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## Age-specific reference ranges are needed to interpret serum methylmalonic acid concentrations in the US population

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### Abstract

**Background:** Serum vitamin B-12 is measured to evaluate vitamin B-12 status. Serum methylmalonic acid (MMA) is a specific functional indicator of vitamin B-12 status, however, concentrations increase with impaired renal function.

**Objective:** Our main objective was to describe the distribution of serum vitamin B-12 and MMA in US adults, and estimate age-specific reference intervals for serum MMA in a healthy subpopulation with replete vitamin B-12 status and normal renal function.

**Design:** We examined cross-sectional data for serum vitamin B-12 and MMA in adults participating in the National Health and Nutrition Examination Survey from 2011–2014. Vitamin B-12 was measured by electrochemiluminescence assay and MMA by isotope-dilution LC-MS/MS.

**Results:** In both bivariate and multivariate analyses, age, race/Hispanic origin, and vitamin B-12 supplement use were generally significantly associated with serum vitamin B-12 and MMA concentrations. Serum MMA concentrations increased with age, particularly in persons  $\geq 70$  y. Non-Hispanic white persons had lower vitamin B-12 and higher MMA concentrations compared with non-Hispanic black persons. Shorter fasting times and impaired renal function were significantly associated with higher serum MMA concentrations, but not with serum vitamin B-12 concentrations after controlling for covariates. The central 95% reference intervals for serum vitamin B-12 and MMA concentrations were widest for persons  $\geq 70$  y compared with younger age groups. Compared with the overall population, the central 95% reference intervals for serum MMA concentrations were considerably narrower for a vitamin B-12 replete subpopulation with normal renal function, but still age-dependent. Serum vitamin B-12 showed little, while serum MMA showed notable increases with impaired renal function.

**Conclusions:** The higher serum MMA concentrations throughout the entire distribution in older persons (especially persons  $\geq 70$  y) who are vitamin B-12 replete and have normal renal function

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indicate the need for age-specific MMA reference intervals to better interpret the vitamin B-12 status in epidemiologic research.

### Keywords

National Health and Nutrition Examination Survey; NHANES; vitamin B-12; MMA; renal function

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## INTRODUCTION

Low vitamin B-12 status has been associated with anemia, various forms of neurological abnormalities, and impaired cognitive function [1]. Most commonly, serum vitamin B-12 concentrations are measured to assess vitamin B-12 status, but often a second indicator, such as serum methylmalonic acid (MMA), is assessed to improve problems with sensitivity and specificity associated with using serum vitamin B-12 alone [2,3]. MMA values tend to be higher in the elderly, but it was generally believed that this reflects inadequate vitamin B-12 status [4–6]. Furthermore, impaired renal function is recognized to lead to higher MMA concentrations [7–9]. Given the co-linearity of age and renal function and MMA, age-specific reference intervals are needed for MMA from a subpopulation pre-selected for vitamin B-12 status and renal function. Prior to this analysis, investigators mostly derived reference intervals from relatively small groups of healthy middle-aged adults, but some used elderly individuals before and/or after vitamin B-12 supplementation to derive so-called “health-related” reference intervals (Table 1). Vogiatzoglou *et al.* showed in a Norwegian population that the difference in MMA concentrations between middle-aged and elderly individuals was only partly explained by vitamin B-12 status and renal function [14], also suggesting the need for age-specific reference intervals.

Using data from the National Health and Nutrition Examination Survey (NHANES) between 1991–1994 and 1999–2004, serum MMA concentrations have been described by demographic variables, use of dietary supplements, and by serum vitamin B-12 and serum creatinine concentrations [15–18]. However, previous analyses did not investigate the need for age-specific reference intervals. Using the most recent NHANES data from 2011–2014, our aim was to describe the distributions of serum vitamin B-12 and MMA concentrations in the adult US population by demographic and other relevant variables and to estimate central 95% reference intervals by age in the overall population compared with a subpopulation with replete vitamin B-12 status and/or normal renal function. The overarching goal of this paper was to bring awareness to the issue of properly defining the reference population when deriving reference intervals and not to investigate the complex relationship of vitamin B-12 status biomarkers, age, and renal function.

## SUBJECTS AND METHODS

### Participants and study design

NHANES is a series of cross-sectional probability samples of the civilian noninstitutionalized population in the United States. Each 2-y cycle collects information to assess the health and nutritional status of a nationally representative sample. Conducted by

the National Center for Health Statistics, Centers for Disease Control and Prevention (CDC), NHANES uses a complex, stratified, multistage probability sample. Participants receive a detailed in-home interview, followed by a physical examination, which includes the collection of a blood sample in a Mobile Examination Center. During 2011–2014, persons aged 80 y and over, non-Hispanic Asian, non-Hispanic black, and Hispanic individuals were oversampled to obtain reliable estimates for these population subgroups [19]. We used data from adult NHANES participants. The unweighted response rates for participants 20 y of age were 67% for the interview component and 64% for the examination component [20]. Participants provided consent and the NHANES protocol was approved by the National Center for Health Statistics Research Ethics Review Board.

### **Biomarker measurement**

Serum vitamin B-12 and MMA have not been continuously monitored in NHANES. Since 2006, the biomarkers were only assessed during a 4-y period between 2011 and 2014. Serum from adult participants (20 y of age for 2011–2012 and 19 y of age for 2013–2014) was collected, processed, and shipped on dry ice to a CDC laboratory for analysis of serum vitamin B-12 and MMA. Vitamin B-12 was measured by use of the Roche cobas electrochemiluminescence assay on the Elecsys E-170 clinical analyzer; the assay achieved good long-term imprecision (CV) of 3–8% at 166–605 pmol/L [21]. Serum MMA was measured by isotope-dilution LC-MS/MS after butanol derivatization [22]; the assay achieved even lower long-term imprecision of 3–5% at 96–560 nmol/L [23].

### **Study variables**

For our main analysis, we categorized the participant data into 4 age groups: 20–39, 40–59, 60–69 y and 70 y. Our analysis further categorized the demographic variables by sex (men and women) and by race/Hispanic origin (Hispanic [Mexican American + other Hispanic], non-Hispanic black, non-Hispanic white, and non-Hispanic Asian; other racial/Hispanic origin groups were included in overall estimates). We reported separate estimates for Mexican Americans to allow comparison to previous reports. We also examined physiologic and lifestyle variables previously shown to be associated with serum vitamin B-12 and MMA concentrations [14,24,25]: use of vitamin B-12-containing dietary supplements (mean daily intake consumed in the past 30 d) (self-reported; no and yes), fasting time (self-reported; <3, 3–<8, and 8 h), and renal function as an indicator of chronic kidney disease (CKD). The latter was determined by calculating the estimated glomerular filtration rate (eGFR [mL/(min x 1.73 m<sup>2</sup>)] using the CKD-EPI creatinine equation [26]. We categorized eGFR according to the National Kidney Foundation classification system [27]: 60 and absence of albuminuria (normal renal function), 60 and presence of albuminuria (CKD stage 1–2), and <60 (CKD stage 3–5).

