logic studies. The II for men and women separately were not different from the II of the reference population. This provides objective evidence that stratified reference intervals according to gender are unnecessary. At 95% confidence, the reference change value of plasma total iPF2α-III was 72.9%, suggesting that relatively large differences between the results of sequential specimens would be required for them to be significantly different.

Our finding of a wide biological variation in the plasma total iPF2α-III concentration has not been reported previously. Helmersson and Basu (9) reported a large variation in the urinary excretion of iPF2α-III in healthy individuals with a mean CV of 42% over 10 consecutive days. Both plasma and urinary iPF2α have been used as markers of in vivo lipid peroxidation, but there is a lack of correlation between plasma and urinary iPF2α concentration (10).

To understand the wide biological variation of plasma total iPF2α-III concentrations in healthy nonsmokers, other factors can also be considered. Diet should not be a contributing factor; it has been reported that diet does not confound plasma total iPF2α-III values in humans (11). Physical exercise can create an imbalance between oxidant and antioxidant concentrations. A recent study showed that extreme endurance exercise is associated with increased production of plasma iPF2α, but moderate exercise such as walking is unlikely to represent a confounding factor (12). Extreme endurance exercise had not been documented in our study group. More studies are required to understand the observed wide variation.

In summary, we present new data relating to plasma total iPF2α-III and the plasma iPF2α-III/AA ratio. To our knowledge, this is the first report on the biological variation of plasma total iPF2α-III. These data are important for the assessment of individuals and the design of studies involving these variables.

References

Specimen Dilution for C2 Monitoring with the Abbott TDxFLx Cyclosporine Monoclonal Whole Blood Assay, JoEtta M. Juenke,1* Paul I. Brown,1 Francis M. Urry,1,2 and Guwendoly A. McMillin1,2 (* ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories Inc., Salt Lake City, UT; 1 Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT; * address correspondence to this author at: ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories Inc., 500 Chipeta Way, Salt Lake City, UT 84108; fax 801-584-5207, e-mail juenkejm@aruplab.com)

Cyclosporine (CsA), a hydrophobic cyclic undecapeptide produced by the fungus Tolypocladium inflatum, is composed of 11 amino acid residues. As a calcineurin inhibitor, CsA inhibits cytokines such as interleukin-2 at the early stage of T-lymphocyte activation. CsA has been shown to improve graft survival in skin, heart, kidney, pancreas, bone marrow, lung, small intestine, and liver transplants (1, 2), but the use of CsA is associated with serious toxic side effects, primarily nephrotoxicity and hepatotoxicity. Other adverse effects include diarrhea, gingival hyperplasia, nausea, vomiting, hirsutism, tremor, and hypertension. CsA is extensively metabolized, and at least 20 metabolites have been identified, the majority of which are considered to be therapeutically inactive (4–7).

CsA monitoring is performed with fluorescence polarization immunoassay, enzyme-based Emit® immunoassays, and chromatographic (HPLC) and tandem mass spectrometry techniques (5–9). Immunoassay techniques have variable cross-reactivities with CsA metabolites, producing overestimated concentrations of CsA in whole blood. Nevertheless, immunoassays offer rapid turnaround times and technical ease and are therefore the most commonly used analytical methods. In a recent College of American Pathologist study, 247 of 292 respondents used the Abbott TDxFLx CsA monoclonal whole blood assay (fluorescence polarization immunoassay) (10, 11).

The process of optimizing immunosuppressant dosing with therapeutic drug monitoring continues to be refined, particularly for CsA. Current evidence suggests that a single concentration of CsA determined with a specimen collected 2 h postdose (C2) better reflects the area under the curve than a conventional predose (trough) concentration, particularly when the microemulsion formulation is used (12). Clinical outcome studies suggest that adjust-
ing the dose on the basis of C2 values may be preferred to adjusting dose on the basis of trough values. For example, the incidence of biopsy-confirmed moderate to severe acute kidney rejection has been reported to be significantly lower in patients managed with C2 values compared with those monitored with trough values (13). When the collection time is shifted from the trough to the C2 value, the measured CsA concentration is 3–6 times higher than the trough concentration. Proposed therapeutic ranges based on C2 values frequently exceed 1000 μg/L and may exceed 2000 μg/L during the first month posttransplantation (10–16).

