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Add Health Wave V Documentation

Add Health
The National Longitudinal Study of Adolescent to Adult Health

Measures of Inflammation and Immune Function

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This document summarizes the rationale, equipment, protocol assays, internal quality control, data cleaning, external quality control, and procedures for the measurement and classification of inflammation at the Wave V home exam. Whenever possible, data collection and methods in Wave V mirrored those of Wave IV to ensure comparability of data between waves, although important inter-Wave differences exist and are grey-highlighted herein. This document is one in a set of Wave V user guides. User guides are also available to describe protocols for the following biological measures in Wave V:

- Anthropometrics
- Cardiovascular Measures
- Medication Use – Home Exam
- Baroreflex Sensitivity & Hemodynamic Recovery
- Glucose Homeostasis
- Lipids
- Renal Function

Additional assays were subsequently performed using archived blood samples from Wave V to mirror those measures that will be analyzed in Add Health Wave VI. None of these additional assays were performed during Wave IV. Those user guides include:

- Hepatic Injury
- Neurodegeneration

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Citation

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1. Introduction

Wave IV measures of inflammation were based on dried blood spots collected using capillary finger prick.[1] In contrast, Wave V measures of inflammation were based on venous blood collected via phlebotomy. The blood was collected by field examiners (FEs) certified in phlebotomy, chilled at 4°C during the remainder of the home exam, centrifuged immediately afterward, aliquoted into transport tubes, and then sent overnight to a laboratory for assay.

Original Assayed Measures of Inflammation

- High Sensitivity C-Reactive Protein [hsCRP] (mg/L)

Moreover, the restricted use Add Health Wave V data include twelve constructed measures designed to facilitate its analysis and interpretation:

- Classification of hsCRP[2]
- Count of common subclinical symptoms[3]
- Count of common infectious or inflammatory diseases
- NSAID/Salicylate medication use in the past 24 hours
- NSAID/Salicylate medication use in the past 4 weeks
- Cox-2 Inhibitor medication use in the past 4 weeks
- Inhaled Corticosteroid medication use in the past 4 weeks
- Corticotropin/Glucocorticoid medication use in the past 4 weeks
- Anti-rheumatic/Anti-psoriatic medication use in the past 4 weeks
- Immunosuppressive medication use in the past 4 weeks
- Any Anti-inflammatory medication use in the past 4 weeks
- Flag Indicating the hsCRP Concentration Type

Subsequent to the original measures of hsCRP, additional measures of inflammation were assayed to mirror those assays that will be performed during Wave VI. Samples were thawed at a later date and assayed for the following inflammatory biomarkers.

Additional Measures of Inflammation

- Interleukin 1 beta [IL-1β] (pg/ml)
- Interleukin 6 [IL-6] (pg/ml)
- Interleukin 8 [IL-8] (pg/ml)
- Interleukin 10 [IL-10] (pg/ml)
- Tumor Necrosis Factor alpha [TNF-α] (pg/ml)
- Cytomegalovirus [CMV] (U/ml)

The restricted use Add Health Wave V data for these additional measures include seven constructed measures designed to facilitate analysis and interpretation:

- Flag Indicating the IL-1β Concentration Type
- Flag Indicating the IL-6 Concentration Type
- Flag Indicating the IL-8 Concentration Type
- Flag Indicating the IL-10 Concentration Type
- Flag Indicating the TNF-α Concentration Type
- Classification of CMV concentration
- CMV Z-transformations

2. General Overview of Data Collection

All Wave V venous blood samples were collected during home exams performed by FEs from two Add Health data collection partners: Examination Management Services, Inc. (2016-2017) and Hooper Holmes, Inc. (2018-2019). All FEs were trained and certified using a custom program specific to the Add Health protocol. FEs used a 7" Samsung Galaxy Tab 4 tablet to record and transmit data. An Add Health data collection application (Open Data Kit or ODK) installed on the tablet guided the FEs through the home exam protocol. In addition, FEs received a series of job aids, both on paper and on the tablet, to serve as quick reference guides when completing the protocol. Each tablet also contained an in-depth Add Health training manual that could be accessed at any time.

FEs conducted home exams among previously consented respondents. All FEs were phlebotomy-certified and had at least two years of experience collecting venous blood. Before home exams, FEs were sent a Visit Supply Kit that included a box for shipping blood to the lab and a Blood Collection Kit containing most required materials for the blood collection. FEs supplied additional materials, as needed (see section 3.2). Protocols for blood collection were dictated to FEs by the handheld 7" Samsung tablet used during all home exams. The tablet gave step-by-step directions for the blood collection and required FEs to enter information about the blood draw for each respondent. All respondents had the option to decline part or all of the blood draw, although declining did not affect their ability to participate in the rest of the home exam. Overall, 91.8% of the respondents agreed to and completed the blood draw. Of the remainder, 6.5% refused, 1.3% agreed but the blood draw was unsuccessful, and < 1% had exams terminated before the blood draw (see the blood draw status variable **H5BLOOD** in the *bdemo5* data set and codebook).

Blood collection was the last step in the home exam. Afterwards, all collection tubes were inverted 8-10 times to distribute the blood and contents of the tubes and then chilled at 4^{o} C (on ice or frozen cold packs) for up to two hours. Subsequent processing involved centrifuging specific tubes then aliquoting serum and plasma into color-coded transport tubes pre-labelled with unique barcode identifiers linking the blood to a particular respondent. Then the transport tubes were packaged in a Styrofoam Box with frozen cold packs and shipped overnight via FedEx to the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont. Overnight shipment enabled receipt by LCBR before 10:30 am the next morning. Upon receipt, LCBR documented the arrival of the transport tubes, evaluated their condition, processed them, and either assayed the specimens or aliquoted and archived them in -80°C freezers.

3. Blood Collection

3.1 Rationale

Venous blood was collected to provide Add Health with the biological specimens necessary to assay and interpret a pre-specified panel of metabolic, hematologic, inflammatory, immune, and renal biomarkers. It also was collected to establish an archive of serum, plasma, whole blood, RNA, and packed cells capable of supporting future assays and ancillary studies including those of the inflammatory biomarkers described herein.

3.2 Equipment

Before exams, FEs were shipped a Visit Supply Kit (Exhibit 1) including (1) a cardboard Shipping Box with an inner Styrofoam Box and two cold packs for shipping collected samples to LCBR, (2) a large Tyvek envelope in which to ship the Shipping Box, and (3) a Blood Collection Kit for collecting blood. The Blood Collection Kit contained:

- Biohazard-labelled Ziploc bag
- Latex-free gloves
- 2”x2” gauze
- Latex-free, Band-Aid type adhesive dressings
- Latex-free, strap tourniquet
- Alcohol prep pads, disposable pipets
- Single-use vacutainer (blood collection) tube holder
- 21-gauge Eclipse straight needle
- 21-gauge butterfly needle
- (3) disposable 3 ml graduated transfer pipets
- (2) 8.5 ml serum separation transport (SST) vacutainer tubes
- (1) 6 ml sodium fluoride/potassium oxalate (NaFl/KOx)-containing vacutainer tube, if needed for the glucose sub-study (see Section 4.1.2.1)
- (1) 3- or 4- ml potassium ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tube
- (1) 10 ml EDTA-containing vacutainer tube
- (1) 10 ml PAXgene vacutainer tube (containing 7.5 ml of preservative)
- (4) 10 ml transport tubes with color coded caps
- Extra barcode labels

BD Biosciences (San Jose, CA) supplied all the vacutainer and transport tubes. As of February 2018, their 3 ml EDTA vacutainer tube (Cat #367835) was no longer available, so Add Health switched to the 4 ml EDTA vacutainer tube (Cat #367844).

