Biological Variation of Plasma F\textsubscript{2}-Isoprostane-III and Arachidonic Acid in Healthy Individuals, Xiongwen Yu, Chung S. Ho, and Christopher W.K. Lam (Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, People’s Republic of China; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, People’s Republic of China; fax 852-2636-5090, e-mail chungshunho@cuhk.edu.hk)

F\textsubscript{2}-Isoprostanes (iPF\textsubscript{2\alpha}) are formed by free radical peroxidation of esterified arachidonic acid (AA) in situ on phospholipids and are subsequently released in free form by the action of phospholipases (1). One of the iPF\textsubscript{2\alpha} isomers, iPF\textsubscript{2\alpha}-III, is recognized as a sensitive and reliable index of lipid peroxidation in vivo. iPF\textsubscript{2\alpha}-III is increased in conditions associated with increased oxidative stress and decreased after dietary supplementation with antioxidants (2). Despite its increasing usage in clinical and nutritional studies, there has been no report on the biological variation in plasma iPF\textsubscript{2\alpha}-III. The aims of this study were (a) to investigate the biological variation of plasma total iPF\textsubscript{2\alpha}-III in healthy individuals over a period of 4 months, and (b) to determine whether the use of the plasma iPF\textsubscript{2\alpha}-III/AA ratio can facilitate interpretation and improve clinical utility.

We recruited 20 healthy nonsmoking Chinese individuals (10 women and 10 men; age range, 31–51 years) for this study. Medical histories were obtained from a questionnaire survey. Comprehensive profiles including commonly used blood and urine tests were performed to exclude common diseases. Other exclusion criteria included pregnancy, hormonal therapy, and body mass index \(\geq 25\). These individuals maintained their usual lifestyle throughout the study and were not taking any other medications (including antioxidant supplements). The study protocol, approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong, was explained thoroughly to the participants before their informed consent was obtained.

Once a month for 4 months, the same phlebotomist collected venous blood from each participant after an overnight fast. Blood (4 mL) was collected into lithium-heparin tubes containing 15 \(\mu\)mol/L indomethacin as a cyclooxygenase inhibitor. Blood samples were centrifuged at 2400 \(\times\) g for 10 min at 4\(^\circ\)C. Aliquots (1 mL) of plasma were transferred to Eppendorf tubes containing 20 \(\mu\)mol/L butylated hydroxytoluene as a free-radical scavenger. The samples were stored at \(-70\)\(^\circ\)C until analysis. Our use of this standardized procedure allowed us to consider preanalytical variation as negligible. Samples from each participant were analyzed once in the same batch after all samples had been collected, thereby eliminating between-run analytical variation.

Plasma total iPF\textsubscript{2\alpha}-III was isolated by immunoaffinity extraction. iPF\textsubscript{2\alpha}-III-d\textsubscript{4} (1 ng as internal standard) was added to 0.5 mL of plasma and hydrolyzed in the presence of 1 mol/L potassium hydroxide at 40 \(\circ\)C for 30 min. The hydrolyzed plasma sample was purified by affinity chromatography on a Gilson ASPEC XL fully automated workstation (Gilson). The affinity column contained anti-iPF\textsubscript{2\alpha}-III antibody immobilized on cyanobromide-activated Sepharose 4B gel supplied by Assays Design Inc. iPF\textsubscript{2\alpha}-III was quantified by isotope-dilution capillary gas chromatography–negative-ion chemical ionization mass spectrometry (GC-NICI-MS) according to the method of Zhao et al. (3).

To measure AA, we added 100 \(\mu\)L of distilled water, 10 \(\mu\)L of concentrated hydrochloric acid, 400 \(\mu\)L of ice-cold Folch solution (choloroform–methanol, 2:1 by volume), and 50 ng of AA-d\textsubscript{8} (internal standard) to 10 \(\mu\)L of hydrolyzed sample. After thorough vortex-mixing, the sample was centrifuged at 2400 \(\times\) g for 5 min. The lower organic layer was transferred to another Eppendorf tube and evaporated under nitrogen. The residue was then dissolved in 200 \(\mu\)L distilled water, and AA was extracted by the addition of 400 \(\mu\)L of hexane. The hexane extracts were dried under nitrogen. AA was analyzed as the pentfluorobenzyl ester by GC-NICI-MS, according to the modified method of Hadley et al. (4).

