DOCUMENTATION

for

Blood, Urine, & Saliva Data

in

MIDUS 3
BIOMARKER PROJECT
(P4)

University of Wisconsin ♦ Institute on Aging
April 2023

INTRODUCTION

This document offers information about tissue samples collected in the MIDUS 3 Biomarker Project (P4). The Table of Contents provides three sections:

Section A is an overview of the data collected and contains information about the constructed administrative variables.

Section B includes standardized protocols for collecting (B-1) and processing (B-2) blood and urine samples. Saliva collection occurs during the Laboratory Challenge Study (part of the Psychophysiology Protocol); its documentation can be accessed separately in "M3_P4 Psychophysiology Data Documentation."

Section C contains descriptions of the biomarker assay methods (C-1). They are listed first by tissue source (ex: I. Blood Assays), then by physiological system assessed (ex: A. Cardiovascular Markers). It includes a table of assay performance characteristics (C-2). Because MIDUS is a longitudinal study, some assays have inevitably changed over the timeline of study phases. In order to achieve harmonization through these changes, method comparison studies were performed resulting in derived crossover regressions. These data adjustments are outlined in section C-3, with corresponding references in C-4.

Data users are also encouraged to review "M3 Biomarker Project Readme Data File Notes." This document provides more thorough information about naming conventions/variables included in the data file. It also includes information about how missing values were handled, and other changes (ex: added variables) that arose over the course of the study.

This document will be periodically revised and updated as researchers continue to work with the MIDUS Biomarker data. If there are suggestions or comments, please contact midus_help@aging.wisc.edu.

Table of Contents

SECTION A-1: OVERVIEW OF DATA FILE AND COLLECTION PROTOCOLS

The Biomarker Project (P4) includes collection of the following tissue samples for biomarker assays:

- Fasting **blood** samples
- 12-hour (overnight) **urine** collection
- **Saliva** collections

Sample types were obtained from three clinical research units (CRUs) located in UCLA, UW-Madison, and Georgetown.

The full participant dataset collection is obtained from the ICPSR database/Colectica website. Once downloaded, the biomarker data is located in the table immediately following the neurological exam data. As described in "M3 Biomarker Project Readme Data File Notes", the naming convention organizes variables according to data type or the method used for data collection. The variable names for the biomarker assay data begin with a unique three-character set, "C4B".

BIOMARKER DATA TYPES

Tissue samples are assayed for cardiovascular, renal, neuroendocrine, inflammatory, antioxidant, bone turnover, and metabolic biomarkers to reflect the functioning of these physiologic systems as follows:

- Fasting blood draws
  - Cardiovascular – glycosylated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides
  - Renal – creatinine
  - Neuroendocrine – dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEA-S)
  - Inflammatory – interleukin 6 (IL-6), soluble interleukin 6 receptor (IL-6 sR), interleukin 8 (IL-8), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), fibrinogen, C-reactive protein (CRP), soluble E-selectin (sE-selectin), and soluble intercellular adhesion molecule 1 (sICAM-1)
  - Antioxidant – *trans*-β-carotene, 13-*cis*-β-carotene, α-carotene, cryptoxanthin, lutein, zeaxanthin, 13-*cis*-lycopene, 9-*cis*-lycopene, *trans*-lycopene, total lycopene, retinol, α-tocopherol, and γ-tocopherol
  - Bone turnover – bone-specific alkaline phosphatase (BAP), n-telopeptide type 1 collagen (NTx), and aminoterminal propeptide type 1 procollagen (P1NP)
  - Metabolic – glucose, insulin, and insulin-like growth factor 1 (IGF-1)
- 12-hour urine collection
  - Neuroendocrine – cortisol, cortisone, norepinephrine, epinephrine, and dopamine
  - Renal – creatinine
- Saliva collections (refer to psychophysiology protocol)
  - Neuroendocrine – cortisol

ADMINISTRATIVE VARIABLES

- C4ZBLOOD, C4ZURINE, C4ZSALIV – indicate if the specified sample type was collected and whether the sample is complete or partial
- The urine collection occurs over a 12-hour period. Some voids may be incomplete for various reasons. Use the following variables to identify problematic cases prior to including urine collection in analyses.
  - C4BUPROB – urine collection period less than 11 hours or greater than 13 hours
  - C4BUSTRT – urine collection start time
  - C4BUEND – urine collection end time
  - C4BUVDYN – any voids not collected
  - C4BUVDN – number of voids not collected
- The following variables indicate details of each saliva collection process. Some collections may be incomplete (C4BINSUFF) for various reasons:
  - C4BINSUFF – number of saliva samples with insufficient saliva in the tube for the assay to be run
  - C4VS1T through C4VS5T – saliva collection times

COMPUTED VARIABLES

The following additional measures were calculated using original biomarker data. Refer to "M3_P4 Documentation for PsychoSocial Constructs and Composite Variables", which details the formation of the following variables:

- C4BLDL – low-density lipoprotein (LDL) cholesterol. This is an indirect measure calculated using the Friedewald formula (see page 11)
- C4BRTHDL – ratio of total cholesterol / HDL cholesterol
- C4BHOMAIR – measure of insulin resistance
- C4BSUCRE – ratio of serum creatinine / urine creatinine
- C4BGFR – glomerular filtration rate (GFR)
- C4BNE12, C4BEPI12, C4BDOP12 – urine norepinephrine, epinephrine, and dopamine adjusted to 12-hour values corresponding to the data collection period
- C4BNOCRE, C4BEPCRE, C4BDOCRE – urine norepinephrine, epinephrine, and dopamine adjusted for urine creatinine
- C4BCLCRE and C4BCOCRE – urine cortisol and cortisone adjusted for urine creatinine
- C4BNECL – ratio of urine norepinephrine / urine cortisol