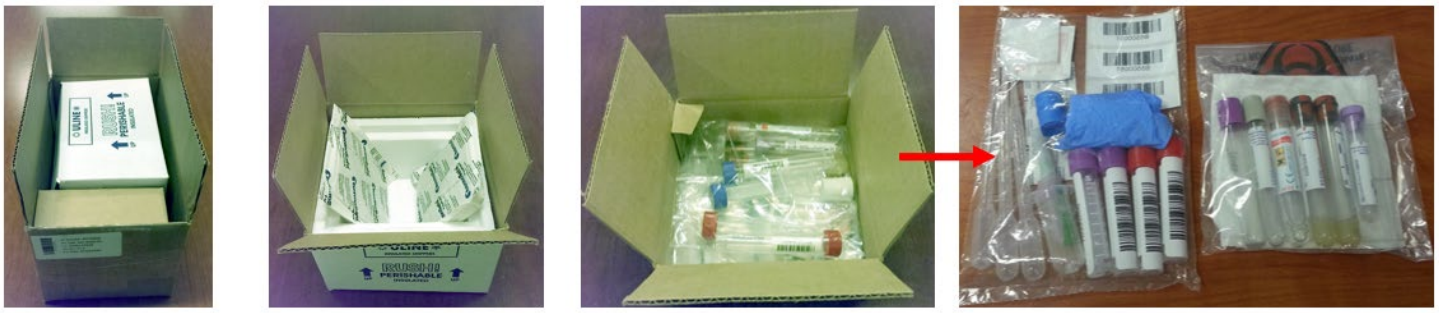


Exhibit 1. Visit Supply and Blood Collection Kits

FEs were responsible for providing ancillary materials for each home exam, including but not limited to a chux-type absorbent under pad, a sharps container, and a cooler with cold packs for keeping samples cold before packaging and shipping them to LCBR.

3.3 General Protocol

3.3.1 Blood Collection

The blood draw was performed as the final stage of the home exam following collection of anthropometric, cardiovascular, and medication information. After confirming respondents were comfortable giving blood, respondents were asked to either sit or recline at their discretion. They also were asked if they had problems in the past with blood collection such as fainting, bleeding, or hard-to-find veins. FEs were instructed to ensure the blood collection area was private, uncluttered, and fully prepared before beginning the blood draw. Preparation involved placing the chux pad, organizing the vacutainer tubes/supplies, preparing the cooler to accept the blood samples, and scanning the barcode located on the outside of the Blood Collection Kit. Scanning it automatically captured a unique, eight-digit code, thereby linking the respondent to the transport tubes / labels within it, the corresponding ODK questionnaire data, and ultimately to LCBR results.

Following standard phlebotomy protocols, FEs asked respondents to identify an arm for collecting blood, applied the tourniquet to that arm, and identified a vein in the antecubital fossa for venipuncture. If no vein appeared suitable, FEs asked to try the opposite arm. Unless respondents had objections, venipuncture was performed on the best potential vein and whole blood was collected, as summarized below:

- Put on nitrile gloves.
- Have the respondent extend his/her arm on the protective pad, palm up and straight at the elbow.
- Inspect the arm. Do not draw blood from an arm that has a rash, open sore, is swollen or shows signs of a recent venipuncture or hematoma. Do not draw blood from an arm that contains an arterial access such as a fistula or shunt.

- Apply the tourniquet several inches above the elbow and palpate for a suitable vein.
- Select a vein that is palpable and well-fixed to surrounding tissue.
- Open the needle assembly unit and attach it to the vacutainer holder.
- Ask the respondent to make a tight fist. Cleanse the area with an alcohol wipe using a circular motion and allow the area to air dry.
- Remove the cover from the needle.
- The vein should be fixed or held taut during the puncture. Push the needle firmly and deliberately into the vein. When firmly in the vein, blood appears in the tubing of the needle assembly past the end of the needle.
- Quickly push the first vacutainer tube (using the sequence in the table above) onto the needle in the holder, puncturing the center of the stopper.
- Release the tourniquet after the flow is established or if the respondent becomes uncomfortable. The respondent may open his/her fist once blood flow is established.
- When the first vacutainer tube is filled to capacity, remove it from the holder and place the next vacutainer tube in the holder.
- Gently invert each vacutainer tube 8-10 times immediately upon removing each one and while filling the next one. Repeat until all the desired vacutainer tubes are filled.
- Place all filled vacutainer tubes directly into a cooler with ice or ice packs.
- When the last vacutainer tube is filled, remove the tourniquet, carefully withdraw the needle, and cover the venipuncture site with a sterile gauze pad.
- Never apply pressure to the gauze until the needle is clear of the puncture site and away from the arm.
- Have the respondent hold the gauze pad with mild pressure and sit quietly for a few minutes.
- Slide the needle safety guard forward to prevent an accidental needle stick. Discard the entire used needle assembly in a sharps container.
- Check the venipuncture site. If it is adequately clotted, remove the gauze and apply a bandage. If after a few minutes, bleeding continues keep direct pressure on the site for 5 minutes.
- Encourage the respondent to sit quietly for a few minutes. Due to a fasting blood draw encourage the respondent to eat a snack if needed.

When the first attempt at blood collection was unsuccessful, FEs were allowed to ask to draw blood from the opposite arm. However, no more than two blood collection attempts were permitted. Moreover, only the antecubital fossa was acceptable for blood draw. FEs were not allowed to collect blood from any other sites, such as the back of the hand.

Either 5 or 6 tubes of blood were collected per respondent, depending on eligibility for a separate glucose sub-study (see Measures of Glucose Homeostasis User Guide, Section 4.1.2.1). Collection order, tube type, and processing information are listed below (Exhibit 2).

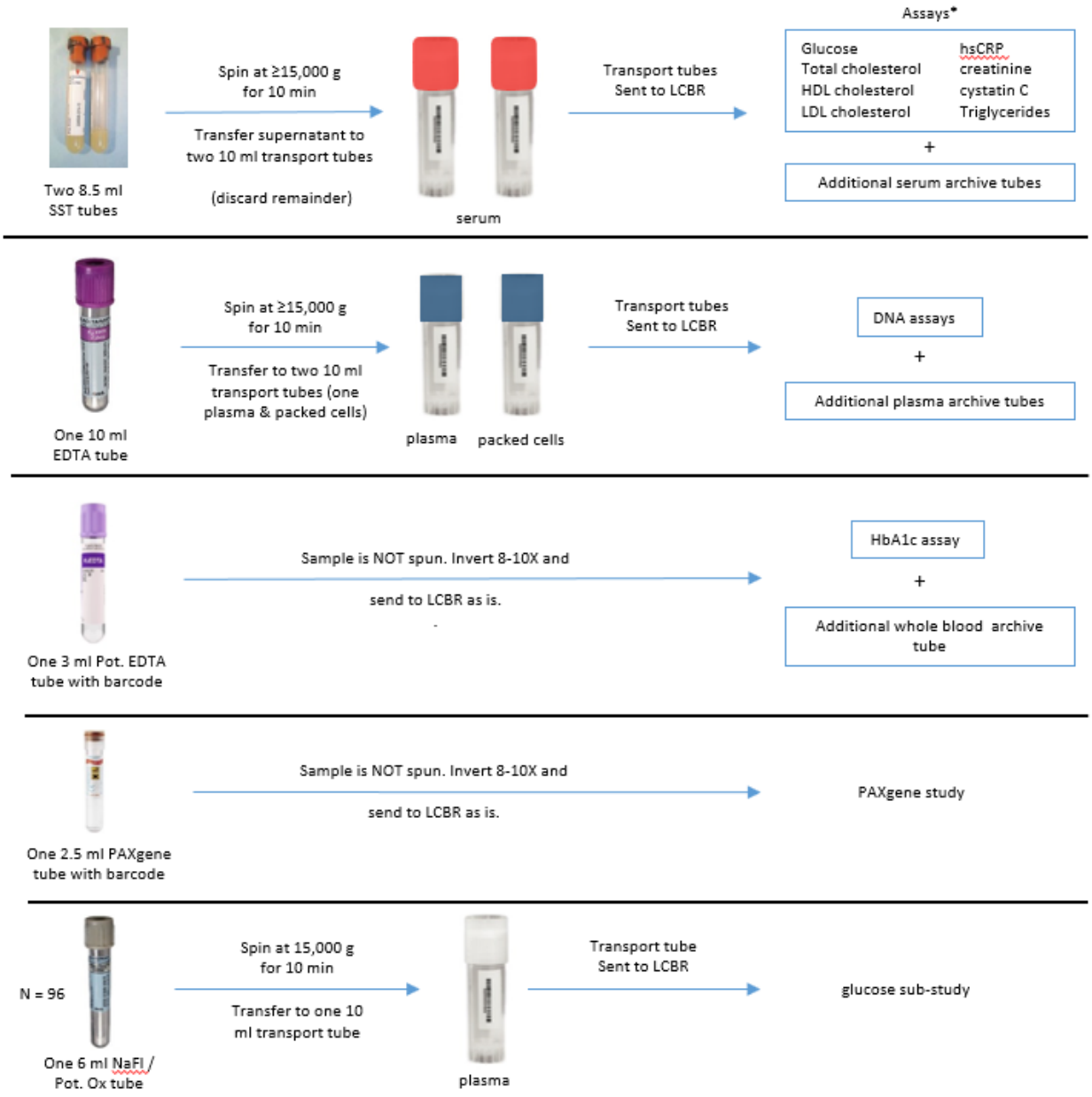
Order	Tube Type	Centrifuged	Resultant supernatant	Resultant precipitate	Use
1	8.5 ml SST	Yes	Serum	Discarded	Assays*: glucose, total cholesterol, high- & low-density lipoprotein-cholesterol, triglycerides, high sensitivity C reactive protein, creatinine & cystatin C
2	10 ml EDTA	Yes	Plasma	RBC/buffy coat	Archival: for future use
3	3 or 4 ml EDTA	No	N/A	N/A	Assay: hemoglobin A1c Archival: for future use
4	8.5 ml SST	Yes	Serum	Discarded	Archival: for future use
5	6 ml NaFl/KOx	Yes	Plasma	Discarded	Assay: glucose sub-study
6	10 ml PAXgene	No	N/A	N/A	Archival: for future use
* Also included IL-1β, IL-6, IL-8, IL-10, TNFα, and CMV, as described below					

