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High Stability of Markers of Cardiovascular Risk in Blood Samples, Erik J. Giltay,1* Johanna M. Geleijnse,1 Evert G. Schouten,1 Martijn B. Katan,2 and Daan Krohnout1 (1 Division of Human Nutrition & Epidemiology, Wageningen University, 6700 EV Wageningen, The Netherlands; 2 Wageningen Center for Food Sciences (M.B.K.), 6700 AN Wageningen, The Netherlands; * address correspondence to this author at: Division of Human Nutrition and Epidemiology, Wageningen University, PO Box 8129, 6700 EV Wageningen, The Netherlands; fax 31-317-483342, e-mail giltay@dds.nl)

Biomarkers representing long-term risk of coronary heart disease are widely used in epidemiologic research. Procedures for blood sampling and processing need to be efficient and cost-effective to enable large sample sizes for greater statistical power and precision. Transfer of blood samples by mail is less expensive, easier to perform, and less time-consuming than fast courier services or asking patients to attend a central laboratory for blood sampling. This approach may be incorporated into routine practice and may reduce the likelihood of missing data and random error (1, 2) and eliminate intercenter variability. Although some studies of many analytes at different temperatures and different time intervals have been reported (3–7) and some studies have mimicked transportation conditions (2, 8), to our knowledge neither the effects of transfer by a next-working-day mail service nor the effects on the fatty acid (FA) composition are known. We therefore investigated the variability and reliability of risk factors for coronary heart disease in blood samples delivered by a next-working-day mail service, which is available within most Western countries.

We studied 20 healthy volunteers (7 men and 13
women) working at our department, with a mean age of 39 years (range, 23–54 years) and a mean (SD) body mass index of 23.7 (4.8) kg/m². The study was approved by the Medical Ethical Review Committee of Wageningen University, and written informed consent was obtained from all participants. One set of three tubes of blood was collected in the morning by one trained phlebotomist from each of eight volunteers after an overnight fast: a 10-mL tube containing 18 mg of EDTA, a 4.0-mL tube containing 8 mg of potassium oxalate and 10 mg of sodium fluoride (as antiglycolytic agents), and a 9.5-mL serum separator (SST) tube (Becton Dickinson). To prevent breakage during transport, polyethylene terephthalate tubes were used. All tubes were kept at room temperature until and during transfer. The study was performed during the fall season with a mean outside temperature of 5 °C (minimum–maximum, −1 to 10 °C).

The baseline set (day 0) was centrifuged (1200 g for 10 min) directly, and plasma, serum, and erythrocytes were stored at −80 °C. All SST tubes containing separation gel were also centrifuged directly to form gel barriers between the serum and cells. This enabled us to test whether prolonged contact of serum with a blood clot and hemolysate potentially increases preanalytical variability. The iron chelator EDTA was used to inhibit iron-catalyzed oxidation of polyunsaturated FAs (PUFAs), especially in hemolyzed plasma samples. Blood samples in specially designed plastic envelopes were posted from eight different postboxes covering a radius of ~100 km. After delivery at our laboratory the following day, tubes were processed identically to the baseline set to yield the “day 1” set. This process was repeated to yield the “day 2” set. One posted envelope (of 40) was delayed by 1 day, and two blood samples were lost because the stoppers of the tubes had opened during transport.

All paired samples were assayed in the same run. Standard assays (Roche Diagnostics) were used to determine total cholesterol (cat no. 1489232), HDL-cholesterol (cat. no. 3038661), triglycerides (cat. no. 1488872), glucose (cat. no. 1448668), and ultrasensitive C-reactive protein (CRP-US latex; cat. no. 1972855) and were performed on an automated analyzer (Hitachi 912; Roche Diagnostics). The intra- and interassay CVs for analytic variation (i.e., measurement error) were, respectively, 0.9% and 1.8% for glucose, 0.8% and 1.7% for total cholesterol, 1.3% and 2.6% for HDL, 1.5% and 1.8% for triglycerides, and 1.4% and 5.7% for CRP. The FA composition was measured in serum cholesteryl esters, in EDTA-plasma cholesteryl esters, and in EDTA-erythrocyte membrane phospholipids, according to a modified gas-liquid chromatographic procedure, as described previously (9, 10). Results for FAs were deemed unreliable when there were high unknown peaks in the chromatograms that led to some missing values. FAs were calculated by summing saturated FAs (SAFAs; n = 9), monounsaturated FAs (MUFAs; n = 8), PUFAs (n = 17), n-6 polyunsaturated FAs (n-6 FAs; n = 6), and n-3 polyunsaturated FAs (n-3 FAs; n = 9) with 12–24 carbon atoms (all cis isomers). Because some minor

Fig. 1. Scatter plots showing the effects of delays of 1 and 2 days, respectively, on concentrations of total and HDL-cholesterol, triglycerides, and C-reactive protein in EDTA plasma and glucose in potassium oxalate/sodium fluoride plasma.

