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Most Blood Biomarkers Related to Vitamin Status, One-Carbon Metabolism, and the Kynurenine Pathway Show Adequate Preanalytical Stability and Within-Person Reproducibility to Allow Assessment of Exposure or Nutritional Status in Healthy Women and Cardiovascular Patients[1–3]

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Abstract

Knowledge of stability during sample transportation and changes in biomarker concentrations within person over time are paramount for proper design and interpretation of epidemiologic studies based on a single measurement of biomarker status. Therefore, we investigated stability and intraindividual vs. interindividual variation in blood concentrations of biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway. Whole blood (EDTA and heparin, $n = 12$) was stored with an icepack for 24 or 48 h, and plasma concentrations of 38 biomarkers were determined. Stability was calculated as change per hour, intraclass correlation coefficient (ICC), and simple Spearman correlation. Within-person reproducibility of biomarkers was expressed as ICC in samples collected 1–2 y apart from 40 postmenopausal women and in samples collected up to 3 y apart from 551 patients with stable angina pectoris. Biomarker stability was similar in EDTA and heparin blood. Most biomarkers were essentially stable, except for choline and total homocysteine (tHcy), which increased markedly. Within-person reproducibility in postmenopausal women was excellent (ICC > 0.75) for cotinine, all-*trans* retinol, cobalamin, riboflavin, α-tocopherol, Gly, pyridoxal, methylmalonic acid, creatinine, pyridoxal 5′-phosphate, and Ser; was good to fair (ICC of 0.74–0.40) for pyridoxic acid, kynurenine, tHcy, cholecalciferol, flavin mononucleotide, kynurenic acid, xanthurenic acid, 3-hydroxykynurenine, sarcosine, anthranilic acid, cystathionine, homoarginine, 3-hydroxyanthranilic acid, betaine, Arg, folate, total cysteine, dimethylglycine, asymmetric dimethylarginine, neopterin, symmetric dimethylarginine, and Trp; and poor (ICC of 0.39–0.15) for methionine sulfoxide, Met, choline, and trimethyllysine. Similar reproducibilities were observed in patients with coronary heart disease. Thus, most biomarkers investigated were essentially stable in cooled whole blood for up to 48 h and had a sufficient within-person reproducibility to allow one-exposure assessment of biomarker status in epidemiologic studies. The Western Norway B Vitamin Intervention Trial was registered at clinicaltrials.gov as NTC00354081. J. Nutr. 144: 784–790, 2014.

Introduction

Modern analytical technologies, in particular methods based on MS, allow accurate and precise measurement of a large number of biomarkers in various matrices, including serum and plasma. In addition, multiplexing capabilities, high-throughput, and low-sample volume consumption of methods based on MS open new

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[3] Supplemental Figures 1–5 are available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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research possibilities for large epidemiologic studies of disease risk using precious specimens stored in biobanks (1–3).

Most prospective cohort studies collected 1 blood sample for each individual and thus rely on a single measurement to obtain biomarker status. Therefore, it is critical that the within-person variance in biomarker concentrations caused by sample handling and storage, as well as natural fluctuations, is small relative to the between-person variance. This reliability can be expressed as the intraclass correlation coefficient (ICC),[11] which is the ratio of within-person variance/total variance (4); therefore, ICC also accounts for preanalytical and analytical variance components.

For large biobanks, centralized sample processing is recommended to reduce cost and secure uniform and optimal sample handling (5). This procedure implies transportation of whole blood to the central facility responsible for separation of serum/plasma and subsequent storage at low temperature. Many but not all clinical chemistry parameters are essentially stable during transportation at 4°C for up to 36 h (6), but such stability has to be investigated for each individual analyte to assess potential bias in biomarker studies caused by preanalytical variability. Conversely, storage is a minor source of error and bias, because a range of analytes, including labile molecules, are stable when stored under conditions currently recommended (i.e., at −80°C or below) (5).

We investigated the stability of 38 biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway in whole-blood samples from 12 men and women for up to 48 h under conditions mimicking those of transportation to the central biobank. We also assessed the reproducibility over a period of 1–2 y of the same biomarkers among 40 postmenopausal women enrolled in the Nurses' Health Study (NHS). Because biomarker concentrations may vary according to lifestyle, nutritional, and clinical status, we also assessed the reproducibility of most biomarkers over 38 mo among 551 patients who had undergone coronary angiography for suspected coronary artery disease.

Participants and Methods

Study populations and sample collection. Analyte stability was assessed from blood samples collected in EDTA and sodium heparin Vacutainers from 12 healthy volunteers (50% male) aged 24–56 y (median of 36 y). Samples from each individual were collected in 3 Vacutainers. The first sample was centrifuged immediately and then divided into aliquots and stored in a liquid nitrogen freezer (≤ −130°C). The second and third samples were stored with ice packs in Styrofoam containers for 24 and 48 h, respectively, before being processed and frozen.

