2 November 2016

1. **Protocol**:
    a. Title: "**Use of NHANES Data to Assess Nutrition Issues Relevant to the Health of DoD Personnel**"
    b. Estimated Start Date and Completion: 30 November 2016 to 31 December 2018
    c. Principal Investigator: Dr. Harris Lieberman, Military Nutrition Division, USARIEM; Associate Investigators: Dr. Victor Fulgoni, III, USARIEM-Henry Jackson Foundation; Dr. Sanjiv Agarwal, USARIEM-ORISE Fellow
    d. Study Location: U.S. Army Research Institute of Environmental Medicine, 10 General Greene Avenue, Bldg. 42, Natick, MA 01760-5007

2. **Primary Purpose/Objectives of the Studies**: The purpose of the proposed studies is to use a free, publicly-available, de-identified database, the National Health and Nutrition Examination Survey (NHANES), which contains de-identified nutrition and health information on a large representative sample of the U.S. population, to assess various nutrition and health issues of direct relevance to DoD Service Members.

3. **Background and significance of the studies**:
    a. The current National Health and Nutrition Examination Survey (NHANES) is a large continuous, population-based, cross-sectional survey conducted by the Center for Disease Control and Prevention (CDC) to monitor health and dietary status of Americans. New datasets are released every two years. The CDC's general description of NHANES is provided here:
    Risk factors, those aspects of a person's lifestyle, constitution, heredity, or environment that may increase the chances of developing a certain disease or condition, will be examined. Physical fitness and activity, weight, and dietary intake will be assessed. The diseases, medical conditions, and health indicators to be studied include but are not limited to: anemia, cardiovascular disease, diabetes, environmental exposures, eye diseases, hearing loss, infectious diseases, kidney disease, nutrition, obesity, oral health, osteoporosis, physical fitness and physical functioning, respiratory disease (asthma, chronic bronchitis, emphysema), and vision. The sample for the survey is selected to represent the U.S. population of all ages. To produce reliable statistics, NHANES over-samples persons 60 and older, African Americans, and Hispanics" [CDC, 2015].

    b. The NHANES 1999–2016 Survey Content Brochure contains a listing of all the variables in NHANES including variables based on responses to questionnaires, from the medical examination, and from laboratory tests conducted as part of NHANES [CDC, 2016]. Because NHANES surveys are conducted using the same state-of-the-art techniques and standardized procedures, very large datasets can be obtained by combining multiple years of data. Additionally there are older NHANES studies (i.e., 1971-1974, 1976-1980, and 1988-1994) which, depending on topic of interest, may also be useful to evaluate.

c. Typically, data from eight NHANES releases (1999-2000, 2001-2002, 2003-2004, 2005-2006, 2007-2008, 2009-2010, 2011-2012, and 2013-2014) (and as appropriate newer data as released) will be combined for analyses. If appropriate, older NHANES datasets may also be used for comparison to more recent data. Study participants will be free-living volunteers who have complete records for analyses of the variables under consideration. Each data release has information on approximately 10,000 subjects, therefore combining data from some or all of the data releases allows for creation of large datasets that allows assessment of various segments of the population (e.g., specific demographic profiles aligned with Service Members, such as individuals who engage in extensive physical activity).

4. **Overall Military Relevance:** Numerous DoD nutrition and health-related topics can be directly addressed using pre-existing, easily assessed NHANES data. Given the large amount of DoD-relevant information in NHANES available at no cost to the DoD, evaluation of specific demographic samples of the free-living American population that corresponds to Service Member demographics (i.e.., age/gender, extensive physical activity, those using dietary supplements, etc.) can be conducted as appropriate. The NHANES datasets can be used to directly address, with high quality data, issues for which no DoD epidemiological data are available.