SECTION B-1: BLOOD AND URINE COLLECTION PROTOCOL

PREPARATION

1. Prepare urine collection supplies

- Two urine collection containers (volume ≥ 2L):
  - CATS collection jug **– ADD ACETIC ACID**
    - Add 25 mL of 50% acetic acid to one urine collection container to acidify urine in preparation for catecholamine tests
    - Place **red** CATS label on side of this container
  - CORT collection jug – **NO ACID**
    - **Do not add acid** to this container
    - Place white CORT label on side of this container
- Hat or urinal

2. Label blood vacutainer tubes with MIDUS ID labels

- Three 10 mL red/black top, serum separator tubes (SST)
- One 4 mL lavender top (EDTA) tube
- One 4 mL lavender top (EDTA) tube, wrapped with aluminum foil
- One 4 mL or 2.7 mL blue top (sodium citrate) tube
- One 4 mL green top (sodium heparin) tube
- One 8 mL red/green top cell preparation tube (CPT)

URINE COLLECTION

*****At the start of the COVID-19 pandemic, urine collection transitioned from inpatient to outpatient, meaning urine collection was no longer overseen by nursing staff, and that the participant was independent during the overnight collection.

At 19:00

- Instruct participant on 12-hour collection
- Provide urine collection device (urinal for men or hat for women) and explain its use
- Post reminder notice in bathroom
- Have participant void – **discard this sample**
  - Record date and time of this void on the collection form (Page 1, #2)
- Instruct participant to collect all urine from then on until 07:00 in specimen hat or urinal **and <u>to call/notify nurse immediately after each void (even during the night)*</u>**

From 19:00 until 07:00

- Pour **one half** of **each void** into each of the two urine collection containers
- Keep both collection containers in the refrigerator throughout the 12-hour collection period
  - Collection containers were placed on ice in the hotel (outpatient setting)
- **Around 21:00-22:00**: Remind participant (later in evening, before they go to bed) to collect urine all night
- **When participant wakes up** and/or when nurse comes in to draw blood: Remind participant to continue collecting all urine until 07:00

At 07:00 – completion of urine collection

- Request final urine void from participant
- Pour **one half** into each of the two urine collection containers

- Inform participant that this is the final urine collection and there is no need to notify staff of any other voids
- On collection form:
    - Record date and time of final void (Page 1, #3)
    - Indicate any uncollected urine and reason (e.g. participant refused) on Page 1, #4
- Take urine to lab for processing and notify technician

BLOOD COLLECTION – COLLECTION OF FASTING BLOOD FROM 06:30-07:00

Preparing participant

- Use **non-dominant arm** if possible
- Make reasonable number of attempts to get needed samples
- Participant should avoid strenuous activity before blood draw but can take a shower

Blood draw order

- Three 10 mL **red/black** top, serum separator tubes (SST)
- First 4 mL **lavender** top tube
    - After draw, ***gently invert*** 3-5 times.
- Second 4 mL **lavender** top tube (foil-wrapped)
    - Cover the vacutainer with aluminum foil before attaching to needle.
    - Slide the foil down slightly during blood draw, leaving the top visible so that one can see the fill line on the tube. After draw, ***gently invert*** 3-5 times. Place tube in ice bath.
- One 4 mL or 2.7 mL **blue** top (citrated) tube
    - Fill tube completely. After draw, ***gently invert*** 3-5 times.
- One 4 mL green top (heparin) tube
    - After draw, ***gently invert*** 3-5 times.
- One 8 mL red/green top cell preparation tube (CPT)

  Note: prevention of backflow

  The BD Vacutainer Cell Prep Tubes (CPT) (Becton Dickinson, Franklin Lakes, NJ) contain chemical additives, thus it is important to prevent possible backflow from the tube with its attendant possibility of adverse reactions to the participant. To guard against backflow, the following precautions should be taken during the draw:
    i. Keep participant's arm in the downward position during the collection procedure.
    ii. Hold the tube upright with the stopper uppermost.
    iii. Release the tourniquet as soon as the blood starts to flow into the tube, or within two minutes of application.
    iv. Make sure the tube contents do not touch the stopper or the end of the needle during the collection procedure.
    v. After draw, gently invert 8-10 times.
- Complete specimen collection form (Page 2)
- Take blood tubes to lab for processing ASAP and record time on form.
    - Place lavender top tube without foil in MIDUS storage box in refrigerator **– do not freeze.**
    - Place lavender top tube with foil **in ice bath.**

SECTION B-2: BLOOD AND URINE PROCESSING PROTOCOL

URINE PROCESSING

- Measure volume in both urine containers and record each on the collection form.
- Sum and record total volume on form (Page. 3, #12).
- From **CORT COLLECTION** bottle (white-labeled jug)
    1. Aliquot **4 mL** into each of **four 5 mL** vials without marker on top.
    2. Record amount aliquotted on form (Page. 3, #13).
- From **CATS COLLECTION** bottle (**red**-labeled jug)
    1. Assess initial pH using a pH strip (Page. 3, #14a).
        - If pH ≥ 5, add 1 mL of acetic acid and mix thoroughly. Re-assess pH.
    2. Repeat above **until pH < 5**. Record final pH on form (Page. 3, #14b).
    3. Once pH < 5, aliquot **4 mL** into each of **two 5 mL** plastic tubes with **red** marker top.
    4. Record amount aliquotted on form (Page. 3, #14c).
- Record time frozen on form (Page. 3, #14d).
- Store at -60 to -80°C.

BLOOD PROCESSING

I. **One lavender top EDTA tube without foil** (whole blood for HbA1c test and DNA extraction)
   a. Refrigerate at 4°C **(DO NOT FREEZE).**

2. **One foil-wrapped lavender top EDTA tube**
   a. Centrifuge 4°C for **20 mins** @ **1000 g (RCF)**. The blue, green, and foil-wrapped lavender tubes may be centrifuged together. On the form, record time centrifugation began.
   b. **Under dim lights**, aliquot **0.5-1 mL** plasma into two yellow-labeled 2 mL cryogenic vials.
   c. On form, indicate number of aliquots filled. If less than two filled, indicate why.
   d. Store at -60 to -80°C.