We investigated the within-person reproducibility over time among postmenopausal women, aged 51–68 y (median of 62 y), enrolled in the NHS. The NHS is an ongoing cohort study that began in 1976 with 121,700 female registered nurses (7). Details about the blood collections in the NHS have been published previously (8). Briefly, in 1989–1990, 32,836 participants arranged to have their blood collected in 2 15-mL sodium heparin tubes. The tubes were placed in Styrofoam containers with an icepack (temperature of ~4°C) and shipped via overnight mail to the central laboratory, where plasma was immediately separated and stored in liquid nitrogen freezers. A subset of 390 postmenopausal participants who returned a blood sample in 1989–1990 provided 2 additional blood samples over the following 1–2 y. For the current investigation, we randomly selected 40 women who provided at least 2 blood samples, each donated after fasting at least 8 h. The NHS was approved by the Committee on the Use of Human Subjects in Research at Harvard School of Public Health and Brigham and Women's Hospital.

Within-person reproducibility was also investigated in a subset of patients enrolled in the Western Norway B Vitamin Intervention Trial (WENBIT) study. The patients had undergone coronary angiography for suspected coronary artery disease between 1999 and 2004. Details of the WENBIT study have been published previously (9). For the current investigation, we selected patients (n = 633) with stable angina recruited at the Haukeland University Hospitals in Bergen, Norway, who were allocated to receiving placebo and had data at baseline and after 1 month, 1 y, and 38 mo of follow-up. Their median (range) age was 62.8 y (31.8–84.7 y), and 75.8% were males. The number of participants with analyte data at all 4 time points varied somewhat by analyte, ranging from 402 to 551. Whole blood was collected into EDTA Vacutainer tubes and immediately centrifuged, and EDTA plasma was stored within 30 min at −80°C until analysis. The WENBIT study was approved by the Regional Committee for Medical and Health Research Ethics, the Data Inspectorate, and the Norwegian Directorate of Health and is registered at clinicaltrials.gov as NCT00354081.

Laboratory analyses. Riboflavin, flavin mononucleotide, pyridoxal 5′-phosphate (PLP), pyridoxal, pyridoxic acid, kynurenic acid, anthranilic acid, 3-hydroxykynurenine, xanthurenic acid, 3-hydroxyanthranilic acid (HAA), neopterin, cotinine (10), free choline, betaine, dimethylglycine, creatinine, methionine sulfoxide, Arg, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), trimethyllysine (11), all-*trans* retinol (vitamin A), cholecalciferol (25-hydroxyvitamin D-3; vitamin D), and α-tocopherol (vitamin E) (12) were determined by liquid chromatography (LC)-MS/MS, and methylmalonic acid (MMA), total homocysteine (tHcy), total cysteine, Met, Ser, Gly, cystathionine, sarcosine, Trp, and kynurenine were determined by GC-MS/MS (2,13). The kynurenine/Trp ratio (KTR) was calculated by dividing the plasma concentration of kynurenine (in nanomoles per liter) by the concentration of Trp (in micromoles per liter). Cobalamin (vitamin B-12) (14) and folate (15) were determined by microbiologic methods. All analyses were performed in the laboratory of Bevital, and the laboratory staff was unaware of the sample identities.

Statistical analyses. Biomarker concentrations in plasma are presented as geometric means with 95% CIs. We examined the within-day precision of the methods for each analyte using blinded replicate samples from 3 large quality-control plasma pools created using discarded plasma from blood donation centers (8 replicates from 1 EDTA plasma pool and 4 replicates each from 2 different heparin plasma pools). Precision was expressed as analytical CV, which in this context is defined as the SD in percentage of the mean. Specifically, we calculated the CV among samples from the same quality-control pool and then averaged CVs across the 3 pools. We calculated changes in concentrations during storage with an icepack after normalizing the value to percentage of the baseline (time 0) concentration, which was defined as 100%. Because storage effects were investigated at only 3 time points (0, 24, and 48 h), degradation and accumulation kinetics were not modeled according to exponential functions (16) or by segmented regression but were summarized in terms of percentage changes (SD) per hour as obtained by simple linear regression. Storage effects were also evaluated by calculating the ICCs, defined as the between-person variance divided by the total variance (4), across all 3 time points for each analyte, using ln-transformed values. To assess whether participant ranking by analyte concentration changed according to storage, correlation of values obtained at 24 and 48 h with baseline values were calculated using the Spearman test (17). Within-person reproducibility for each analyte was assessed by determination of ICC (95% CI) from repeated participant samples using ln-transformed values and a random-effects mixed model with participant identification as the random variable. An ICC < 0.40 is considered as poor reproducibility, 0.40–0.75 as fair-to-good reproducibility, and ≥0.75 as excellent reproducibility (4).