Exhibit 2. Tubes of Blood Collected

3.3.2 Blood Processing

The venous blood draw concluded the home exam. After cleaning up all supplies and equipment, FEs left the exam sites and were allowed a maximum of two hours before processing the blood which was chilled at 4^{o} C (on ice or frozen cold packs) in the interim.

All FEs centrifuged specific blood collection tubes, including the 8.5 ml SST, 10 ml EDTA, and when collected, the 6 ml NaFl/KOx vacutainer tubes. The 3-4 ml EDTA vacutainer tube used for the HbA1c assay was *not* centrifuged. FEs centrifuged tubes for ≥ 10 min at ≥ 1300 g, depending on the capabilities of their centrifuge. After centrifugation, FEs used the graduated transfer pipettes included in the Blood Collection Kit to aliquot serum from the SST and (separately, when collected) plasma from the NaFl/KOx vacutainer tubes into 10 ml, round bottom, skirted transport tubes (BD Biosciences, NJ). FEs aliquoted as much supernatant as possible into the transport tubes but avoided disturbing the precipitate layer. A red cap identified transport tubes containing serum from the SST vacutainer tubes, a blue cap identified transport tubes containing plasma from the 10 ml EDTA vacutainer tube, and a white cap identified the transport tube containing plasma from the Na/Fl/KOx tube. Transport tubes were chilled at 4^{o} C (on ice or frozen cold packs) until packaged for shipment to LCBR. Exhibit 3 demonstrates the complete blood processing protocol.



* Also included IL-1β, IL-6, IL-8, IL-10, TNFα, and CMV, as described below

Exhibit 3. Blood Processing Protocol

After processing the blood, FEs took a loose barcode label provided in the Blood Collection Kit and affixed it to a paper manifest designed to accompany the transport tubes to LCBR. The loose barcode label matched the barcode labels on the transport tubes and the Shipping Box. FEs recorded all vacutainer tubes that were collected and identified all difficulties during blood draw or processing on the manifest as well as in the tablet. The barcode-labelled manifest was designed to be scanned on arrival at LCBR to associate it with an individual respondent's transport tubes.

3.3.3 Shipment of Samples

Immediately before shipment, FEs removed two cold packs from the freezer, sandwiched the transport tubes between them, enclosed the sandwich within the Styrofoam Box, placed the manifest on top of the Styrofoam Box, sealed the cardboard Shipping Box around it, put the cardboard Shipping Box inside the Tyvek envelope, applied a pre-printed FedEx shipping label to the envelope, carried it to a FedEx office, and handed it to a FedEx representative (*in person*) for Priority Overnight shipment to LCBR with arrival the following morning. FEs were not permitted to leave shipments at unattended FedEx drop boxes.

When overnight shipment was impossible, FEs noted this on the manifest and held unboxed transport tubes in a refrigerator approved for biological specimens or cooler with enough cold packs to keep them chilled at 4^{o} C overnight without risk of freezing (or thawing), as is possible on wet or dry ice. The transport tubes were packaged and shipped the next day using freshly frozen cold packs.

3.3.4 Receipt of Samples at LCBR

LCBR technicians specifically trained for Add Health Wave V received and immediately processed samples each morning. They unpacked the shipping boxes one at a time, evaluated the volume and quality of each transport tube, and entered them into a custom-made laboratory information management system (LIMS) program.

After re-centrifuging the serum samples for hsCRP assays at 4° C for 10 min at 30,000 g, the technicians aspirated the supernatant, discarded all remaining precipitate, transferred the aspirate to pre-labelled tubes, and placed them in a biospecimen refrigerator for archival (in 1 ml aliquots at -80° C) or assay (200 ul aliquot). The LCBR technicians entered all aliquot information into the LIMS system.

3.3.5 Preparation of Samples for Inflammation Assays

Assays of hsCRP were performed immediately on samples after arrival at LCBR and followed the procedure outlined in section 3.3.4. Assays for all additional inflammatory biomarkers were performed using archived serum samples stored originally at the time of initial processing. A 1 ml archive tube of serum from each respondent was selected, thawed in a 36°C water bath for 10 minutes, and re-vortexed on the day their sample was to be assayed. Samples were prepared as stated in specific assay sections below. Afterwards, the remainder of the sample was refrozen and re-archived at -80°C. All cytokine assays were performed within a range of 293-1338 days after the samples were initially archived (mean=732 days, SD=303 days). All CMV assays were performed within a range of 613-1380 days after the samples were initially archived (mean=971 days, SD=202 days).

4. Assay and Internal Quality Control

4.1 High Sensitivity C-Reactive Protein **[H5CRP]**

4.1.1 Rationale

CRP is produced by the liver in response to inflammation. It also is a fairly stable protein that can be sensitively measured with precision using standardized laboratory procedures[2]. Moreover, in asymptomatic, intermediate-risk men aged ≤ 50 years and women ≤ 60 years, measurement of hsCRP may be useful in cardiovascular risk assessment.[4]

4.1.2 Assay

All hsCRP assays were run on the same day of sample arrival at LCBR using the Siemens BNII / BN Prospec System (Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany) and an hsCRP-specific particle enhanced immunonephelometric assay. Serum from venous blood collected using the SST vacutainer tubes was introduced into the Siemens system by placing sample vials holding 200 µl of serum into an automatic sampling tray, after which all processes were automatically performed and results output by the Siemens system. All samples were automatically run at a sample dilution of 1:20 with N diluent.

The Siemens system read barcodes on the vials to automatically determine which assays to run. In addition to hsCRP, Cystatin C also was run using the Siemens system. Only the hsCRP assay is described below. Assay protocols for Cystatin C can be found in the Add Health Renal Function User Guide.