3. **One blue top sodium citrate tube**
   a. Keep on ice after inversion. Centrifuge within 15 minutes of draw.
   b. Centrifuge 4°C for **20 mins** @ **1000 g (RCF)**. The blue, green, and foil-wrapped lavender tubes may be centrifuged together. On the form, record time centrifugation began.
   c. Aliquot **0.5-1 mL** plasma into each of two blue-labeled 2 mL cryogenic vials.
   d. On form, indicate number of aliquots filled of each type. If none filled or fewer than number specified, indicate why.
   e. Store at -60 to -80°C.

4. **One green top sodium heparin tube**
   a. Centrifuge within 30 minutes from time of draw.
   b. Centrifuge 4°C for **20 mins** @ **1000 g (RCF)**
   c. Aliquot 1 mL plasma into each of two 2 mL cryovials (**clear** caps) labeled HDLF.
   d. On form, record time centrifugation began.
   e. Store at -60 to -80°C.

5. **Three red/black marble top SST tubes**
   a. Let stand for 15-30 minutes after draw (maximum of two hours allowed between draw and centrifugation).
   b. Centrifuge 4°C for **20 mins** @ **1000 g (RCF)**
   c. From SSTs, aliquot 1 mL serum into each labeled 2 mL vial as follows:
      "**ARUP"** – three vials
      White – two vials
      Green – two vials
      Orange – five vials
   d. On form, indicate number of aliquots filled of each type. If fewer than target was obtained, indicate why.
   e. Store at -60 to -80°C.

6. **One red/green marble top CPT tube**
   a. Invert 8-10 times immediately prior to centrifugation.
   b. Centrifuge **at room temp** for **20 mins** @ **1800 g (RCF)** without brake, at room temperature.
   c. Using a transfer pipette, discard the clear plasma from the uppermost layer. Avoid disturbing the whitish/cloudy cell layer below the plasma that contains the peripheral blood mononuclear cells (PBMC).
   d. Pipette the cell layer into a 3.6 mL round-bottom cryovial. **Re-centrifuge at room temp** for **5 mins** @ **1200 g (RCF)**
   e. Without disturbing the white cell pellet at the bottom of the tube, remove as much of the remaining fluid as possible and discard. Only the cell pellet should remain.
   f. Label the tube "**PBMC**"
   g. Store at -60 to -80°C.

SECTION C-1: BIOMARKER ASSAY DESCRIPTIONS

Overview

The laboratory assays of tissue samples collected as part of the MIDUS Biomarker Project (Project 4) are described below, including details about assay method and performance characteristics. Assays are listed first by tissue source (blood, urine, saliva), and then by physiological system assessed (cardiovascular, inflammatory, etc.). Each section begins with a brief overview of sample collection and processing. Information about each assay was provided by the lab performing the assay or was developed by the MIDUS Biocore. A summary table listing all the assays along with the specimen type, assay type, and performance characteristics follows these descriptions. For several assays that had a substantial method change during or since MIDUS 2, information about the change and any adjustments to the data are provided in the Data Adjustments section. This section ends with a list of the references cited.

I. Blood Assays

Sample Collection

Fasting blood samples are collected from each participant before breakfast on Day 2 of their hospital stay. To ensure consistency, all samples are collected and processed at the CRU using standardized procedures described in Section B. Frozen samples are stored in a -60°C to -80°C freezer until shipped on dry ice to the MIDUS Biocore Lab. Samples are subsequently stored in a -70°C freezer until assayed.

- *Fresh whole blood* samples are refrigerated and shipped to the MIDUS Biocore Lab weekly and assayed for HbA1c.

- *Frozen serum and plasma* in 1 mL aliquots are shipped to the MIDUS Biocore Lab monthly for the following biomarker assays:

    - Cardiovascular – total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides
    - Renal – creatinine
    - Neuroendocrine – DHEA and DHEA-S
    - Inflammatory – IL-6, IL-6 sR, IL-8, IL-10, TNF-α, fibrinogen, CRP, sE-selectin, and sICAM-1
    - Antioxidant – *trans-ß*-carotene, 13-*cis-ß*-carotene, α-carotene, cryptoxanthin, lutein, zeaxanthin, 13-cis-lycopene, 9-cis-lycopene, trans-lycopene, total lycopene, retinol, α-tocopherol, and γ-tocopherol
    - Bone turnover – BAP, NTx, and P1NP
    - Metabolic – glucose, insulin, and IGF-1

A. Assay Details – Cardiovascular Markers

The HbA1c and lipid panel assays are performed at Meriter Labs (Madison, WI) using a Roche Cobas 6000 c501 analyzer (Roche Diagnostics, Indianapolis, IN) [1].

***Glycosylated hemoglobin**:*

HbA1c is assayed on the Cobas analyzer using the Tina-Quant Hemoglobin A1c kit (Roche Diagnostics, Indianapolis, IN). The instrument measures total hemoglobin concentration of EDTA-anticoagulated whole blood colorimetrically and also measures the concentration of glycosylated hemoglobin (HbA1c) using a turbidometric inhibitor procedure. It then divides the HbA1c concentration by the total hemoglobin concentration and reports this as a percentage [1]. The HbA1c assay changed over time, see Data Adjustments (section C-3) for more information.

Lipid panel:

Total cholesterol: The Cobas total cholesterol assay uses enzymes to convert the cholesterol to a colored product and measures absorbance of the product [1].

HDL cholesterol: The Cobas HDL assay depletes *ß*-lipoproteins from the serum by precipitation using polyethylene glycol, and then measures HDL (the *α*-fraction) as described above for total cholesterol [1].

Triglycerides: The Cobas triglyceride assay adds enzymes to the serum to obtain a colored product, and measures absorbance of the product [1].

LDL cholesterol: An estimation of LDL cholesterol level (LDL) is calculated using the Friedewald formula (below). This equation uses direct measurements of total cholesterol (TC), triglycerides (TG), and HDL cholesterol (HDL).