5. **Project 1: Establishing Red Blood Cell (RBC) Reference Intervals with NHANES Data [This project is described extensively to provide an example of the procedures to be used for all the proposed projects.]**
    a. **Primary Purpose/Objectives of the Study**: The purpose of the proposed study is to update red blood cell (RBC) reference intervals with a large representative sample of the U.S. population using a publicly-available database – the National Health and Nutrition Examination Survey (NHANES) – and to evaluate extended population sub-groups.

    b. **Significance of the study**: Anemia, which is diagnosed by the Complete Blood Count (CBC), [RBC parameters are key components of CBC] is a global health problem affecting about 25% of the population worldwide with highest prevalence among preschool-aged children and pregnant women [de Benoist et al., 2008]. In a survey of female U.S. Army personnel, the prevalence of iron deficiency anemia immediately following basic combat training has been reported to be about 21%, with higher prevalence among Hispanic and African-American military personnel than among Caucasian military personnel [McClung et al, 2006]. Subsequent USARIEM research has replicated and extended these findings [Karl et al., 2009; McClung et al 2009a,b]. Anemia is identified by the presence of low hemoglobin levels, however other CBC measures, in particular the RBC parameters, are considered as surrogate measures of iron availability and are essential for optimal classification, monitoring and management of anemia [Brugnara et al, 2013; Buttarello, 2016].

CBC reference intervals are the upper and lower limits for normal test results and are typically defined as 95% (± 2.5% upper and lower limits) of the measured values from a healthy population. They provide baseline values to allow health care providers to interpret a patient's clinical laboratory test results and help in patient diagnosis and management [Dixon, 1997; Leach, 2014]. CBC parameters are known to vary with demographic factors such as age, sex and ethnicity, and lifestyle factors such as smoking [Ceriotti et al, 2009; Cheng et al, 2004]. Existing reference values are derived from a broad range of sources, including published research, individual laboratory studies, manufacturers' studies and convenience samples of various populations [Friedberg et al, 2007]. According to the Clinical Laboratory Standards Institute (CLSI) guidelines, reference interval values can be developed based on at least 120 healthy reference individuals, a minimal requirement to achieve statistical significance [Clinical and Laboratory Standards Institute, 2008]. A critical issue for establishment of valid reference values is collection of data from a well-defined "healthy" population, however, these are often difficult to obtain [Ceriotti et al, 2009]. Most individuals participating in NHANES are healthy and those with illnesses can readily be identified. Therefore, NHANES provides an optimal database for this study. Furthermore, since demographic and lifestyle factors like age, gender, race, smoking habits, etc., affect CBC values, larger sample sizes and stratification of the population studied may be required.

c. **Military Relevance:** Red Blood Cell (RBC) laboratory values are among the most important clinical laboratory tests used by health care providers for diagnosing diseases, monitoring various medical conditions and assessing nutritional status [Horvath, 2013]. As mentioned previously the prevalence of iron deficiency anemia immediately following basic combat training has been reported to be about 21% with a higher prevalence among Hispanic and African-American military personnel than among Caucasian military personnel [McClung et al, 2006]. Having up-to-date and age-specific reference values for men and women will aid in assessing abnormal lab values and designing future DoD clinical studies. Equivalent data are not available in DoD epidemiologic databases. Additionally, given available information in NHANES we plan to evaluate reference values for highly active individuals as measured with standardized physical activity questions.

d. **Study Design/Methods:** To date only a limited number of studies have used NHANES data to derive reference intervals. NHANES III (1988-1994) data were previously used to derive CBC reference intervals by age, gender and race [Cheng et al, 2004]. However, these are based on data collected over 25 years ago and an update is appropriate. NHANES data for thyroid function are widely-cited [Spencer et al, 2007]. Another study reported seasonal trends in blood counts using NHANES 1999-2012 [Liu & Taioli, 2015].

Piecewise regression will be used to determine breakpoints across ages and define population sub-groups where different reference values are needed based on the presence of a statistically significant differences ($P<0.05$). This procedure will objectively define the number of age/gender groups needed to for reference values. Analyses will be conducted using SAS 9.2 (SAS Institute, Inc.; Cary, NC) and SUDAAN release 11.0 (Research Triangle Institute; Research Triangle Park, NC). Appropriate weighting factors will be used to adjust for oversampling of selected groups and survey non-response to ensure estimates are nationally representative. The percent of the population below and above existing (current) and the piecewise regression-derived reference ranges will be assessed using age- and gender-specific cutoff values.