A sample size of 40 people with 2 samples per person provides excellent power to estimate relatively high ICCs (4), which are of most

[11] Abbreviations used: ADMA, asymmetric dimethylarginine; HAA, 3-hydroxyanthranilic acid; ICC, intraclass correlation coefficient; KTR, kynurenine/Trp ratio; MMA, methylmalonic acid; NHS, Nurses' Health Study; PLP, pyridoxal 5′-phosphate; SDMA, symmetric dimethylarginine; tHcy, total homocysteine; WENBIT, Western Norway B Vitamin Intervention Trial.

scientific interest, e.g., the confidence interval width will be less than ±0.2 for an ICC ≥ 0.65. However, this sample size provides only modest power to estimate lower ICCs, e.g., the CI width for an estimated ICC of 0.35 will be ±0.28, indicating that a true ICC as high as 0.63 is consistent with the data. Thus, low ICC estimates in the NHS population should be interpreted cautiously because they could reflect a chance finding of low between-person variability among the selected 40 participants.

Approximate estimates of the within- and between-person CVs were determined by taking the square root of the within- and between-person variance components from the random-effects mixed model on the ln-transformed scale (4). The program R version 2.15.1 (18) was used for statistical analyses, and the package "ICC" was used to calculate ICC.

Results and Discussion

Principal findings. We investigated stability of 38 biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway in chilled whole blood for up to 48 h and within-person reproducibility of biomarkers over 1–2 y in healthy postmenopausal women (NHS) and over 38 mo (for 32 biomarkers) in patients with stable coronary heart disease (WENBIT study). Most biomarkers were essentially stable, although pyridoxal, choline, tHcy, Arg, and HAA (in EDTA plasma) changed >1% per hour during storage. Within-person reproducibilities obtained from the NHS samples was fair to excellent for most (34 of 38) biomarkers with an ICC ≥ 0.75 for PLP, pyridoxal, riboflavin, cobalamin, Ser, Gly, all-*trans* retinol, α-tocopherol, MMA, creatinine, and cotinine and an ICC < 0.40 for choline, Met, methionine sulfoxide, and trimethyllysine. Similar within-person reproducibilities were obtained from the WENBIT samples.

Stability. Among the biomarkers investigated, most were stable in cooled whole blood (i.e., change <1% per hour) (**Fig. 1**; **Supplemental Fig. 1**). The fat-soluble vitamins were stable in cooled blood (Fig. 1) as demonstrated previously for all-*trans* retinol and less so for α-tocopherol (19). tHcy and choline increased markedly, and for choline, the increase was more pronounced in heparin (Supplemental Fig. 1) than EDTA blood (Fig. 1). This is in agreement with published results and is explained by egress of homocysteine from intact blood cells (20) and enzymic conversion of phosphatidylcholine to free choline (21) catalyzed by phospholipase D, a calcium-dependent enzyme (22) inhibited by EDTA. Pyridoxal and Arg declined markedly, which for pyridoxal may reflect cellular uptake (23). HAA is known be unstable (24), and the concentration decreased markedly, in particular in the presence of EDTA. Otherwise, stability was similar in EDTA (Fig. 1) and heparin blood (Supplemental Fig. 1).

Unstable biomarkers (choline, tHcy, Arg) had low ICC (<0.1) across storage time from 0 to 48 h. However, for choline and tHcy in particular, values at 24 and 48 h correlated well ($\rho \geq 0.5$) with values at baseline (Fig. 1; Supplemental Fig. 1). This is explained by a parallel increase in blood concentrations as a function of time across samples (data not shown) and implies that participants were equally ranked by concentrations of these biomarkers in samples subjected to similar processing, although the absolute value of the biomarker changed markedly after a 24–48 h delay in processing. If there are variable transportation time and temperatures of whole blood in a study, this may bias results involving choline and tHcy.

We previously investigated stability in isolated sera and plasmas at room temperature over days or during storage at −20°C for decades for most biomarkers included in the present study (25). In general, most biomarkers that were stable under these conditions are also stable in chilled whole blood, but there are notable exceptions (summarized in **Supplemental Fig. 2**). The rapid degradation of folate in EDTA plasma (26) was not

