of glomerular filtration rate? *Lancet* 2000;356:1624–5.
3. Randers E, Erlandsen EJ. Serum cystatin C as an endogenous marker of renal function—a review. *Clin Chem Lab Med* 1999;37:389–95.
4. Tenstad O, Roald AB, Grubb A, Aukland K. Renal handling of radiolabelled human cystatin C in the rat. *Scand J Clin Lab Invest* 1996;56:409–14.
5. Khyse-Anderson J, Schmidt C, Nordin G, Andersson B, Nilsson-Ehle P, Lindstrom V, et al. Serum cystatin C, determined by a rapid, automated particle enhanced turbidimetric method, is a better marker than serum creatinine for glomerular filtration rate. *Clin Chem* 1994;40:1921–6.
6. Simonson O, Grubb A, Thysell H. The blood serum concentration of cystatin C (γ -trace) as a measure of the glomerular filtration rate. *Scand J Clin Invest* 1985;45:97–101.
7. Newman DJ, Thakkar H, Edwards RG, Wilkie M, White T, Grubb A, et al. Serum cystatin C measured by automated immunoassay: a more sensitive marker of changes in GFR than serum creatinine. *Kidney Int* 1995;47:312–8.
8. Helin I, Axenram M, Grubb A. Serum cystatin C as a determinant of glomerular filtration rate in children. *Clin Nephrol* 1998;49:221–5.
9. Bökenkamp A, Domanetzki M, Zinck R, Schumann G, Byrd D, Brodehl J. Cystatin C—a new marker of glomerular filtration rate in children independent of age and height. *Pediatrics* 1998;101:875–81.
10. Ravnskov U. Serum β_2 -microglobulin and glomerular function. *N Engl J Med* 1976;294:611.
11. Braren V, Goddard J, Brill AB, Touya JJ. β_2 -Microglobulin as renal diagnostic agent. *Urology* 1979;13:624–8.
12. Shea PH, Maher JF, Horak E. Prediction of glomerular filtration rate by serum creatinine and β_2 -microglobulin. *Nephron* 1981;29:30–5.
13. Forman DT. β_2 microglobulin—an immunogenetic marker of inflammatory and malignant origin. *Ann Clin Lab Sci* 1982;12:447–52.
14. Roiter I, Da Rin G, De Menis E, Foscolo GC, Legovini P, Conte N. Increased serum β_2 -microglobulin concentrations in hyperthyroid states. *J Clin Pathol* 1991;44:73–4.
15. Kreisman SH, Hennessey JV. Consistent reversible elevations of serum creatinine levels in severe hypothyroidism. *Arch Intern Med* 1999;159:79–82.
16. Verhelst J, Berwaerts J, Marescau B, Abs R, Neels H, Mahler C, et al. Serum creatine, creatinine, and other guanidino compounds in patients with thyroid dysfunction. *Metabolism* 1997;46:1063–7.
17. Capasso G, De Tommaso G, Pica A, Anastasio P, Capasso J, Kinne R, et al. Effects of thyroid hormones on heart and kidney functions. *Miner Electrolyte Metab* 1999;25:56–64.
18. Newman D. More on cystatin C. *Clin Chem* 1999;45:2556–7.
19. Bökenkamp A, Domanetzki M, Zinck R, Schumann G, Byrd D, Brodehl J. Cystatin C serum concentrations underestimate glomerular filtration rate in renal transplant patients. *Clin Chem* 1999;45:1866–8.

Quantification of Glutamine in Dried Blood Spots and Plasma by Tandem Mass Spectrometry for the Biochemical Diagnosis and Monitoring of Ornithine Transcarbamylase Deficiency, Minh-Uyen Trinh,¹ Jennifer Blake,¹ J. Rodney Harrison,¹ Rosemarie Gerace,¹ Enzo Ranieri,¹ Janice M. Fletcher,^{1,2} and David W. Johnson^{1*} (¹ Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Rd., North Adelaide, South Australia 5006, Australia; ² University of Adelaide, North Terrace, Adelaide, South Australia 5000, Australia; * author for correspondence: fax 61-8-81617100, e-mail david.johnson@adelaide.edu.au)

A notable deficiency in the use of tandem mass spectrometry (MS/MS) for newborn screening is the inability to screen for urea cycle defects. The most common of these,

with an incidence of 1 in 14 000 births (1), is the inherited X-linked disorder ornithine transcarbamylase deficiency (OTCD). A majority (60%) of hemizygous males risk death from hyperammonemic coma during the first week of life. The remainder, including 10% of heterozygous females, exhibit lethargy, vomiting episodes, and behavioral problems during childhood. The severity of the disorder and the potential for correction of OTCD by liver transplantation and gene therapy (2) provide adequate justification for newborn screening.