8. Project 2: Association of vitamin D status with anemia

a. This project will examine the relationship of vitamin D status (serum 25-hydroxy vitamin D) with anemia by examining its association with iron status [ferritin, transferrin saturation (limited data on men; larger data set on women), erythrocyte protoporphyrin (limited data on men; larger data set on women), red blood cell distribution width, and body iron (limited data on men; larger data set on women), folate status, and vitamin B12 status. Various definitions of anemia available in the published literature will also be used. Vitamin D status is recognized as an important DoD nutrition issue especially for female service members and is a major focus of interest of USARIEM's nutrition research program [See work conducted by: Andersen et al., 2010; Gaffney-Stomberg et al., 2014; McClung and Gaffney-Stomberg 2016; Lutz et al., 2012; McClung et al., 2014; Montain et al., 2013]. Suboptimal vitamin D intake and diminished vitamin D status are particularly important during periods of intense physical activity including military basic training because compromised bone health contributes to the development of stress fractures [Andersen et al, 2010]. Decrements in vitamin D status during US Army basic combat training have been documented in female Soldiers [Andersen et al, 2010].

b. Linear regression will be used to determine if vitamin D status as a continuous variable is related to iron status markers and presence of anemia. Given controversy over optimal vitamin D levels, multiple levels (i.e., 20 and 30 ng/ml) will be used to define vitamin D deficiency. Logistic regression will also be used to assess whether presence of vitamin D deficiency impacts iron status. Analyses will be conducted using SAS 9.2 (SAS Institute, Inc.; Cary, NC) and SUDAAN release 11.0 (Research Triangle Institute; Research Triangle Park, NC). Appropriate weighting factors will be used to adjust for oversampling of selected groups and survey non-response to ensure estimates are nationally representative.

9. Project 3: The association of demographic characteristics and intake of various food groups and nutrients with markers of metabolic syndrome and Type II diabetes

a. This project will examine demographic factors (e.g., age, gender, race/ethnicity, socioeconomic status, smoking, alcohol intake, etc.) and intake variables (nutrients, food groups, micronutrients and dietary supplements) and their association with fasting blood glucose, glycohemoglobin and insulin levels (and as appropriate other biochemical markers of metabolism and stress). Fasting blood glucose, glycohemoglobin and insulin levels are the primary predictors of metabolic syndrome and Type II diabetes. Overweight, obesity, metabolic syndrome and Type II diabetes are increasing problems in the DoD [Clark and Taubman 2016; Rush et al., 2016] as they are in the general population.

b. Analyses will be conducted using SAS 9.2 (SAS Institute, Inc.; Cary, NC) and SUDAAN release 11.0 (Research Triangle Institute; Research Triangle Park, NC). The MAXimum R^2 option in SAS procedures allows for examining every possible combination of variables of interest. However, a MAXimum R^2 option is not available in the SAS SurveyReg procedure used to analyze complex survey data, such as NHANES; therefore, a macro will be developed to assess every possible combination of the 16 variable while accounting for the survey weights in NHANES (SAS version 9.2; SAS Institute). This method will identify the best one-variable model producing the greatest percentage of variation explained (R^2), the best two-variable model, and so forth. The MAXimum R^2 approach, unlike other standardized stepwise methods, evaluates the possible switching of the order of variables entered into the model, which can affect the model results. These analyses will result in evaluation of 65,536 separate regression models, which is equivalent to 2 to the power of 16 (number of variables). From each set of models that contained a specified number of variables (1 through 16), the model with the highest adjusted R^2 will be chosen as the best model. Adjusted R^2 values will be used to compare the various models because the number of variables in the model affects the R^2. The adjusted R^2 adjusts for the number of explanatory terms in the model including nutrients and the other covariates age, sex, and ethnicity.