In the hsCRP assay, polystyrene particles coated with monoclonal antibodies specific to human CRP were aggregated when mixed with serum containing CRP. These aggregates scattered a beam of light passed through the sample. The intensity of the scattered light was proportional to the concentration of the relevant protein in the sample. The result was evaluated by comparison with a standard of known concentration.[5]

Reference curves were generated by multi-point calibration. Serial dilutions of N Rheumatology Standard SL were automatically prepared by the instrument using N Diluent. The exact measuring range depended upon the concentration of the protein in each lot of N Rheumatology Standard SL. The analytical sensitivity of the assay was determined by the lower limit of the reference curve and therefore depended upon the concentration of the protein in the N Rheumatology Standard SL. Lower and upper limits of detection for the Add Health samples were from 0.160 - 19,480 mg/L for measurements performed using a sample dilution of 1:20. The following reagents and materials were used in the assay and supplied by Siemens Healthcare (Newark, DE).

- CardioPhase hsCRP; Cat. # OQIY21
- N Supplementary Reagent/Precipitation; Cat. # OUMU15
- N/T Rheumatology Control SL/1; Cat. # OQDB13
- N/T Rheumatology Control SL/2; Cat. # OQDC13
- Apolipoprotein Control Serum CHD; Cat. #OUPH07
- N Rheumatology Standard SL; Cat. # OQKZ13

- BNII Additive; Cat. # OQKY61
- N Diluent; Cat. # OUMT65
- Reaction Buffer; Cat. # OUMS65

4.1.3 Internal Quality Control

The Siemens system was maintained daily by inspecting all tubing, connections, and syringes for leaks, kinks, cracks, or contamination. Reaction buffers, N diluent, and wash solutions were also replaced daily.

N/T Rheumatology Controls SL/1 and SL/2 and Apolipoprotein Control Serum was assayed after each establishment of a reference curve, the first use of a reagent vial as well as with each run of samples. LCBR also ran EDTA and serum controls as specimen samples each time a new standard curve was established. In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E050115). The other pool was a serum normal donor pool (Lot #S042715). LCBR periodically assayed both pools over the course of Wave V. The plasma and serum hsCRP concentration mean (coefficient of variation) based on those assays was 4.6 mg/L (6.3%) and 7.3 mg/L (7.9%), respectively. When hsCRP concentrations exceeded acceptable parameters, the Siemens system was investigated and repaired.

4.2 Cytokine Assays - IL-1β, IL-6, IL-8, IL-10, TNF-α **[H5IL1B, H5IL6, H5IL8 H5IL10, H5TNFA]**

4.2.1 Rationale

Cytokines are small proteins produced by many cells of the immune system. Interleukins (IL) and tumor necrosis factors (TNF) are two of the common types. When cytokines bind cellular receptors, they can trigger humoral or cellular immune responses. For example, pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and TNF-α) can stimulate inflammation and activate immune cells, whereas anti-inflammatory cytokines (e.g., IL-6 and IL-10) can inhibit and suppress them. Although their actions and interactions are often complex, cytokines are therefore important in health and disease. As such, cytokines may serve as biomarkers of the latter, providing insight into pathophysiology when they are measured accurately and reliably.[6,7,8,9]

4.2.2 Assay Principle

Samples were analyzed for Il-1β, IL-6, IL-8, IL-10, and TNF-α using a highly sensitive electrochemiluminescent immunoassay on the MesoScale Diagnostics (MSD) platform (V-PLEX Human Cytokine; K15049G-2) and read using an MSD QuickPlex 120 (Meso Scale Diagnostics, Rockville, MD). This platform allowed for the simultaneous quantification of multiple analytes in a single assay. Each kit included the following components: 96-well plate, pre-coated with capture antibody arrays in each well; detection antibody conjugated with an electrochemiluminescent label; calibrators; and diluents and buffers to be used as needed.

The assay principle was as follows: Capture antibodies for each target were printed as arrays in discrete spots at the bottom of each test well in a 96-well plate. Following the addition of sample or controls and capture of the target, detection antibody with an electrochemiluminescent label was added to the wells and an electrical impulse generated light in proportion to bound target at each spot in the array. The MSD QuickPlex 120 then measured the intensity of emitted light, which was proportional to the

amount of analyte present in the sample[10] (Exhibit 4). This method had a wide dynamic range, low background, high sensitivity, and it required only a small volume of sample to generate results for multiple analytes in a single assay.

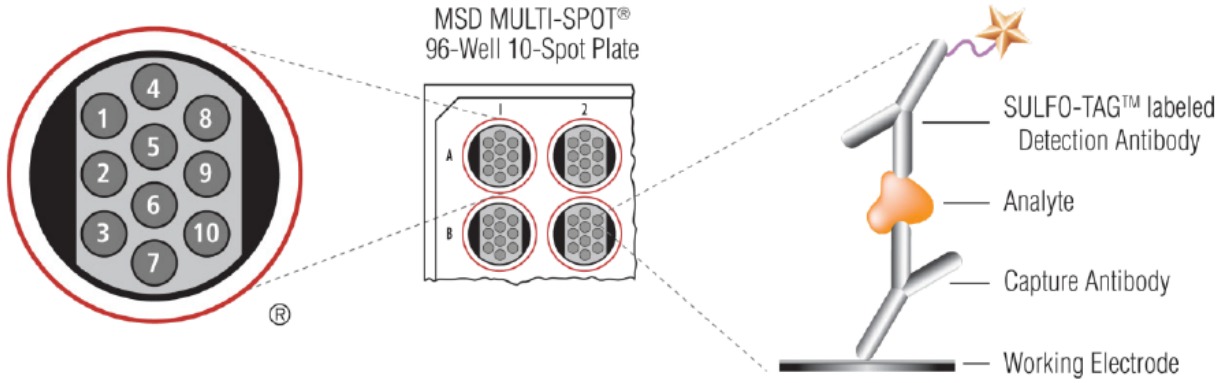


Exhibit 4. VPlex Cytokine Assay Scheme

4.2.3 Assay Calibration and Control Preparation

A multi-analyte lyophilized calibrator supplied in the kit (MSD, C0049-2) was used that yielded the highest recommended calibration concentration. This powder was reconstituted in 1 ml of MSD Diluent 2, and then this calibration solution was serially diluted in Diluent 2 in a 1:4 ratio to make a total of 7 calibrators, plus a zero calibrator. These calibrators were used throughout the assay period and were rerun on a continual basis to ensure consisted machine calibration. The calibrators were stable through -80°C freeze-thaw cycles, so only one lot was used throughout the testing period.

MSD also supplied a proinflammatory human control pack (MSD, C4049-1) which contained 3 controls of known concentrations of all cytokines detected by the VPlex Panel. As with the calibrators, these controls were supplied as a lyophilized powder, and were reconstituted in 250 µl of MSD Diluent 2. Again, the controls were stable through multiple freeze-thaw cycles, and thus only one lot was required throughout the testing period. Mean values for the 3 controls are listed in Exhibit 5.

CONTROL	IL1-β	IL-6	IL-8	IL-10	TNF-α
1	218	273	224	136	92.5
2	51.8	48.4	33.8	25.3	16.5
3	11.9	8.11	6.31	3.81	2.13

Exhibit 5. Mean Values of Human Control Samples

4.2.4 Assay Protocol

Serum samples were diluted at 1:2 ratio according to manufacturer recommendations. Calibration standards and controls were prepared as stated above. All MSD diluents, detection antibodies, wash

buffers, phosphate-buffered saline, and read buffers were prepared according to manufacturer recommendations.[10]

For the assay, the pre-coated 96-well plates were washed 3 times with 150 µl wash buffer, and then 60 µl of samples, controls, or calibrators were added to each well. The plates were sealed with an adhesive plate seal and incubated at room temperature for 2 hours while shaking. Plates were then washed again 3 times with 150 µl wash buffer, and 250 µl of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature for 2 hours while shaking. The plates were then washed again 3 times with 150 µl wash buffer, and 150 µl of 2X read buffer was added to each well. The plate was then analyzed on the MSD QuickPlex 120. A 4-parameter logistic curve was generated to fit the signal and calibrator concentrations, and the concentrations of samples were calculated based on the standard curve for each analyte. The following table lists the calibration range for each analyte and the corresponding detectable range for each analyte for all samples run at a 1:2 dilution. (Exhibit 6).

