Application of blood concentration biomarkers in nutritional epidemiology: example of carotenoid and tocopherol intake in relation to chronic disease risk

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ABSTRACT

Background: Biomarkers provide potential to objectively measure the intake of nutrients and foods, and thereby to strengthen nutritional epidemiology association studies. However, there are only a few established intake biomarkers, mostly based on recovery of nutrients or their metabolites in urine. Blood concentration measures provide a potential biomarker source for many additional nutritional variables, but their use in disease-association studies requires further development.

Objective: The aim of this study was to apply recently proposed serum-based carotenoid and tocopherol intake biomarkers and to examine their association with the incidence of major cardiovascular diseases, cancers, and diabetes in a subset of Women's Health Initiative (WHI) cohorts.

Methods: Serum concentrations of α- and β-carotene, lutein plus zeaxanthin (L + Z), and α-tocopherol were routinely measured at baseline in a subset of 5488 enrollees in WHI cohorts. Intake biomarkers for these 4 micronutrients, obtained by combining serum concentrations with participant characteristics, were recently proposed using a 153-woman feeding study within WHI. These biomarker equations are augmented here to include pertinent disease risk factors and are associated with subsequent chronic disease incidence in this WHI subset.

Results: HRs for a doubling of micronutrient intake differed only moderately from the null for the outcomes considered. However, somewhat lower risks of specific cardiovascular outcomes, breast cancer, and diabetes were associated with a higher intake of α- and β-carotene, lower risk of diabetes was associated with higher L + Z intake, and elevated risks of certain cardiovascular outcomes were associated with a higher intake of α-tocopherol. These patterns remained following the exclusion of baseline users of dietary supplements.

Conclusions: Concentration biomarkers can be calculated from blood specimens obtained in large epidemiologic cohorts and applied directly in disease-association analyses, without relying on self-reported dietary data. Observed associations between carotenoid and tocopherol biomarkers and chronic disease risk could be usefully evaluated further using stored serum specimens on the entire WHI cohort. This study was registered at www.clinicaltrials.gov as NCT00000611. *Am J Clin Nutr* 2019;109:1189–1196.

Keywords: biomarker, cancer, cardiovascular disease, carotenoid, diabetes, diet, measurement error, tocopherol

Introduction

Several recent publications (1–4) have emphasized the potential of biomarkers to objectively assess intake of nutrients, foods, and dietary patterns, and to strengthen nutritional epidemiology research, which to date has almost exclusively relied on self-reported dietary data. A recent editorial (5) argued that it is "now imperative to move these biomarkers into practice" while also cautioning that biomarkers are needed that "can aid dietary assessment by acting as objective measures of intake," rather than just serving as correlates of intake. The few established intake biomarkers, for total energy (6), protein (7), sodium, and potassium (8, 9), rely on participant-burdensome collection

This study is supported by National Cancer Institute grants R01 CA119171 and R01 CA10921. The Women's Health Initiative is supported by the National Heart, Lung and Blood Institute (contracts HHSN268201100046C, HHSN268201600001C, HHSN268201600002C, HHSN268201600003C, HHSN268201600004C, and HHSN271201600004C).

Supplemental Tables 1–5 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: CABG/PCI, coronary artery bypass graft or percutaneous coronary intervention; CHD, coronary heart disease; CR, confidence region; CT, clinical trial; CVD, cardiovascular disease; DLW, doubly labeled water; FFQ, food-frequency questionnaire; L + Z, lutein plus zeaxanthin; NPAAS, Nutrition and Physical Activity Assessment Study; OS, observational study; WHI, Women's Health Initiative

Received April 24, 2018. Accepted for publication November 28, 2018.

First published online March 27, 2019; doi: https://doi.org/10.1093/ajcn/nqy360.

of 24-h urine specimens. These collections are rarely included in epidemiologic cohort studies. Furthermore, the energy-assessment biomarker requires doubly labeled water (DLW) dosing, rather than being derivable from stored specimens.

Blood concentrations provide a potential source for intake biomarker development for many other dietary components (10). At a minimum, such concentrations need to be rescaled to reflect intake, and the rescaling factor may depend on such study participant characteristics as BMI, age, and sex. Recently our group proposed intake biomarkers for several micronutrients based on serum nutrient concentrations and relevant study participant characteristics using data from a controlled feeding study among 153 Women's Health Initiative (WHI) enrollees (11). Here we consider the methodology for, and the findings from, application of these biomarkers to cardiovascular disease (CVD), cancer, and diabetes incidence in WHI cohorts.