### **Statistical analysis**

Statistical analyses were carried out using SAS for Windows software version 9.4 (SAS Institute, Cary, NC) and SAS callable SUDAAN software version 11 (RTI, Research triangle Park, NC) to account for the complex survey design. We used 2- and 4-y Mobile Examination Center survey weights to account for the unequal probabilities of selection and adjustment for non-response. SUDAAN uses Taylor series linearization to calculate variance

estimates. This analysis was undertaken to examine whether serum MMA concentrations differed among subpopulations defined by demographic attributes, renal function and/or vitamin B-12 status. The research was performed following a pre-specified data analysis plan that was approved by all authors prior to conducting the statistical analysis. We excluded pregnant and/or lactating women from all analyses ( $n = 80$  in 2011–2012 and  $n = 104$  in 2013–2014). Of these women, some had missing serum vitamin B-12 and MMA measurements ( $n = 7$  in 2011–2012 and  $n = 4$  in 2013–2014). Our analytical sample of participants 20 y consisted of 4,804 (2011–2012) and 5,216 (2013–2014) participants with complete serum vitamin B-12 data and 4,807 (2011–2012) and 5,213 (2013–2014) participants with complete serum MMA data (Supplemental Figure 1). Since this is primarily a descriptive paper, we used available-case analysis (“pairwise deletion”) to handle missing or incomplete data (e.g., B-12 supplement use, renal function) to maximize the amount of information available. This means that a participant was deleted if at least 1 of a pair of variables was missing for that specific analysis (or association). Both serum B-12 and MMA concentrations were right skewed; therefore, a log transformation was used to make the data more symmetric to facilitate interpretation and statistical inference through the use of geometric mean. The weighted mean of the log-transformed data were back-transformed to the original scale to report the geometric mean concentration (and 95% CI) of serum B-12 and MMA for each study variable by survey period (2011–2012 and 2013–2014) for participants 20 y. The geometric means were compared across the categories of the study variables within each survey cycle on the basis of Wald  $F$  tests. In addition, Satterthwaite  $F$  tests were used to assess whether at least 1 of the adjusted geometric means across the categories was significantly different after controlling for additional covariates (age group, sex, race/Hispanic origin, vitamin B-12 supplement use, fasting time, and renal function). Lastly, the geometric means were also compared across the 2 survey cycles for each category of the study variables using Wald  $F$  test ( $P_{Trend}$  value). Significance was defined as a 2-sided  $P$  value of  $<0.05$  with no adjustment for multiple comparisons.

Since there were limited statistically significant differences for both serum B-12 and MMA across the 2 cycles, we combined the 4 y of NHANES data from 2011–2014 for participants 20 y. We calculated the median (95% CI) and central 95% reference intervals for serum vitamin B-12 and MMA for all participants and by age group. In addition, to demonstrate the effect of age, B-12 status, and renal function on serum MMA concentrations, we presented the weighted empirical cumulative distributions stratified by age group, as well as median and 95% reference intervals for the adult subpopulation with normal renal function (free of CKD) and/or vitamin B-12 replete (serum vitamin B-12  $\geq 300$  pmol/L). We chose this vitamin B-12 cutoff based on previous work demonstrating that these concentrations likely indicate adequate vitamin B-12 status ( $>296$  pmol/L [17] and  $>287$  pmol/L [28]). We conducted an exploratory analysis to see how high serum MMA concentrations were on the other side of the spectrum, i.e., in persons with impaired renal function and/or vitamin B-12 deficiency (serum vitamin B-12  $<200$  pmol/L). This cutoff has been used to indicate low serum vitamin B-12 concentrations [29], “metabolically significant vitamin B-12 deficiency” [30,31], and is within the range of 148–221 pmol/L defined as “marginal vitamin B-12 deficiency” [32].

In order to provide relevant data to policy makers, we calculated the geometric mean and selected percentiles for serum vitamin B-12 and MMA by Dietary Reference Intake (DRI) age groups for participants  $\geq 19$  y (data on 19 y old persons were only available for 2013–2014).

## RESULTS

Among the US population  $\geq 20$  y, approximately one-third each were young adults (20–39 y), middle-aged adults (40–59 y), and older adults (60–69 y and  $\geq 70$  y), half were women, and the race/Hispanic origin percentages were approximately 15%, 11%, 67%, and 5% for Hispanic, non-Hispanic black, non-Hispanic white, and non-Hispanic Asian, respectively (Table 2). Approximately 35% of the population used a dietary supplement containing vitamin B-12, approximately half fasted for  $\geq 8$  h, and approximately 80% had normal renal function.

### Serum vitamin B-12 and MMA concentrations for 2011–2012 and 2013–2014 by variable categories

We observed no difference in serum vitamin B-12 ( $P=0.47$ ) or MMA ( $P=0.06$ ) concentrations for adults  $\geq 20$  y between 2011–2012 and 2013–2014 (Table 3). When we grouped data by demographic or other variables, we found no differences in serum vitamin B-12 or MMA concentrations between the 2 survey cycles, except for a small decrease in serum vitamin B-12 for 20–39 y olds (2.9%) and small increases in serum MMA for non-Hispanic blacks (5.6%), non-Hispanic whites (5.1%), and non-users of vitamin B-12 containing supplements (4.7%).

Age and race/Hispanic origin were significantly associated with serum vitamin B-12 and MMA concentrations in both survey periods, while sex was only significantly associated with serum vitamin B-12 concentrations in 2013–2014 (Table 3). For the most part, these observations were unchanged after we controlled for other covariates (age group, sex, race/Hispanic origin, vitamin B-12 supplement use, fasting time, and renal function). However, during the 2013–2014 survey cycle, age was no longer significantly associated with serum vitamin B-12. We observed increasing serum vitamin B-12 and MMA concentrations with age, especially in persons  $\geq 70$  y. Serum vitamin B-12 concentrations were approximately 13% higher in persons  $\geq 70$  y compared with 20–39 y olds. For MMA, the age difference was more pronounced: persons  $\geq 70$  y had approximately 60% higher MMA concentrations compared with the youngest age group. Non-Hispanic white persons had lower vitamin B-12 (approximately 10%) and higher MMA (approximately 25%) concentrations compared with non-Hispanic black persons, while other race/Hispanic origin groups had intermediate concentrations.