LDL = TC - HDL - TG/5

When a participant's triglyceride level exceeded 400 mg/dL, then 400 mg/dL was substituted in the equation for the TG value.

B. Assay Details – Neuroendocrine Markers

DHEA and DHEA-S assays are performed by Associated Regional & University Pathologists (ARUP, Salt Lake City, UT).

DHEA:

During MIDUS 2, DHEA was assayed using a radioimmunoassay (RIA) kit (cat# DSL8900, Diagnostic Systems Laboratories, Webster, TX). The radioimmunoassay used competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of (I-125)-labeled DHEA bound to the antibody is inversely proportional to the concentration of the unlabeled DHEA present. The separation of free and bound antigen was achieved using a double antibody system [2].

On May 18, 2009, ARUP began using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to assay DHEA. MIDUS 2 data collection ended on May 31, 2009; thus the final 75 samples for MIDUS 2 (as well as all subsequent MIDUS samples) were assayed using LC-MS/MS. This method uses the Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA) to fractionate the serum and the API 5500 Mass Spectrometer (Applied Biosystems, Waltham, MA) for detection [3]. The DHEA assay changed over time, see Data Adjustments (section C-3) for more information.

DHEA-sulfate:

This assay is an electrochemiluminescent immunoassay and is run on a Roche Cobas e602 analyzer (Roche Diagnostics, Indianapolis, IN) [3].

C. Assay Details – Renal Markers

Serum creatinine assays are performed by Meriter Laboratories (Madison, WI).

Serum creatinine:

This assay is run on a Roche Cobas 6000 c501 Analyzer (Roche Diagnostics, Indianapolis, IN) and is an enzymatic colorimetric assay, in which the instrument measures absorbance on the colored end product of an enzymatic reaction [1].

D. Assay Details – Inflammatory Markers

IL-6, IL-6 sR, and a proinflammatory cytokine panel of IL-10, IL-6, IL-8, and TNF-α are assayed in the MIDUS Biocore laboratory (University of Wisconsin–Madison, Madison, WI). The fibrinogen, CRP, sE-selectin, and sICAM-1 assays are

performed at the Laboratory for Clinical Biochemistry Research (University of Vermont, Burlington, VT) by the team of Dr. Russell Tracy.

IL-6 by ELISA:

At the beginning of MIDUS 3, IL-6 was measured using the Human IL-6 Quantikine High-Sensitivity ELISA Kit (cat# HS600B, R&D Systems, Minneapolis, MN). This is a sandwich ELISA using a microplate pre-coated with a monoclonal antibody specific for IL-6. Standards and samples are pipetted into the wells, and any IL-6 present is bound by the immobilized antibody. After washing away the unbound substances, an alkaline phosphatase-labeled detection antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate (NADPH) is added, which is converted to NADH. After an incubation period, an amplifier solution containing two additional enzymes whose actions are coupled to the cycling of NADH and NAD+ (diaphorase and alcohol dehydrogenase) and their respective substrates (iodonitrotetrazolium-violet and ethanol) are added. The diaphorase converts the iodonitrotetrazolium-violet to formazan, a red-colored product. The reaction is stopped at a predetermined endpoint with sulfuric acid, and the absorbance, which is proportional to the concentration of IL-6 in the sample, is read at 490nm using a Dynex MRXe plate reader (Magellan Biosciences, Chantilly, VA) [4].

Since May, 2019, IL-6 is measured using the reformulated Human IL-6 Quantikine® High-Sensitivity ELISA Kit (cat# HS600C, R&D Systems, Minneapolis, MN). The new kit is a similar sandwich ELISA which uses a biotinylated detection antibody, followed by streptavidin-bound horseradish peroxidase, followed by its substrate, tetramethylbenzidine. This substrate produces a blue color, which changes to yellow at the end of the incubation period when sulfuric acid is added. The absorbance is read at 450nm using the Dynex MRXe plate reader (Magellan Biosciences, Chantilly, VA) [5].

IL-6 soluble receptor (IL-6 sR):

Concentration of IL-6 sR is measured using the Human IL-6R alpha Quantikine® ELISA Kit (cat# DR600, R&D Systems, Minneapolis, MN). This is a sandwich ELISA using a microplate pre-coated with a monoclonal antibody specific for IL-6 sR. Standards and samples are pipetted into the wells, and any IL-6 sR is bound by the immobilized antibody. After washing away the unbound substances, a horseradish peroxidase-labeled detection antibody specific for IL-6 sR is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate (tetramethylbenzidine) is added to the wells, and a blue-colored product develops. This reaction is stopped at a predetermined endpoint using sulfuric acid, which changes the color to yellow. Absorbance is read at 450 nm using a Dynex MRXe plate reader (Magellan Biosciences, Chantilly, VA), and is positively correlated to the concentration of IL-6 sR in the samples [6].

IL-6, IL-8, IL-10, and TNF-α by immunoelectrochemiluminescence:

These cytokines are measured using a V-PLEX Plus Proinflammatory Panel 1 Human Kit (cat# K15049G, Meso Scale Discovery, Rockville, MD). This technology employs a 96-well multispot plate, with each spot pre-coated with a capture antibody for a particular cytokine. These spots are connected to an electrode surface at the bottom of the plate. Serum samples are pipetted into wells, where the cytokines are allowed to adhere to their corresponding antibody spots. After washing off nonadherent samples, a solution containing detection antibodies tagged with ruthenium(II) tris-bipyridine-(4-methylsulfonate) N-hydroxysuccinimide ester (MSD Sulfo-tag™) is pipetted into the wells and allowed to adhere to the immobilized cytokines. After washing off unbound antibodies, a buffer is added, and the plate is loaded into an imager (MSD QuickPlex SQ120) where a voltage applied to the plate electrodes causes the captured Sulfo-tag to emit light. The instrument measures the intensity of the emitted light to provide a quantitative measure of analytes in each sample [7].