OTCD patients have low blood citrulline because of reduced conversion from carbamoyl phosphate. Citrulline is one of the amino acids routinely measured in MS/MS newborn-screening programs. Unfortunately, many protein-restricted newborns also have low blood citrulline (3). A more selective amino acid metabolite for OTCD is glutamine. The derivatization procedure used in many MS/MS screening programs (4), which uses butanol–hydrogen chloride, destroys glutamine. Approximately one-half of the glutamine is converted to glutamic acid dibutyl ester and is indistinguishable from that formed from endogenous glutamic acid in the blood. The surviving glutamine butyl ester is deaminated in acidic solution to a protonated form of pyroglutamic acid butyl ester in the electrospray source of the MS/MS. Again it is not possible to distinguish this pyroglutamic acid from what is already present in the blood. As a secondary consequence, the measurements of glutamic and pyroglutamic (and by analogy, aspartic) acids in blood spots after derivatization are grossly inaccurate. MS/MS newborn-screening programs that do not derivatize amino acids avoid solvolysis of glutamine and of pyroglutamic acid to glutamic acid. During electrospray ionization-MS/MS analysis, however, glutamine is again indistinguishable from pyroglutamic acid. Resolution is possible by separation with time-consuming liquid chromatography (5), which is unsuited to rapid screening programs.

Formamidene butyl esters of amino acids afford more stable ions than the corresponding butyl esters during electrospray ionization-MS/MS analysis (6). We have further optimized this derivatization method to quantify glutamine in dried blood spots from newborns and in plasma to monitor OTCD patients on treatment. Milder derivatization conditions minimized amide derivatization and solvolysis. The preparation of formamidene isobutyl esters increased MS/MS signal intensity. Additionally, glutamic and pyroglutamic acids were simultaneously quantified to determine the fate of glutamine in stored samples.

Amino acids were extracted with methanol from 3-mm dried blood spots or 2 μ L of plasma adsorbed on a 3-mm filter-paper disk for 15 min. [$^2\text{H}_5$]Glutamine (2 nmol), [$^2\text{H}_3$]glutamic acid (1 nmol), and [$^2\text{H}_3$]pyroglutamic acid (1 nmol) were added as internal calibrators. The amino acids were treated with 240 μ L of dimethylacetal dimethylformamide–acetonitrile–methanol (2:5:5 by volume) at room temperature for 5 min, excess reagents were evaporated, and the residue was treated with isobutanol–3 mol/L–hydrogen chloride (200 μ L) at room tempera-

ture for 10 min and then was evaporated to dryness. Isobutanol affords a 50% increase in ion intensity during MS/MS analysis relative to *n*-butanol. The derivatives were dissolved in 2 mL of acetonitrile–water–formic acid (50:50:0.025 by volume).

An Applied Biosystems/MDS Sciex Model API3000 tandem mass spectrometer equipped with a TurboIon-spray source (temperature, 100 °C) was used for analysis. An Agilent HP1100 LC pumped acetonitrile–water–formic acid (50:50:0.025 by volume) at a flow rate of 160 μ L/min into the TurboIon-spray via a Gilson 233 autosampler fitted with a 20- μ L injection loop. Samples were injected from a 96-well tray at 2-min intervals. Multiple-reaction monitoring experiments with six ion pairs, representing a neutral loss of 73 atomic mass units (amu; for glutamine) and a neutral loss of 102 amu (for glutamic and pyroglutamic acids), and the corresponding set for the three labeled internal calibrators were used for data acquisition.

Calibration curves were constructed from the analyses of derivatized mixtures of the three amino acids. Statistical analysis revealed response linearity for glutamine (0–2.5 mmol/L; $y = 0.999x - 0.09 \mu\text{mol/L}$; $R^2 = 0.9999$), glutamic acid (0–1.25 mmol/L; $y = 0.999x + 0.26 \mu\text{mol/L}$; $R^2 = 1$), and pyroglutamic acid (0–1.25 mmol/L; $y = 0.992x + 6.33 \mu\text{mol/L}$; $R^2 = 0.9997$). The imprecision in the measurement of an amino acid calibrator containing glutamine (1 mmol/L) was 2.3% (intraassay; $n = 9$) and 2.9% (interassay; $n = 12$). The imprecision in the measurement of glutamine in dried blood spots prepared from an adult blood sample (0.3 mmol/L) was 7.4% (intraassay; $n = 9$) and 12% (interassay; $n = 10$).

