DOCUMENTATION

for

BLOOD, URINE, AND SALIVA DATA

in

MIDUS 2 BIOMARKER PROJECT (P4)

University of Wisconsin ♦ Institute on Aging
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INTRODUCTION

This document provides an overview of the tissue samples collected in the MIDUS 2 Biomarker Project (P4). It describes the protocols for collecting and processing tissue samples, and provides information about the construction and usage of related administrative and constructed variables. In addition, it contains descriptions of the biomarker assay methods including details about assay ranges, reference ranges, and inter-, and intra- assay covariations.

Data users are also encouraged to review the "M2 P4 Biomarker Project Data File Notes". This document provides information about naming conventions, as well as administrative and filter variables included in the data file. It also includes information about how we handled missing values and other issues that arose over the course of the study. For example, there are instances when variables were added or sections of an instrument were expanded for data entry purposes to accommodate additional information provided by the respondent.

This document will be periodically revised and updated as more information is gathered and researchers continue to work with the MIDUS 2 Biomarker data. If there are suggestions or comments, please submit a message through the MIDUS HelpDesk (http://midus.wisc.edu/helpdesk.php).

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SECTION A: OVERVIEW OF DATA AND COLLECTION PROTOCOLS

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

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

The biomarker data appear in the data file immediately following the Physical Exam data. As described in "M2 P4 Biomarker Project 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 the unique 3-character set "B4B".

All sample types are obtained at all three clinical research units (CRUs) located in UCLA, UW-Madison, and Georgetown. The remainder of this section provides general information and indicates where additional details can be found.

Revision in The 2024 Update Release

Three new urine assay measures are added to this update release, all as indicators for kidney health. One new blood assay, suPAR, is added as a biomarker associated with immune and inflammatory reaction.

- B4BALBUMIN: Urine albumin (mg/dL)
- B4BUACR: Albumin-Creatinine Ratio (uACR)
- B4BCYSTATINC: Urine Cystatin C (mg/L)
- B4BSUPAR: Soluble urokinase plasminogen activator receptor (suPAR)

Albumin and cystatin C concentrations were measured from aliquots of frozen 12-hour urine collection samples stored from MIDUS 2. The concentration of suPAR was measured from frozen serum samples stored from MIDUS 2. Detailed description of these assays can be found in Section C below.

Biomarker Data

The biomarkers reflect functioning of the hypothalamic-pituitary-adrenal axis, the autonomic nervous system, the immune system, cardiovascular system, musculoskeletal system, antioxidants, and metabolic processes. Our tissue specimens allow for assessment of multiple indicators within these major systems as follows:

- Fasting Blood Draws
    - Cardiovascular – Cholesterol Panel (Total, HDL, LDL) and Triglycerides
    - Hormone markers – DHEA and 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), soluble intercellular adhesion molecule 1 (sICAM-1), and soluble urokinase plasminogen activator receptor (suPAR)
    - Bone – BSAP (Bone Specific Alkaline Phosphatase), P1NP (aminoterminal propeptide type 1 procallagen), and NTx (n-teleopeptide type 1 collagen)

    - Antioxidant – All *trans*-beta-carotene, 13-*cis*-beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, lycopene, retinol, alpha-tocopherol, and gamma-tocopherol
    - Creatinine
    - Glucose Metabolism – Glycosylated hemoglobin (HA1c), glucose, insulin, and IGF-1 (Insulin-like Growth Factor-1)
- 12-Hour Urine
    - *Neuroendocrine* – Cortisol, Epinephrine, Norepinephrine, Dopamine, Creatinine, Albumin, and Cystatin C
- Saliva (Experimental Protocol)
    - *Neuroendocrine* – Cortisol

Administrative Variables

In addition to the biomarker assay results, this section of the data file also includes the following administrative, filter or computed variables:

- B4ZBLOOD, B4ZURINE, B4ZSALIVA – 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 and requires that participants notify nursing staff after each void. Thus, some voids are missed or incomplete for various reasons. The following variables can be used to identify problematic cases that data users may wish to examine more closely prior to including them in analyses
    - B4BUPROB – Urine collection period less than 11 hours or greater than 13 hours
    - B4BUSTRT – Urine collection: Start time
    - B4BUEND – Urine collection: End time
    - B4BUVDYN – Any voids not collected
    - B4BUVDN – Number of voids not collected

Computed Variables

The urine biomarker data were also used to create several additional measures. Details about the creation of the following variables can be found in the "Documentation for Psychosocial Constructs and Composite Variables":

- B4BNE12, B4BEPI12, B4BDOP12 – Epinephrine, Norepinephrine, and Dopamine adjusted to 12-hour values corresponding to the data collection period
- B4BNOCRE, B4BEPCRE, B4BDOCRE – Epinephrine, Norepinephrine, and Dopamine adjusted for creatinine
- B4BGFR – Glomerular filtration rate
- B4BUACR – Albumin-Creatinine Ratio (uACR)

Undetectable Assay Values: Sometimes lab values are reported as ">" or "<" than some value because the actual value falls outside the valid range for the assay. When this occurs, the reported value is replaced with a value 'one unit' below the minimal or maximal detectable score. For example:

If the lower limit was < 1.0, then we could change all of those scores to 0.9.
If the highest possible score was 120 for a particular test and was not normally reported with decimal values, then all the > 120 would be converted to 121.