LDL-cholesterol values are calculated. C-reactive protein concentrations are shown on a logarithmic scale. ○ represent means (or geometric means for C-reactive protein), and error bars represent 95% confidence intervals. Reliability coefficients are calculated for the three time points combined. To transform mmol/L (SI) into mg/L (MGH), divide by 0.0555 for glucose; by 0.0259 for total cholesterol, HDL-cholesterol, and LDL-cholesterol; and by 0.0113 for triglycerides.
Table 1. Variability and reliability of FAs in cholesteryl esters and erythrocyte membrane phospholipids after time delays of 1 and 2 days compared with baseline values. 

<table>
<thead>
<tr>
<th>FAs in cholesteryl esters from EDTA plasma</th>
<th>Day 1 vs day 0</th>
<th>Day 2 vs day 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) on day 0</td>
<td>Mean (SD)</td>
<td>Reliability</td>
</tr>
<tr>
<td>(baseline)</td>
<td>CV, %</td>
<td>coefficient</td>
</tr>
<tr>
<td>SAFAs</td>
<td>-0.28 (0.54)</td>
<td>2.8</td>
</tr>
<tr>
<td>MUFAs</td>
<td>-0.18 (0.43)</td>
<td>1.5</td>
</tr>
<tr>
<td>PUFAs</td>
<td>0.44 (1.02)</td>
<td>1.1</td>
</tr>
<tr>
<td>n-6 FAs</td>
<td>0.42 (1.03)</td>
<td>1.2</td>
</tr>
<tr>
<td>n-3 FAs</td>
<td>0.03 (0.11)</td>
<td>2.8</td>
</tr>
<tr>
<td>C20:5n-3 (EPA)</td>
<td>0.04 (0.11)</td>
<td>5.4</td>
</tr>
<tr>
<td>C22:6n-3 (DHA)</td>
<td>0.00 (0.01)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

FAs in cholesteryl esters from serum

| Mean (SD) on day 0                        | Mean (SD)     | Reliability   |
| (baseline)                                | CV, %         | coefficient   |
| SAFAs                                     | -0.39 (0.29)  | 1.6           | 0.78          | -0.06 (0.56)  | 2.9           | 0.82          |
| MUFAs                                     | -0.30 (0.76)  | 2.6           | 0.91          | 0.02 (0.35)   | 1.2           | 0.98          |
| PUFAs                                     | 0.57 (0.94)   | 1.1           | 0.94          | 0.28 (0.89)   | 1.0           | 0.96          |
| n-6 FAs                                   | 0.52 (0.94)   | 1.1           | 0.97          | 0.23 (0.93)   | 1.1           | 0.98          |
| n-3 FAs                                   | 0.04 (0.13)   | 3.4           | 1.00          | 0.05 (0.17)   | 4.5           | 1.00          |
| C20:5n-3 (EPA)                            | 0.05 (0.09)   | 4.5           | 1.00          | 0.06 (0.09)   | 4.4           | 1.00          |
| C22:6n-3 (DHA)                            | 0.01 (0.04)   | 4.3           | 0.99          | 0.02 (0.05)   | 5.5           | 0.98          |

FAs in erythrocyte membrane phospholipids from EDTA blood

| Mean (SD) on day 0                        | Mean (SD)     | Reliability   |
| (baseline)                                | CV, %         | coefficient   |
| SAFAs                                     | 0.47 (1.01)   | 1.6           | 0.55          | 0.40 (1.33)   | 2.1           | 0.46          |
| MUFAs                                     | 0.11 (0.66)   | 2.5           | 0.85          | -0.01 (0.64)  | 2.5           | 0.84          |
| PUFAs                                     | 0.57 (0.94)   | 1.1           | 0.94          | 0.28 (0.89)   | 1.0           | 0.96          |
| n-6 FAs                                   | 0.09 (0.96)   | 2.9           | 0.89          | 0.23 (0.93)   | 1.1           | 0.98          |
| n-3 FAs                                   | 0.34 (0.28)   | 2.1           | 0.95          | -0.51 (0.41)  | 3.0           | 0.89          |
| C20:5n-3 (EPA)                            | 0.02 (0.09)   | 7.9           | 0.99          | -0.01 (0.04)  | 3.4           | 1.00          |
| C22:6n-3 (DHA)                            | -0.12 (0.22)  | 3.9           | 0.97          | -0.20 (0.28)  | 4.8           | 0.94          |

The delays were attributable to shipping samples by next-working-day mail service.

Relative percentages (i.e., percentages by weight of total FAs).

Significant at **P < 0.005; P < 0.0005; P < 0.05.

EPA; eicosapentaenoic acid; DHA, docosahexaenoic acid.

For day 0, n = 18; for day 1 vs day 0, n = 14 pairs; for day 2 vs day 0, n = 17 pairs.