Pertinent serum concentrations were routinely assessed in an ongoing fashion in a subset of WHI cohorts, permitting a fairly straightforward application of corresponding intake biomarkers. Chronic disease-association analyses in this subset provide a fresh look at certain carotenoid and tocopherol intakes in relation to chronic disease risk, and have implications more generally for the further development of a biomarker-based approach to nutritional epidemiology.

Subjects and Methods

During 1993–1998, a total of 68,132 women enrolled in the randomized, controlled Clinical Trial (CT) and 93,676 women enrolled in the prospective Observational Study (OS) within the WHI. All women were postmenopausal and in the age range of 50–79 y at enrollment at 40 US clinical centers. All women provided core questionnaires including medical history, reproductive history, family history, personal habits, and dietary supplements (used by about 40% of enrollees), along with a fasting blood sample, at baseline (12). Serum aliquots were stored at −80°C until pulled for analyses.

Serum samples were analyzed on an ongoing basis on random subsets, oversampled for minorities, of both the CT (approximate 5.8% sample) and OS (approximate 1% sample). Analysis on this combined subcohort ($n = 5488$) included concentrations of α- and β-carotene, lutein plus zeaxanthin (L + Z), and α-tocopherol, which were among the micronutrients for which serum-based biomarkers were recently proposed (11). Carotenoids and tocopherols were both measured by HPLC. Interbatch CVs for laboratory QC samples were <6.0% for each analyte.

The Nutrition and Physical Activity Assessment Study (NPAAS) Feeding Study (2010–2014) enrolled 153 WHI women in the Seattle area. Women were provided food and most beverages over a 2-wk feeding period, with individualized diets that were intended to approximate their usual diets so that blood and urine concentrations would stabilize quickly, and intake variations in the study cohort would be substantially retained during the feeding period. Biomarkers proposed for the nutritional intakes studied here are based primarily on corresponding serum nutrient concentrations (adjusted for serum cholesterol), with the inclusion of readily available study participant characteristic measures. The biomarker equations in (11) are as follows:

$$\begin{aligned}\log(\alpha-\text{carotene}) = {} & 6.362 + 1.241 \times \log(\text{serum}\,\alpha-\text{carotene}) \\ & + 0.082 \times \text{BMI} - 0.325 \\ & \times \text{spring season indicator} \\ & - 0.534 \times \text{summer season indicator} \\ & - 0.258 \times \text{fall season indicator} \qquad (1)\end{aligned}$$

$$\begin{aligned}\log(\beta-\text{carotene}) = {} & 8.478 + 0.624 \times \log(\text{serum}\,\beta-\text{carotene}) \\ & + 0.050 \times \text{BMI} \qquad (2)\end{aligned}$$

$$\begin{aligned}\log(\text{L}+\text{Z}) = {} & 7.426 + 1.101 \times \log(\text{serum L}+\text{Z}) \\ & - 0.028 \times \text{age} + 0.049 \times \text{BMI} \\ & + 0.593 \times \text{white race indicator} \qquad (3)\end{aligned}$$

$$\begin{aligned}\log(\alpha-\text{tocopherol}) = {} & 2.885 + 2.077 \\ & \times \log(\text{serum}\,\alpha-\text{tocopherol}) \\ & + 0.510 \times \text{dietary supplement use indicator} \qquad (4)\end{aligned}$$

In these equations, age in years was centered at 75.36, and BMI at 26.39 kg/m^2, and units were μg/d for the carotenoids and mg/d for α-tocopherol.

Outcome ascertainment and disease categories

Clinical outcomes were reported biannually in the CT and annually in the OS, by self-administered questionnaire throughout the time period from enrollment to the end of the intervention period (8 April 2005), and annually thereafter in both cohorts. An initial report of CVD or invasive cancer during cohort follow-up was confirmed by a review of medical records and pathology reports by physician-adjudicators. In addition, coronary heart disease (CHD), stroke, and all deaths were centrally reviewed by expert investigator committees, and all cancers except nonmelanoma skin cancer were centrally coded using the NCI's Surveillance, Epidemiology and End Results (SEER) procedures (13).