Extremely high and low values, therefore, are curtailed. Consequently, the variance on the tails of the distribution is truncated. This is only problematic if a high percent of values falls in this category. The following assays included values outside the detectable range values, and they were adjusted as indicated:

- Cardiovascular measures:
    - HDL (B4BHDL) values > 120 mg/dL were adjusted to 121 mg/dL then LDL (B4BLDL) and the ratio of Total /HDL Cholesterol (B4BRTHDL) were computed
    - DHEAS (B4BDHEAS) values < 1 ug/dL were adjusted to 0.9 ug/dL
- Inflammation measures:
    - E-Selectin (B4BSESEL) values < 0.1 ng/mL were adjusted to 0.09 ng/mL
    - ICAM (B4BICAM) values < 45 ng/mL were adjusted to 44 ng/mL
- Urine Cortisol: there was variability in the minimum detectable level over time, thus (per recommendation of the BioCore Director), all cortisol values reported as "<" were adjusted to 0.019 ug/dL
- Urine Epinephrine values < 0.05 ng/mL adjusted to 0.009 ng/mL
- Dopamine: these values are more variable, thus, they require a different adjustment than the imputation rule, above. Thus, any values reported as ">" were adjusted to 450 ng/mL

Tissue Sample Collection and Processing

Standardized protocols for collecting and processing tissue samples were implemented at all three sites. Copies of the blood and urine collection and processing protocols followed by GCRC staff appear in Section B.

Details about the saliva sample collection are included in the documentation for the Laboratory Challenge Study conducted during the GCRC stay (see "M2 P4 Psychophysiology Protocol documentation").

Biomarker Assay Descriptions

Details about the methods used to assay the tissue samples appear in Section C. This section includes details about assay method and sensitivity for inclusion in manuscripts, as well as information about any changes in assay method and subsequent corrections to the data. The assays are listed first by tissue source (blood, urine or saliva) and then by physiological system assessed. A table summarizing assay ranges, reference ranges, and intra- and inter-assay co-variations appears next, followed by a list of references cited throughout this section.

SECTION B: TISSUE COLLECTION AND PROCESSING PROTOCOLS

BLOOD AND URINE COLLECTION PROTOCOL

PREPARATION

1. Prepare urine collection supplies

- Two urine collection containers (to hold at least 2 liters each)
  - CATS COLLECTION bottle **– ADD ACETIC ACID**
    - Add 25ml of 50% acetic acid to 1 urine collection container to acidify urine collected for catecholamine tests
    - Place **red** CATS label from packet on side of this container
  - CORT collection bottle – **NO ACID**
    - **Do not add acid** to this container!
    - Place **white** CORT label from packet (if MIDUS staff prepare labels) on side of collection container
- Hat or urinal

2. Label blood tubes with MIDUS ID labels

- Three 10-ml SST vacutainers (red/black, serum separator tubes)
- One 4-ml lavender top (EDTA tube) (6-ml at UW only)
- One 4-ml lavender top (EDTA tube), wrapped with aluminum foil
- One 4-ml or 2.7 ml blue top (sodium citrate tube)

URINE COLLECTION

At 1900:

- Instruct subject on 12-hour collection
- Provide urinal (men) or hat (women) and explain use
- Post reminder notice in bathroom
- Have subject void – DO NOT SAVE THIS SAMPLE
- Record date and time of void
- Instruct subject to collect all urine from then on until 0700 in specimen hat or urinal ***AND to call/notify nurse immediately after each void (even during the night)***

From 1900 until 700

- Pour **ONE HALF** of **EACH VOID** into each of the 2 urine collection containers
- Keep both collection containers in the refrigerator throughout the 12hr collection period
- **Sometime around 2100-2200***:* Remind subject (later in evening, before they go to bed) to collect urine all night
- **When subject wakes up** and /or when nurse comes in to draw blood): Remind subject to continue collecting all urine until 0700

At 0700 – Completion of urine collection

- Request final urine void from subject
- Pour **ONE HALF** into each of the 2 urine collection containers

- Let subject know that this is the end of the urine collection and there is no need to notify staff of any other voids
- Record date and time of final void
- Indicate any missed voids or urine not collected and reason urine not collected (e.g., subject refused or other reasons)
- Take urine to lab for processing and notify technician

BLOOD COLLECTION – COLLECTION OF FASTING BLOOD FROM 0630-0700

Preparing subject

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

Blood draw order

- Three 10-ml **red/black SST** tubes
- First 4-ml **lavender** (6-ml at UW only)
    - After draw, ***gently invert*** 3-5 times
- Second 4-ml **lavender** (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 up to what point in the tube the blood should be drawn

After draw, ***gently invert*** 5 times

- One 4-ml or 2.7-ml **blue** (citrated) tube
    - After draw, ***gently invert*** 3-5 times
- Complete specimen collection form
- Take blood tubes to lab for processing ASAP and record time on form
    - Place *lavender tube without foil* in MIDUS storage box in refrigerator **- DO NOT FREEZE**
    - Place *lavender tube with foil* **in ice bath**

BLOOD AND URINE PROCESSING PROTOCOL

URINE PROCESSING

- Measure volume in each of the 2 urine containers and record each on the form
- Calculate and record total volume on form
- From **CORT COLLECTION** bottle
    - Aliquot **11 ml** into each of **(2) 13-ml** plastic tubes with **white** labels
    - Aliquot **4 ml** into each of **(2) 5-ml** vials with **white** labels
- From **CATS COLLECTION** bottle (the one with RED labels)
    - Assess pH
    - If the pH is 5 or more, then add 1 ml of acetic acid and mix thoroughly with urine
    - Continue adding additional 1ml increments of acetic acid (and re-checking pH after each addition) until you get pH below 5. **Final pH must be below 5!** Record final pH on form
    - Once pH is below 5, aliquot **11 ml** into each of **(2) 13-ml** plastic tubes with **red** labels
- Store all urine samples in MIDUS box in GCRC freezer (-60 to –80) for MIDUS staff pick-up