Users of vitamin B-12 containing supplements had approximately 40% higher serum vitamin B-12 concentrations in both survey periods (statistically significant with and without controlling for other covariates), and approximately 5% lower MMA concentrations (statistically significant in 2013–2014 without and in both survey periods with controlling for other covariates) (Table 3). Shorter fasting time was significantly associated with approximately 10% higher MMA concentrations in both survey periods, but showed no

association with serum vitamin B-12 after controlling for other covariates. Similarly, impaired renal function was significantly associated with approximately 10–15% higher serum vitamin B-12 and approximately 50–70% higher MMA concentrations in both survey periods, but for vitamin B-12 the association was no longer significant after controlling for other covariates.

### **Serum vitamin B-12 and MMA concentrations for 2011–2014 by DRI age groups**

To provide data for policy makers interested in vitamin B-12 status by DRI age groups corresponding to intake categories, we calculated the geometric mean concentrations and percentile distributions for serum vitamin B-12 and MMA concentrations for participants 19 y (Table 4). The older age group (> 51 y) had higher serum vitamin B-12 and MMA concentrations compared with the younger age group (19–50 y), similar to what we observed with the 3 age group categories. We observed higher MMA concentrations for the older age group across the entire distribution and higher vitamin B-12 concentrations for the upper half of the distribution.

### **Reference intervals for serum vitamin B-12 and MMA concentrations for 2011–2014 by age groups**

The central 95% reference interval for serum vitamin B-12 concentrations (overall group) was wider for persons 60–69 y (152–1,530 pmol/L,  $n = 1,166$ ) and > 70 y (137–1,720 pmol/L,  $n = 1,548$ ) compared with the 2 younger age groups (20–39 y: 167–840 pmol/L,  $n = 3,375$ ; 40–59 y: 160–1,150 pmol/L,  $n = 3,431$ ) (Table 5). We observed the same pattern for serum MMA concentrations (overall group); participants > 70 y showed a reference interval of 92.5–758 nmol/L ( $n = 1,551$ ). However, as we limited this group to a subpopulation with normal renal function (85.9–571 nmol/L,  $n = 672$ ) and further to a subpopulation also being vitamin B-12 replete (84.3–317 nmol/L,  $n = 491$ ), the central 95% reference intervals narrowed considerably. Nonetheless, even in the subpopulation with replete vitamin B-12 status and normal renal function, the reference interval for the 20–39 y age group was lower compared with the reference intervals for the 40–59 y, 60–69 y, and > 70 y age groups. We noted similar patterns for the medians, with higher MMA concentrations with increasing age even in the subpopulation with replete vitamin B-12 status and normal renal function. We also noted similar patterns for the entire distribution of MMA concentrations (Figure 1).

We observed a selective impact of vitamin B-12 status *vs.* renal function by age group (Supplemental Figure 2). In the 20–39 y (panel A) and 40–59 y (panel B) age groups, excluding persons with low vitamin B-12 status shifted the MMA distributions lower compared to excluding persons with impaired renal function. In the 60–69 y (panel C) and > 70 y age groups (panel D), both of these approaches resulted in a similar shift of the MMA distributions, but the combined approach (excluding persons with low vitamin B-12 status and impaired renal function) had an additive effect and resulted in an even bigger shift to lower MMA concentrations, especially in the oldest age group.

We stratified our analytic sample by age and renal function to illustrate the effect of impaired renal function on the relationship between age and biomarker concentrations (Figure 2). At the geometric mean, serum vitamin B-12 (panel A) showed small and

consistent age differences for persons with normal *vs.* impaired renal function: 40–59 y olds had 1.6% (normal) and 2.1% (impaired) higher concentrations, 60–69 y olds had 9.4% (normal) and 4.4% (impaired) higher concentrations, and 70 y olds had 12% (normal) and 9% (impaired) higher concentrations compared with 20–39 y olds. Serum vitamin B-12 concentrations for people with normal *vs.* impaired renal function were significantly different in the overall population ( $P = 0.0072$ ), but not when stratified by age, 20–39 y ( $P = 0.30$ ), 40–59 y ( $P = 0.39$ ), 60–69 y ( $P = 0.74$ ), and 70 y ( $P = 0.97$ ). On the other hand, serum MMA (panel B) showed large and inconsistent age differences for persons with normal *vs.* impaired renal function: compared with 20–39 y olds, 40–59 y olds had 14% (normal) and 27% (impaired) higher concentrations, 60–69 y olds had 21% (normal) and 45% (impaired) higher concentrations, and 70 y olds had 39% (normal) and 75% (impaired) higher concentrations. Serum MMA concentrations were significantly different in persons with normal *vs.* impaired renal function ( $P = 0.0001$ ), with the exception of those aged 20–39 y ( $P = 0.30$ ).

### **MMA concentrations in persons with impaired renal function and/or vitamin B-12 deficiency**

Not surprisingly, MMA concentrations were much higher at the opposite side of the spectrum where participants showed unhealthy characteristics (Supplemental Table 1). MMA median concentrations were generally higher in vitamin B-12 deficient persons compared with persons with impaired renal function. We also observed increasing concentrations with increasing age group. However, most of the 97.5<sup>th</sup> percentile estimates were subject to greater uncertainty due to the small cell size, as can be seen by the large 95% CIs.

## **DISCUSSION**

Serum MMA concentrations have been described by age and/or vitamin B-12 status in several moderate sample size studies of healthy middle-aged and elderly Americans and Europeans (mostly from Scandinavian countries) [4–6,10–17]. Earlier work from NHANES 1999–2004 using a large, nationally representative sample of the racially-diverse US population concluded that the “age-related increase in serum MMA concentrations is likely to be due to a concomitant decline in kidney function and vitamin B-12 status” [18]. Our analysis extends this work by better defining these 2 variables relative to MMA concentrations and thus providing helpful information to both epidemiologists as well as clinicians. We confirmed the previously reported positive association between serum MMA and age [4–6,15,16,18] and the inverse association between serum MMA and renal function [7–9]. While serum vitamin B-12 also showed a weak inverse association with renal function, the association was no longer significant after we adjusted for other covariates. The important finding of our study was that serum MMA concentrations were higher as age increased even in a reference population of vitamin B-12 replete persons with normal renal function, confirming the observations made by Vogiatzoglou *et al.* in the Norwegian population [14], and suggesting that age-specific reference intervals should be used when interpreting serum MMA concentrations. However, since the reference intervals in the 40–59

y and 60–69 y age groups are similar, it may suggest these 2 age groups do not need separate reference intervals for MMA.