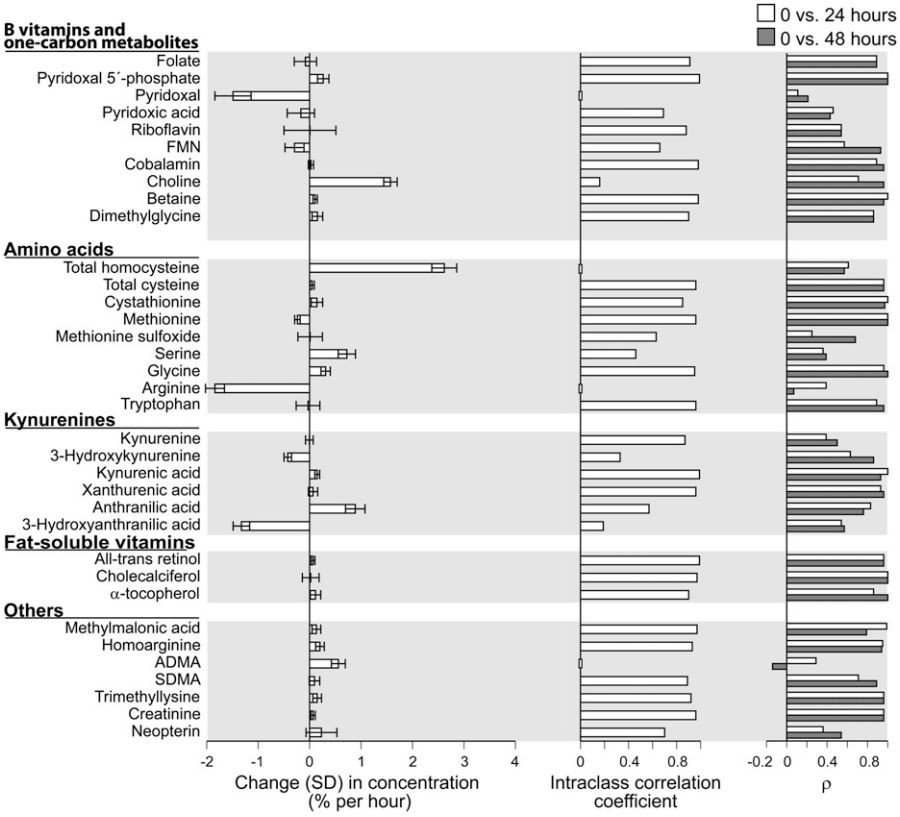

FIGURE 1 Stability of biomarkers in EDTA blood stored in a Styrofoam container with an icepack for 24 or 48 h. Biomarkers (pyridoxal, choline, total homocysteine, Arg, and hydroxyanthranilic acid) that changed substantially over time (left panel) had a low intraclass correlation coefficient (<0.2) across the storage time (middle panel), but for some (choline and total homocysteine), concentrations at 24 or 48 h still showed a fair-to-good ($\rho >$ 0.4) correlation with values at time 0 (right panel). This reflects a parallel increase in concentrations over time and implies that the ranking of the values at fixed time points was maintained. ADMA decreased moderately during storage, but both intraclass correlation coefficient and ranking were low, which is explained by the low between-person variability for ADMA. n = 12. ADMA, asymmetric dimethylarginine; FMN, flavin mononucleotide; SDMA, symmetric dimethylarginine.

observed in cold EDTA blood (Fig. 1; Supplemental Fig. 1). Furthermore, PLP is dephosphorylated to pyridoxal in serum, citrate plasma, and heparin plasma but not EDTA plasma at room temperature and in frozen serum, leading to a concurrent increase in pyridoxal (25). However, in cold blood, PLP was stable and pyridoxal decreasing on storage (Fig. 1; Supplemental Fig. 1). tHcy is stable in serum and plasma, and Arg is stable in plasma and slightly increasing in serum (25) in contrast to the marked changes in these biomarkers observed in whole blood, which probably reflect cellular transport (20). The marked decrease in Met and its recovery as methionine sulfoxide that occur in serum samples during prolonged storage at −20°C was not observed in serum, plasma at room temperature (25), or whole blood (Fig. 1; Supplemental Fig. 1).

Within-person reproducibility in NHS participants. The quality control CVs, medians, and Spearman correlations of values at 2 visits, within-person and between-person CVs (percentage), and ICCs for the 38 biomarkers measured in 40 NHS samples are listed in **Table 1**. The within-person reproducibility over 1–2 y was excellent (ICC range of 0.95–0.75) for the following biomarkers (in decreasing order of ICCs): cotinine, all-*trans* retinol, cobalamin, riboflavin, α-tocopherol, Gly, pyridoxal, MMA, creatinine, PLP, and Ser. Within-person reproducibility was good to fair (ICC range of 0.74–0.40) for pyridoxic acid, kynurenine, tHcy, vitamin D, flavin mononucleotide, kynurenic acid, xanthurenic acid, 3-hydroxykynurenine, sarcosine, KTR, anthranilic acid, cystathionine, homoarginine, HAA, betaine, Arg, folate, total cysteine, dimethylglycine, ADMA, neopterin, SDMA, and Trp and was poor (ICC range of 0.39–0.15) for methionine sulfoxide, Met, choline, and trimethyllysine (Table 1).

The ICC values and Spearman correlation coefficients between the first and second collection showed similar variation across analytes (**Supplemental Fig. 3**). Furthermore, the ICC was 0.71 for tHcy and 0.29 for choline (Table 1), despite a substantial increase in both tHcy and choline during storage as also indicated by higher concentrations of tHcy and choline than expected (27) in healthy women. Together, these observations emphasize the advantage of uniform preanalytical sample handling, in particular for unstable analytes.

A few biomarkers, including total cysteine, Trp, ADMA, and SDMA, with low within-person CVs of <10% had only moderate ICCs (<0.6) because the between-person CV was low (Table 1). The low interindividual biologic variation of ADMA has been reported previously by others (28) who have emphasized the importance of accurate and precise analytical methods to obtain reproducible results that allow meta-analyses and implementation of ADMA in clinical diagnostics.