BLOOD PROCESSING

- Keep foil-wrapped lavender in ice bath for 30-45 minutes
- **Blue** citrated tube
    - As soon as possible centrifuge vacutainers at 4°C for **15** minutes at **2000-3000 rpm** and record time begun
    - From **blue**, aliquot 1ml plasma into each of 2 **blue** labeled 2-ml vials
    - Store in MIDUS storage box in GCRC freezer (-60 to -80 degrees) for MIDUS staff pick up and record time frozen
- 3 10-ml **SST** tubes
    - Let stand for 15-30 minutes after draw (maximum of 2 hours allowed between draw and centrifuging)
    - Centrifuge vacutainers at 4°C for 20 minutes at 2000-3000 rpm and record time begun
    - From **SSTs**, aliquot 1ml sera into each labeled 2-ml vial as follows:
        Red – 2 vials
        White – 2 vials
        Green – 3 vials
        Orange – 5 vials
    - Store in MIDUS storage box in GCRC freezer (-60 to -80 degrees) for MIDUS staff pick up and record time frozen
- **Foil-wrapped** lavender tube
    - **Under dim lights**, centrifuge plasma at 4°C for **15** minutes at **1000g** and record time begun. Record time centrifuge begun
    - Aliquot **0.5 ml** plasma into **2 yellow** labeled 2-ml vials
    - Store in MIDUS storage box in GCRC freezer (-60 to -80 degrees) for MIDUS staff pick up and record time frozen

SECTION C: BIOMARKER ASSAY DOCUMENTATION

BIOMARKER ASSAY DESCRIPTIONS

Overview

The following provides information about laboratory assays of tissue samples collected as part of the MIDUS 2 Biomarker Project (Project 4) including details about assay method and sensitivity for inclusion in manuscripts. If the assay method changed during the 5-year data collection period, information about the change and any corrections to the data are also provided. 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 the assays 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 sensitivity appears at the end of this section.

I. Blood Assays

A: 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 GCRC 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 -65° C freezer until assayed.

- *Fresh whole blood* samples are refrigerated and shipped to the MIDUS BioCore Lab weekly and assayed for Hemoglobin A1c

- *Frozen serum and plasma* in 1 mL aliquots are shipped to the MIDUS BioCore Lab monthly for the following biomarker assays:
  - Cardiovascular markers – Cholesterol Panel
  - Hormone markers – DHEA and DHEA-S
  - Inflammation markers – IL-6, s-IL6-r, C-Reactive Protein, Fibrinogen, E-Selectin, ICAM, and suPAR
  - Anti-Oxidant markers – All trans-beta-carotene, 13-cis-beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, lycopene, retinol, alpha-tocopherol, and gamma-tocopherol
  - Bone Turnover markers – BSAP (Bone Specific Alkaline Phosphatase), NTx (n-teleopeptide type 1 collagen), and P1NP (aminoterminal propeptide type 1 procollagen)
  - Creatinine
  - Glucose metabolism markers - glucose, insulin, and IGF-1

B: Assay Details

B1: Assay Details - Cardiovascular Markers

The Hemoglobin A1c and Cholesterol panel (lipid) assays are performed at Meriter Labs (GML) (Madison, WI) using a Cobas Integra® analyzer (Roche Diagnostics, Indianapolis, IN).

***Glycosylated Hemoglobin**:*

The instrument hemolyzes the EDTA-anticoagulated whole blood specimen using a diluent with low osmotic pressure; the free hemoglobin is then degraded by pepsin, releasing the heme, which is converted to a brownish-green chromophore (alkaline hematin D-575). The color intensity is proportional to the hemoglobin concentration in the sample and is determined by monitoring the increase in absorbance at 552 nm.

Hemoglobin A1c is measured using monoclonal antibodies attached to latex particles, which bind the ß-N-terminal fragments of HbA1c. Remaining free antibodies are agglutinated with a synthetic polymer carrying multiple copies of the ß-N-terminal structure of HbA1c. The change in turbidity is inversely related to the quantity of bound glycopeptides and is measured turbidometrically at 552 nm.

Percent HA1c is determined by dividing the concentration of HA1c by the total hemoglobin.[1]

The HA1c assay changed twice during the data collection.

- On May 22, 2006, the calibration protocol at the reference lab was modified. The results of assays done after that date are adjusted, as follows, to bring the new values in line with the existing data:

  Adjusted value = 1.107(new value) - 0.380

- On February 19, 2007, the reference lab introduced a new enhanced reagent. The results of assays done after that date are adjusted, as follows, to bring them in line with the pre-May 22, 2006 data:

  Adjusted value = 1.266(new value) - 1.354

Cholesterol Panel (Lipid assays):

Cholesterol: The instrument uses the enzyme cholesterol esterase to cleave cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, this hydrogen peroxide effects the coupling of phenol and 4-aminoantipyrine to form a red quinone-imine dye. The color intensity of the dye is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance at 512 nm.[3]

HDL-cholesterol: Serum is first mixed with synthetic polyanions, which adsorb to the surfaces of the other lipoproteins (LDL, VLDL, and chylomicrons); they are thereby transformed into detergent-resistant forms, whereas HDL is not. After solubilizing the HDL with detergent, it is measured as described above for total cholesterol.[5]

The HDL assay was re-standardized by Roche Diagnostics on August 6, 2007. The results of assays done after that date are adjusted, as follows, to bring the new values in line with the existing data:

Adjusted value = 1.1423(new value) - 0.9028

Triglycerides: Triglycerides are hydrolyzed by the enzyme lipoprotein lipase to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase. The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide effects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red-colored quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of tryglycerides in the sample.[4]

LDL-cholesterol: An estimation of LDL-cholesterol level is made using the Fridewald formula from direct measurements of total cholesterol (TC), triglycerides (TG), and HDL-cholesterol. The Fridewald formula is as follows:

LDL = TC - HDL - TG/5

The Fridewald formula begins to be unreliable when triglycerides are elevated (≥ 350 mg/dl). Meriter adopts a conservative approach and will not report it if the subject's triglycerides level is ≥350 mg/dl. Therefore, when the triglycerides level is ≥ 350 mg/dl the BioCore lab will calculate LDL using the above formula.