Analyte	Calibrator Range (pg/ml)	Detectable Range (pg/ml)
IL-1β	0.005 - 578	0.01 - 1156
IL-6	0.025 - 752	0.05 - 1504
IL-8	0.03 - 560	0.06 - 1120
IL-10	0.02 - 383	0.04 - 766
TNF-α	0.02 - 342	0.04 - 685

Exhibit 6. Standard Curve and Detectable Range of Cytokines

Data reduction software at LCBR extrapolated values for some data points beyond the detectable range of the standard curve. The final reported analyte values for each analyte are listed below (Exhibit 7).

Analyte	Reported Range (pg/ml)
IL-1β	0.001 - 403.8
Il-6	0.021 - 1274
IL-8	2.43 - 3419
IL-10	0.021 - 57.92
TNF-α	0.13 - 2955

Exhibit 7. Cytokine Data Ranges as Reported by LCBR

4.2.5 Internal Quality Control

All assays were run by the same technicians, using a single lot number of reagents from MSD and the same set of calibrators and controls that were manufactured at the beginning of the assay period. The MSD Quick-Plex 120 is a maintenance free, self-check analyzer with built in status monitor. The status monitor maintained and recorded the functional status of the onboard camera which must operate at -25°C. Operational equipment was relayed to end user as a constant blue "okay" light. If the unit failed

self-maintenance checks, the analyzer displayed a red light and would not function. A calibration curve was generated on each kit plate and was evaluated by running the human control pack listed above that had defined ranges for each level (low, high) of kit QC per analyte on the panel. Quality control data was run every 36 samples.

In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E050115). The other pool was a serum normal donor pool (Lot #S042715). LCBR periodically assayed both pools over the course of the assay period. The plasma and serum cytokine concentration mean (coefficient of variation) based on those assays is listed in the table below (Exhibit 8). When cytokine concentrations exceeded acceptable parameters, the MSD analyzer was investigated and repaired.

Assay	Serum Mean (pg/ml)	Serum (%CV)	Plasma Mean (pg/ml)	Plasma (%CV)
IL-1β	0.6	8.17	N/A	N/A
IL-6	2.88	5.22	1.59	5.28
IL-8	67.46	4.27	5.73	4.76
IL-10	0.42	5.62	0.15	12.49
TNF-α	2.99	5.91	2.48	6.07

Exhibit 8. QC Plasma and Serum Control Values

4.3 Cytomegalovirus **[H5CMVZ]**

4.3.1 Rationale

The cytomegalovirus (CMV) belongs to the family Herpesviridae, which includes Herpes simplex 1 and 2, varicella zoster, and Epstein-Barr DNA viruses. CMV is responsible for a range of infections in humans. CMV infections can be acquired before birth (congenital), at birth (perinatal) or later in life (postnatal). CMV infections are frequent and occasionally severe in immunosuppressed individuals such as patients with AIDS, HIV, cancer, or organ transplants. Such infections may represent reactivation of latent virus or primary infection introduced by blood transfusion or transplanted organ.[11,12,13,14]

4.3.2 Multiple Assay Approach

Initial CMV assays were performed using an enzyme immunoassay (EIA) kit from Diamedix (ERBA Diagnostics, Miami Lakes, FL). However, after 1,345 samples were analyzed using this kit, Diamedix discontinued its production. After thorough research by LCBR, the remaining 3,470 assays were performed using an enzyme-linked immunosorbent assay (ELISA) kit from Creative Diagnostics (Creative Diagnostics, Shirley, NY). Specific protocols for each assay, as well as data conversion protocols follow in the sections below.

4.3.2.1 Diamedix Assay Principle

The Diamedix Immunosimplicity® *Is*-CMV IgG Test Kit (720-320, Miami Lakes, FL) was an EIA procedure intended for the qualitative and semi-quantitative detection of antibodies to CMV antigen. The results were objective and reported in EU/ml, which were traceable to in-house reference materials.[15]

Diluted samples were incubated with CMV antigen bound to the solid surface of a 96-plate well. If IgG antibodies against CMV were present in the samples, they bound to the antigen forming antigen-antibody complexes. Residual sample was eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) was added to bind to these complexes. Unbound conjugate was removed by aspiration and washing. Substrate was then added and incubated. In the presence of bound enzyme, the substrate was converted to an end product. The absorbance of this end product was read spectrophotometrically at 450 nm (reference 600-630 nm) and was directly proportional to the concentration of IgG antibodies to CMV antigen present in the sample.

4.3.2.2 Diamedix Assay Protocol

Diamedix assays were performed as previously described by Dowd, et. al.[16] A summary is provided here as follows. Thawed serum samples were initially diluted 1:10 using sample diluent supplied by the kit. Then these samples were further diluted 1:101 by adding 5 µl of sample to 500 µl of diluent. Three standards for creating calibration curves were provided in the kit, with known values of 0, 10, and 160 EU/ml. Three controls were also included: a high positive, a low positive, and a negative control. All other reagents were prepared according to the manufacturer's specifications.

All components were allowed to come to room temperature and gently mixed prior to using. 100 µl of samples, standards, and controls were added to respective wells in each 96-well plate. The plates were incubated uncovered at 37°C for one hour. Each well was washed 3 times with wash solution included at 37°C for one hour, and then washed 3 times with wash buffer. 100 µl of substrate was then added and plates were incubated as before, but not washed. At the end of the incubation, 100 µl of stop solution was added to each well. The plate was then inserted into a Biotek ELx808IU plate reader and the absorbance was read at 450 nm, using a reference wavelength of 600 nm.

The calibration curve of this assay ranged from 0 - 160 EU/ml. With samples prediluted 1:10, this gave a working detectable range of 0 - 1600 EU/ml. High and low controls had mean values (%CV) of 74.90 (5.52%) and 16.60 (5.03%) EU/ml.

4.3.2.3 Internal Quality Control

The BioTek reader was maintained following manufacturer specifications, which included cleaning exposed surfaces, cleaning wavelength filters, lubricating robotic components, and cleaning lamp contacts.

Quality control for the Diamedix kit was performed by running the high and low controls provided in the kit (Section 4.3.2.2) at regular intervals to ensure consistency across all runs. LCBR also ran a "citrate mean" (a pooled sample from several plasma samples) for comparison with the Creative Diagnostics kit (Section 4.3.2.6). The mean (%CV) for the citrate sample on the Diamedix kit was 53.51 EU/ml (5.48%).

4.3.2.4 Creative Diagnostics Assay Principle

The Creative Diagnostics assay was a semi-quantitative solid phase ELISA based on the sandwich principle.[17] Wells in a 96 well plate were coated with antigen. Specific antibodies of the sample binding to the antigen coated wells were detected by a secondary enzyme conjugated antibody specific for human IgG. After the substrate reaction, the intensity of the color developed was proportional to the amount of IgG-specific antibodies detected. The color was then read using a Biotek ELx808IU plate reader (Biotek, Santa Clara, CA). The results of the samples were then compared to a standard curve using reagents provided in the kit.

4.3.2.5 Creative Diagnostics Assay Protocol

Thawed serum samples were initially diluted 1:5 using sample diluent supplied by the kit. Then these samples were further diluted 1:101 by 5 µl of sample to 500 µl of diluent. A negative control, plus three standards for creating calibration curves were provided in the kit, with known typical values of 1, 10, 30, and 90 U/ml. All other reagents were prepared according to the manufacturer's specifications.