Fibrinogen:

Fibrinogen antigen is measured in EDTA plasma using the BNII nephelometer (N Antiserum to Human Fibrinogen; Siemens, Malvern, PA). The amount of fibrinogen present in the sample is quantitatively determined by immunochemical reaction. Complexes formed between antigen and antibody molecules scatter light passing

through the sample. The intensity of the scattered light is proportional to the concentration of the antigen (fibrinogen) in the sample [8].

C-reactive protein (CRP):

During MIDUS 2, CRP was measured in plasma using the BN II nephelometer (Dade Behring, Deerfield, IL) utilizing a particle-enhanced immunonephelometric assay. Polystyrene particles are coated with monoclonal antibodies to CRP which, in the presence of antigen (CRP) agglutinate and cause an increase in the intensity of scattered light. The increase in scattered light is proportional to the amount of CRP in the sample.

Since 2016 (including all of MIDUS 3), CRP assays are performed by immunoelectrochemiluminescence using the V-PLEX Plus Human CRP Kit (cat# K151STG, Meso Scale Discovery, Rockville, MD) on the MSD platform using frozen serum [8]. The CRP assay changed over time, see Data Adjustments (section C-3) for more information.

Soluble E-selectin (sE-selectin):

sE-selectin, also known as endothelial leukocyte adhesion molecule-1 (ELAM-1) and CD62E, is measured in serum by sandwich ELISA using the Quantikine ELISA Human E-Selectin/CD62E Immunoassay Kit (cat# DSLE00, R&D Systems, Minneapolis, MN). The sE-Selectin assay utilizes two antibodies directed against different epitopes on the sE-Selectin molecule. The amount of sE-selectin bound is determined colorimetrically [9].

Soluble intercellular adhesion molecule 1 (sICAM-I):

The sICAM-1 assay is a sandwich ELISA using the Quantikine ELISA Human ICAM-1/CD54 Immunoassay Kit (cat# DCD540, R&D Systems, Minneapolis, MN). sICAM-1 in the serum is sandwiched by an immobilized monoclonal antibody and the enzyme-linked monoclonal antibody. The amount of ICAM-1 present is determined by colorimetric reaction [10].

E. Assay Details – Antioxidant Markers

Antioxidant assays were performed in the clinical and analytical core laboratory (Nutrition Evaluation Laboratory) at the Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA.

Trans-ß-carotene, 13-cis-ß-carotene, α-carotene, cryptoxanthin, lutein, zeaxanthin, 13-cis-lycopene, 9-cis-lycopene, trans-lycopene, total lycopene, retinol, α-tocopherol, *and* ***γ-tocopherol***:

Plasma samples were exacted with chloroform/methanol followed by hexane (using echinenone and retinyl acetate in ethanol for internal standards) for simultaneous determination of the markers by a reverse-phase gradient HPLC method with UV/VIS detection (2695 Alliance HPLC system with the 2996 photodiode array programmable detector, Waters Corporation, Milford, MA) according to a modification of the methods previously described [11,12]. A C30 carotenoid column (YMC America, Devens, MA) was used for separation of metabolites [13].

F. Assay Details – Bone Turnover Markers

Bone-specific alkaline phosphatase (BAP), n-telopeptide type 1 collagen (NTx), and aminoterminal propeptide type 1 procollagen (P1NP) assays are performed by Dr. Neil Binkley's laboratory at the University of Wisconsin–Madison (Madison, WI).

Aminoterminal propeptide type 1 procollagen (P1NP):

UniQ P1NP RIA (cat# 67034, Aidian, Espoo, Finland) was used for all P1NP analyses. This is a competitive radioimmunoassay [14].

Bone-specific alkaline phosphatase (BAP):

MicroVue BAP kits (cat# 8012, Quidel Corporation, San Diego, CA) were used for all BAP analyses. This procedure is a sandwich ELISA [15].

n-Telopeptide type 1 collagen (NTx):

Osteomark NTx kits (cat# 9021, Alere Scarborough, Inc., Scarborough, ME) were used for all NTx analyses. This assay is a competitive-inhibition sandwich ELISA. Assay values are reported in nanomoles bone collagen equivalents (nmoles BCE) per liter (nmoles BCE/L) [16].

G. Assay Details – Metabolic Markers

These assays are performed at ARUP laboratories (Salt Lake City, UT).

Glucose:

This assay is run on the Roche Cobas c502 analyzer (Roche Diagnostics, Indianapolis, IN) and is an enzymatic colorimetric assay [3].

Insulin:

This assay is performed on the ADVIA Centaur (Siemens, Chicago, IL) and is a two-site sandwich immunoassay using direct chemiluminescent technology [3].

Insulin-like growth factor 1 (IGF-1):

This assay is run on the DiaSorin Liaison® XL (DiaSorin, Stillwater, MN) and is a two-site sandwich immunoassay using a chemiluminescent detection system [3].

II. Urine Assays

Sample Collection

12-hour overnight (19:00 – 07:00) urine samples were obtained from each participant. Details on collection and processing can be found in Sections B-1 and B-2.

Urine samples are assayed at the University of Wisconsin–Madison's Institute for Clinical and Translational Research (ICTR, Madison, WI) for the following biomarkers: creatinine, glucocorticoids (cortisol, cortisone), and catecholamines (norepinephrine, epinephrine, dopamine).

A. Assay Details – Renal Marker

Urine creatinine assays are performed at UW-Madison's ICTR (Madison, WI) for MIDUS 3. During MIDUS 2, the assay was completed at Mayo Clinic Laboratories (Rochester, MN) using an enzymatic colorimetric method.

Creatinine:

Urine creatinine is measured by a kinetic colorimetric assay known as the Jaffe reaction. The urine is mixed with an alkaline picrate solution, which reacts with creatinine to produce an orange-yellow end-product that is measured colorimetrically at 520nm [17]. The urine creatinine assay changed over time (see Data Adjustments section C-3 for more information).