If the subject's triglyceride level is > 400 mg/dl, then the BioCore lab will use 400 mg/dl as the upper limit for triglycerides when calculating LDL-cholesterol.[17]

B2. Assay Details – Hormone Markers

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

DHEA:

This was a radioimmunoassay performed using kit #DSL8900 from Diagnostic Systems Laboratories (Webster, TX). The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a nonradioactive antigen for a fixed number of antibody binding sites. The amount of [I125]-labeled DHEA bound to the antibody is inversely proportional to the concentration of the unlabeled DHEA present. The separation of free and bound antigen is easily and rapidly achieved using a double antibody system.[7]

On May 18, 2009, ARUP began using LC-MS/MS (Liquid Chromatography tandem Mass Spectrometry) to assay DHEA. Data collection ended on May 31, 2009; thus the final 75 samples were assayed under the new method. The data from this final set of assays were adjusted, as follows, to bring the new values in line with the existing data:

Adjusted value = 1.6145(new value) + 0.4668

DHEA-Sulfate:

This assay is performed with a Roche Modular Analytics E170 analyzer, using an Elecsys® kit (Roche Diagnostics, Indianapolis, IN).

By incubating the serum with a DHEA-S specific biotinylated antibody, an immunocomplex is formed, the amount of which is dependent upon the DHEA-S

concentration in the specimen. After addition of strepavidin-coated microparticles and a DHEA-S derivative labeled with a ruthenium complex, the still-vacant sites of the biotinylated antibodies become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into a measuring cell where the microparticles are magnetically captured onto the surface of an electrode. Unbound substances are removed. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.[18]

B3. Assay Details – Inflammation Markers

Cytokines and Il-6sr are assayed in the MIDUS BioCore Laboratory (University of Wisconsin, Madison, WI). The ICAM, E-Selectin, Fibrinogen and CRP assays are performed at the Laboratory for Clinical Biochemistry Research (University of Vermont, Burlington, VT).

IL6, IL8, IL10, and TNFα by Immunoelectrochemiluminescence: Note, these assays were completed using stored MIDUS 2 samples, after this set of assays was added to the MIDUS Refresher Biomarker protocol.

These cytokines were measured using a V-plex Custom Human Cytokine Kit (catalog #K151A0H-2) manufactured by Meso Scale Diagnostics, Rockville, MD. This technology employs a 96-well multispot plate, with each spot precoated 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 to be measured 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 special buffer is added, and the plate is loaded into an imager (Meso Scale Discovery Sector Imager Model #HTS24), 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.[23]

IL6 by ELISA:

IL6 is measured using the Quantikine® High-sensitivity ELISA kit #HS600B (R & D Systems, Minneapolis, MN). This is a sandwich ELISA using a microplate precoated with a monoclonal antibody specific for IL6. Standards and samples are pipetted into the wells, and any IL6 present is bound by the immobilized antibody. After washing away, the unbound substances, an alkaline phosphatase-labeled detection antibody specific for IL6 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 IL6 in the sample, is read at 490 nm using a Dynex MRXe plate reader (Magellan Biosciences, Chantilly, VA).[10]

Soluble Receptors for IL6 (IL6-sr):

Concentration of IL6-sr is measured using the Quantikine® ELISA kit #DR600 (R & D Systems, Minneapolis, MN). This is a sandwich ELISA using a microplate precoated with a monoclonal antibody specific for IL6-sr. Standards and samples are pipetted into the wells, and any IL6-sr is bound by the immobilized antibody. After washing away, the unbound substances, a horseradish peroxidase-labeled detection antibody specific for IL6-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 proportional to the concentration of IL6-sr in the samples.[12]

sICAM-I (Human Soluble Intercellular Adhesion Molecule-1):

sICAM-1 is measured by an ELISA assay (Parameter Human sICAM-1 Immunoassay; R&D Systems, Minneapolis, MN). sICAM-1 is sandwiched by an immobilized monoclonal antibody and the enzyme-linked monoclonal antibody. The amount of ICAM-1 present is determined by colorimetric reaction.

There were lot-to-lot changes in the sICAM-I assay over the course of the data collection period. Correction factors were applied to the data at the Tracy lab.

Soluble E-Selectin (sE-Selectin):

sE-selectin, also known as endothelial leukocyte adhesion molecule-1 (ELAM-1) and CD62E, is measured using a high sensitivity ELISA assay. (Parameter Human sE-Selectin Immunoassay; 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.

There were lot-to-lot changes in the sE-Selectin assay over the course of the data collection period. Correction factors were applied to the data at the Tracy lab.

Fibrinogen:

Fibrinogen antigen is measured using the BNII nephelometer (N Antiserum to Human Fibrinogen; Dade Behring Inc., Deerfield, IL). 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.

C-reactive protein (CRP):

CRP is measured using the BNII nephelometer from Dade Behring utilizing a particle enhanced immunonepholometric assay. Polystyrene particles are coated with monoclonal antibodies to CRP, which, in the presence of antigen (CRP) agglutinate to cause an increase in the intensity of scattered light. The increase in scattered light is proportional to the amount of CRP in the sample.

UPDATE - Samples falling below the assay range for CRP by this method were re-assayed by immunoelectrochemiluminescence using a high-sensitivity assay kit (Meso Scale Diagnostics #K151STG).[24]

Soluble urokinase plasminogen activator receptor (suPAR): Note, suPAR assays were completed using stored MIDUS 2 samples.

suPAR (soluble urokinase plasminogen activator receptor) is a biomarker associated with immune and inflammatory reactions. suPAR levels typically increase with age. Healthy individuals have suPAR levels falling in the range of 2 to 4 ng/mL. MSD's R-Plex Human U-PAR Assay kit[25] was utilized for the quantification of this biomarker. Blank MSD GOLD 96-well Small Spot Streptavidin plates were coated with R-Plex human U-PAR capture antibodies and left to incubate. After three washes to remove any unbound capture antibodies not fixated to the plate, thawed and diluted serum samples were added and incubated. After an additional three washes to remove any unbound sample antigens from the well, detection antibody was added which was conjugated with "SULFO-TAG" electrochemiluminescent labels and incubated. This detection antibody attaches to antibody-antigen complexes and allows for quantification of the desired analyte. The last incubation step was followed by another wash step to remove unbound detection antibodies. Read buffer was then added to the plate and analyzed with the MESO QuickPlex SQ 120 machine.