In contrast to studies in European populations that found similar or lower serum vitamin B-12 concentrations in older compared with younger persons [5,8,14], when combining the 2 cycles together, we found slightly higher serum vitamin B-12 concentrations in persons 60–69 y and 70 y compared with the 2 younger age groups, even when controlling for covariates like vitamin B-12 supplement use (data not shown). However, when we stratified our NHANES 2011–2014 sample by vitamin B-12 supplement use, we observed increasing serum vitamin B-12 concentrations with age (20–39, 40–59, 60–69, and 70 y) in supplement users (429, 466, 533, and 543 pmol/L), but similar concentrations in non-users (357, 342, 338, and 341 pmol/L) (data not shown). A previous NHANES analysis showed increased consumption frequency of dietary supplements containing vitamin B-12 by older persons [33], supporting our observation.

Race/Hispanic origin was significantly associated with serum vitamin B-12 and MMA concentrations, and it remained significant after we adjusted for other covariates. Previous reports from earlier NHANES surveys [16,34] also showed that non-Hispanic blacks and Mexican Americans had higher serum vitamin B-12 concentrations compared to non-Hispanic whites. The mean serum MMA concentrations were significantly lower in non-Hispanic blacks compared with non-Hispanic whites, as noted both in the pre-folic acid fortification [15] and post-fortification periods [16,18]. A new enzymatic polymorphism that showed a strong association with elevated MMA concentrations was recently reported in the white Irish population [35] that may partially explain the higher MMA concentrations in non-Hispanic whites. Our study also reports the first nationally representative serum vitamin B-12 and MMA concentrations for non-Hispanic Asians. Similar to Hispanics, this population group showed intermediate concentrations for both biomarkers, between those observed for non-Hispanic blacks and non-Hispanic whites. An earlier investigation in an elderly community-dwelling multiethnic population also found higher serum vitamin B-12 concentrations in Asian Americans compared with Whites [36]. A recent study conducted in a convenience sample of healthy young Canadian women reported similar serum vitamin B-12 and plasma MMA concentrations for women of South Asian vs. European ethnicity [37].

The upper end of the age-specific central 95% reference interval for serum MMA in persons who were vitamin B-12 replete and had normal renal function (254, 293, 281, 317 nmol/L in 20–39, 40–59, 60–69, and 70 y, respectively) was much lower compared with the “apparently healthy” overall population (304, 442, 497, 758 nmol/L in 20–39, 40–59, 60–69, and 70 y, respectively). This shows that MMA cutoffs derived from epidemiologic data are too high if parameters such as renal function and vitamin B-12 status are not considered. As such, the study of Erdogan *et al.* [6] found a higher reference interval of 60–360 nmol/L in “apparently healthy” US adults with unknown clinical history. Moreover, when the authors derived representative ranges by age group from close to 5,000 MMA tests performed in 2006 using a nonparametric approach following exclusion of the top 10% of values, they also arrived at much higher upper end MMA cutoffs (from 260 nmol/L for 11–20 y to 480 nmol/L for 71 y) compared with our study. Our age-specific MMA reference intervals for



vitamin B-12 replete persons with normal renal function were in agreement with some of the published reference intervals in other populations [4,5,10,14], but not with all [5,11,12,14]. The higher reference intervals found in some studies may be due to the lack of consideration for impaired renal function.

There is still debate on what the right serum vitamin B-12 cutoff is to denote adequate vitamin B-12 status [17,28,38]. Vogiatzoglou *et al.* defined individuals with serum vitamin B-12  $\geq 400$  pmol/L as vitamin B-12 replete, while individuals with serum vitamin B-12  $< 200$  pmol/L and  $\geq 200$  pmol/L but  $< 400$  pmol/L were considered as low/low-normal and intermediate, respectively [14]. A frequently used serum vitamin B-12 cutoff to separate possible “subclinical deficiency” from repletion is 296 pmol/L [39]. In a modeling analysis of a MMA-derived change point for serum vitamin B-12, Bailey *et al.* found that serum vitamin B-12 concentrations  $> 287$  pmol/L likely indicate adequate vitamin B-12 status [28]. Thus, we chose the rounded cutoff of 300 pmol/L to define vitamin B-12 repletion.

Our exploratory analysis in a unhealthy subgroup of US adults with impaired renal function and/or vitamin B-12 deficiency provides new insights that are helpful to clinicians, however the results have to be interpreted with caution due to the small sample size. Nonetheless, we observed the same kind of age pattern in this subgroup as in the healthy subgroup.

Our group previously demonstrated that serum vitamin B-12 concentrations did not change appreciably from pre- (NHANES 1988–1994) to post-folic acid fortification (NHANES 1999–2004) [40]. In this current analysis, we verified that during the 15-y post-fortification period (NHANES 1999–2014) serum vitamin B-12 concentrations continued to be similar (Supplemental Figure 3 and Supplemental Text 1). Serum MMA concentrations showed a small but significant increase over the same time period. The mean increase was 9.5%, but it varied by age group: 20–39 y: 4.8%; 40–59 y: 14.2%; 60–69 y: 14.9% and  $\geq 70$  y: 7.8%. Whether this small increase is due to secular changes in intake of foods rich in vitamin B-12, fortification practices, or dietary supplements, other confounding factors such as renal function, or unknown sampling biases between the NHANES surveys, is not known. However, this small change is not considered to be of public health concern.

Our analysis is subject to some limitations. While NHANES is a large probability sample designed to estimate information on the nutritional status in selected groups of the U.S. population, it is a cross-sectional study. For this reason, any associations identified between serum vitamin B-12 or MMA concentrations with other variables need to be interpreted with caution as there may be other factors that were not investigated and that may impact the relationship. While we had limited missing data, any missing values, if they are not at random, can bias the reported estimates. The strengths of our paper are the large sample size that allowed us to provide age-specific MMA reference intervals as well as nationally representative estimates for different race-Hispanic origin groups and the first data for non-Hispanic Asians.