CVD categories considered are CHD, defined as nonfatal myocardial infarction plus coronary death; coronary artery bypass graft or percutaneous coronary intervention (CABG/PCI); stroke, defined as ischemic plus hemorrhagic stroke; and total CVD, defined as CHD and CABG/PCI plus stroke. Cancer categories considered are invasive breast cancer and total invasive cancer. Prevalent (treated) diabetes at baseline was self-reported during eligibility screening. Incident diabetes during follow-up was documented by self-report at each annual contact. These sources have been shown to be consistent with periodic medication inventories of oral diabetes agents and insulin in WHI cohorts (14). The outcome categories just listed are the subset of

outcomes considered in a previous report (15) on energy intake and activity-related energy expenditure in relation to chronic disease outcomes, but with restriction to outcomes having at least 100 cases during follow-up in the previously mentioned subcohort.

For the present analyses, CHD and invasive breast cancer can be considered as coprimary outcomes. These were primary outcomes in the WHI hormone therapy and dietary modification trials. Other cardiovascular disease categories, total cancer, and diabetes outcomes can be considered as secondary outcomes.

Following the intervention period, WHI participants had the opportunity to enroll in additional follow-up through 30 September 2010, and subsequently for additional open-ended follow-up, with more than 80% of women doing so on each occasion. Cancer, diabetes, and mortality (including National Death Index matching) outcomes through 31 December 2013 are included here. Follow-up for CVD incidence is included only through 30 September 2010, because self-reports for most WHI participants were not adjudicated after that date. The mean follow-up duration here is 11.0 y for CVD incidence, and 13.2 y for cancer and diabetes.

Statistical methods

Log-transformed intake biomarker equations from the 4 micronutrients were augmented to include pertinent disease risk factors (as elaborated below), and biomarker intake values were calculated for members of the CT/OS subset and entered into Cox regression models (16), along with disease-specific potential confounding factors. A linear modeling of log-HR on log-intake is assumed, and this implies a fixed HR for a fractional increase in intake. To be specific, we present HR estimates for a doubling in intake. Baseline hazard rates in the Cox model analyses were stratified on age in 5-y categories, race/ethnicity because of the oversampling, on cohort (CT or OS), and, in the CT, were further stratified on participation in the WHI dietary modification trial (intervention, comparison, or not randomized) and on participation in the WHI hormone therapy trials (estrogen, estrogen placebo, estrogen plus progestin, estrogen plus progestin placebo, not randomized).

In addition to the estimated log-transformed intake biomarker, the regression variables in the Cox model included a set of outcome-specific potential confounding factors. CVD outcome analyses included age (linear), race/ethnicity, family income, education, cigarette smoking history, alcohol consumption, leisure physical activity, height, weight, any supplement use, prior menopausal hormone use, hypertension, cardiovascular disease in first-degree relative, personal history of cancer, and personal or family history of diabetes. Invasive cancer analyses included these same variables, exclusive of prevalent CVD and of family history of CVD or diabetes, and inclusive of family history of breast cancer, family history of colorectal cancer, and personal history of colon polyp removal. Diabetes incidence analyses included the same variables as the CVD analyses except for family history of myocardial infarction or stroke. The pre-enrollment use of pertinent medications was also considered for inclusion in disease-risk models. A list of the potential confounding variables considered for each outcome category is provided in **Supplemental Table 1**. Missing data rates were generally low for modeled variables, and participants were excluded from outcome-specific analyses if any covariate was missing. Participants having CVD, invasive cancer, or treated diabetes before enrollment were excluded from respective CVD, cancer, or diabetes analyses.

Disease occurrence time for a "case" developing a study outcome was days from enrollment to diagnosis, and censoring time for "noncases" was days from enrollment to the earlier of date of death without the outcome under study, last contact, or either 30 September 2010 for CVD outcomes or 31 December 2013 for cancer and diabetes outcomes.

The estimated log-micronutrient intake values can be thought of as estimating actual short-term log-intake, plus random error that is uncorrelated with actual log-intake or with the potential confounding factors. Because of this error component, a "sandwich-type" variance estimator is needed for the log-HR parameter estimates (17–19). These modeling issues are an important aspect of the use of serum concentration-based intake estimates, and more detailed developments and justifications follow.