B4. Assay Details – Anti-oxidant Markers

The total trans-beta-carotene, 13-cis-beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein, zeaxanthin, lycopene, retinol, alpha-tocopherol, and gamma-tocopherol assays are performed at the Antioxidants Research Laboratory (Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA).

Plasma samples (1.0 mL) are extracted with chloroform:methanol, and internal standards for carotenoids, retinoids, and tocopherols are added, along with saline. A reverse-phase gradient HPLC method with UV/VIS and fluorescence detection is used for the simultaneous determination α- and γ-tocopherol, α-carotene, *cis*- and *trans*-β-carotene, β-cryptoxanthin, lutein, *cis*- and *trans*-lycopene, and zeaxanthin, in addition to retinol, according to a modification of the methods described by Ribaya-Mercado et al. (1992) and.Yeum et al. (1998).[19]

Citations:

Ribaya-Mercado JD, Fox JG, Rosenblad WD, Blanco MC, Russell RM. ß-Carotene, retinol and retinyl ester concentrations in serum and selected tissues of ferrets fed ß-carotene. *Journal of Nutrition* 1992;122:1898-1903.

Yeum KJ, Ahn SH, de Paiva SAR, Lee-Kim YC, Krinsky NI, Russell RM. Correlation between carotenoid concentrations in serum and normal breast adipose tissue of women with benign breast tumor or breast cancer. *Journal of Nutrition* 1998;128:1920-6.

B5. Assay Details – Bone Turnover Markers

The Bone Turnover assays (Bone Specific Alkaline Phosphatase, n-teleopeptide type 1 collagen, and aminoterminal propeptide type 1 procollagen), are performed in Dr. Neil Binkley's laboratory at the University of Wisconsin (Madison, WI).

n-teleopeptide type 1 collagen (NTx):

Osteomark® kits (Wampole Laboratories, Princeton, NJ) were used for all NTx analyses. This assay provides a quantitative measure of cross-linked N-telopeptides of type 1 collagen (NTx) in human serum as an indicator of bone resorption. This assay is a competitive-inhibition enzyme-linked immunosorbent assay (ELISA). The NTx epitope is adsorbed onto a 96 well microplate. Diluted samples are added to the microplate wells, followed by a horseradish peroxidase labeled monoclonal antibody. NTx in the study specimen competes with the NTx epitope in the microplate well for antibody binding sites. Following a wash step, the amount of labeled antibody bound is measured by colorimetric generation of a peroxide substrate. Absorbance is determined spectrophotometrically, and NTx concentration is calculated using a standard calibration curve. Assay values are reported in nanomoles bone collagen equivalents (nm BCE) per liter (nM BCE/L).

Aminoterminal propeptide type 1 procollagen (P1NP):

Orion Diagnostica RIA (catalog # 67034) was used for all P1NP analyses. This assay provides a quantitative measure of the concentration of aminoterminal propeptide of type 1 procollagen (P1NP) in human serum as an indicator of bone formation. Specifically, a sample containing an unknown amount of the substance to be assayed is mixed with a standard amount of a radioactively labeled derivative of the same substance. The labeled and unlabeled antigens are then allowed to compete for the limited number of high affinity binding sites on the antibody. The amount of radioactive antigen in the antigen-antibody complex is reversely proportional to the amount of unlabeled antigen in the reaction mixture. After separation of the free antigen from the antibody-antigen complex, the residual radioactivity is counted and the actual concentration is calculated with the aid of a standard curve based on known amounts of unlabeled antigen analyzed in parallel with the unknown.

Bone Specific Alkaline Phospatase (BSAP):

Metra BAP ELISA kits (catalog number 8012, Quidel Corporation, San Diego, CA) were used for all BSAP analyses. This procedure is an immunoassay in a microtiter strip format that utilizes a monoclonal anti-BSAP antibody coated on the strip to capture BSAP in the serum sample. The enzyme activity of the captured BSAP is subsequently detected with a pNPP substrate.

B6. Assay Details – Serum Creatinine

Creatinine is measured using a Cobas Integra® analyzer (Roche Diagnostics, Indianapolis, IN).

Serum Creatinine:

This assay employs the enzymes creatininase, creatinase, and sarcosine oxidase which react with creatinine and produce hydrogen peroxide as one of the end products. This reacts with aminophenazone and HT1B to form a quinine imine chromogen as an end product. The absorbance of this colored end product is measured at 552 nm and is directly proportional to the creatinine concentration in the serum.[9]

B7. Assay Details - Glucose Metabolism

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

Glucose:

This is an enzymatic assay using hexokinase to convert glucose to glucose-6-phosphate, and then glucose-6-phosphate dehydrogenase to convert glucose-6-

phosphate to gluconate-6-phosphate. This second reaction is coupled to the reduction of NADP+ to NADPH. The rate of NADPH formation is directly proportional to the glucose concentration and can be measured photometrically. This test is performed on an automated analyzer (Roche Modular Analytics P).[13]

Insulin:

The ADVIA Centaur Insulin assay (Siemens) is a two-site sandwich immunoassay using direct chemiluminescent technology which uses constant amounts of two antibodies. The first antibody is a monoclonal mouse anti-insulin antibody labeled with acridinium ester. The second antibody is a monoclonal mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles. A direct relationship exists between the amount of insulin present in the patient sample and the relative light units (RLUs) detected by the system. This assay is performed on a Siemens Advia Centaur analyzer.[14]

Insulin-like Growth Factor-1 (IGF-1):

IGF-1 is measured using a two-site sandwich immunoassay using antibody-coated beads for the first step and an enzyme-labeled antibody for the second step. Following the removal of unbound labeled antibody, a substrate is added, initiating a chemiluminescent reaction. A direct relationship exists between the concentration of IGF-1 in the sample and the light detected by the system. This assay is performed on a Siemens Immulite 2000 analyzer.[20]

II. Urine Assays

A. Sample Collection:

12-hour overnight (7:00 pm – 7:00 am) urine samples are collected from each participant during the GCRC visit. Prior to collecting the first sample GCRC staff prepare two containers. The first, for catecholamine assay, has 25 mL of 50% acetic acid added to it and is labeled “CATS – ACID”. The second, for cortisol assay, is labeled "No ACID". Each void is split between two urine collection containers. Details can be found in Section B.