In summary, the age-specific MMA reference intervals reported in this study will be a useful tool for epidemiologists and public health officials to monitor the vitamin B-12 status of the population over time or to compare the vitamin B-12 status of different populations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

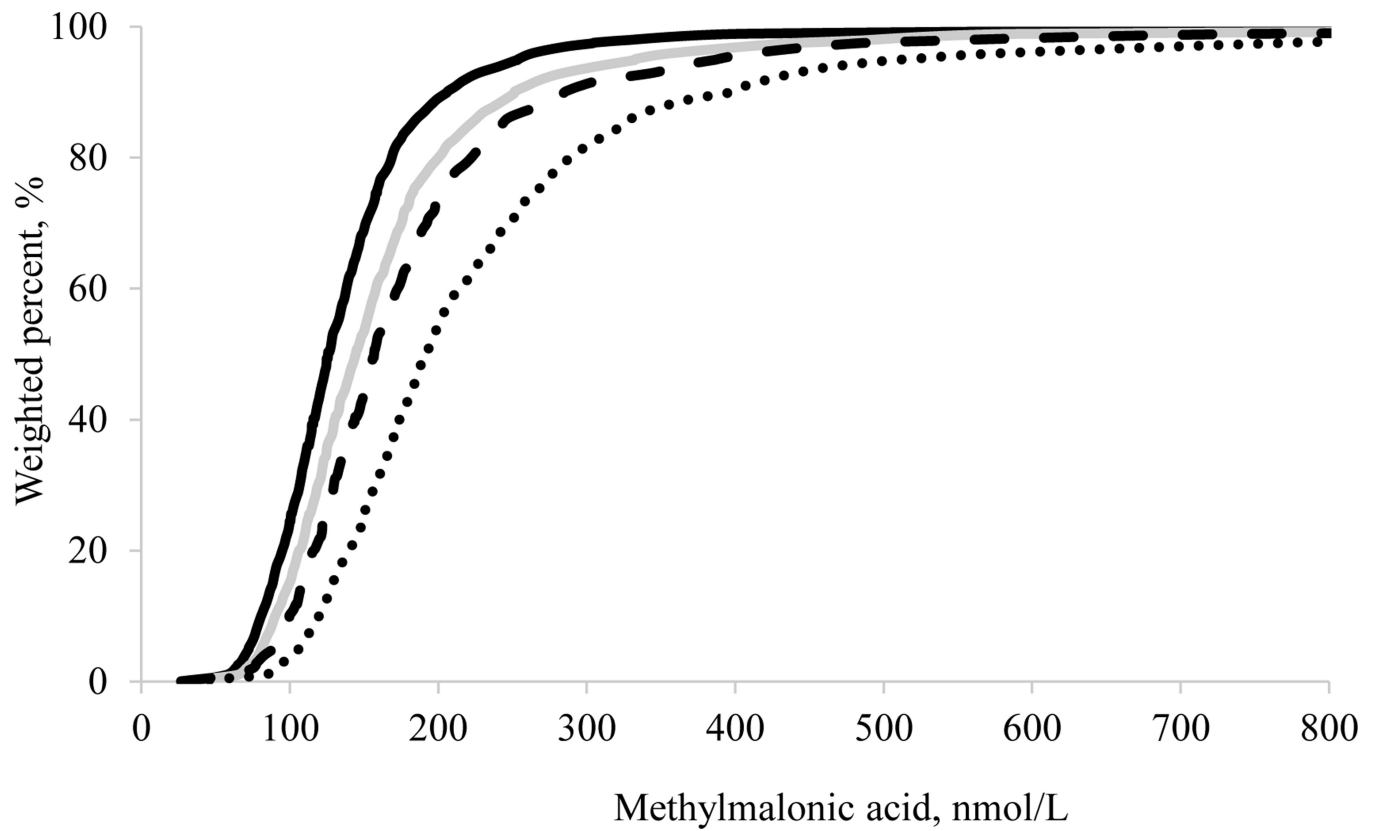
We thank Gwendolyn Gabey and Donna LaVoie (CDC's National Center for Environmental Health) for performing the serum MMA and vitamin B-12 analysis. Author contributions: CMP developed the study concept and design. MRS performed the statistical data analysis. EMM, CMP, and YA conducted the data interpretation. EMM wrote the initial draft, which was modified based on critical review from all coauthors. CMP has primary responsibility for all content. All authors read and approved the final manuscript. None of the authors declared any personal or financial conflict of interest.

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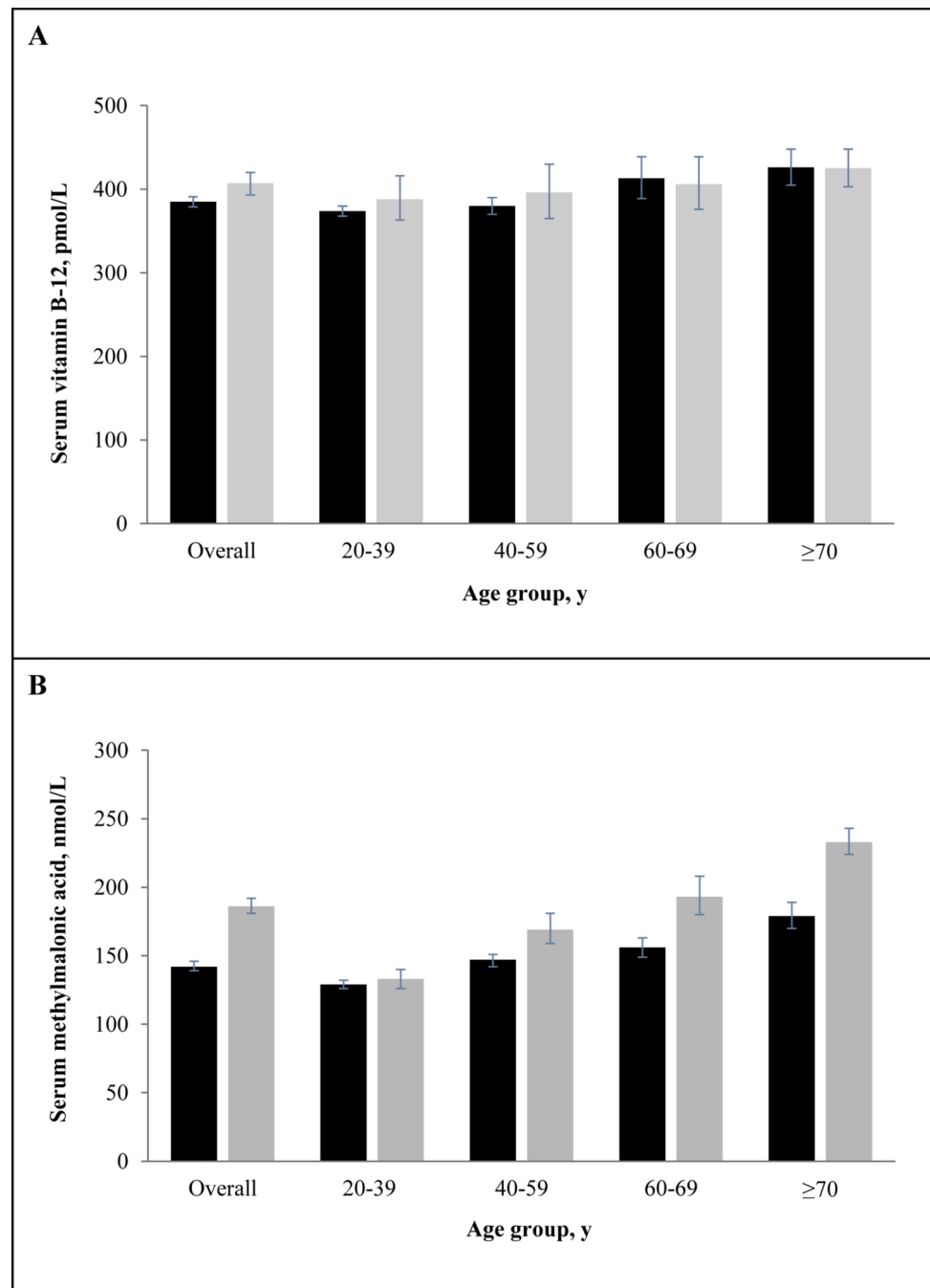
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**Figure 1.**