Intake biomarker measurement error methods

Denote by z a participant's log-transformed mean daily intake of a nutritional variable of interest, and by x a corresponding log-transformed serum concentration measure. At a minimum, a serum concentration for this nutritional variable needs to be rescaled to reflect dietary intake, and the rescaling may depend on participant characteristics. Let $v = (v_1, v_2, \ldots)'$ denote a numerically coded set of potentially relevant characteristics. Linear regression of z on (x, v) in the NPAAS feeding study can capture this possibly complex rescaling and yield log-transformed intake estimates of the form

$$\hat{z} = \hat{\beta}_0 + \hat{\beta}_1 x + \hat{\beta}_2' v \quad (5)$$

where $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2 = (\hat{\beta}_{21}, \hat{\beta}_{22}, \ldots)'$ are estimated regression coefficients from the linear regression. The central biomarker assumption is that actual log-transformed intake can be written as

$$z = b_0 + b_1 x + b_2' v + e \quad (6)$$

where the error term e is independent of (x, v). This is primarily a subject-matter assumption that presumes the absence of other dietary factors or study participant characteristics that correlate with the residual from regression of z on (x, v). The assumption can be partially checked in a feeding study context by examining the impact of entering additional variables into the above linear model. For a biomarker to be efficient, the variance of the error term in this linear model should be small relative to that for z, or equivalently the fraction of variation explained (R^2) by the linear model should be fairly large. Hence, to put forward a novel nutritional biomarker from a feeding study such as that conducted in WHI, one needs to argue for the absence of important omitted variables from the linear model given above, and one needs also to show that the biomarker R^2 satisfies a suitable criterion. We used an R^2 cutoff of 0.36 or larger in putting forward the serum-based nutrient biomarkers considered here (11). Our rationale was as follows: even when the targeted mean daily intake pertains to the actual feeding period in a

biomarker development feeding study (2 wk for the NPAAS feeding study), there may be considerable random variation in the feeding study actual consumption assessments. That is, we do not have available the precise intake values z for feeding study participants, but rather have a possibly noisy estimate thereof, for example, based on lack of precise information on the nutrient composition of provided food, inaccuracies in the nutrient database that convert food intake to nutrient intake, and actual intakes that may depart somewhat from food provided. For example, in the NPAAS feeding study, participants were asked to return unconsumed food, but some such food may not have been returned, or participants may have consumed and not reported some food other than that provided during the feeding period. Collectively, these sources, in conjunction with possible imprecision in established biomarkers, led to estimated R^2 values of about 50% for the DLW assessment of log-transformed energy intake (even after adjustment for minor weight variation during the 2-wk feeding period) and about 40% for the log-transformed urinary nitrogen biomarker of protein intake (11). These values provide benchmarks for novel biomarker identification, with the idea that those with $R^2 \geq 36\%$ correlate about as closely with the somewhat noisy estimates of actual (log-transformed) intake as do these other well-established intake biomarkers. The R^2 criterion was developed for use in the WHI feeding study context, and different criteria may be needed for biomarker development in other contexts.

The partial R^2 for adding x to a linear model that already includes v is another pertinent statistic for biomarker development. This feature is particularly relevant to disease-association applications, because disease-association analyses typically condition on components of v that are disease risk factors, and association analyses can therefore be expected to be imprecise if this partial R^2 is small. We have not imposed a specific criterion for this partial R^2, but small values (e.g., only a few percent) suggest that the potential biomarker would not be able to be regarded as deriving primarily from the related blood concentration measurement.

Under standard linear model assumptions, one can write

$$z = \hat{z} + \hat{e} \tag{7}$$

where the error term $\hat{e}$ is uncorrelated with $\hat{z}$. Hence, z has the structure of the biomarker $\hat{z}$ plus uncorrelated error component, and therefore plausibly adheres to a so-called Berkson error model. It follows under a joint normality assumption and a rare disease assumption that $\hat{z}$ can be used in place of z in the Cox models described above, essentially without biasing HR estimates for z in relation to the study outcome (17–19). This statement also assumes that disease-risk confounding factors for the study outcome have been included as needed in the biomarker specification model. The biomarker equations listed above for the 4 micronutrients were augmented, for each outcome category, by a stepwise inclusion of the potential confounding factors listed in Supplemental Table 1. HR parameter estimates are, of course, reduced in efficiency by the noise component of the biomarker, compared with analyses using the actual short-term log-transformed intake z. A sandwich-type variance estimator (17–19) acknowledges the additional variance in log-HR parameter estimates that is attributable to this error component $\hat{e}$. Augmented biomarker equations for each micronutrient are listed in **Supplemental Table 2**, separately for CVD, cancer, and diabetes analyses. A $P < 0.10$ criterion was used for inclusion of these potential confounding factors in biomarker equations.