B. Assay Details – Neuroendocrine Markers

Urine cortisol & cortisone assays are performed at UW-Madison's ICTR (Madison, WI) for MIDUS 3. During MIDUS 2, these assays were done at Mayo Clinic Laboratories (Rochester, MN).

Cortisol & cortisone:

Urine cortisol and cortisone are measured simultaneously using liquid chromatography-tandem mass spectrometry (LC-MS/MS) using lab-developed methodology. Quality control was measured using three pools of human urine [17].

Catecholamine assays are performed at UW-Madison's ICTR (Madison, WI) for MIDUS 3. During MIDUS 2 these assays were done at Mayo Clinic Laboratories (Rochester, MN).

Norepinephrine, epinephrine, dopamine:

Urine norepinephrine, epinephrine, and dopamine are quantified by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) using lab-developed methodology using a Dionex Ultimate 3000 electrochemical detection system (Thermo Scientific, Waltham, MA) [17,18].

III. Saliva Assays

Sample Collection

Saliva samples are collected during the Psychophysiology Challenge Protocol to complement assessments of stress reactivity based on heart rate variability. Five samples are collected: pre-protocol, baseline, post-cognitive challenge, immediately post-orthostatic challenge, and 30 minutes post-protocol. The timing of saliva collections is represented by variables C4VS1T through C4VS5T in the data file. Samples are then frozen and shipped to the MIDUS Biocore lab for storage and subsequent cortisol assay. See "M3_P4 Psychophysiology Data Documentation" for details.

A. Assay Details – Neuroendocrine Marker

Salivary cortisol is assayed at UW-Madison's Institute for Clinical and Translational Research (ICTR) as described below. During MIDUS 2, the cortisol assays were performed by Dresden Lab Service (Dresden, Saxony, Germany) with IBL Cortisol ELISA kits (IBL-International GmbH, Hamburg, Germany).

The samples for this assay are collected on cotton swabs in salivettes (cat# 51.1534, Sarstedt, Nümbrecht, Germany) and frozen at -60°C to -80°C. At the time of assay, they are thawed and centrifuged at 3000rpm for 5min, resulting in a particulate-free, clear fluid of low viscosity. At the start of the COVID-19 pandemic, saliva was required to be heat-inactivated before assaying (65°C for 30min). ICTR tested and found that the treatment has no effect on the cortisol levels.

Salivary cortisol:

Salivary cortisol concentrations were determined using radioimmunoassay (cat# 0722110, MP Biomedicals, Solon, OH) per manufacturer instructions. Quality control was measured using two pools of human saliva [17]. The salivary cortisol assay changed over time, see Data Adjustments (section C-3) for more information.

SECTION C-2: ASSAY PERFORMANCE CHARACTERISTICS SUMMARY

P4 Bioassays from blood, urine, and saliva samples. Markers are ordered by physiologic system as described in Section A-1.

Assay	Specimen	Assay Type	Performed at	Assay Range	Variability	Reference Range
HbA1c	Whole blood	Immunoturbidometric	Meriter	4.2–20.1% [19]	inter-assay CV: 1.70% [26]	4.0–5.6% [19]
Total cholesterol	Serum	Enzymatic colorimetric	Meriter	3.86–8000 mg/dL [20]	inter-assay CV: 2.25% [26]	<200 mg/dL [20]
Triglycerides	Serum	Enzymatic colorimetric	Meriter	8.85–4425 mg/dL [21]	inter-assay CV: 1.30% [26]	<150 mg/dL [21]
HDL cholesterol	Serum	Enzymatic colorimetric	Meriter	3.09–150 mg/dL [22]	inter-assay CV: 1.15% [26]	≥40 mg/dL [22]
LDL cholesterol	Serum	Calculated	Univ. of Wisconsin (MIDUS Biocore)	N/A	inter-assay CV: 3.94% [26]	Optimal: <100mg/dL [27] Near optimal: 100–129 mg/dL Borderline high: 130–159 mg/dL
DHEA	Serum	HPLC-MS/MS	ARUP	0.05–40 ng/mL [23]	inter-assay CV: 3.33% [3] intra-assay CV: 3.34% [3]	Ages ≥40: 0.63–4.70 ng/mL [23] Postmenopausal: 0.60–5.73 ng/mL
DHEA-S	Serum	Immunoelectro-chemiluminescent	Associated Regional & University Pathologists (ARUP)	1–1000 µg/dL [24]	inter-assay CV: 1.4–4.1% [3] intra-assay CV: 1.0–1.6% [3]	Age Male Female [24] 45-54 44–331 35–256 55-64 52–295 19–205 65-74 34–249 9–246 ≥75 16–123 12–154
Creatinine	Serum	Enzymatic colorimetric	Meriter	0–122 mg/dL [25]	inter-assay CV: 2.14% [26]	Male: 0.50–1.20 mg/dL [25] Female: 0.50–1.10 mg/dL
IL-6	Serum	ELISA	Univ. of Wisconsin (MIDUS Biocore)	0.156–10 pg/mL [4]	inter-assay CV: 19.14% [26] intra-assay CV: 1.99% [26]	0.45–9.96 pg/mL [4]
IL-6 sR	Serum	ELISA	Univ. of Wisconsin (MIDUS Biocore)	31.2–2000 pg/mL [6]	inter-assay CV: 13.35% [26] intra-assay CV: 1.50% [26]	13,547–44,942 pg/mL [6]
IL-6	Serum	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS Biocore)	0.633–488 pg/mL [7]	inter-assay CV: 12.41% [26] intra-assay CV: 4.69% [26]	0.16–27.2 pg/mL [7]
IL-8	Serum	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS Biocore)	0.591–375 pg/mL [7]	inter-assay CV: 16.48% [26] intra-assay CV: 3.25% [26]	1.48–1720 pg/mL [7]
IL-10	Serum	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS Biocore)	0.298–233 pg/mL [7]	inter-assay CV: 14.56% [26] intra-assay CV: 7.29% [26]	0.06–3.08 pg/mL [7]
TNF-α	Serum	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS Biocore)	0.690–248 pg/mL [7]	inter-assay CV: 17.19% [26] intra-assay CV: 4.07% [26]	0.10–1.75 pg/mL [7]
Fibrinogen	Plasma	Nephelometry	Univ. of Vermont (Tracy Lab)	51–964 mg/dL [8]	inter-assay CV: 1.00% [8]	200–400 mg/dL [28]