To evaluate the imprecision of iPF\textsubscript{2\alpha}-III and AA measurements, we processed two plasma samples, collected from healthy laboratory staff, according to the same procedure as study samples. These samples were used for internal quality control and were included in each batch analysis. Within-run imprecision was estimated from 12 determinations within a single batch. Between-run imprecision was estimated from nine determinations over different batches. All data were processed with SPSS 10.0 for Windows (SPSS Inc.). Data distribution was evaluated by the Shapiro–Wilks test. The distribution of plasma total iPF\textsubscript{2\alpha}-III and AA and the iPF\textsubscript{2\alpha}-III/AA ratio were gaussian, and the data are expressed as the mean (SD). Para-
metric analysis methods were used, and statistical significance was set at $P < 0.05$.

Before analyzing the data to establish biological variation, we used the Cochran and Reed tests to exclude outliers (5). The Cochran test ruled out 2 of 80 values for plasma total iPF$_{2\alpha}$-III and 1 of 80 values for plasma AA. One female participant was excluded when the Reed test was applied to the plasma total iPF$_{2\alpha}$-III data set. The between-run CV is expressed as $CV_A$. Using ANOVA with the participant as a random effect, we obtained the overall mean value and the within- ($CV_I$) and between-subject ($CV_G$) biological variation (5). Because duplicate analyses of the samples were not performed, the effect of within-run imprecision was eliminated from $CV_I$ by use of the within-run precision data from the internal quality-control procedure. The desirable quality specification for analytical imprecision ($CV_D$) was calculated as $0.50CV_I$. The reference change value for detecting a significant change within individuals was calculated as: $1.96 \times 2^{1/2}$ ($CV_A^2 + CV_I^2)^{1/2}$. The index of individuality (II) was determined as: $(CV_A^2 + CV_I^2)^{1/2}/CV_G$ (5).

The distributions of plasma total iPF$_{2\alpha}$-III for all participants are shown in Fig. 1. Individuals 1–10 were men and 11–19 were women. According to the Student $t$-test, there was no significant difference in plasma total iPF$_{2\alpha}$-III concentrations between sexes ($P = 0.277$), indicating that sex-specific reference intervals were not required. The loss of AA was observed to correlate with the formation of iPF$_{2\alpha}$ in an in vitro study (6). However, this study also showed no statistically significant correlation between total iPF$_{2\alpha}$-III and AA in plasma ($r = 0.161; P = 0.173$).

The biological variation components of plasma total iPF$_{2\alpha}$-III and AA and the iPF$_{2\alpha}$-III/AA ratio for both sexes and in the reference population are summarized in Table 1. The mean plasma AA and total iPF$_{2\alpha}$-III and concentrations in healthy individuals reported here are in accordance with results reported for earlier studies in our laboratory and other studies when the same technique was used (7). The $CV_I$ and $CV_G$ for plasma total iPF$_{2\alpha}$-III were high (25% and 20%, respectively). The plasma iPF$_{2\alpha}$-III/AA ratio had also been used for the assessment of oxidative stress in vivo (7). Because individuals could have different plasma AA concentrations (the substrate for iPF$_{2\alpha}$-III formation), the plasma total iPF$_{2\alpha}$-III/AA ratio might reduce variation and facilitate interpretation. The $CV_I$ and $CV_G$ of plasma AA (14% and 24%, respectively) were similar and comparable to those obtained for other fatty acids (8). However, our study indicated that the plasma total iPF$_{2\alpha}$-III/AA ratio does not reduce variation ($CV_I$ and $CV_G$ were 27% and 38%, respectively).

The within- and between-run CVs were 7.0% and 7.8% for plasma total iPF$_{2\alpha}$-III and 3.7% and 5.9% for plasma AA, respectively. The best current strategy for defining desirable standards of analytical imprecision is based on biological variation (5). In our study, $CV_D$ was 13% for plasma total iPF$_{2\alpha}$-III and 7% for AA. Thus, the precision of the methods used in this study was better than the desirable specifications.