The analytical technique selected for monitoring C2 concentrations of CsA must have a sufficiently large analytical measurement range (AMR) to accommodate concentrations of at least 2000 μg/L. With exception of the CEDIA® Cyclosporine Plus immunoassay, currently available immunoassays do not meet this criterion. Even the CEDIA assay is unable to cover the analytical range of 25–2000 μg/L in one assay; the range must be split into two separate calibrations. Dilution of C2 specimens will theoretically provide a practical and rapid accommodation. However, dilution protocols are not well controlled among laboratories, as evidenced by poor performance in proficiency testing samples with target values >1000 μg/L (17). The AMR of the TDxFLx assay is 25–1500 μg/L. In this study, we evaluated three dilution protocols with the TDxFLx assay to assess performance in monitoring of CsA with C2 concentrations.

The dilution protocol recommended by Abbott (18) is a twofold dilution, prepared before assay set up, with equal amounts of patient specimen and calibrator A. Calibrator A is a whole-blood matrix with no CsA added. This dilution is mixed and set aside to equilibrate before testing of the sample. After an equilibration of 10 min at room temperature, 150 μL of the diluted sample is transferred to a corresponding centrifuge tube, and 50 μL of the solubilization reagent is added; 300 μL of the whole blood precipitation reagent is then added to each tube. The tubes are capped and vortex-mixed for 10 s, until no unmixed portion of sample and precipitation reagent is visible at the bottom of the tube. The tubes are then centrifuged for 5 min at 9500g, until a clear supernatant and hard compact pellet of denatured protein are obtained. At least 150 μL of supernatant is decanted into the sample well of a TDxFLx sample cartridge. The sample is then analyzed and the result multiplied by 2.

Results vary depending on the amount of time the sample is allowed to equilibrate (data not shown), a condition that is not defined in the package insert. Results may be variable in part because of differences in equilibration and linearity of dilution among cross-reacting CsA metabolites.

In this study, we compared the recommended dilution protocol against two dilution protocols in which the diluent is composed of the assay solubilization and precipitation reagents. In these modified dilution protocols, the specimen volume is not changed, but the solubilization and precipitation reagents are doubled or tripled, yielding a 1.85- or 2.5-fold dilution, respectively. The advantages of these two modified dilution protocols over the recommended protocol include (a) elimination of the variable sample equilibration step and (b) improved efficiency because the dilution occurs during routine assay set up as opposed to before set up. Incorporating the dilution into the routine assay set up eliminates the need for additional tubes and thereby reduces the opportunity for error. Delivery of the solubilization and precipitation reagents can be automated as well. We found that the modified dilutions are preferred by our technical staff because of the visible difference in volume, which clearly identifies any diluted samples. The 2.5-fold dilution protocol will extend the upper limit of the AMR to 3750 μg/L, which should meet the needs of the C2 and other timed specimens.

For the 1.85-fold dilution protocol, 150 μL of specimen is transferred to a corresponding centrifuge tube, followed by 100 μL of the solubilization reagent and 600 μL of the precipitation reagent. For the 2.5-fold dilution protocol, 150 μL of specimen is transferred to a corresponding centrifuge tube, followed by 150 μL of the solubilization reagent and 900 μL of the precipitation reagent. The remaining sample preparation and assay steps are identical to those described for the Abbott twofold dilution as stated in the package insert. Results are multiplied by 1.85 and 2.5 for specimens prepared with the protocols for the 1.85- and 2.5-fold dilutions, respectively.

To compare the effect of dilution on assay performance, we compared the results obtained with each dilution protocol with results obtained with undiluted samples using three controls (UTAK) and de-identified patient samples. Prepared samples were mixed for 10 min before testing and were analyzed with a TDxFLx instrument. For the UTAK controls, 25 values were obtained for each concentration, after specimen preparation with each of the three dilution schemes. These results were compared with values obtained without dilution (n = 25) for each concentration. The mean, imprecision (expressed as CV), range, and mean accuracy are shown in Table 1. The performance data are similar to those published previously for this assay (10). The data obtained with any of the three dilution protocols were consistent with those obtained without dilution, suggesting that assay performance is not compromised by dilution.

We then evaluated the effect of the dilution protocols on assay performance with 30 CsA-containing (33–2600 μg/L) whole-blood samples from patients that were de-identified according to corporate protocol. Results for the nine patients with concentrations <1500 μg/L were correlated with results obtained without dilution to demonstrate that dilution of patient samples did not affect the accuracy of the assay. Patient data were stratified at 1500 μg/L because this is the upper limit of the analytical measurement range defined for the Abbott assay to identify those specimens that require dilution. Pairs of results obtained with undiluted samples and results obtained with each of the three dilution protocols were compared
by calculating Spearman rank correlation coefficients. This analysis yielded coefficients of 0.95 for each pair ($P = 0.0001$), indicating that results obtained with dilution and without dilution were highly correlated.