Assay	Specimen	Assay Type	Performed at	Assay Range	Variability	Reference Range
CRP	Serum	Immunoelectro-chemiluminescent	Univ. of Vermont (Tracy Lab)	0.01–3860 µg/mL [8]	inter-assay CV: 6.14% [8]	<8 µg/mL [29]
sE-selectin	Serum	ELISA	Univ. of Vermont (Tracy Lab)	0.09–80 ng/mL [8]	inter-assay CV: 2.47% [8]	17.9–79.2 ng/mL [9]
sICAM-1	Serum	ELISA	Univ. of Vermont (Tracy Lab)	1.92–1000 ng/mL [8]	inter-assay CV: 0.33% [8]	98.8–320 ng/mL [10]
P1NP	Serum	RIA	Univ. of Wisconsin (Binkley Lab)	5–250 µg/L [14]	inter-assay CV: 5.5–9.5% [14] intra-assay CV: 3.2–9.6% [14]	Premenopausal female: [14] 19–83 µg/mL Postmenopausal female: 16–96 µg/mL Male: 22–87 µg/mL
BAP	Serum	ELISA	Univ. of Wisconsin (Binkley Lab)	0–140 U/L [15]	inter-assay CV: 5.2–7.6% [15] intra-assay CV: 3.9–5.8% [15]	Premenopausal female: [15] 11.6-29.6 U/L Postmenopausal female: 14.2-42.7 U/L Male: 15.0-41.3 U/L
NTx	Serum	ELISA	Univ. of Wisconsin (Binkley Lab)	3.2–40 n*M* BCE (bone collagen equivalents) [16]	inter-assay CV: 6.9% [16] intra-assay CV: 4.6% [16]	Premenopausal female: [16] 7.7–19.3 n*M* BCE Male: 8.1–24.8 n*M* BCE
***trans*-β-Carotene**	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 3.5% [13] intra-assay CV: <5% [13]	None established [13]
13-*cis*-β-Carotene	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 10% [13] intra-assay CV: <5% [13]	None established [13]
α-Carotene	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 4.5% [13] intra-assay CV: <5% [13]	None established [13]
Cryptoxanthin	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 4% [13] intra-assay CV: <5% [13]	None established [13]
Lutein	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 4% [13] intra-assay CV: <5% [13]	None established [13]
Zeaxanthin	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 6% [13] intra-assay CV: <5% [13]	None established [13]
Lycopene	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 0.2 picomole [13]	inter-assay CV: 6.5% [13] intra-assay CV: <5% [13]	None established [13]
Retinol	Plasma	HPLC/colorimetric	Tufts Univ. (Johnson Lab)	min 2 picomoles [13]	inter-assay CV: 3.5% [13] intra-assay CV: <5% [13]	None established [13]
α-Tocopherol	Plasma	HPLC/fluorometric	Tufts Univ. (Johnson Lab)	min 2.7 picomoles [13]	inter-assay CV: 5% [13] intra-assay CV: <5% [13]	None established [13]

Assay	Specimen	Assay Type	Performed at	Assay Range	Variability	Reference Range
γ-Tocopherol	Plasma	HPLC/fluorometric	Tufts Univ. (Johnson Lab)	min 2.7 picomoles [13]	inter-assay CV: 4.5% [13] intra-assay CV: <5% [13]	None established [13]
Glucose (fasting)	Serum	Enzymatic colorimetric	ARUP	2–750 mg/dL [3]	inter-assay CV: 0.5% [3] intra-assay CV: 0.2–0.5% [3]	<100 mg/dL [30]
Insulin	Serum	Immunochemiluminescent	ARUP	1–300 mU/L [3]	inter-assay CV: 2.4–3.6% [3] intra-assay CV: 1.5–2.7% [3]	3–25 µIU/mL [31]
IGF-1	Serum	Immunochemiluminescent	ARUP	10–1200 ng/mL [3]	inter-assay CV: 4.0–6.5% [3] intra-assay CV: 2.1–4.2% [3]	Age Range (ng/mL) [32] 25-39 114–492 40-54 90–360 ≥55 71–290
Creatinine	Urine	Colorimetric	ICTR	10–200 mg/dL [17]	inter-assay CV: 13.46% [17] intra-assay CV: 3.38% [17]	Male: 800–2000 mg/24h [33] Female: 600–1800 mg/24h
Cortisol	Urine	HPLC-MS/MS	ICTR	0.0293–15 µg/dL [17]	inter-assay CV: 11.72–14.78% [17] intra-assay CV: 4.50–5.31% [17]	10–100 µg/24h [34]
Cortisol/ creatinine ratio	Urine	Calculated	Univ. of Wisconsin (MIDUS Biocore)	N/A	N/A	Male: <32 µg/g [35] Female: <24 µg/g Pregnant: <59 µg/g
Cortisone	Urine	HPLC-MS/MS	ICTR	0.0293–15 µg/dL [17]	inter-assay CV: 12.31–16.71% [17] intra-assay CV: 6.22–11.41% [17]	Male: 17–141 µg/24h [36] Female: 15–122 µg/24h
Norepinephrine	Acidified urine	HPLC-ECD	ICTR	0.0975–50 µg/dL [17]	inter-assay CV: 10.27–16.63% [17] intra-assay CV: 9.64–15.81% [17]	0–100 µg/24h [37]
Epinephrine	Acidified urine	HPLC-ECD	ICTR	0.0975–50 µg/dL [17]	inter-assay CV: 12.65–19.42% [17] intra-assay CV: 8.53–13.47% [17]	0–15 µg/24h [37]
Dopamine	Acidified urine	HPLC-ECD	ICTR	0.0975–50 µg/dL [17]	inter-assay CV: 10.50–16.11% [17] intra-assay CV: 7.36–10.62% [17]	65–400 µg/24h [37]
Cortisol	Saliva	RIA	ICTR	0.69–68.98 n*M* [17]	inter-assay CV: 14.27–18.83% [17] intra-assay CV: 2.79–5.19% [17]	07:00–09:00 hours: [38] 2.76–20.69nM