Participants provided written informed consent for the overall WHI and for their NPAAS activities, and protocols were approved by the Institutional Review Boards at the Fred Hutchinson Cancer Research Center, and at each participating clinical center.

Results

Study participant characteristics, serum concentration measures, and disease incidence in the WHI subcohort

Supplemental Figure 1 shows participant flow for this research project. **Supplemental Table 3** shows participant characteristics for the 5488 CT and OS women in the cohort subset having serum analytes routinely measured. The blood concentration data used here derived from baseline serum measurements only. **Table 1** lists the geometric means and 95% confidence regions for serum concentrations of the 4 nutrients under study in this subset. Corresponding summary statistics for nutrient intake estimates were calculated using the biomarker equations from Lampe et al. (11), and these are also included in Table 1.

Serum concentration-based biomarkers in relation to chronic disease incidence

Following baseline exclusions for prevalent disease and for missing data on modeled covariates, there were 3780 women included in CVD analyses, of whom 154 developed CHD, an additional 172 had CABG or PCI, 124 had a stroke, and a total of 370 experienced a CVD event (including CABG/PCI) during the defined follow-up periods.

Following exclusions, there were 3686 women included in (invasive) cancer analyses, of whom 176 were diagnosed with breast cancer and 473 with invasive cancer overall (excludes nonmelanoma skin cancer) during cohort follow-up. There were 3693 women included in diabetes analyses, of whom 644 reported first taking either oral diabetes medications or insulin during cohort follow-up.

Table 2 shows estimated HRs (95% CIs) for a doubling in nutrient intake for each of the disease categories just mentioned. HRs are mostly not far from the null value of 1. HRs for an increase in α-carotene or β-carotene, however, are below 1 for CABG/PCI, breast cancer, and diabetes incidence, and an increase in L + Z is associated with a decrease in certain cardiovascular outcomes, and in diabetes incidence. In contrast, an increase in α-tocopherol intake, which in this population derived substantially from the use of dietary supplements, was associated with an increase in CABG/PCI.

These analyses were repeated, excluding participants who were users of dietary supplements at baseline (see Supplemental Table 1). After adding this exclusion, there were 2114 participants in CVD analyses, of whom 86, 101, 65, and 207 experienced CHD, CABG/PCI, stroke, and total CVD during cohort follow-up; there were 2095 participants in cancer analyses, of whom 104 and 267 experienced breast cancer and total invasive cancer during cohort follow-up; and there were 2043 participants in

TABLE 1 Geometric mean and 95% CR for serum concentration and for biomarker-estimated intake of carotenoids and tocopherol in a Women's Health Initiative serum biomarker substudy ($N = 5488$) enrolled during 1993–1998[1]

	Serum concentration (μg/mL)								
	Clinical trial			Observational study			Combined		
Nutrient	N	Geometric mean	95% CR	N	Geometric mean	95% CR	N	Geometric mean	95% CR
α-Carotene	4414	0.058	0.011, 0.268	1060	0.077	0.015, 0.311	5474	0.061	0.012, 0.288
β-Carotene	4414	0.236	0.048, 1.205	1060	0.259	0.054, 1.336	5474	0.241	0.049, 1.177
L + Z	4416	0.199	0.081, 0.476	1060	0.208	0.083, 0.523	5476	0.201	0.080, 0.486
α-Tocopherol	4415	14.89	7.55, 37.06	1061	16.72	8.92, 39.22	5476	15.23	7.79, 36.86
Biomarker-estimated nutrient intake									
α-Carotene (μg/d)	4391	500.9	65.8, 3139.6	1042	650.9	99.8, 3620.7	5433	526.7	70.9, 3204.3
β-Carotene (μg/d)	4391	5540.6	2045.5, 14,493.8	1042	5319.8	1895.6, 13,785.6	5433	5497.6	2002.9, 14,225.8
L + Z (μg/d)	4393	3703.8	1206.7, 10,925.8	1042	3824.7	1262.8, 10,942.6	5435	3726.7	1218.9, 10,929.6
α-Tocopherol (mg/d)	4412	20.5	5.1, 160.7	1061	31.0	7.0, 192.6	5473	22.2	5.3, 167.2

[1]CR, confidence region (2.5- and 97.5-sample percentiles); L + Z, lutein plus zeaxanthin.