To determine whether a diagnosis of OTCD can be made in archived blood spots, we evaluated the stability of glutamine over time. The analyses of neonatal blood spots (five at each time point) stored at ambient temperature (15–25 °C) for up to 10 years are shown in Fig. 1. We observed a rapid decrease in glutamine, a moderate decrease in glutamic acid, and a rapid increase in pyroglutamic acid. A duplicate dried blood spot from an OTCD patient stored at –20 °C in a sealed plastic bag for 6 months, however, retained 98% of its original glutamine concentration. We analyzed blood spots from seven OTCD patients, together with control blood spots of identical age and storage conditions, for glutamine (Table 1). One patient was newly diagnosed, and the other six samples were from an archived collection stored at ambient temperature. Apart from the oldest sample, in which glutamine was unmeasurable, increased glutamine was observed for all of the OTCD patients except for one heterozygous female.

We also measured glutamine in freshly collected plasma samples ($n = 36$) referred to our laboratory for amino acid analysis and including some from OTCD patients on treatment. These samples were also analyzed for glutamine on a Waters 600E HPLC-based amino acid analyzer. Statistical analysis of the comparison (see Fig. 2 in the Data Supplement accompanying the online version of this Technical Brief at <http://www.clinchem.org/>

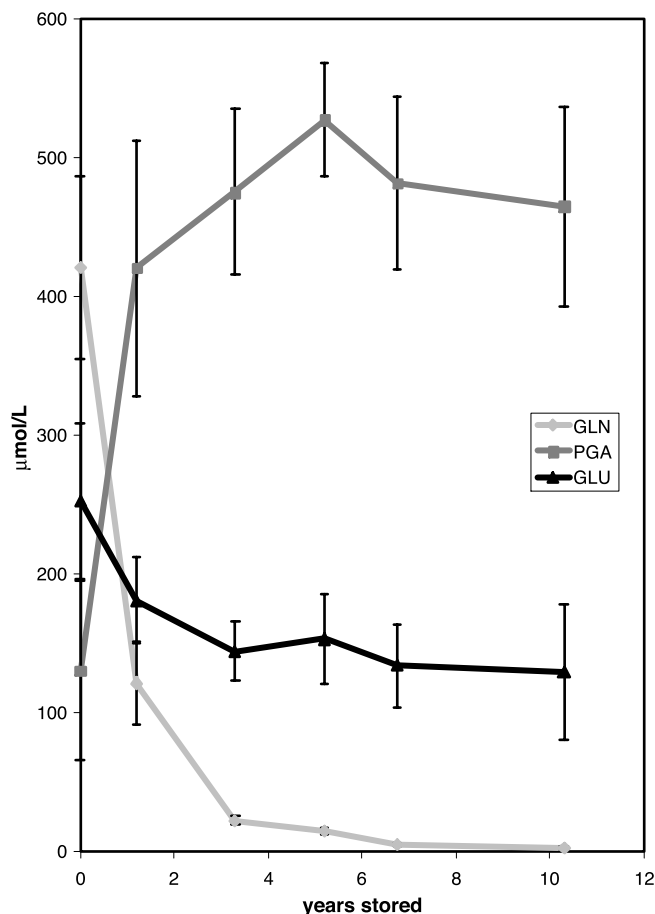


Fig. 1. Concentrations of glutamine (GLN), glutamic acid (GLU), and pyroglutamic acid (PGA) in dried blood spots kept at ambient temperature.

content/vol49/issue4/) afforded excellent correlation ($y = 1.02x - 4.39 \mu\text{mol/L}$; $R^2 = 0.88$).