Weighted empirical cumulative distributions (ECDF) for serum methylmalonic acid concentrations in US persons  $\geq 20$  y of age who are vitamin B-12 replete and have normal renal function by age group, NHANES 2011–2014. Black solid line: 20–39 y ( $n = 2,178$ ); grey solid line: 40–59 y ( $n = 1,988$ ); large dashed line: 60–69 y ( $n = 824$ ); small dashed line: 70 y ( $n = 491$ ).



**Figure 2.** Serum vitamin B-12 (panel A) and methylmalonic acid (panel B) geometric mean concentrations by age group and renal function in US persons  $\geq 20$  y of age, NHANES 2011–2014. Black bars represent normal renal function and grey bars represent impaired renal function (stage 1–5 chronic kidney disease). Error bars represent 95% CI. Sample sizes for B-12 and normal vs. impaired renal function: overall ( $n = 7,719$  vs. 2,212); 20–39 y ( $n = 3,040$  vs. 309); 40–59 y ( $n = 2,837$  vs. 575); 60–69 y ( $n = 1,172$  vs. 479), and  $\geq 70$  y ( $n = 670$  vs. 849). Sample sizes for MMA and normal vs. impaired renal function: overall ( $n = 7,718$

vs. 2,213); 20–39 y ( $n = 3,038$  vs. 309); 40–59 y ( $n = 2,835$  vs. 575); 60–69 y ( $n = 1,173$  vs. 479); and 70 y ( $n = 672$  vs. 850).

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**Table 1.**

Selected published reference intervals for serum or plasma methylmalonic acid

Author (year) [Reference]	Population	Central 0.95 reference interval, nmol/L	Additional information
Allen 1990 [10]	Healthy U.S. middle-aged adults	73–271	Men and women, <i>n</i> =50; 18–65 y
Rasmussen 1990 [11]	Healthy Danish middle-aged adults	50–370	Men and women, <i>n</i> =58; 40–68 y (median: 53 y)
Rasmussen 1996 [4]	Healthy Danish middle-aged adults before and after vitamin B-12 supplementation for 1 wk (in a few cases for 2 wk)	80–280	Men ( <i>n</i> =109) and women ( <i>n</i> =126); 20–84 y (men: median: 50 y) and 20–85 y (women; median: 49 y); all but 1 subject had plasma creatinine concentrations within the reference interval for healthy subjects
Joosten 1996 [5]	Healthy Belgian, Dutch, and German middle-aged adults Healthy Dutch elderly living at home Healthy German elderly after B-vitamin supplementation for 3 wk	62–247 72–476 55–278	Men and women, <i>n</i> =99; 19–55 y (mean: 30 y) Men and women, <i>n</i> =64; 65–88 y (mean: 76 y); no participant had creatinine clearance <30 mL/min Men and women, <i>n</i> =143; 65–96 y (mean: 75 y); no participant had creatinine clearance <30 mL/min
Lewerin 2003 [12]	Swedish elderly with and without B-vitamin supplementation Total study group at baseline Healthy elderly at baseline Healthy elderly after B-vitamin supplementation for 4 mo	110–480 120–380 20–340	Men and women, <i>n</i> =209; 70–88 y (women) and 70–93 y (men) (overall median: 76 y) <i>n</i> =208 <i>n</i> =123 <i>n</i> =78 (vitamin B-12 replete)
Milman 2007 [13]	Healthy Danish pregnant women 18 wk gestation 32 wk gestation 39 wk gestation 8 wk post-partum	40–290 50–340 60–360 80–350	Women ( <i>n</i> =434) with a normal pregnancy 37 wk <i>n</i> =413 <i>n</i> =390 <i>n</i> =250 <i>n</i> =160
Vogiatzoglou 2009 [14]	Norwegian middle-aged adults: Unselected Vitamin B-12 200 pmol/L Vitamin B-12 400 pmol/L Norwegian elderly: Unselected Vitamin B-12 200 pmol/L Vitamin B-12 400 pmol/L	100–320 100–300 100–280 110–490 110–410 100–360	Men and women, <i>n</i> =3,684; 47–49 y <i>n</i> =3,684 <i>n</i> =3,568 <i>n</i> =1,306 (vitamin B-12 replete) Men and women, <i>n</i> =3,262; 71–74 y <i>n</i> =3,262 <i>n</i> =3,043 <i>n</i> =1,058 (vitamin B-12 replete)
Erdogan 2010 [6]	Healthy US adults US persons tested for MMA (unknown clinical history) 0–10 y 11–20 y 21–30 y 31–40 y 41–50 y 51–60 y 61–70 y 71 y	60–360 0–510 30–260 50–330 50–400 50–400 50–420 50–440 50–480	Men ( <i>n</i> =16) and women ( <i>n</i> =24) Males and females ( <i>n</i> =4,944); highest 10% of results disregarded (potentially unhealthy persons) <i>n</i> =28 <i>n</i> =39 <i>n</i> =165 <i>n</i> =287 <i>n</i> =545 <i>n</i> =813 <i>n</i> =918 <i>n</i> =2149