TABLE 2 HRs and 95% CIs for a doubling in micronutrient intake in relation to the incidence of CVD ($N = 3780$), cancer ($N = 3686$), and diabetes ($N = 3693$) during follow-up of a Women's Health Initiative subcohort[1]

	α-Carotene		β-Carotene		Lutein plus zeaxanthin		α-Tocopherol	
	HR[2]	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
CVD (cases, n)								
Coronary heart disease (154)	0.94	(0.83, 1.06)	1.05	(0.80, 1.38)	1.04	(0.81, 1.33)	1.10	(0.98, 1.25)
CABG/PCI (172)	0.86	(0.78, 0.95)	0.77	(0.60, 0.97)	0.83	(0.66, 1.04)	1.15	(1.03, 1.28)
Stroke (124)	0.96	(0.85, 1.09)	1.03	(0.80, 1.33)	0.74	(0.55, 0.99)	0.92	(0.79, 1.06)
Total CVD (370)	0.92	(0.85, 0.99)	0.93	(0.79, 1.10)	0.83	(0.71, 0.98)	1.08	(0.99, 1.17)
Cancer (cases, n)								
Invasive breast cancer (176)	0.88	(0.77, 1.00)	0.68	(0.51, 0.91)	0.90	(0.69, 1.17)	0.99	(0.87, 1.11)
Total invasive cancer (473)	0.94	(0.88, 1.02)	0.85	(0.72, 1.02)	1.04	(0.89, 1.20)	1.04	(0.97, 1.13)
Diabetes (644 cases)	0.91	(0.85, 0.97)	0.72	(0.63, 0.82)	0.80	(0.70, 0.92)	1.05	(0.98, 1.13)

[1]Follow-up period from enrollment during 1994–1998 to 30 September 2010 for CVD, and to 31 December 2013 for cancer and diabetes. Women's Health Initiative subcohort composed of 5488 women for whom core analytes including the micronutrients considered here and total serum cholesterol were routinely measured using blood specimens collected at baseline. Women having a history of CVD, invasive cancer, or diabetes, or having missing covariates used to control confounding were excluded from respective CVD, cancer, and diabetes analyses. CABG/PCI, coronary artery bypass graft or percutaneous coronary intervention; CVD, cardiovascular disease.

[2]HRs were calculated using Cox regression with detailed baseline stratification of hazard rates, and with modeled covariates including log-transformed biomarker intake and pertinent confounding factors. CIs are calculated using a sandwich procedure that acknowledges random error in the biomarker intake estimates.

diabetes analyses, of whom 396 developed incident diabetes during cohort follow-up. As shown in **Table 3**, the HRs of Table 2 are little changed following this exclusion, although the precision of HR estimates is reduced. Also, α-tocopherol intake becomes positively associated with total invasive cancer after excluding baseline supplement users.

These HR analyses were repeated with BMI added to the list of potential confounding factors, with essentially no change in results from those shown in Tables 2 and 3. See **Supplemental Tables 4** and **5** for details of these analyses.

Discussion

Use of serum-based biomarkers in nutritional epidemiology

Serum-based intake biomarkers may provide objective intake assessments following a rescaling procedure that acknowledges possible dependence on such study participant characteristics as BMI, age, race, and seasonality, among others. The major criterion for an acceptable biomarker is that the residual error following intake estimation is unrelated to either actual (short-term) consumption or study participant variables needed to control confounding. The plausibility of this criterion being met needs to be considered carefully for a serum-based measure, in the context of the study population and the disease outcome(s) under consideration. Under this criterion, the magnitude of the remaining error relative to the corresponding actual intake variation essentially determines the biomarker efficiency. We propose an $R^2 \geq 36\%$ for use in this feeding study context (11) based on R^2 values for established energy and protein biomarkers. This criterion may be more difficult to satisfy if serum measurements derive from hepatic or renal conversion of pertinent dietary intakes, rather than from the nutritional variable under study itself. Hence, whether blood-based measures can lead to suitable dietary intake biomarkers for a broad range of nutrients and foods, through the conduct of human feeding studies, remains to be determined.