All components were allowed to come to room temperature and gently mixed prior to use. 100 µl of samples and standards were added to respective wells in each 96-well plate. The plates were covered with foil and incubated at room temperature for one hour. Each well was washed 3 times with wash solution included in the kit, and then 100 µl of enzyme conjugate was added to each well. Plates were covered and incubated again at room temperature for one hour, and then washed 3 times with wash buffer. 100 µl of tetramethylbenzidine (TMB) substrate was then added and plates were incubated for 20 minutes in the dark but uncovered. At the end of the incubation, plates were not washed, and 100 µl of TMB stop solution was added to each well. The plate was then inserted into a Biotek ELx808IU plate reader and the absorbance was read at 450 nm, using a reference wavelength of 600 nm.

As stated above, initial assays were run using a 1:5 sample dilution. The calibration curve for this dilution provided detectable limits of 5 - 450 U/ml. If assay results were either above or below the normal detectable limits, then the sample was rerun using either a 1:10 or no sample dilution. The detectable limits of each dilution are shown in Exhibit 9.

Sample Dilution	Detectable Range (U/ml)
none	1 - 90
1:5	5 - 450
1:10	10 - 900

Exhibit 9. Creative Diagnostics Detectable Ranges

4.3.2.6 Internal Quality Control

The Biotek reader was maintained following manufacturer specifications, which included cleaning exposed surfaces, cleaning wavelength filters, lubricating robotic components, and cleaning lamp contacts.

In addition to the normal quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E050115). The other pool was a serum normal donor pool (Lot #S042715). LCBR periodically assayed both pools over the course of the assays, and each was run at normal concentration and then at a 1:5 dilution. The plasma and serum index mean (coefficient of variation) for the CMV Assay were 65.135 (6.45%) and 54.692 (8.07%) for neat and 121.718 (12.65%) and 102.564 (13.25%) for 1:5 diluted samples, respectively. The pooled citrate sample was also run and had a mean (%CV) of 66.672 U/ml (13.14%) when run at a 1:5 dilution and 39.213 U/ml (12.06) when run neat.

4.3.3 Inter-Conversion Between Diamedix and Creative Diagnostics Data

Four samples were selected at random from those 1,345 respondents originally assayed using the Diamedix kit within each of 24 strata defined by race/ethnicity (non-Hispanic white; non-Hispanic black; Hispanic; other), sex (male; female), and CMV concentration tertiles (≤ 6.59, 6.62-126.67, and ≥ 126.78 EU/mL). Collectively, the stratified, random sample included 4 × 24 = 96 respondents. These 96 samples were rerun using the Diamedix Kit using the same parameters and protocols as when they were originally run. The same 96 samples were then immediately run using the Creative Diagnostics kit, again using the same parameters and protocols that were used for all other samples run by this kit. The Creative Diagnostics concentrations (U/ml) and average Diamedix CMV concentrations (EU/ml) were z-transformed by subtracting their respective means and then dividing by their standard deviations. The association between the z-transformed values (Exhibit 10) was modeled using Deming regression,[18,19] i.e. Creative Diagnostics CMV = $\alpha + \beta \times$ (Diamedix CMV) (Pearson r = 0.94). Estimates of α and β and their 95% confidence intervals were 0 (-0.072, 0.072) and 1 (0.838, 1.162), respectively. Although the z-transformed Diamedix CMV and z-transformed Creative Diagnostics CMV concentrations were therefore treated as interchangeable, those based on the Diamedix assay were flagged (H5CMVDIA=1). The final z-transformed CMV concentrations (H5CMVZ) ranged from -0.94 to 8.82, in standard deviation units.

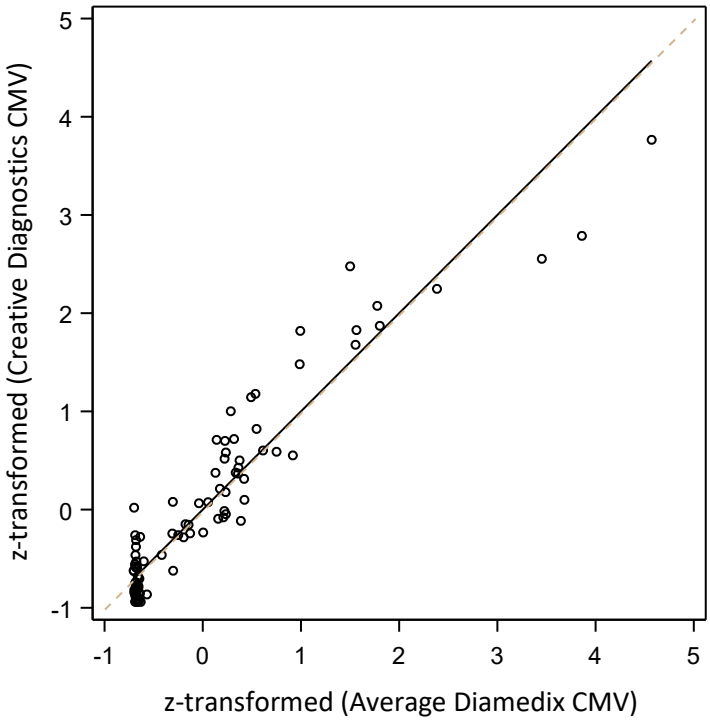


Exhibit 10. Plot of z-transformed Creative Diagnostics CMV vs z-transformed Diamedix CMV Concentrations

5. External Quality Control

5.1 Reliability of hsCRP

Within a race/ethnicity- and sex-stratified random sample of 94 Add Health respondents among whom venous blood was collected twice, on average 14.3 (95% confidence interval: 13.0-15.6) days apart, typically by the same FE and at approximately the same time of day, the reliability of hsCRP (mg/L) was estimated as an intra-class correlation coefficient (ICC, 95% confidence interval) (Exhibit 11). The estimate suggests that the home exam venous blood collected at Add Health Wave V yields a more reliable measure of hsCRP than the dried capillary whole blood spots collected at Wave IV.

Measure (mg/L)	**N**	**ICC**	**95% CI**
hsCRP	94	0.82	(0.75, 0.89)

Exhibit 11. Reliability of hsCRP

5.2 Reliability of the Cytokines - IL-1β, IL-6, IL-8, IL-10 and TNF-α

Within a race/ethnicity- and sex-stratified random sample of 105 Add Health respondents among whom venous blood was collected twice, on average 14.3 (95% confidence interval: 13.1-15.5) days apart, typically by the same FE and at approximately the same time of day, the reliability of IL-1β, IL-6, IL-8, IL-10 and TNF-α (pg/ml) were estimated as intra-class correlation coefficients (ICC, 95% confidence interval) before and after natural log transformation (Exhibit 12). The estimates suggest that the home exam venous blood collected at Add Health Wave V yields a highly reliable measure of IL-10; slightly less reliable measures of IL-6 and TNF-α; and poor reliability of IL-1β and IL-8 that can be modestly increased by log transformation.

Measure (pg/ml)	Untransformed [N: ICC (95% CI)]	Log-Transformed [N: ICC (95% CI)]
IL-1β	53: 0.15 (-0.11, 0.41)	53: 0.47 (0.26, 0.68)
IL-6	105: 0.68 (0.57, 0.78)	105: 0.78 (0.70, 0.85)
IL-8	105: 0.13 (-0.06, 0.32)	105: 0.54 (0.40, 0.67)
IL-10	105: 0.94 (0.92, 0.96)	105: 0.77 (0.69, 0.85)
TNF-α	105: 0.77 (0.69, 0.85)	105: 0.81 (0.75, 0.88)

Exhibit 12. Reliability of Cytokine Assays

5.3 Reliability of CMV

Within a race/ethnicity- and sex-stratified random sample of 105 Add Health respondents among whom venous blood was collected twice, on average 14.3 (95% confidence interval: 13.1-15.5) days apart, typically by the same FE and at approximately the same time of day, the reliability of z-transformed CMV (standard deviation units) was estimated as an intra-class correlation coefficient (ICC, 95% confidence interval) (Exhibit 13). The estimate suggests that the home exam venous blood collected at Add Health Wave V yields a highly reliable measure of CMV.