Sample sizes and percentages for serum vitamin B-12 and methylmalonic acid by variable categories in US persons 20 y of age, NHANES 2011–2014<sup>1</sup>

**Table 2.**

Variable categories	Serum vitamin B-12				Serum MMA			
	2011–2012	2013–2014 <sup>2</sup>	2011–2012	2013–2014 <sup>2</sup>	2011–2012	2013–2014 <sup>2</sup>	2011–2012	2013–2014 <sup>2</sup>
	n	%	n	%	n	%	n	%
All	4,804	-	5,216	-	4,807	-	5,213	-
Age, y								
20–39	1,670	35.2	1,705	35.2	1,670	35.1	1,703	35.2
40–59	1,608	38.8	1,823	37.4	1,608	38.8	1,821	37.4
60–69	800	14.0	866	14.8	800	14.0	867	14.8
70	726	12.0	822	12.6	729	12.1	822	12.6
Sex								
Male	2,424	49.1	2,535	49.3	2,424	49.1	2,534	49.3
Female	2,380	50.9	2,681	50.7	2,383	50.9	2,679	50.7
Race-ethnicity								
Hispanic	974	14.3	1,172	14.7	974	14.3	1,174	14.8
Mexican American	481	7.8	709	9.2	481	7.8	711	9.2
Non-Hispanic Black	1,220	10.8	1,026	10.8	1,220	10.8	1,024	10.8
Non-Hispanic White	1,805	67.4	2,279	66.6	1,807	67.4	2,275	66.6
Non-Hispanic Asian	670	5.0	588	5.2	671	5.0	589	5.3
B-12 supplement use								
No	3,303	66.1	3,514	64.1	3,304	66.0	3,515	64.1
Yes	1,467	33.9	1,672	35.9	1,469	34.0	1,668	35.9
Fasting								
<3 h	1,347	29.4	1,689	35.5	1,347	29.4	1,688	35.5
3–<8 h	982	18.7	983	16.8	983	18.7	980	16.8
8 h	2,475	51.9	2,544	47.7	2,477	51.9	2,545	47.7
Renal function								
Normal	3,730	82.2	3,989	80.1	3,731	82.2	3,987	80.1
CKD stage 1–2	613	10.6	693	11.8	614	10.6	693	11.8
CKD stage 3–5	424	7.3	482	8.1	425	7.3	481	8.1

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<sup>1</sup>Values are weighted percentages. Pregnant and lactating women were excluded; available-case analysis was used to handle missing or incomplete data (e.g., B-12 supplement use, renal function). CKD, chronic kidney disease; MMA, methylmalonic acid.

<sup>2</sup>While NHANES 2013–2014 collected samples from participants 19 y of age, we only included data for participants 20 y of age.

**Table 3.** Serum vitamin B-12 and methylmalonic acid concentrations by variable categories in US persons 20 y of age, NHANES 2011–2014<sup>1</sup>

Variables	Serum vitamin B-12, pmol/L			Serum MMA, nmol/L		
	2011–2012	2013–2014 <sup>2</sup>	<i>P</i> <sub>Trend</sub> value <sup>3</sup>	2011–2012	2013–2014 <sup>2</sup>	<i>P</i> <sub>Trend</sub> value <sup>3</sup>
All	391 (382, 400)	387 (380, 394)	0.47	147 (143, 152)	153 (149, 158)	0.06
Age, y						
20–39	381 (373, 388)	370 (362, 378)	0.0457	127 (123, 132)	131 (127, 136)	0.21
40–59	377 (365, 390)	387 (374, 400)	0.27	147 (141, 153)	153 (146, 161)	0.13
60–69	423 (390, 459)	402 (380, 424)	0.27	159 (149, 169)	170 (162, 179)	0.07
70	431 (411, 452)	421 (396, 447)	0.50	206 (199, 214)	207 (198, 216)	0.93
<i>P</i> <sub>crude</sub> value <sup>4</sup>	<0.0001	0.0034		<0.0001	<0.0001	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	0.0464	0.12		<0.0001	<0.0001	
Sex						
Male	385 (371, 399)	373 (364, 383)	0.16	149 (145, 154)	153 (150, 156)	0.19
Female	397 (387, 406)	400 (393, 408)	0.49	145 (138, 152)	153 (147, 160)	0.08
<i>P</i> <sub>crude</sub> value <sup>4</sup>	0.12	0.0001		0.22	0.93	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	0.69	0.0024		0.08340	0.52	
Race-ethnicity <sup>6</sup>						
Hispanic	396 (385, 407)	393 (376, 411)	0.81	128 (121, 135)	127 (123, 132)	0.91
Mexican American	406 (392, 421)	404 (374, 436)	0.89	120 (114, 127)	123 (118, 128)	0.41
Non-Hispanic black	425 (408, 442)	408 (394, 423)	0.12	124 (121, 128)	131 (127, 136)	0.0160
Non-Hispanic white	384 (371, 397)	380 (371, 390)	0.63	157 (152, 162)	165 (159, 171)	0.0277
Non-Hispanic Asian	407 (385, 431)	401 (377, 426)	0.70	134 (127, 141)	138 (131, 146)	0.38
<i>P</i> <sub>crude</sub> value <sup>4</sup>	0.0012	0.0060		<0.0001	<0.0001	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	<0.0001	0.0002		<0.0001	<0.0001	
B-12 supplement use <sup>7</sup>						
No	350 (344, 357)	344 (337, 351)	0.18	149 (143, 154)	156 (151, 162)	0.0476
Yes	481 (458, 505)	475 (463, 488)	0.64	143 (138, 149)	147 (143, 152)	0.21