10. Project 4: Association of demographic and intake variables with hormones and hormone-binding globulins and other biomarkers

a. This project will assess demographic variables (e.g., age, gender, race/ethnicity, socioeconomic status, smoking etc.), nutrient intake (e.g., total and types of fats, dietary fiber, protein, vitamins, dietary supplements, etc.) and food group intakes (total vegetables, whole grains, solid fats, added sugars, etc.) associated with various hormones and hormone-binding globulins including thyroid hormones, testosterone, estradiol, sex hormone binding globulin (only limited data are available for several hormones) and various other biomarkers of stress and health status. Previous work indicates intake of certain nutrients/foods (e.g., dietary fiber/wheat bran) may affect hormones that regulate weight regulation and ability to respond to a variety of stressors. Our laboratory and others have documented substantial changes in various hormones in military exercises and during deployment due to activation of the

Hypothalamic-Pituitary-Adrenal (HPA) axis. [See for example Morgan et al, 2000; Lieberman et al, 2016].

b. Linear regression will be used to determine if demographic and intake variables are associated with hormones and hormone-binding globulins, etc. based on the presence of a statistically significant association ($P<0.05$). Analyses will be conducted using SAS 9.2 (SAS Institute, Inc.; Cary, NC) and SUDAAN release 11.0 (Research Triangle Institute; Research Triangle Park, NC). Appropriate weighting factors will be used to adjust for oversampling of selected groups and survey non-response to ensure estimates are nationally representative.

11. Project 5: Assess impact of macronutrient distribution, especially dietary protein, on health risk/disease prevention.

a. This project will assess intake and relative variation in macronutrient distribution across numerous demographic groups (e.g., age/gender, higher/lower socioeconomic status; race/ethnicity, etc.). One hypothesis to be examined is that dietary protein distribution has the smallest relative variation as compared to the other macronutrient distributions. Intake and variation in micronutrient and dietary supplement consumption will also be evaluated. Protein intake in the diet and in dietary supplements are of great interest to the DoD and a major focus of USARIEM's research program [Pasiakos et al., 2013a; 2013b]. Protein intake and its biological consequences are of direct relevance to DoD service members since it is an important determinate of physical performance. Protein supplements are taken by very large numbers of Service Members unlike the civilian population who rarely take these supplements [Austin et al., in press; Austin et al., 2016; Knapik et al., 2014]. Furthermore, the proportion of Soldiers regularly using such supplements significantly increases during deployments - from 32% of to 48% [Austin et al, 2016]. This study will assess the variation in protein intake in the U.S. population to determine the biological limitations of protein supplementation and relate protein intake to ration acceptability and safety. Other dietary supplements will also be examined.

b. Analyses will be conducted using SAS 9.2 (SAS Institute, Inc.; Cary, NC) and SUDAAN release 11.0 (Research Triangle Institute; Research Triangle Park, NC). Appropriate weighting factors will be used to adjust for oversampling of selected groups and survey non-response to ensure estimates are nationally representative. The distribution of the percentage of energy from carbohydrate, fats, and protein will be determined and characterized via standard statistics [e.g., mean, standard deviation, measures of skewness/kurtosis, and test statistics for normality (Kolmogorov-Smirnov, Cramer von Mises, and Anderson-Darling] for numerous demographic sub-groups based on age, gender, race/ethnicity, socioeconomic status, and physical activity levels. Given differences in mean values of percentage of energy of the macronutrients, the coefficient of variation (standard deviation

as percentage of mean) will also be used to discern differences in distributions.

12. Data Collection/Analysis (all studies): All data to be used have previously been collected and de-identified and are publically available. We will conduct retrospective review/analyses of an existing national public database. The database is accessible to the public through the Centers for Disease Control website on the World Wide Web ("National Health and Nutrition Examination Survey;" http://cdc.gov/NCHS/nhanes.htm) and does not contain any personal identifiers.

13. Risks (all studies): There are no risks to this study. All data to be used have previously been collected and de-identified and are publically available. Similar requests using NHANES databases (Research Protocol R10-02, "A Retrospective Study of Biochemical Markers, Nutritional Status and Mood" and Research Protocol R11-01 "A Retrospective Study of Demographic and Lifestyle Factors Associated with Caffeine and Dietary Supplement Use") were approved by the USARIEM IRB and Commander.

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