13-mL aliquots of both acidified and untreated urine are stored in a -60^{o} C to -80^{o} C freezer and then shipped to the MIDUS BioCore lab on a monthly basis for neuroendocrine markers: catecholamines (epinephrine, norepinephrine, dopamine) and corticosteroids (cortisol, cortisone), as well as creatinine.

B: Assay Details

B1. Assay Details - Catecholamines

Catecholamine assays are performed at the Mayo Medical Laboratory (Rochester, MN).

Epinephrine, Norepinephrine, Dopamine:

High-Pressure Liquid Chromatography (HPLC) is used for Urinary Free Catecholamine Fractionation which includes unconjugated epinephrine, norepinephrine, and dopamine.

An aliquot of a 24-hour urine collection preserved in acid is extracted with ethyl acetate to remove acidic metabolites. A 1.0 mL aliquot of the extracted urine is absorbed on aluminum oxide at an alkaline pH and eluted with acid. The specimen is further purified by complexing with boric acid gel. The catecholamines are removed by washing with

boric acid. An aliquot of the boric acid eluate is injected onto a high-performance reverse-phase paired ion-chromatography column where the catecholamines are resolved into individual components. The catechol is oxidized electronically to an O-quinone. The current generated at the detector is converted by amplifier to a voltage signal and an XY recording is generated (Jiang and Machacek, 1987; Moyer et al, 1979).

Citations:

Jiang NS, Machacek D: Measurement of catecholamines in blood and urine by liquid chromatography with amperometric detection. In *Progress in HPLC*. Vol. 2. Edited by Parvez. VNU Science Press, 1987, pp 397-426

Moyer TP, Jiang NS, Tyce GM, Sheps SG: Analysis for urinary catecholamines by liquid chromatography with amperometric detection: methodology and clinical interpretation of results. *Clinical Chemistry* 1979;(25), 256-263

B2. Assay Details – Cortisol

Urine cortisol & cortisone assays are performed at the Mayo Medical Laboratory (Rochester, MN).

Cortisol & Cortisone:

Enzymatic Colorimetric Assay and Liquid Chromatography-Tandem Mass Spectrometry(LC-MS/MS) are used for Urine Free, Random, Cortisol/Cortisone assay.

Deuterated cortisol [d(3)-cortisol] is added to a 0.1-mL urine specimen as an internal standard. Cortisol, cortisone, and d(3)-cortisol are extracted from the specimens using on-line turbulent flow HPLC and analyzed by liquid chromatography-tandem mass spectrometry using multiple reaction monitoring in positive mode. The following ion pairs are used for analysis: Cortisol (363.0/121.1); Cortisone (361.0/163.0); d(3)-Cortisol (366.0/121.2). A calibration curve, generated from stripped urine spiked standards, is included with each batch of patient specimens (Taylor et al, 2002).

Citations:

Taylor RL, Machacek DA, Singh RJ: Validation of a high-throughput liquid chromatography-tandem mass spectrometry method for urinary cortisol and cortisone. *Clinical Chemistry* 2002; 48:1511-1519)

B3. Assay Details – Creatinine

Urine creatinine assays are performed at the Mayo Medical Laboratory (Rochester, MN).

Creatinine:

Enzymatic Colorimetric Assay is used for urine creatinine assay. This assay employs the enzymes creatininase, creatinase, and sarcosine oxidase which react with creatinine and produce hydrogen peroxide as one of the end products. The liberated hydrogen peroxide is measured via a modified Trinder reaction using a colorimetric indicator. Optimization of the buffer system and the colorimetric indicator enables the creatinine concentration to be quantified both precisely and specifically. (Package Insert: Roche Diagnostics,Indianapolis IN, 2004)

B4. Assay Details – Albumin and Cystatin C

Urine albumin and cystatin C are assayed in the MIDUS BioCore Laboratory (University of Wisconsin – Madison) using stored MIDUS 2 samples.

Albumin and Cystatin C:

Albumin and cystatin C are both biomarkers of kidney health and can be found in the blood and detected in urine. High levels of urinary albumin (albuminuria) can indicate poor kidney filtration since this organ normally prevents the flow of albumin into the urine. High levels of urinary cystatin C can indicate that the kidney tubules are not reabsorbing and catabolizing cystatin C. Albumin and cystatin C concentrations were measured from aliquots of frozen 12-hour urine collection samples derived from the M2 and MR1 phases of MIDUS. The BioCore designed a multiplex sandwich immunoassay that simultaneously tested these two analytes through electrochemiluminescent technology. MSD's R-Plex capture antibodies for both cystatin C[29] and albumin[27] were coated to the U-Plex Development SECTOR Plates (Meso Scale Discovery; Rockville, MD). Capture antibodies were attached to opposite ends of each individual well to help reduce signal overlap. Following three rinses with MSD wash buffer, thawed urine samples were diluted and added to the coated plate. After sample incubation and additional wash step, detection antibodies were added to each well that were conjugated with "SULFO-TAG" electrochemiluminescent labels. After a 1-hour incubation with the detection antibody, the plate was rinse 3 times with MSD wash buffer. Read buffer was then added to the plate and analyzed with the MESO QuickPlex SQ 120 instrument.

III. Saliva Assays

A. Sample Collection:

Saliva samples are collected during the Psychophysiology Challenge Protocol to complement assessments of stress reactivity based on Heart Rate Variability. Four samples are collected (baseline, post-cognitive challenge, immediately post-orthostatic challenge, and 30-minute post-orthostatic challenge). Samples are then frozen and shipped to the MIDUS BioCore lab for storage and subsequent Cortisol assay. See in the Psychophysiology Data documentation for details.