Variables	Serum vitamin B-12, pmol/L			Serum MMA, mmol/L		
	2011–2012	2013–2014 <sup>2</sup>	<i>P</i> <sub>Trend</sub> value <sup>3</sup>	2011–2012	2013–2014 <sup>2</sup>	<i>P</i> <sub>Trend</sub> value <sup>3</sup>
<i>P</i> <sub>crude</sub> value <sup>4</sup>	<0.0001	<0.0001		0.08	0.0011	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	<0.0001	<0.0001		<0.0001	<0.0001	
Fasting time, h						
<3	400 (386, 415)	390 (381, 399)	0.18	158 (152, 164)	164 (159, 170)	0.12
3–<8	400 (379, 422)	395 (380, 410)	0.68	153 (146, 159)	159 (153, 165)	0.15
8	382 (374, 391)	382 (371, 394)	1.00	139 (134, 144)	143 (138, 149)	0.26
<i>P</i> <sub>crude</sub> value <sup>4</sup>	0.0183	0.38		<0.0001	<0.0001	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	0.25	0.38		<0.0001	<0.0001	
Renal function						
Normal	387 (376, 397)	383 (376, 391)	0.61	140 (136, 145)	145 (140, 150)	0.17
CKD stage 1–2	388 (366, 411)	388 (361, 418)	0.98	152 (144, 161)	159 (153, 165)	0.21
CKD stage 3–5	452 (423, 484)	421 (390, 454)	0.14	235 (219, 251)	249 (234, 264)	0.19
<i>P</i> <sub>crude</sub> value <sup>4</sup>	0.0019	0.0420		<0.0001	<0.0001	
<i>P</i> <sub>adj</sub> value <sup>5</sup>	0.20	0.64		<0.0001	<0.0001	

<sup>1</sup>Values are weighted geometric means (95% CI). Serum vitamin B-12 was measured by Roche E-170 immunoassay and serum MMA by HPLC-MS/MS. See Table 2 for sample sizes. Pregnant and lactating women were excluded; available-case analysis was used to handle missing or incomplete data. CKD, chronic kidney disease; MMA, methylmalonic acid.

<sup>2</sup>While NHANES 2013–2014 collected samples from participants 19 y of age, we only included data for participants 20 y of age.

<sup>3</sup>Test compares the 2 NHANES cycles with no covariate adjustment.

<sup>4</sup>Wald *F* *P* value tests the null hypothesis of no difference for unadjusted geometric means for each variable separately.

<sup>5</sup>Satterthwaite *F* *P* value tests the null hypothesis of no difference for covariate-adjusted (age, sex, race-ethnicity, vitamin B-12 supplement use, fasting time, and renal function) geometric means.

<sup>6</sup>Hispanic sub-group represents the sum of MA and other Hispanic ethnicity; *P* values for race-ethnicity show comparison of Hispanic, NH White, NH Black, NH Asian, and other (not shown).

<sup>7</sup>Vitamin B-12 containing dietary supplements.

Serum vitamin B-12 and methylmalonic acid concentrations by Dietary Reference Intake age groups in US persons 19 y of age, NHANES 2011–2014<sup>1</sup>

**Table 4.**

Analyte	Age group	n <sup>2</sup>	Geometric mean (95% CI)	Percentiles										
				2.5	5	10	25	50	75	90	95	97.5		
B-12, pmol/L														
	19–50 y	5,457	374 (369, 380)	163	190	219	281	369	489	633	752	885		
	51 y	4,694	408 (399, 418)	153	176	213	282	392	561	791	1040	1440		
	P value		0.0064											
MMA, nmol/L														
	19–50 y	5,455	133 (131, 136)	67.5	74.4	83.0	103	129	165	214	263	338		
	51 y	4,696	175 (171, 179)	79.8	90.3	103	127	164	220	317	420	558		
	P value		<0.0001											

<sup>1</sup> NHANES 2011–2012 collected samples from participants 20 y of age, while NHANES 2013–2014 collected samples from participants 19 y of age. Serum vitamin B-12 was measured by Roche E-170 immunoassay and serum MMA by HPLC-MS/MS. Pregnant and lactating women were excluded. P values are based on Wald F test. B-12, vitamin B-12; MMA, methylmalonic acid.

<sup>2</sup> Sample size

**Table 5.**

Serum MMA and vitamin B-12 median, central 95% reference intervals, and corresponding sample sizes by age group in US persons 20 y of age, NHANES 2011–2014<sup>1</sup>

Age group	Serum B-12, pmol/L		Serum MMA, nmol/L			
	Overall <sup>2</sup>	Overall <sup>2</sup>	Normal renal function <sup>3</sup>	B-12 replete <sup>4</sup>	B-12 replete & normal renal function <sup>5</sup>	
<i>Median (95% CI)</i>						
All	378 (372, 384)	144 (141, 147)	138 (134, 141)	135 (132, 139)	130 (127, 133)	
Age, y						
20–39	373 (364, 381)	126 (123, 128)	125 (123, 128)	121 (118, 124)	120 (118, 124)	
40–59	365 (357, 378)	144 (140, 148)	142 (138, 146)	133 (129, 139)	133 (128, 138)	
60–69	398 (375, 420)	157 (150, 164)	151 (143, 158)	149 (143, 154)	143 (134, 149)	
70	416 (401, 428)	191 (186, 197)	168 (162, 175)	180 (175, 184)	161 (151, 167)	
<i>2.5<sup>th</sup> – 97.5<sup>th</sup> Percentile</i>						
All	158 – 1,140	70.6 – 451	69.6 – 391	67.9 – 328	67.2 – 281	
Age, y						
20–39	167 – 840	65.1 – 304	65.5 – 297	63.3 – 256	63.5 – 254	
40–59	160 – 1,150	72.8 – 442	73.0 – 403	70.5 – 324	70.5 – 293	
60–69	152 – 1,530	77.0 – 497	76.4 – 430	74.0 – 327	72.4 – 281	
70	137 – 1,720	92.5 – 758	85.9 – 571	90.1 – 447	84.3 – 317	
<i>Sample size</i>						
All	10,020	10,020	7,718	7,109	5,481	
Age, y						
20–39	3,375	3,373	3,038	2,420	2,178	
40–59	3,431	3,429	2,835	2,393	1,988	
60–69	1,666	1,667	1,173	1,170	824	
70	1,548	1,551	672	1,126	491	

<sup>1</sup> Values are weighted percentiles and sample sizes. While NHANES 2013–2014 collected samples from participants 19 y of age, we only included data for participants 20 y of age. Serum vitamin B-12 was measured by Roche E-170 immunoassay and serum MMA by HPLC-MS/MS. B-12, vitamin B-12; CKD, chronic kidney disease; MMA, methylmalonic acid.

<sup>2</sup> Pregnant and lactating women were excluded.

<sup>3</sup> Pregnant and lactating women, participants with CKD stage 1–5, and participants with missing or incomplete CKD stage information were excluded.

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<sup>4</sup> Pregnant and lactating women, participants with serum B-12 <300 pmol/L, and participants with missing serum vitamin B-12 data were excluded.

<sup>5</sup> Pregnant and lactating women, participants with CKD stage 1-5 and serum B-12 <300 pmol/L, and participants with missing/incomplete CKD stage information and/or missing serum vitamin B-12 data were excluded.