Measure (standard deviations)	N	ICC	95% CI
CMV	105	0.98	(0.98, 0.99)

Exhibit 13. Reliability of CMV

6. Constructed Measures

6.1 Classification of hsCRP **[H5CCRP]**

The classification of hsCRP concentrations among Add Health respondents was constructed without regard to fasting status, in accordance with the American Heart Association/Centers for Disease Control (AHA/CDC) clinical and public health practice recommendations regarding markers of inflammation and cardiovascular disease.[2] In keeping with the recommendations, classes of hsCRP were defined to approximate tertiles in the adult population, as tabulated below, although it should be noted that in many populations, only 5% of hsCRP concentrations exceed 10 mg/L (Exhibit 14):

Classification	hsCRP (mg/L)	AHA/CDC Class
1	< 1	Low
2	1 - 3	Average
3	> 3	High

Exhibit 14. Classification of hsCRP

6.2 Count of Common Subclinical Symptoms **[H5SUBCLN]**

High hsCRP concentrations, particularly those exceeding 10 mg/L, should trigger searches for non-cardiovascular (e.g. infectious or inflammatory) diseases capable of seriously confounding hsCRP-based estimates of cardiovascular disease risk. Subclinical sources of infection or inflammation identified in Question 14 of the Wave V home exam also have potential to confound hsCRP-based estimates of cardiovascular disease risk in apparently healthy populations. Common symptoms identified by items H5Q014A - H5Q014G were therefore counted and categorized as previously described [3,21,22] for investigation or control of potential confounding in hsCRP analyses (Exhibit 15).

Code	Symptom Count*
0	0
1	1
2	2
3	≥ 3

* Subclinical sources of infection or inflammation in the last 2 weeks include the following: cold or flu-like symptoms [variable: H5Q014A = 1], Q014b fever [variable: H5Q014B = 1], night sweats [variable: H5Q014C = 1], nausea or vomiting or diarrhea [variable: H5Q014D = 1], blood in stool or feces or urine [variable: H5Q014E = 1], frequent urination [variable: H5Q014F = 1], and skin rash or abscess [variable: H5Q014G = 1].

Exhibit 15. Count of Common Subclinical Symptoms

6.3 Count of Common Infectious or Inflammatory Diseases **[H5INFECT]**

Although the infectious and inflammatory diseases identified in Question 13 of the Wave V home exam are not included in the symptom count tabulated above, they too may confound hsCRP-based estimates of cardiovascular disease risk. Therefore, responses to items H5Q013A - H5Q013F from this question were counted and categorized for investigation or control of potential confounding in hsCRP analyses (Exhibit 16). Please note that at Wave IV, the analogous constructed variable (C_INFECT) included two other items in the count, namely "Asthma/Chronic Bronchitis/Emphysema" and "Hepatitis C," which were not captured in the Wave V home exam.

Code	Disease Count*
0	0
1	1
2	2
3	≥ 3

* Infectious and inflammatory diseases in the last 4 weeks include the following: gum disease/tooth loss [variable: H5Q013A = 1], active infection [variable: H5Q013B = 1], injury [variable: H5Q013C = 1], acute illness [variable: H5Q013D = 1], surgery [variable: H5Q013E = 1], and active seasonal allergies [variable: H5Q013F = 1].

Exhibit 16. Count of Common Infectious or Inflammatory Diseases

6.4 Medication Use Variables [**H5CRP1 - H5CRP8]**

Use of anti-inflammatory medications (and/or the diseases for which they are being taken) also may confound hsCRP-based estimates of cardiovascular disease risk. These exposures were captured at Wave V in the home exam medication inventory, and for salicylates/nonsteroidal anti-inflammatory drugs (NSAIDs), in questions 69 and 70 of the home exam. [22] They should be used cautiously in the investigation or control of potential confounding in hsCRP analyses because the typical intermittency and brevity of anti- inflammatory medication use (e.g. for headache, menstrual cramps, muscle ache, etc.) and their short half-lives in the circulation reduce ability to accurately define exposure. Moreover, selection biases often threaten the study of non-randomized medication exposures. Respondents used ≥ 1 medication identified by ≥ 1 of the following questions, coded therapeutic classes, or active ingredients. (Exhibit 17)

Question / Class	Label	Variable
Q069=yes Q070=yes	Salicylate past 24 hours or Non-Steroidal Anti-Inflammatory Drug (NSAID) past 24 hours	H5CRP1
057-058-061 057-058-062	NSAIDs past 4 weeks or Salicylate past 4 weeks or Any oral medication that contains NSAID or Salicylate as an active ingredient* in a combination medication past 4 weeks	H5CRP2
057-058-278	Cyclooxygenase-2 (COX-2) Inhibitor past 4 weeks	H5CRP3
122-130-296	Inhaled Corticosteroids past 4 weeks	H5CRP4
097-098-300 097-098-301	Corticotropin or Glucocorticoid past 4 weeks	H5CRP5
105-192-*** 105-270-***	Anti-rheumatic or Anti-psoriatic past 4 weeks	H5CRP6
254-104-*** 254-257-***	Immunosuppressive agents or Immunosuppressive monoclonal antibodies past 4 weeks	H5CRP7
Any of the above	Any of the above anti-inflammatories	H5CRP8

*Active Ingredients:

NSAIDS

- Bromfenac
- Diclofenac
- Diflunisal
- Etodolac
- Fenoprofen
- Flurbiprofen
- Ibuprofen
- Indomethacin
- Ketoprofen
- Ketorolac
- Meclofenamate
- Mefenamic Acid
- Meloxicam
- Nabumetone
- Naproxen
- Oxaprozin
- Piroxicam
- Sulindac
- Tolmetin

Salicylates

- Aspirin
- Choline salicylate
- Magnesium salicylate
- Salsalate
- Sodium salicylate
- Thiosalicylate

Exhibit 17. Anti-Inflammatory Medications

6.5 Concentration Type Variables

All assay concentrations were categorized based on their corresponding limits of detection (LODs) and extrapolation beyond them, then flagged as tabulated below (Exhibits 18-24). *Warning: Users should recognize extrapolated concentrations as such and exercise caution when working with them. Moreover, theoretical and computational recommendations for properly analyzing left- and right-censored concentrations falling beyond limits of detection under both frequentist and Bayesian frameworks can be found elsewhere.*[10]

6.5.1 Flag Indicating the hsCRP Concentration Type [**H5CRPFL**]

Code	Description
1	hsCRP concentration is below the lower LOD (< 0.160 mg/L) – missing
2	hsCRP concentration is below the lower LOD (< 0.160 mg/L), but extrapolated
3	hsCRP concentration is within the LODs (0.160 - 19,480 mg/L)
4	hsCRP concentration is above the upper LOD (> 19,480 mg/L), but extrapolated
5	hsCRP concentration is above the upper LOD (> 19,480 mg/L) – missing

Exhibit 18. Flag Indicating the hsCRP Concentration Type

6.5.2 Flag Indicating the IL-1β Concentration Type [**H5IL1BFL**]

Code	Description
1	IL-1β concentration is below the lower LOD (< 0.010 pg/ml) – missing
2	IL-1β concentration is below the lower LOD (< 0.010 pg/ml), but extrapolated
3	IL-1β concentration is within the LODs (0.010 - 1156 pg/ml)
4	IL-1β concentration is above the upper LOD (> 1156 pg/ml), but extrapolated
5	IL-1β concentration is above the upper LOD (> 1156 pg/ml) – missing

Exhibit 19. Flag Indicating the IL-1β Concentration Type

6.5.3 Flag Indicating the IL-6 Concentration Type [**H5IL6FL**]

Code	Description
1	IL-6 concentration is below the lower LOD (< 0.050 pg/ml) – missing
2	IL-6 concentration is below the lower LOD (< 0.050 pg/ml), but extrapolated
3	IL-6 concentration is within the LODs (0.050 - 1504 pg/ml)
4	IL-6 concentration is above the upper LOD (> 1504 pg/ml), but extrapolated
5	IL-6 concentration is above the upper LOD (> 1504 pg/ml) – missing