B: Assay Details

B1. Assay Details – Saliva Cortisol

Salivary Cortisol:

The samples for this assay are collected on cotton swabs in salivettes (Sarstedt Cat. #51.1534) and frozen. At the time of assay, they are thawed and centrifuged at 3000 rpm for 5 min, resulting in a particulate-free, clear fluid of low viscosity. Concentrations of free cortisol (the only type found in saliva) were determined using an immunoluminescence assay (kit #RE62011 manufactured by IBL International, Hamburg, Germany). Briefly, this assay uses a detection antibody conjugated to an agent capable of oxidizing luminol to 3-aminophthalic acid; this reaction emits light, which can be quantified, and which is proportional to the concentration of cortisol in the sample.[21]

ASSAY SENSITIVITY SUMMARY TABLE

A. Biomarker Bioassays from blood samples (specimen type)

Assay	Assay Type	Performed at	Assay Range[22]	Variability	Reference Range
HA1c (blood)	Immunoturbidometric	Meriter	2.4-75.8 mg/dL	inter-assay CV: 1.1-3.4% intra-assay CV: 0.43%	4.0-5.9%[28]
Total Cholesterol (serum)	Enzymatic colorimetric	Meriter	0-800 mg/dL	inter-assay CV: 2.65% intra-assay CV: 0.51-0.81%	150-199mg/dL[31]
Triglycerides (serum)	Enzymatic colorimetric	Meriter	0-875 mg/dL	inter-assay CV: 1.01% intra-assay CV: 1.6%	Male: 40-160mg/dL[28] Female: 35-135mg/dL[28]
HDL-cholesterol (serum)	Enzymatic colorimetric	Meriter	0-155 mg/dL	inter-assay CV: 6.52% intra-assay CV: 1.1-1.4%	Male: >45mg/dL[28] Female: >55mg/dL[28]
LDL-cholesterol (serum)	Calculated	Univ. of Wisconsin (MIDUS Biocore Lab)	N/A	inter-assay CV: 10.11%	<130mg/dL[28]
DHEA-sulfate (serum)	Immunoelectro-chemiluminescent	Associated Regional & University Pathologists (ARUP)	0.1-1000 µg/dL[6] min 1 µg/dL	inter-assay CV: 2.9% intra-assay CV: 0.8-3.8%	Males: 1.3-5.5mg/mL[31] Females: 0.6-3.3mg/mL[31]
DHEA (serum)	RIA	ARUP	0.2-2.26 ng/mL min 0.1 ng/mL	inter-assay CV: 5.47% intra-assay CV: 2.7-3.8%	Ages ≥40: 0.63-4.70 ng/mL [28,32] Postmenopausal: 0.60-5.73 ng/mL[28,32]
Creatinine (serum)	Enzymatic colorimetric	Meriter	0-30.5 mg/dL	inter-assay CV: 0.00%	Male: 0.6-1.2mg/dL[28] Female: 0.5-1.1mg/dL[28]
IL-6 (serum)	ELISA	Univ. of Wisconsin (MIDUS BioCore Lab)	0.156-10 pg/mL min 0.156 pg/mL	inter-assay CV: 12.31% intra-assay CV: 3.25%	0.45-9.96pg/mL[10, 11]
IL6-sr (serum)	ELISA	Univ. of Wisconsin (MIDUS BioCore Lab)	31.2-2000 pg/mL min 31.2 pg/mL	inter-assay CV: 6.78-7.34% intra-assay CV: 1.39%	13,547-44,942 pg/mL[12]
IL-6 (serum)	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	1.58-488 pg/mL min 0.06 pg/mL	inter-assay CV: 5-15 % intra-assay CV: 4.73 %	0.16-27.2 pg/mL[11, 23]
IL-8 (serum)	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	1.13-375 pg/mL min 0.04 pg/mL	inter-assay CV: 6-7 % intra-assay CV: 2.88 %	1.48-1720 pg/mL[23]
IL-10 (serum)	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	0.68-233 pg/mL min 0.03 pg/mL	inter-assay CV: 11-14 % intra-assay CV: 5.78 %	0.06-3.08 pg/mL[23]
TNF-α (serum)	Immunoelectro-chemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	0.69-248 pg/mL min 0.04 pg/mL	inter-assay CV: 7 % intra-assay CV: 3.19 %	0.1-1.75 pg/mL[23]
Fibrinogen (citrated plasma)	Immunoturbidometric	Univ. of Vermont (Tracy Lab)	60-1200 mg/dL	inter-assay CV: 2.6% intra-assay CV: 2.7%	200-400 mg/dL[8, 28]
CRP (citrated plasma)	Immunoturbidometric	Univ. of Vermont (Tracy Lab)	0.175-1100 µg/mL min 0.15 µg/mL	inter-assay CV: 2.1-5.7% intra-assay CV: 2.3-4.4%	<1.0 mg/dL[28]