SECTION C-3: MIDUS 3 DATA ADJUSTMENTS TO REFLECT ASSAY CHANGES

Overview:

MIDUS biomarker collection began with the MIDUS 2 phase in 2004. Since that time, there have been several substantial method changes to modernize some of the biomarker assays. To allow for comparison of values across study phases, method comparison analyses of those changes were performed. Samples were measured using both the old and new methods and the results were compared to derive a regression curve. For assays listed below that underwent substantial changes, harmonization adjustments are offered for comparison of published MIDUS 2 values with MIDUS 3.

I. Glycosylated hemoglobin (HbA1c)

During MIDUS 2, the HbA1c assay at Meriter labs underwent a change in calibration protocol on May 21, 2006. The current assay performed by Meriter Labs is NGSP-certified, the current standard for clinical HbA1c measurement in the USA. As a shift in values was noted following this change, duplicate samples were assayed using both protocols. A regression formula was derived to harmonize the MIDUS 2 values with the current technique used in the MIDUS 3.

Due to the noted shift in values, researchers interested in comparing MIDUS 2 HbA1c values to MIDUS 3 HbA1c values may choose to apply the following regression adjustment:

(MIDUS 3-harmonized HbA1c) = ((MIDUS 2 HbA1c) + 0.380) / 1.107 [39]

II. Dehydroepiandrosterone (DHEA)

During most of MIDUS 2, DHEA was assayed at ARUP Laboratories (Salt Lake City, UT) by radioimmunoassay (RIA). On May 18, 2009 ARUP changed their DHEA assay from RIA to HPLC-MS/MS. Duplicate RIA-assayed samples were retested using HPLC-MS/MS and a comparison regression was derived. The regression harmonizes MIDUS 2 values with the current technique used in MIDUS 3.

Due to the considerable changes in technique, researchers interested in comparing the MIDUS 2 DHEA values to MIDUS 3 DHEA values may choose to apply the following regression adjustment:

(MIDUS 3-harmonized DHEA) = ((MIDUS 2 DHEA) - 0.4668) / 1.6145 [40]

III. C-reactive protein (CRP)

In MIDUS 2, CRP was assayed with a nephelometric assay. Since 2016, all CRP assays have been moved to the MSD immunoelectrochemiluminescent platform. Regression analysis was performed comparing nephelometry to MSD values. The regression harmonizes MIDUS 2 with the current technique used in MIDUS 3.

Due to the considerable changes in technique, researchers interested in comparing MIDUS 2 CRP values to MIDUS 3 CRP values may choose to apply the following regression adjustment:

(MIDUS 3-harmonized CRP) = ((MIDUS 2 CRP) + 0.0841) / 0.7828 [41]

IV. Urinary creatinine

During MIDUS 2, urinary creatinine was assayed by Mayo Labs using an enzymatic colorimetric method. For all subsequent MIDUS phases, urine creatinine assays were performed by UW–Madison's ICTR using a kinetic colorimetric Jaffe reaction. A regression analysis comparing samples run using both methods was performed. The regression harmonizes MIDUS 2 values with the current technique used in MIDUS 3.

Due to the considerable changes in technique, researchers interested in comparing MIDUS 2 urine creatinine data to MIDUS 3 urine creatinine data may choose to apply the following regression adjustment:

(MIDUS 3-harmonized urine creatinine) = ((MIDUS 2 urine creatinine) + 5.4888) / 1.0019 [42]

Researchers that prefer to adjust MIDUS 2 urine creatinine values to MIDUS 3-harmonized (as shown above) should also recalculate the following urine creatinine-dependent calculated variables:

Ratio of serum creatinine / urine creatinine

Glomerular filtration rate (mL/min for 1.73 m^2 surface) =
urine volume (mL) * urine creatinine (mg/dL) / (serum creatinine (mg/dL) * 720 mins)

Ratio of urine cortisol (µg) / urine creatinine (g) = 1000 mg/g * urine cortisol (µg/dL) / urine creatinine (mg/dL)

Ratio of urine cortisone (µg) / urine creatinine (g) = 1000 mg/g * urine cortisone (µg/dL) / urine creatinine (mg/dL)

Ratio of urine norepinephrine (µg) / urine creatinine (g) = 1000 mg/g * urine norepinephrine (µg/dL) / urine creatinine (mg/dL)

Ratio of urine epinephrine (µg) / urine creatinine (g) = 1000 mg/g * urine epinephrine (µg/dL) / urine creatinine (mg/dL)

Ratio of urine dopamine (µg) / urine creatinine (g) = 1000 mg/g * urine dopamine (µg/dL) / urine creatinine (mg/dL)

V. Salivary cortisol

During MIDUS 2, salivary cortisol was measured by Dresden Lab Service with IBL Cortisol ELISA kits (IBL-International GmbH, Hamburg, Germany). For subsequent MIDUS phases, salivary cortisol assays have been run by UW-Madison's ICTR using radioimmunoassay kits from MP Biomedical (Solon, OH). Comparison studies were performed and regression equations derived to harmonize across methods. The regression harmonizes MIDUS 2 values with the current technique used in MIDUS 3.

Due to the considerable changes in technique, researchers interested in comparing MIDUS 2 salivary cortisol data to MIDUS 3 salivary cortisol may choose to use the following regression adjustment:

(MIDUS 3-harmonized salivary cortisol) = ((MIDUS 2 salivary cortisol) + 0.0544) / 0.7629 [43]

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