Exhibit 20. Flag Indicating the IL-6 Concentration Type

6.5.4 Flag Indicating the IL-8 Concentration Type [**H5IL8FL**]

Code	Description
1	IL-8 concentration is below the lower LOD (< 0.060 pg/ml) – missing
2	IL-8 concentration is below the lower LOD (< 0.060 pg/ml), but extrapolated
3	IL-8 concentration is within the LODs (0.060 - 1120 pg/ml)
4	IL-8 concentration is above the upper LOD (> 1120 pg/ml), but extrapolated
5	IL-8 concentration is above the upper LOD (> 1120 pg/ml) – missing

Exhibit 21. Flag Indicating the IL-8 Concentration Type

6.5.5 Flag Indicating the IL-10 Concentration Type [**H5IL10FL**]

Code	Description
1	IL-10 concentration is below the lower LOD (< 0.040 pg/ml) – missing
2	IL-10 concentration is below the lower LOD (< 0.040 pg/ml), but extrapolated
3	IL-10 concentration is within the LODs (0.040 - 766 pg/ml)
4	IL-10 concentration is above the upper LOD (> 766 pg/ml), but extrapolated
5	IL-10 concentration is above the upper LOD (> 766 pg/ml) – missing

Exhibit 22. Flag Indicating the IL-10 Concentration Type

6.5.6 Flag Indicating the TNF-α Concentration Type [**H5TNFAFL**]

Code	Description
1	TNF-α concentration is below the lower LOD (< 0.040 pg/ml) – missing
2	TNF-α concentration is below the lower LOD (< 0.040 pg/ml), but extrapolated
3	TNF-α concentration is within the LODs (0.040 - 685 pg/ml)
4	TNF-α concentration is above the upper LOD (> 685 pg/ml), but extrapolated
5	TNF-α concentration is above the upper LOD (> 685 pg/ml) – missing

Exhibit 23. Flag Indicating the TNF-α Concentration Type

6.5.7 Flag Indicating the CMV Concentration Type [**H5CMVFL**]

Code	Description
1	CMV concentration is below the lower LOD – missing
2	CMV concentration is below the lower LOD, but extrapolated
3	CMV concentration is within the LODs
4	CMV concentration is above the upper LOD, but extrapolated
5	CMV concentration is above the upper LOD– missing

Exhibit 24. Flag Indicating the CMV Concentration Type

6.6 Classification of CMV [**H5CCMV**]

To assess the effect of changing from the Diamedix to the Creative Diagnostic CMV assay (Section 4.3.2) on estimated CMV seroprevalence, the two assays were compared in the race/ethnicity-, sex-, and CMV concentration tertile-stratified random sample of 96 respondents introduced above (Section 4.3.3). CMV seroprevalence as estimated by the Creative Diagnostic assay was found to be markedly higher than other population samples in the same age range. In contrast, CMV seroprevalence as estimated by the Diamedix assay was found to be commensurate with other population samples in the same age range and when compared with CMV seroprevalence at Wave IV, consistent with temporal expectations.[23,24,25] We therefore identified CMV seropositivity thresholds among the 1,345 samples assayed using the Diamedix CMV kit and then applied the corresponding percentiles to the 3,470 samples assayed using the Creative Diagnostics kit, yielding a standard classification of z-transformed CMV concentrations across all assayed samples (Exhibit 25):

Code	Percentile	Interpretation
1	≤ 40.19st	Negative for anti-CMV IgG
2	40.20th–46.69th	Equivocal for anti-CMV IgG
3	≥ 46.70st	Positive for anti-CMV IgG

Exhibit 25. Classification of CMV Concentrations

7. The Inflammation and Immune Function Data Files (bcrp5.xpt & binflam5.xpt)

7.1. Structure

The structure of both disseminated inflammation and immune function data files are flat. This means that they are respondent-level data files, where each respondent has one and only one record. The respondent's identifying number (the AID variable) will appear in each data file only once. To combine the two data files, they can simply be merged using the AID variable.

7.2. Contents

7.2.1 Original inflammation and immune function data file (bcrp5.xpt)

The original inflammation and immune function data file (bcrp5.xpt) includes the variables below, which are described in the corresponding codebook documentation that also contains frequencies.

Variable Name	Variable Description
AID	Respondent Identifier
H5CRP	hsCRP assay result (mg/L)
H5CCRP	hsCRP AHA/CDC classification
H5Q013A	Q013a Gum disease/tooth loss in last 4 weeks
H5Q013B	Q013b Active infection in last 4 weeks
H5Q013C	Q013c Injury in last 4 weeks
H5Q013D	Q013d Acute illness in last 4 weeks
H5Q013E	Q013e Surgery in last 4 weeks
H5Q013F	Q013f Active seasonal allergies in last 4 weeks
H5INFECT	Q013 Count of infectious/inflammatory diseases
H5Q014A	Q014a Cold or Flu-like symptoms in last 2 weeks
H5Q014B	Q014b Fever in last 2 weeks
H5Q014C	Q014c Night sweats in last 2 weeks
H5Q014D	Q014d Nausea or vomiting or diarrhea in last 2 weeks
H5Q014E	Q014e Blood in stool or feces or urine in last 2 weeks
H5Q014F	Q014f Frequent urination in last 2 weeks
H5Q014G	Q014g Skin rash or abscess in last 2 weeks
H5SUBCLN	Q014 Count of subclinical symptoms
H5CRP1	Flag indicating NSAID/Salicylate medication use (24 hours)
H5CRP2	Flag indicating NSAID/Salicylate medication use (4 weeks)
H5CRP3	Flag indicating Cox-2 Inhibitor medication use (4 weeks)
H5CRP4	Flag indicating Inhaled Corticosteroid medication use (4 weeks)
H5CRP5	Flag indicating Corticotropin/Glucocorticoid medication use (4 weeks)
H5CRP6	Flag indicating Anti-rheumatic/Anti-psoriatic med use (4 weeks)
H5CRP7	Flag indicating Immunosuppressive medication use (4 weeks)
H5CRP8	Flag indicating any Anti-Inflammatory medication use (4 weeks)

7.2.2 Additional inflammation and immune function data file (binflam5.xpt)

The additional inflammation and immune function data file (binflam5.xpt) includes the variables below, which are described in the corresponding codebook documentation that also contains frequencies.

Variable Name	Variable Description
AID	Respondent Identifier
H5CRPFL	Flag indicating hsCRP concentration type
H5CMVZ	Z-transformed Cytomegalovirus concentration (CMV, standard deviations)
H5CCMV	CMV Classification
H5CMVDIA	Flag indicating CMV Diamedix assay
H5CMVFL	Flag indicating CMV concentration type
H5IL1B	Interleukin 1 beta concentration (IL-1β, pg/ml)
H5IL1BFL	Flag indicating IL-1β concentration type
H5IL6	Interleukin 6 concentration (IL-6, pg/ml)
H5IL6FL	Flag indicating IL-6 concentration type
H5IL8	Interleukin 8 concentration (IL-8, pg/ml)
H5IL8FL	Flag indicating IL-8 concentration type
H5IL10	Interleukin 10 concentration (IL-10, pg/ml)
H5IL10FL	Flag indicating IL-10 concentration type
H5TNFA	Tumor Necrosis Factor alpha concentration (TNF-α, pg/ml)
H5TNFAFL	Flag indicating TNF-α concentration type

7.3 Use

Despite attempts to harmonize methods across Waves IV and V, important inter-Wave differences in protocols, biospecimens, assays, and data quality exist, as grey-highlighted above. Their existence suggests that the measures of inflammation markers described in Sections 1-6 may not be readily comparable from wave to wave. Caution should therefore be exercised when leveraging repeated measures of inflammation from Wave IV-V, whether they are primary measures or constructed classifications. Indeed, the merit of pre-analytical z-transformation or quantile-based classification of Wave IV-V biomarkers (inflammation) and the potential pitfall otherwise associated with equating values in their original units across visits should be carefully considered before using these data.

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