Within-person reproducibility in WENBIT participants. The ICCs and the variance components for most analytes studied in NHS samples were also assessed among WENBIT participants (n = ~550) donating samples at 4 visits for up to 38 mo (**Table 2**). The ICCs in the WENBIT were similar to those in the NHS, although somewhat lower for most B vitamins, amino acids, and kynurenines and equal or even higher than in NHS samples for choline, betaine, dimethylglycine, MMA, SDMA, creatinine, KTR, and neopterin (**Supplemental Fig. 4**). For choline, the relatively high ICC in the WENBIT probably reflects optimal sample handling (as indicated by a geometric mean for choline of ~10 μmol/L) (Table 2). Higher prevalence of reduced renal function, cellular immune activation, metabolic syndrome, and coronary heart disease in WENBIT patients than in healthy NHS participants might have increased the between-person CVs for creatinine, SDMA (29), MMA (30), neopterin, KTR (31,32), betaine (33), or dimethylglycine (34), thus increasing the ICC.

We also investigated the change in ICC in the WENBIT samples for each analyte by narrowing the time period between sample collections from 38 mo to 1 y or 28 d. The ICC values for most biomarkers were remarkably stable across different periods, and only 4 biomarkers increased >10% when reducing the time span from 38 mo to 28 d, i.e., folate (from 0.51 to 0.71), ADMA (from 0.51 to 0.60), tHcy (from 0.72 to 0.85), and cystathionine (from 0.60 to 0.68) (**Supplemental Fig. 5**).

Comparison with published reliability data. Reproducibility over time has been assessed previously in healthy individuals for some biomarkers that were investigated in the present study. Compared with reproducibility data obtained among the NHS participants, similar ICC values for Arg, Met, and Trp and lower values for Gly and Ser have been reported from a study based on European Prospective Investigation into Cancer and Nutrition–Potsdam samples collected 4 mo apart (35). One study based on samples collected over 30 mo demonstrated similar ICC for tHcy (36), whereas a higher ICC of 0.90 for tHcy over 2–5 y was reported from a European Prospective Investigation into Cancer and Nutrition–Dutch study, which also reported lower reproducibility for folate, vitamin B-6, vitamin B-12, and α-tocopherol (37). A recent study on intra-individual variations of 19 biomarkers related to one-carbon metabolism (38) demonstrated similar reproducibility for most biomarkers as we observed for NHS participants, except for better reproducibility for choline and DMG and lower for folate, cobalamin, tHcy, and cystathionine. Reproducibility over 2–4 y has been investigated previously for fat-soluble vitamins in postmenopausal women participating in the NHS (8), whereas similar ICCs were reported for α-tocopherol and 25-hydroxyvitamin D and lower ICC for all-*trans* retinol compared with the values obtained in the present study. Thus, our results are essentially in agreement with published data on within-person reproducibility for 21 biomarkers, which demonstrate fair-to-excellent reproducibility.

In conclusion, most biomarkers investigated were essentially stable in whole blood stored with ice for up to 48 h, and biomarkers that were stable under these conditions were generally found to be stable in plasma and serum, with notable exceptions for folate (in EDTA plasma stored for days at 23°C) and PLP (in heparin plasma for days at 23°C) (Supplemental Fig. 2). Biomarkers that are unstable in whole blood, such as choline and tHcy, maintain ranking only if samples are undergoing uniform preanalytical handling. Thus, biomarker stability should be assessed under conditions close to those occurring during sample transportation and handling.

Most biomarkers also have a fair-to-excellent within-person reproducibility over 1–2 y in healthy postmenopausal women and a similar reproducibility over 38 mo in patients with stable angina pectoris. However, some differences in reproducibility were noted between healthy women and patients with coronary artery disease. Such differences may partly be explained by increased between-person variance for biomarkers reflecting clinical conditions that are common among cardiovascular patients, such as renal dysfunction, inflammation, and metabolic syndrome. Consequently, a precise or valid correction of regression dilution bias in epidemiologic research based on a

TABLE 1 Concentrations and within-person reproducibility of biomarkers in plasma samples collected at 2 time points 1–2 y apart from 40 postmenopausal women in the Nurses' Health Study[1]