The II value for plasma total iPF$_{2\alpha}$-III was 1.31, indicating that it has low individuality and that conventional reference values can be useful in clinical and epidemio-

### Table 1. Mean (SD) estimated analytical variation ($CV_A$), within- ($CV_I$) and between-subject ($CV_G$) biological variation, and derived indices for plasma total iPF$_{2\alpha}$-III, AA, and iPF$_{2\alpha}$-III/AA ratio.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Group</th>
<th>Mean (SD)</th>
<th>$CV_A$, %</th>
<th>$CV_I$, %</th>
<th>$CV_G$, %</th>
<th>II</th>
<th>RCV,* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>iPF$_{2\alpha}$-III, nmol/L</td>
<td>All (n = 19)</td>
<td>0.51 (0.16)</td>
<td>7.8</td>
<td>25</td>
<td>20</td>
<td>1.31</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td>Men (n = 10)</td>
<td>0.55 (0.17)</td>
<td></td>
<td>26</td>
<td>18</td>
<td>1.43</td>
<td>74.2</td>
</tr>
<tr>
<td></td>
<td>Women (n = 9)</td>
<td>0.46 (0.14)</td>
<td></td>
<td>24</td>
<td>18</td>
<td>1.40</td>
<td>70.6</td>
</tr>
<tr>
<td>AA, mmol/L</td>
<td>All (n = 20)</td>
<td>0.34 (0.09)</td>
<td>5.9</td>
<td>14</td>
<td>24</td>
<td>0.60</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>Men (n = 10)</td>
<td>0.34 (0.10)</td>
<td></td>
<td>14</td>
<td>26</td>
<td>0.54</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td>Women (n = 10)</td>
<td>0.34 (0.09)</td>
<td></td>
<td>14</td>
<td>23</td>
<td>0.63</td>
<td>40.7</td>
</tr>
<tr>
<td>iPF$_{2\alpha}$-III/AA, mmol/mol AA</td>
<td>All (n = 19)</td>
<td>1.61 (0.74)</td>
<td>9.8</td>
<td>27</td>
<td>38</td>
<td>0.74</td>
<td>78.9</td>
</tr>
<tr>
<td></td>
<td>Men (n = 10)</td>
<td>1.81 (0.90)</td>
<td></td>
<td>28</td>
<td>42</td>
<td>0.69</td>
<td>81.7</td>
</tr>
<tr>
<td></td>
<td>Women (n = 9)</td>
<td>1.39 (0.47)</td>
<td></td>
<td>25</td>
<td>22</td>
<td>1.17</td>
<td>72.4</td>
</tr>
</tbody>
</table>

* RCV, reference change value. Minimum difference between values within an individual considered statistically significant ($P < 0.05$).
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 iPF$_{2\alpha}$-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 iPF$_{2\alpha}$-III concentration has not been reported previously. Helmersson and Basu (9) reported a large variation in the urinary excretion of iPF$_{2\alpha}$-III in healthy individuals with a mean CV of 42% over 10 consecutive days. Both plasma and urinary iPF$_{2\alpha}$ have been used as markers of in vivo lipid peroxidation, but there is a lack of correlation between plasma and urinary iPF$_{2\alpha}$ concentration (10).

To understand the wide biological variation of plasma total iPF$_{2\alpha}$-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 affect iPF$_{2\alpha}$ levels. Exercise such as walking is unlikely to represent a confounding factor (12). Extreme exercise was not 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 iPF$_{2\alpha}$-III and the plasma iPF$_{2\alpha}$-III/AA ratio. To our knowledge, this is the first report on the biological variation of plasma total iPF$_{2\alpha}$-III. These data are important for the assessment of individuals and the design of studies involving these variables.

**References**


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Specimen Dilution for C2 Monitoring with the Abbott TDxFLx Cyclosporine Monoclonal Whole Blood Assay, JoEtta M. Juenke,*, Paul I. Brown,† Francis M. Urry,‡ and Gwendolyn A. McMillin.† (1 ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories Inc., Salt Lake City, UT; 2 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 (1–4).

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 turn-around 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-