The 1.85- and 2.5-fold dilutions were then compared directly with the manufacturer-suggested 2-fold dilution (“gold standard”) for all 30 specimens; the 2-fold dilution scheme was regressed on the 1.85- and 2.5-fold dilution schemes, respectively. Simple linear regression yielded the following equations: $y = 1.01x + 23.0$ µg/L ($r = 0.9925$) for the 1.85-fold dilution scheme; and $y = 0.98x + 46.6$ µg/L ($r = 0.9941$) for the 2.5-fold dilution scheme. These data are shown in Fig. 1. The percentage agreement between the recommended protocol (2-fold) and the modified protocols (1.85- and 2.5-fold) was determined from the ratios of results obtained with each protocol. The mean percentage agreement with the 2-fold protocol (95% confidence interval) was 101.8% (99.4–104.1%) for the 1.85-fold protocol and 99.7% (97.9–101.4%) for the 2.5-fold protocol. Because the sample and the dilution scheme are both factors that may affect the result, the data were also analyzed by two-way ANOVA. Results from the ANOVA suggested that the differences between the three dilutions are not significant ($P = 0.0561$).

This work supports the use of any of the three described dilution schemes to sufficiently extend the AMR to support measurement of C2 CsA concentrations without compromise in accuracy or precision. The Abbott-recommended twofold dilution requires standardization and validation of the equilibration step and must be performed before assay set up. The modified 1.85- and 2.5-fold dilutions prevent the bias that may occur with variable equilibration in the recommended dilution protocol and may be more efficient for a busy laboratory because they are prepared during assay set up.

We acknowledge and thank Clinton J. Thompson, Department of Family & Preventive Medicine, University of Utah, for consultation and assistance in the statistical analyses.

### References


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### Table 1. Range, mean, mean accuracy, and imprecision comparison for three dilution protocols using UTAK controls (n = 25 per concentration)

<table>
<thead>
<tr>
<th>Dilution protocol</th>
<th>Target concentration</th>
<th>Range, µg/L</th>
<th>Mean, µg/L</th>
<th>Accuracy, %</th>
<th>CV, %</th>
<th>Range, µg/L</th>
<th>Mean, µg/L</th>
<th>Accuracy, %</th>
<th>CV, %</th>
<th>Range, µg/L</th>
<th>Mean, µg/L</th>
<th>Accuracy, %</th>
<th>CV, %</th>
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<tr>
<td></td>
<td>150 µg/L</td>
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<tr>
<td>Undiluted</td>
<td></td>
<td>125–175</td>
<td>157</td>
<td>105</td>
<td>9.4</td>
<td>558–658</td>
<td>612</td>
<td>102</td>
<td>5.6</td>
<td>1125–1362</td>
<td>1258</td>
<td>105</td>
<td>5.2</td>
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<tr>
<td>1.85-fold</td>
<td></td>
<td>144–175</td>
<td>161</td>
<td>108</td>
<td>19</td>
<td>596–658</td>
<td>626</td>
<td>104</td>
<td>7</td>
<td>1269–1345</td>
<td>1313</td>
<td>109</td>
<td>5.1</td>
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<tr>
<td>2-fold</td>
<td></td>
<td>144–194</td>
<td>163</td>
<td>109</td>
<td>16</td>
<td>560–689</td>
<td>633</td>
<td>106</td>
<td>7.4</td>
<td>1301–1489</td>
<td>1378</td>
<td>110</td>
<td>5.3</td>
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<tr>
<td>2.5-fold</td>
<td></td>
<td>144–175</td>
<td>161</td>
<td>108</td>
<td>16</td>
<td>615–695</td>
<td>644</td>
<td>107</td>
<td>9.4</td>
<td>1277–1436</td>
<td>1350</td>
<td>111</td>
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<td></td>
<td>600 µg/L</td>
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<td></td>
<td>1200 µg/L</td>
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</table>

Fig. 1. CsA concentrations (µg/L) in whole blood determined for 30 deidentified patient specimens by three dilution protocols.