Biomarker	QC CV[2]	Geometric mean (95% CI)		P^3	ρ	Within-person CV[4]	Between-person CV[4]	ICC (95% CI)[5]
		First collection	Second collection					
	%					%	%	
B vitamins and one-carbon metabolites								
Folate, *nmol/L*	5.5	28.5 (22.5, 36.1)	31.4 (24.5, 40.2)	0.37	0.55	47.3	58.9	0.61 (0.37, 0.77)
Pyridoxal 5′-phosphate, *nmol/L*	3.3	57.4 (45.0, 73.2)	59.1 (46.4, 75.2)	0.73	0.66	36.3	66.3	0.77 (0.61, 0.87)
Pyridoxal, *nmol/L*	5.6	25.1 (19.2, 32.8)	26.5 (20.7, 33.8)	0.51	0.66	35.9	71.5	0.80 (0.65, 0.89)
Pyridoxic acid, *nmol/L*	6.5	37.7 (29.2, 48.6)	40.5 (32.3, 50.8)	0.41	0.51	38.7	64.8	0.74 (0.56, 0.85)
Riboflavin, *nmol/L*	7.4	26.2 (21.7, 31.5)	28.5 (23.7, 34.2)	0.08	0.82	21.3	54.0	0.87 (0.76, 0.93)
Flavin mononucleotide, *nmol/L*	8.8	4.67 (4.11, 5.31)	5.10 (4.56, 5.71)	0.06	0.74	20.9	31.5	0.69 (0.49, 0.83)
Cobalamin, *pmol/L*	2.7	469 (425, 518)	461 (414, 514)	0.53	0.87	11.6	30.2	0.87 (0.77, 0.93)
Choline, *μmol/L*	3.7	16.4 (15.5, 17.4)	15.3 (14.6, 16.2)	0.05	0.33	14.8	9.4	0.29 (−0.02, 0.55)[6]
Betaine, *μmol/L*	4.1	36.1 (33.9, 38.5)	36.4 (33.7, 39.3)	0.82	0.64	13.5	17.5	0.63 (0.40, 0.79)
Dimethylglycine, *μmol/L*	7.8	3.05 (2.78, 3.35)	2.96 (2.73, 3.22)	0.48	0.53	18.4	20.5	0.55 (0.30, 0.74)
Sarcosine, *μmol/L*	4.6	1.09 (0.96, 1.25)	1.13 (1.00, 1.26)	0.57	0.73	21.9	32.0	0.68 (0.48, 0.82)
Amino acids								
Total homocysteine, *μmol/L*	6.5	11.1 (10.2, 12.0)	11.0 (10.1, 12.0)	0.87	0.74	14.2	22.4	0.71 (0.52, 0.84)
Total cysteine, *μmol/L*	5.7	325 (312, 338)	330 (318, 344)	0.34	0.49	8.1	9.2	0.56 (0.31, 0.74)
Cystathionine, *μmol/L*	3.1	0.15 (0.14, 0.17)	0.16 (0.14, 0.18)	0.49	0.53	21.0	28.8	0.65 (0.43, 0.80)
Met, *μmol/L*	1.9	25.4 (24.2, 26.6)	26.1 (25.0, 27.2)	0.30	0.31	11.1	8.4	0.36 (0.06, 0.60)
Methionine sulfoxide, *μmol/L*	8.2	1.07 (0.99, 1.15)	1.04 (0.96, 1.13)	0.58	0.32	19.0	14.5	0.37 (0.07, 0.61)
Ser, *μmol/L*	2.5	116 (110, 122)	111 (105, 117)	0.01	0.71	8.7	15.2	0.76 (0.59, 0.86)
Gly, *μmol/L*	2.8	304 (280, 330)	290 (267, 314)	0.03	0.82	10.2	23.1	0.84 (0.71, 0.91)
Arg, *μmol/L*	4.8	48.2 (41.5, 56.1)	50.1 (44.1, 57.0)	0.52	0.67	26.9	34.3	0.62 (0.39, 0.78)
Trp, *μmol/L*	3.3	67.1 (64.6, 69.7)	67.1 (64.0, 70.4)	0.99	0.50	9.8	9.2	0.47 (0.19, 0.68)
Kynurenines								
Kynurenine, *μmol/L*	2.2	1.55 (1.45, 1.67)	1.59 (1.49, 1.69)	0.43	0.70	11.5	18.0	0.71 (0.52, 0.84)
3-Hydroxykynurenine, *nmol/L*	3.7	34.2 (31.4, 37.2)	36.0 (32.8, 39.6)	0.14	0.65	15.8	23.2	0.68 (0.48, 0.82)
Kynurenic acid, *nmol/L*	4.6	44.7 (38.8, 51.5)	47.8 (42.0, 54.4)	0.21	0.65	23.6	35.1	0.69 (0.49, 0.82)
Xanthurenic acid, *nmol/L*	7.3	8.45 (6.97, 10.2)	9.08 (7.63, 10.81)	0.32	0.68	32.2	47.4	0.68 (0.48, 0.82)
Anthranilic acid, *nmol/L*	4.8	15.7 (14.0, 17.7)	17.0 (15.4, 18.8)	0.07	0.63	19.8	28.2	0.67 (0.46, 0.81)
3-Hydroxyanthralinic acid, *nmol/L*	6.9	26.5 (23.8, 29.6)	29.0 (26.0, 32.2)	0.05	0.63	20.5	27.3	0.64 (0.42, 0.79)
Fat-soluble vitamins								
All-*trans* retinol, *μmol/L*	3.8	2.30 (2.16, 2.45)	2.32 (2.18, 2.45)	0.62	0.82	6.8	17.7	0.87 (0.77, 0.93)
Cholecalciferol, *nmol/L*	7.0	68.5 (62.9, 74.7)	69.3 (63.6, 75.4)	0.75	0.66	14.4	22.4	0.71 (0.51, 0.83)
α-Tocopherol, *μmol/L*	2.4	35.6 (31.9, 39.6)	37.7 (33.7, 42.1)	0.04	0.75	12.7	32.1	0.86 (0.76, 0.93)
Others								
Methylmalonic acid, *μmol/L*	4.9	0.16 (0.15, 0.18)	0.17 (0.16, 0.18)	0.11	0.78	12.0	22.9	0.79 (0.63, 0.88)
Homoarginine, *μmol/L*	6.7	1.59 (1.41, 1.78)	1.65 (1.47, 1.85)	0.43	0.57	21.3	28.8	0.65 (0.43, 0.80)
ADMA, *μmol/L*	7.2	0.61 (0.58, 0.63)	0.61 (0.59, 0.64)	0.69	0.47	8.6	9.5	0.55 (0.30, 0.74)
SDMA, *μmol/L*	5.8	0.60 (0.58, 0.63)	0.60 (0.58, 0.63)	0.96	0.51	8.7	8.7	0.50 (0.23, 0.70)
Creatinine, *μmol/L*	2.8	71.4 (67.8, 75.3)	72.9 (69.5, 76.4)	0.23	0.74	7.4	13.7	0.77 (0.62, 0.87)
Trimethyllysine, *μmol/L*	6.3	0.63 (0.56, 0.72)	0.65 (0.58, 0.73)	0.81	0.38	34.7	14.6	0.15 (−0.16, 0.44)[6]
KTR, *nmol/μmol*		23.1 (21.6, 24.8)	23.6 (21.9, 25.5)	0.49	0.65	13.1	18.8	0.67 (0.47, 0.81)
Neopterin, *nmol/L*	6.8	15.3 (13.5, 17.3)	16.7 (15.1, 18.5)	0.11	0.53	24.6	25.7	0.52 (0.26, 0.71)
Cotinine,[7] *nmol/L*	2.2	2.21 (1.11, 4.40)	2.48 (1.24, 4.98)	0.27	0.44	46.9	211.4	0.95 (0.91, 0.97)