Unfortunately, this method cannot be used as a substitute for the butylation method used in MS/MS newborn-screening programs, primarily because acylcarnitines are not fully derivatized at room temperature. It is doubtful that the incidence of OTCD justifies a separate newborn dried blood spot screen for glutamine with this method. One obvious role of the method is high-risk screening of

all newborns with a familial history of OTCD. Another is as a second-tier screening method to reexamine dried blood spots with a low citrulline concentration. Our experience suggests that performing glutamine analysis on the lowest 2% of citrulline results should ensure the flagging of female OTCD heterozygotes. Since our MS/MS newborn screening program was introduced 4 years ago, dried blood spots from two diagnosed OTCD patients have been analyzed. A male had a citrulline concentration of $6 \mu\text{mol/L}$ (below the 0.1 percentile), and a heterozygous female had a citrulline concentration of $12 \mu\text{mol/L}$ (between the 1st and 2nd percentiles).

This study demonstrates the instability of glutamine and, to a lesser extent, glutamic acid in dried blood spots under typical storage conditions. Both are deaminated or dehydrated to pyroglutamic acid. Glutamine is not hydrolyzed to glutamic acid as is the case with plasma. After 1 year, pyroglutamic acid reaches a long-term stable concentration of $\sim 500 \mu\text{mol/L}$ (see Fig. 1) in dried blood spots from newborns. This is the case even in archived OTCD dried blood spots that initially contained much higher newborn glutamine concentrations.

When glutamine measurements are used as a second-tier screen, dried blood spots need to be analyzed within 2 weeks of collection to avoid more than a 5% decrease in concentration. This period can be extended if the dried blood spot samples are stored at a low temperature. Archived dried blood spots <10 years of age can be analyzed for glutamine if they are compared with age-matched and storage-condition-matched control blood spots.

This method for the quantification of glutamine in plasma supplements the measurements of plasma ammonia and urine orotic acid commonly used to monitor patients with urea cycle defects. It is a superior alternative to the use of an amino acid analyzer to monitor OTCD patients on treatment. Sample analysis time, without HPLC separation, is much shorter (2 vs 120 min), and the required sample volume is much smaller (2 vs $125 \mu\text{L}$). Analysis can also be performed on dried blood spots from less invasive fingerprick samples that are sent from remote locations by post. Any other essential α -amino acids can be quantified simultaneously, with detection limits comparable to those of glutamine, by adding the appro-

Table 1. Glutamine concentration in newborn dried blood spots from OTCD patients and age-matched controls.

OTCD patient	Sex	Age of blood spot	Glutamine, $\mu\text{mol/L}$	n	Age-matched controls	
					Glutamine, $\mu\text{mol/L}$	
					Mean	SD
1	M	20 years 1 month	0	5	0	0
2	M	10 years 5 months	9.6	5	3.0	1.1
3	F	6 years 9 months	40.3	5	5.2	1.6
4	M	5 years 10 months	218	5	10.3	1.1
5	F	5 years 2 months	46.4	5	14.8	2.2
6	F	3 years 4 months	25.5	5	22.5	3.2
7	M	2 days	1515	53	490	144

priate isotope-labeled internal calibrators. Amino acids containing amide and imine groups, such as citrulline, arginine, and asparagine, exhibited poor detection limits with the original formamidene derivatization method (6). The milder derivatization conditions and the use of isobutanol in this modified method improved MS/MS analysis of these amino acids dramatically.

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References

1. Brusilow SW, Maestri NE. Urea cycle disorders: diagnosis, pathophysiology, and therapy. *Adv Pediatr* 1996;43:127–70.
2. Batshaw ML, Robinson MB, Ye X, Pabin C, Daikhin Y, Burton BK, et al. Correction of ureagenesis after gene transfer in an animal model and after liver transplantation in humans with ornithine transcarbamylase deficiency. *Pediatr Res* 1999;46:588–93.
3. Batshaw ML, Berry GT. Use of citrulline as a diagnostic marker in the prospective treatment of urea cycle disorders. *J Pediatr* 1991;118:914–7.
4. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW. Rapid diagnosis of maple syrup urine disease in blood spots from newborns by tandem mass spectrometry. *Clin Chem* 1995;41:62–8.
5. Qu J, Chen W, Luo G, Wang Y, Xiao S, Ling Z, Chen G. Rapid determination of underivatized pyroglutamic acid, glutamic acid, glutamine and other relevant amino acids in fermentation media by LC-MS-MS. *Analyst* 2002;127:66–9.
6. Johnson DW. Analysis of amino acids as formamidene butyl esters by electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2001;15:2198–205.