Significant at **P < 0.0005; **P < 0.005; **P < 0.05.

SAFAs were not assessed, the sums of the mean SAFAs, MUFAs, and PUFAs concentrations added up to ~97%.

Intra- and interassay CVs were, on average, 20%, respectively, for FAs in cholesteryl esters and somewhat lower for FAs in erythrocyte membrane phospholipids (3.0% and 2.9%, respectively).

The reliability coefficient (i.e., intraclass correlation coefficient with a one-way random effects model with single measure reliability in SPSS) was computed, which reflects the correlation and agreement of test values. These indices allowed comparison of our results with many other reports on variability and reliability.

Shown in Fig. 1 are the plasma values for glucose, lipids, and C-reactive protein. The CV for the paired difference was small (≤3.2% for all), and the reliability was high (≥0.97; P ≤0.005) for all) at day 1 vs day 0. A delay of 2 days yielded a somewhat larger CV (≥4.9% for all) and lower reliability (≥0.90% for all). These results were similar for serum values (data not shown). For individual FAs that compose ~1%, the mean CV for the paired difference in cholesteryl esters was 3.4% and 2.9% for day 1 and day 2, respectively, and in phospholipids the mean CV was 3.3% and 4.2% for day 1 and day 2, respectively. The CV for the paired difference tended to be higher for minor FAs that constituted <1% of total FAs.
stable, showing a relative decrease in cholesteryl ester FAs and increase in phospholipid FAs. In both cholesteryl esters and phospholipids, very-long-chain n-6 FAs and n-3 FAs were the most reliable and stable. Eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), both n-3FAs of marine origin, were highly reliable in all fat fractions (Table 1). Although the proportion of n-3 PUFAs was lower in cholesteryl esters than in erythrocyte membrane phospholipids (2.8% vs 9.4%), the variability was larger in cholesteryl esters than in erythrocyte membrane phospholipids (SD, 1.7% vs 1.4%).

Mailing blood samples offers a cost-effective approach and enables the study of large numbers of samples (13). In practice, blood specimens can be sampled by phlebotomists at individuals’ homes without the need for strict preanalytic procedures (i.e., direct centrifugation, separation, dispensing, and freezing). This approach ensures lower numbers of missing values, high comparability of groups, and thereby, a high internal validity of the study. We also tested whether direct centrifugation of SST tubes provided extra advantages, which was not the case.

Although the systematic error for some of these analytes (glucose, lipids, and C-reactive protein) was statistically significant, the degree of error was small (~3%), which is in accordance with studies that found a high stability during storage (2–8). EDTA-plasma cholesteryl esters, serum cholesteryl esters, and phospholipids in EDTA-erythrocyte membranes yielded similar reliability coefficients. Major FAs and their composites were found to be especially reliable, but saturated FAs and minor FAs that constitute <1% of total FAs were less so. The CVs in the present study (~4–5%) were small when balanced first against the intra- and interassay measurement errors (~2–5%) and second against the within-person variability of FAs over time (~9%) (14, 15). Essential n-6 and n-3 PUFAs were especially reliable, both in EDTA plasma and serum and, to a somewhat lesser extent, in erythrocyte-membrane phospholipids. These FAs are not synthesized endogenously, but their circulating concentrations depend on the amounts in foods and reflect dietary intake well (16, 17).

We conclude that after a delay of 1 or 2 days in blood processing, glucose, lipids, C-reactive protein, and individual FAs adequately rank individuals according to baseline values. These analytes are generally stable after next-working-day mail delivery at room temperature; this procedure may therefore be suitable for many epidemiologic investigations. For the FA composition, use of EDTA plasma is the most practical and reliable, whereas for glucose, lipids, and C-reactive protein, plasma and serum are equivalent. Mailing blood samples offers a cost-effective approach for risk factor assessment with acceptable stability and reliability.

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Detection of Donor-specific DNA Polymorphisms in the Urine of Renal Transplant Recipients, Ying Li,1 Deirdre Hahn,2 Xiao Yan Zhong,3 Peter D. Thomson,2 Wolfgang Holzgreve,4 and Sinuhe Hahn1* (1 University Women’s Hospital/Department of Research, University of Basel, CH 4031 Basel, Switzerland; 2 Division of Paediatric Nephrology, University of the Witwatersrand and Johannesburg Hospital, Johannesburg, South Africa; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women’s Hospital/Department of Research, Schanzenstrasse 46, CH 4031 Basel, Switzerland; fax 41-61-325-9399, e-mail shahn@unbs.ch)

Recently, a novel form of chimerism, termed urinary DNA chimerism, has been described in kidney transplant recipients in that cell-free DNA from the donor kidney was detected in the recipient’s urine (1). Quantitative analysis of this urinary donor-derived DNA has indicated that it may serve as a new marker to monitor kidney