[1] ADMA, asymmetric dimethylarginine; ICC, intraclass correlation coefficient; KTR, kynurenine/Trp ratio; QC, quality control; SDMA, symmetric dimethylarginine.
[2] From 16 blinded replicates from 3 quality-control plasma pools.
[3] Paired *t* test comparing geometric mean at first vs. second blood collection.
[4] Within- and between-person CVs were estimated by taking the square root of the within- and between-person variance components from random-effects mixed model on the ln-transformed scale (4).
[5] Calculated using ln-transformed analyte values.
[6] A lower value for 95% CI < 0 is often explained by the intraindividual variation being large compared with the interindividual variation and indicates that the computed ICC is not significantly different from 0.
[7] Cotinine values below the detection limit (1 nmol/L) were set to 1.

single biomarker measurement (39) should be assessed using ICC values determined from a population with demographic and clinical characteristics similar to those of the study population.

Biomarker stability during sample collection, transportation, and storage reflects the chemical structure of the actual compound. Low stability is a major source of preanalytical variability, which should be minimized by adequate sample handling

TABLE 2 Concentrations and within-person reproducibility of biomarkers in plasma samples collected at 4 visits over 3.5 y from cardiovascular patients enrolled in the Western Norway B Vitamin Intervention Trial[1]

Biomarker	Participants/ time points	Geometric mean (95% CI)		P^2	Within-person CV[3]	Between-person CV[3]	ICC (95% CI)[4]
		First visit	Last visit				
	n/n				%	%	
B vitamins and one-carbon metabolites							
Folate, *nmol/L*	551/4	10.6 (10.2, 11.0)	11.8 (11.2, 12.4)	0.0001	37.6	38.4	0.51 (0.47, 0.55)
Pyridoxal 5′-phosphate, *nmol/L*	545/4	40.6 (39.1, 42.3)	41.7 (40.0, 43.4)	0.70	30.4	39.0	0.62 (0.59, 0.66)
Pyridoxal, *nmol/L*	545/4	9.79 (9.43, 10.2)	10.6 (10.2, 11.1)	0.001	35.3	31.2	0.44 (0.40, 0.48)
Pyridoxic acid, *nmol/L*	545/4	26.1 (25.2, 27.1)	28.6 (27.3, 29.8)	0.0001	36.7	31.3	0.42 (0.38, 0.47)
Riboflavin, *nmol/L*	545/4	12.5 (11.8, 13.2)	11.5 (10.8, 12.2)	0.02	39.1	59.7	0.70 (0.67, 0.73)
Cobalamin, *pmol/L*	551/4	334 (323, 345)	328 (316, 340)	0.06	19.9	36.7	0.77 (0.75, 0.80)
Choline, *μmol/L*	551/4	9.52 (9.34, 9.70)	10.5 (10.3, 10.7)	<0.0001	18.1	15.8	0.43 (0.39, 0.47)
Betaine, *μmol/L*	551/4	38.2 (37.3, 39.2)	41.3 (40.3, 42.2)	<0.0001	17.4	24.0	0.66 (0.62, 0.69)
Dimethylglycine, *μmol/L*	551/4	4.09 (3.98, 4.20)	4.27 (4.16, 4.38)	<0.0001	17.1	28.3	0.73 (0.70, 0.76)
Amino acids							
Total homocysteine, *μmol/L*	551/4	10.6 (10.4, 10.9)	10.5 (10.3, 10.8)	0.46	15.7	25.5	0.73 (0.70, 0.75)
Total cysteine, *μmol/L*	551/4	289 (286, 292)	291 (288, 294)	0.02	7.2	10.5	0.68 (0.65, 0.71)
Cystathionine, *μmol/L*	545/4	0.27 (0.26, 0.28)	0.32 (0.30, 0.34)	<0.0001	39.3	48.2	0.60 (0.56, 0.64)