Exercise-induced Myocardial Ischemia Is Accompanied by Increased Serum Creatine Concentrations, Youri E.C. Taes,^{1,2} Norbert H. Lameire,² Marc L. De Buyzere,¹ Amir Shoja,³ Guy De Backer,⁴ and Joris R. Delanghe^{1*} (¹ Laboratory Clinical Chemistry, ² Renal Division, Department of Internal Medicine, ³ Department of Internal Medicine, and ⁴ Cardiac Rehabilitation Centre, University Hospital Ghent, 9000 Ghent, Belgium; * address correspondence to this author at: Department of Clinical Chemistry 2P8, University Hospital Ghent, De Pintelaan 185, 9000 Ghent, Belgium; fax 32-9-240-4985, e-mail Joris.Delanghe@rug.ac.be)

Coronary artery disease remains the leading cause of mortality in adults in the westernized world (1, 2). Coronary artery disease can present itself as a wide range of diseases, from uncomplicated stable angina, to unstable angina, to myocardial infarction. The diagnosis of myocardial ischemia in patients with stable angina depends on the patient's history and various technical investigations, such as electrocardiography (ECG) and stress testing. Exercise ECG is a simple and inexpensive investigation with diagnostic, prognostic, and functional information in selected populations (3). Exercise testing has an overall sensitivity of 66% and a specificity of 84% (4), but its diagnostic performance is much lower in asymptomatic individuals and females. The diagnosis and grading of unstable angina or myocardial infarction depend on biochemical markers together with clinical and

electrocardiographic data (5). The currently used routine biochemical markers for the assessment of myocardial infarction or unstable angina require tissue necrosis to be detected in plasma. Serum and urine creatine concentrations have been described as markers for myocardial infarction. In contrast to other markers, creatine is a nonprotein nitrogenous compound, present in heart and skeletal muscle. It has a considerably lower molecular mass (131 Da) compared with conventional markers; release into the circulation therefore occurs at an early stage of myocardial ischemia, allowing rapid diagnosis (6, 7). We hypothesized that creatine could be released from ischemic myocardial tissue without the necessity of tissue necrosis. This study investigates the relationship between serum creatine and electrocardiographic indices during exercise testing.

We recruited 47 male Caucasians with a mean (SD) age of 61 (10) years from patients referred for exercise testing. Patients referred for detection of exercise-induced arrhythmias or with major arrhythmias (pacemaker rhythm, ventricular tachycardia, or conduction disorders) during exercise, excessive increase (>250 mmHg systolic, >120 mmHg diastolic) or decrease (>10 mmHg systolic blood pressure) in blood pressure during exercise, myocardial infarction <6 months, and symptomatic heart failure were excluded from the study. This study was approved by the local ethics committee. All participants underwent exercise testing on an electromagnetically braked bicycle ergometer with stepwise incremental workloads [50 W + 25 W/2 min (41 individuals); 50 W + 50 W/2 min (6 individuals)]. ECG recordings were acquired during exercise and during recovery. ST-segment depression was quantified 60 ms (ST60) after the J-point in lead V5. ST-segment depression ≥ 1.5 mm (0.15 mV) was considered to be diagnostic for myocardial ischemia. ST-segment depression was adjusted to heart rate (ST/HR index) (8). Exercise was stopped on achieving the age-matched maximal exercise level, severe ST-depression >2.0 mm (0.2 mV), or severe symptoms (incapacitating fatigue, grade III/IV angina, severe dyspnea).

The rate–pressure product [heart rate \times systolic blood pressure (mmHg \cdot beats \cdot min⁻¹)] was determined at peak exercise. Fasting blood samples were taken at rest before exercise and 10 min after termination of exercise. Coronary angiography was performed as a clinical diagnostic procedure in 19 participants (40% of study population). Serum creatine concentration (CV = 2.2% at 230 μ mol/L and 3.0% at 58 μ mol/L) was determined by an enzymatic assay as described previously (6). Creatine kinase (CK) catalytic activity was determined according to the IFCC (9) at 37 °C on a Modular analyzer using CK-NAC reagents (Roche Diagnostics). CK-MB was measured with an immunoinhibition assay. Total serum glutathione (CV = 2.4% at 1 μ mol/L and 3.6% at 0.5 μ mol/L) was determined according to the method of Griffith (10). Myoglobin was measured immunoturbidimetrically, and cardiac troponin T was measured with an electrochemiluminescence immunoassay (Roche Diagnostics). Plasma lactate was measured using commercial reagents on a