Results obtained with the modified dilution protocols (1.85-fold dilution (•) and 2.5-fold dilution (○)) were compared with the results obtained with the manufacturer’s recommended dilution protocol (2-fold).
Second-Trimester Maternal Serum Invasive Trophoblast Antigen: A Marker for Down Syndrome Screening, Raj Pandian,1* Laurence A. Cole,2 and Glenn E. Palomaki,3 (1) Quest Diagnostics Nichols Institute, San Juan Capistrano, CA; 2 University of New Mexico, Albuquerque, NM; 3 Foundation for Blood Research, Scarborough, ME; * address correspondence to this author at: Quest Diagnostics Nichols Institute, 35608 Ortega Hwy., San Juan Capistrano, CA 92690; e-mail Raj.M.Pandian@questdiagnostics.com

More than 2 million pregnancies in the United States are screened for Down syndrome each year, most often in the second trimester, by a test using three biochemical markers in combination with maternal age (1). The “triple” test includes measurements of serum α-fetoprotein (AFP), unconjugated estriol (uE3), and human chorionic gonadotropin (hCG). In the general pregnancy population, the triple test can identify ~65% of Down syndrome pregnancies at a 5% false-positive rate (2, 3). Recently, a fourth test, dimeric inhibin-A (DIA), was added to form the “quadruple” test, which has a higher sensitivity of up to 75% (4, 5). Combining first-trimester biochemical (free β-subunit of hCG and pregnancy-associated plasma protein A) and ultrasound markers can yield higher performance (6–8), but the highest performance reported to date is from a combination of both first- and second-trimester markers into the “integrated” test (9). The search continues for even better markers that can be used to both increase the detection rate and reduce false positives. One such marker was originally identified as hyperglycosylated hCG. Cole et al. (10) used a specific antibody to measure hyperglycosylated hCG [recently called invasive trophoblast antigen (ITA)] in random urine specimens from women with Down syndrome pregnancies. When these authors used only the creatinine-corrected ITA multiple of the median (MoM), 78% of cases were detected at a false-positive rate of 5% (10). The current practice of Down syndrome screening is based on serum samples. Although collecting random urine is not difficult, the additional sample type may be inconvenient and adds expense beyond the measurements of ITA and creatinine. In addition, obtaining a serum sample for AFP analysis would still be required to screen for open neural tube defects. We therefore investigated the possibility of using maternal serum ITA as a Down syndrome marker. To examine whether serum ITA would improve current screening, we also determined the concentrations of other second-trimester serum markers (11).

Blood samples were collected in plain (red-top) evacuated tubes from pregnant women between 14 and 22 weeks of gestation. Samples from women with a Down syndrome pregnancy (defined by fetal karyotype after amniocentesis) were collected at the time of initial counseling (at Yale University; courtesy of Maurice J. Mahoney, MD). Samples from women undergoing second-trimester serum screening (University of Connecticut; courtesy of Peter Benn, PhD) were collected. All were shown to have singleton pregnancy outcomes. Oral consent and demographic and pregnancy-related information were obtained at the time of blood collection. Serum was separated by centrifugation within 4 h of phlebotomy and remained frozen for 3 years at −80 °C. The samples were quickly thawed at Quest Diagnostics and tested immediately, without access to clinical information. ITA concentrations were determined in California by an automated immunochemiluminometric assay as described previously (12). The detection limit of the ITA assay is 0.2 μg/L with a calibration range up to 300 μg/L. The intra- and interassay imprecisions (CVs), as determined by the use of three controls, were <3.5% and <7.4%, respectively. The serum samples were also assayed on the same day for DIA by an assay from Diagnostic System Laboratories and for AFP, hCG, and uE3 on an automated analyzer (Immulite 2000) from Diagnostics Products Corporation. All assays were performed according to the manufacturer’s instructions. The intra- and interassay CVs for all four analytes were <8% and <13%, respectively. Data analysis was performed at the Foundation for Blood Research in Scarborough, ME. Confidence intervals were computed using True Epistat. Estimates of screening performance were modeled using gaussian distributions and the maternal age distributions for the United States in 2000 (13). Maternal serum samples were available for 16 Down syndrome and 84 control pregnancies. Fig. 1 shows the ITA results in case and control pregnancies on the logarithmic y axis vs the gestational age (based on ultrasound dating) on the x axis. The concentration of ITA in control pregnancies vs gestational age fit a log-linear regression well, with a mean decrease of 19% per week. This pattern is distinctly different from that found for hCG during the same period of pregnancy (14, 15). The geometric mean ITA concentration in the Down syndrome pregnancies...