Met, *μmol/L*	551/4	27.1 (26.5, 27.6)	27.6 (27.1, 28.2)	0.14	20.8	13.8	0.30 (0.26, 0.35)
Methionine sulfoxide, *μmol/L*	551/4	0.94 (0.91, 0.97)	0.99 (0.95, 1.02)	0.21	36.0	26.0	0.34 (0.30, 0.39)
Ser, *μmol/L*	477/4	93.2 (91.5, 95.0)	92.4 (90.4, 94.3)	0.13	14.1	19.5	0.66 (0.62, 0.69)
Gly, *μmol/L*	551/4	202 (198, 206)	204 (200, 208)	0.32	11.8	21.4	0.77 (0.74, 0.79)
Arg, *μmol/L*	551/4	44.8 (43.9, 45.7)	45.9 (45.0, 46.8)	0.03	19.7	17.1	0.43 (0.39, 0.47)
Trp, *μmol/L*	402/4	58.5 (57.4, 59.6)	58.0 (56.9, 59.2)	0.16	14.3	15.6	0.54 (0.49, 0.59)
Kynurenines							
Kynurenine, *μmol/L*	545/4	1.62 (1.59, 1.65)	1.72 (1.69, 1.76)	<0.0001	15.2	20.8	0.65 (0.62, 0.68)
3-Hydroxykynurenine, *nmol/L*	543/4	29.0 (28.1, 29.9)	31.4 (30.4, 32.5)	<0.0001	28.9	31.9	0.55 (0.51, 0.59)
Kynurenic acid, *nmol/L*	545/4	48.1 (46.7, 49.6)	54.4 (52.6, 56.2)	<0.0001	22.7	32.2	0.67 (0.63, 0.70)
Xanthurenic acid, *nmol/L*	545/4	13.9 (13.4, 14.4)	16.2 (15.6, 16.8)	<0.0001	33.5	32.5	0.48 (0.44, 0.53)
Anthranilic acid, *nmol/L*	543/4	13.8 (13.4, 14.2)	14.8 (14.3, 15.3)	<0.0001	23.9	29.9	0.61 (0.57, 0.65)
3-Hydroxyanthralinic acid, *nmol/L*	543/4	33.9 (32.8, 35.0)	37.8 (36.6, 39.2)	<0.0001	32.1	28.7	0.44 (0.40, 0.49)
Others							
Methylmalonic acid, *μmol/L*	551/4	0.17 (0.16, 0.17)	0.18 (0.17, 0.18)	<0.0001	15.5	33.3	0.82 (0.80, 0.84)
ADMA, *μmol/L*	551/4	0.71 (0.70, 0.72)	0.71 (0.70, 0.72)	0.67	12.9	13.1	0.51 (0.47, 0.55)
SDMA, *μmol/L*	551/4	0.54 (0.53, 0.55)	0.56 (0.55, 0.57)	<0.0001	12.3	20.6	0.74 (0.71, 0.76)
Creatinine, *μmol/L*	551/4	73.6 (72.4, 74.9)	74.7 (73.3, 76.0)	0.007	9.6	19.4	0.80 (0.78, 0.82)
KTR, *nmol/μmol*	396/4	26.8 (26.1, 27.5)	29.4 (28.5, 30.3)	<0.0001	17.2	29.0	0.74 (0.71, 0.77)
Neopterin, *nmol/L*	545/4	8.16 (7.97, 8.37)	8.65 (8.42, 8.88)	<0.0001	19.1	27.4	0.67 (0.64, 0.70)
Cotinine,[5] *nmol/L*	545/4	9.09 (7.14, 11.6)	7.24 (5.53, 9.47)	0.25	103.9	298.5	0.89 (0.88, 0.90)

[1] ADMA, asymmetric dimethylarginine; ICC, intraclass correlation coefficient; KTR, kynurenine/Trp ratio; SDMA, symmetric dimethylarginine.
[2] From paired *t* test comparing geometric mean at first vs. last visit.
[3] Within- and between-person CVs were determined by taking the square root of the within- and between-person variance components from random-effects mixed model on the ln-transformed scale (4).
[4] Calculated using ln-transformed analyte values.
[5] Continine values below the detection limit (1 nmol/L) were set to 1.

based on stability data for each biomarker. For stable biomarkers determined by a precise method, within-person reproducibility mainly reflects the biologic variability over time due to changing pathophysiologic processes and altered lifestyle, including nutrition. Knowledge on within-person reproducibility is paramount for adequate study design, data interpretation, and statistical analysis, including adjustment for important confounders.

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