Assay	Assay Type	Performed at	Assay Range[22]	Variability	Reference Range
CRP (serum)	Immunoelectro-chemiluminescent	Univ. of Vermont (Tracy Lab)	0.014-216 µg/mL min 10^{-6} µg/mL	inter-assay CV: 4.72-5.16 % intra-assay CV: 2.2-4.1	<1.0 mg/dL[28]
suPAR	Electrochemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	0.01-50 ng/mL	inter-assay CV: 4.96% intra-assay CV: 2.65%	2-4 ng/mL[26]
sE-Selectin (serum)	ELISA	Univ. of Vermont (Tracy Lab)	0.47-10.52 ng/mL min 0.1 ng/mL	inter-assay CV: 5.7-8.8% intra-assay CV: 4.7-5.0%	17.9-79.2 ng/mL[34]
sICAM-1 (serum)	ELISA	Univ. of Vermont (Tracy Lab)	2.73-49.55 ng/mL min 0.35 ng/mL	inter-assay CV: 5.0%	98.8-320 ng/mL[38]
P1NP (serum)	RIA	Univ. of Wisconsin (Binkley Lab)	5-250 µg/L min 2 µg/L	inter-assay CV: 2.46% intra-assay CV: 6.5%	Premenopausal female: 19-101 µg/L[28] Postmenopausal female: 16-96 µg/L[28] Male: 22-105 µg/L[28]
sBAP (serum)	ELISA	Univ. of Wisconsin (Binkley Lab)	0-140 U/L min 0.7 U/L	inter-assay CV: 4.46-6.26% intra-assay CV: 5.8%	Premenopausal female: 11.6-29.6 U/L[39] Postmenopausal female: 14.2-42.7 U/L[39] Male: 15.0-41.3 U/L[39]
sNTx (serum)	ELISA	Univ. of Wisconsin (Binkley Lab)	3.2-40 n*M* BCE (bone collagen equivalents)	inter-assay CV: 7.75-9.06% intra-assay CV: 4.6%	Female: 6.2-19.0 nM BCE[28] Male: 5.4-24.2 nM BCE[28]
***trans*-β-Carotene (EDTA-plasma)**	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.03 µ*M*	inter-assay CV: 1-5% intra-assay CV: 7-11%	None established
13-*cis*-β-Carotene (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.03 µ*M*	inter-assay CV: 6-16% intra-assay CV: 9-13%	None established
α-Carotene (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.01 µ*M*	inter-assay CV: 2-4% intra-assay CV: 7-11%	None established
β-Cryptoxanthine (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.03 µ*M*	inter-assay CV: 5% intra-assay CV: 7-11%	None established
Lutein (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.03 µ*M*	inter-assay CV: 4-6% intra-assay CV: 6-8%	None established
Zeaxanthine (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.01 µ*M*	inter-assay CV: 5-7% intra-assay CV: 6-10%	None established
Lycopene (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.02 µ*M*	inter-assay CV: 4-8% intra-assay CV: 8-12%	None established
Retinol (EDTA-plasma)	HPLC/colorimetric	Tufts Univ. (Blumberg Lab)	min 0.04 µ*M*	inter-assay CV: 2-4% intra-assay CV: 3-7%	32.5-78µg/dL[2, 33]
α-Tocopherol (EDTA-plasma)	HPLC/fluorometric	Tufts Univ. (Blumberg Lab)	min 1.18 µ*M*	inter-assay CV: 4-6% intra-assay CV: 6-10%	5.5-17mg/L[33]

Assay	Assay Type	Performed at	Assay Range[22]	Variability	Reference Range
γ-Tocopherol (EDTA-plasma)	HPLC/fluorometric	Tufts Univ. (Blumberg Lab)	min 0.11 μ*M*	inter-assay CV: 2-6% intra-assay CV: 6-10%	None established
Fasting glucose	Enzymatic colorimetric	ARUP	2-750 mg/dL min 2 mg/dL	inter-assay CV: 1% intra-assay CV: 1%	18-59 yr: 74-106mg/dL[28] 60-90 yr: 82-115mg/dL[28] >90 yr: 75-121mg/dL[28]
Fasting insulin	Immunochemiluminescent	ARUP	1-300 uIU/mL min 1 uIU/mL	inter-assay CV: 2.4-4.6% intra-assay CV: 2.5-4.0%	6-26 μU/mL[28]
IGF-1	Immunochemiluminescent	ARUP	25-1600 ng/mL[15] min 17.6 ng/mL[15]	inter-assay CV: 4.4-6.8% intra-assay CV: 2-5%[24]	42-110ng/mL[28]

B. Biomarker Bioassays from urine and saliva samples (specimen type)

Assay	Assay Type	Performed at	Assay Range	Variability	Reference Range
Creatinine (urine)	Enzymatic colorimetric	Mayo	min 2 mg/dL	inter-assay CV: 0.85%	Male: 800–2000 mg/24h [37] Female: 600–1800 mg/24h[37]
Cortisol/creatinine ratio (urine)	HPLC, mass spectrometry	Mayo	N/A	inter-assay CV: 5.25%	Male: <32 μg/g[35] Female: <24 μg/g[35] Pregnant: <59 μg/g[35]
Cortisol (urine)	calculated	Univ. of Wisconsin (MIDUS BioCore Lab)	min 0.08 μg/dL	inter-assay CV: 6.1%	<100 μg/24hr[28]
Cortisone/creatinine ratio (urine)	HPLC, mass spectrometry	Mayo	N/A	inter-assay CV: 2.01%	N/A
Cortisone (urine)	calculated	Univ. of Wisconsin (MIDUS BioCore Lab)	min 0.08 μg/dL	inter-assay CV: 2.86%	Male: 17–141 μg/24h [36] Female: 15–122 μg/24h[36]
Albumin (urine)	Electrochemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	0.000084-2 mg/dL	inter-assay CV: 6.26-7.34% intra-assay CV: 7.64%	<2 mg/dL[28]
Cystatin C (urine)	Electrochemiluminescent	Univ. of Wisconsin (MIDUS BioCore Lab)	0.00036-0.46 mg/L	inter-assay CV: 4.35-5.56% intra-assay CV: 9.2%	0.03-0.18 mg/L[30]
Norepinephrine (acidified urine)	HPLC, electrochemical	Mayo	min 0.1 ng/mL	inter-assay CV: 6.7-6.9% intra-assay CV: 8%[37]	<100μg/24hr[28]
Epinephrine (acidified urine)	HPLC, electrochemical	Mayo	min 0.1 ng/mL	inter-assay CV: 7.8-7.9% intra-assay CV: 8%[37]	<20 μg/24hr[28]
Dopamine (acidified urine)	HPLC, electrochemical	Mayo	min 0.1 ng/mL	inter-assay CV: 6.0-7.6% intra-assay CV: 8%	65-400 μg/24hr[28]
Cortisol (saliva)	Immunochemiluminescent	Dresden Lab Service, Dresden, Germany (Kirschbaum Lab)	min 0.43 n*M*[16]	inter-assay CV: 15% intra-assay CV: 3%	7am-9am: 100-750ng/dL[28] 3pm-5pm: <401ng/dL[28] 11pm-midnight: <100ng/dL[28]

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