Direct biomarker application and 2-step indirect data-analysis procedures

Previous disease-association analyses in WHI cohorts (e.g., 15) have used intake biomarkers in a 2-step indirect process. In the first step, log-transformed urinary recovery biomarkers were regressed linearly on log-transformed self-reported intake using food-frequency questionnaire (FFQ), 4-d food record data, or 24-h dietary recall data and pertinent study participant characteristics in nutrition biomarker substudies (20, 21) within WHI cohorts. Resulting linear equations were then applied to dietary self-report and study participant data to generate calibrated intake estimates throughout WHI cohorts. The strength of the relation between actual intake and calibrated intake can, therefore, be reduced by random error in the relation between actual intake and biomarker, and by random error in the relation between biomarker and calibrated self-report assessment. Hence, this 2-step approach is inherently weaker than the direct biomarker application approach presented in this paper. However, the 2-step approach can be cost-effective if both the biomarker and the self-report data relate fairly strongly to actual intake, because biomarker values then need only be determined in moderate-sized substudies. WHI nutrition

TABLE 3 HRs and 95% CIs for a doubling in micronutrient intake in relation to the incidence of CVD ($N = 2114$), cancer ($N = 2095$), and diabetes ($N = 2043$) during follow-up of a Women's Health Initiative subcohort with restriction to participants not taking dietary supplements at baseline[1]

	α-Carotene		β-Carotene		Lutein plus zeaxanthin		α-Tocopherol	
	HR	95% CI[2]	HR	95% CI	HR	95% CI	HR	95% CI
CVD (cases, *n*)								
Coronary heart disease (86)	0.93	(0.80, 1.07)	0.96	(0.64, 1.44)	0.93	(0.66, 1.32)	0.99	(0.80, 1.22)
CABG/PCI (101)	0.89	(0.78, 1.02)	0.77	(0.55, 1.06)	0.83	(0.60, 1.15)	1.10	(0.94, 1.30)
Stroke (65)	0.93	(0.77, 1.12)	0.76	(0.52, 1.11)	0.94	(0.65, 1.35)	0.97	(0.78, 1.20)
Total CVD (207)	0.92	(0.83, 1.01)	0.84	(0.66, 1.05)	0.86	(0.70, 1.06)	1.03	(0.91, 1.16)
Cancer (cases, *n*)								
Invasive breast cancer (104)	0.88	(0.75, 1.04)	0.77	(0.52, 1.13)	0.99	(0.69, 1.43)	1.12	(0.94, 1.34)
Total invasive cancer (267)	0.92	(0.84, 1.01)	0.88	(0.69, 1.12)	1.05	(0.84, 1.31)	1.15	(1.03, 1.28)
Diabetes (396 cases)	0.89	(0.82, 0.96)	0.67	(0.56, 0.79)	0.74	(0.61, 0.89)	1.10	(0.99, 1.23)

[1] Follow-up period from enrollment during 1994–1998 to 30 September 2010 for CVD, and to 31 December 2013 for cancer and diabetes. Women's Health Initiative subcohort composed of 2575 participants for whom core analytes including the macronutrients considered here and total serum cholesterol were routinely measured using blood specimens collected at baseline, exclusive of participants using dietary supplements at baseline. Participants having a history of CVD, invasive cancer, or diabetes, or having missing covariates used to control confounding were excluded from respective CVD, cancer, and diabetes analyses. CABG/PCI, coronary artery bypass graft or percutaneous coronary intervention; CVD, cardiovascular disease.

[2] HRs were calculated using Cox regression with detailed baseline stratification of hazard rates, and with modeled covariates including log-transformed biomarker intake and pertinent confounding factors. CIs were calculated using a sandwich procedure that acknowledges random error in the biomarker intake estimates.

biomarker substudies had samples sizes of 544 in CT (20) and 450 in OS (21), whereas corresponding disease-association studies apply to the typically much larger cohorts having dietary self-report data. However, with biomarkers like those applied here, which are relatively weak in the sense that dietary self-report data can help explain variation in feeding study intake (z) beyond that explained by (x, v), the biomarker equations in the first step need to be further augmented to avoid bias in disease-association estimates. The authors plan to further develop and illustrate this 2-step approach elsewhere.

The 2-step approach can be useful and efficient with a strong biomarker such as the DLW energy assessment and a corresponding strong calibration equation. Importantly, however, the DLW method for energy assessment is intimately tied to the 2-step procedure, because it requires an active protocol of DLW dosing that is impractical, in terms of both cost and logistics, for application to all participants in large epidemiologic cohorts. An alternative objective energy intake assessment that relies only on stored specimens in conjunction with other inexpensive measurements could provide a very valuable step in work toward an overall objective intake measures approach to nutritional epidemiology.

Carotenoids and tocopherols, and chronic disease risk

Although based on only a subcohort and a moderate number of incident disease events over a long follow-up period, the direct biomarker application analyses presented here (Tables 2 and 3) do not suggest major associations between dietary intake (including supplements) for any of the 4 nutrients considered, and any of the chronic disease outcome classes listed. On the other hand, there are several nominally significant associations. These associations could be studied further in the larger WHI cohorts using stored serum specimens. Specifically, nearly all WHI women have serum stored at baseline and 1 subsequent time point (year 1 in CT, year 3 in OS), and at multiple subsequent times for women in the subcohort analyzed here. These resources could support, for example, matched case-control analyses of intake biomarkers in relation to shorter-term subsequent disease incidence, using more specifically defined disease categories.

Although there are perhaps few well-developed hypotheses linking the micronutrients studied here to chronic disease risk, a combination of dietary self-report associations and serum concentration associations (without calibration) were viewed as sufficient 35 y ago for related large supplementation trials (22–24), aimed primarily at lung cancer risk reduction, to be initiated. These trials, which primarily focused on β-carotene and α-tocopherol supplementation, did not demonstrate cancer risk reduction, but rather provided some evidence for lung-cancer risk elevation with β-carotene supplementation (22, 23) and some evidence of ischemic heart-disease risk elevation with α-tocopherol supplementation. The former (lung cancer) association was not considered here, whereas the latter (heart disease) elevation is supported by Tables 2 and 3, especially when the analysis includes dietary supplement users. The associations noted here of α-tocopherol intake with both CVD and cancer merit further evaluation in the larger WHI cohort, or other cohorts, using objective intake measures.

In summary, the present analyses suggest some chronic disease benefits at higher intake of the carotenoids studied, and some chronic disease risks at higher intake of α-tocopherol. As usual with observational studies, we were are unable to fully assess the adequacy of our attempts to avoid confounding. Additional study limitations include the facts that significance levels are not adjusted for multiple comparisons, and that the analyses presented use a so-called "complete case" approach to missing data. The latter, however, is not likely to influence study results because most exclusions were due to prevalent outcomes at baseline, rather than to missing covariate or outcome data.

Overall, these findings can be expected to have reliability exceeding that for other published observational epidemiology reports on these associations, because of our use of objective intake measures. With the present paper, the authors hope to stimulate additional biomarker development activities for other nutrients, foods, and dietary patterns by the nutrition research community, for example using serum metabolomic profiling, and to stimulate discussion concerning criteria that should be satisfied by novel intake biomarkers.

The authors thank the following investigators in the WHI Program: Program Office (National Heart, Lung, and Blood Institute, Bethesda, Maryland): Jacques E Rossouw, Shari Ludlam, Dale Burwen, Joan McGowan, Leslie Ford, and Nancy Geller. Clinical Coordinating Center: Women's Health Initiative Clinical Coordinating Center (Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA): Garnet L Anderson, Ross L Prentice, Andrea Z LaCroix, and Charles L Kooperberg. Investigators and Academic Centers: (Brigham and Women's Hospital, Harvard Medical School, Boston, MA): JoAnn E Manson; (MedStar Health Research Institute/Howard University, Washington, DC): Barbara V Howard; (Stanford Prevention Research Center, Stanford, CA): Marcia L Stefanick; (The Ohio State University, Columbus, OH): Rebecca Jackson; (University of Arizona, Tucson/Phoenix, AZ): Cynthia A Thomson; (University at Buffalo, Buffalo, NY): Jean Wactawski-Wende; (University of Florida, Gainesville/Jacksonville, FL): Marian C Limacher; (University of Iowa, Iowa City/Davenport, IA): Robert M Wallace; (University of Pittsburgh, Pittsburgh, PA): Lewis H Kuller; (Wake Forest University School of Medicine, Winston-Salem, NC): Sally A Shumaker. Women's Health Initiative Memory Study (Wake Forest University School of Medicine, Winston-Salem, NC): Sally A Shumaker.

The authors' responsibilities were as follows—RLP, MP, MLN, LFT, YH, CZ, and JWL: designed the research; MP, YH, CZ, and RLP: analyzed the data; RLP, MP, MLN, LFT, YH, and JWL: drafted the manuscript; CZ, JEM, YM-R, and GLA: provided a critical review; RLP: had primary responsibility for the final content; and all authors read and approved the final manuscript. None of the authors report a conflict of interest related to this study.

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