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Add Health Wave IV Documentation

Measures of Inflammation and Immune Function: Addendum 1

Report prepared by

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Citation

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1. Introduction

During Wave IV, Add Health collected biological specimens from a large, nationally representative sample of young adults. Given the size of the Wave IV sample, its geographic distribution, and in-home setting of the respondent interviews, biological specimen collection involved practical, relatively non-invasive, cost-efficient and innovative methods. These methods included collection of capillary whole blood via finger prick by trained and certified field interviewers, it's *in situ* desiccation, then shipment, assay and archival of dried blood spots. The collection of capillary whole blood followed the collection of cardiovascular and anthropometric measures (Entzel et al., 2009) and saliva (Smolen et al., 2013). It preceded the collection of data on respondent use of prescription and select over-the-counter medications (Tabor et al., 2010). Further details on the design of Add Health Waves I-IV, are available elsewhere (Harris, 2019), as are descriptions of the previously assayed biomarkers (Whitsel et al., 2012a; 2012b; 2013).

After Wave V (in 2019-2020), the archived dried blood spots were assayed for seven additional measures of inflammation and immune function by two Add Health ancillary studies: [1] "*Life Course Process of Alzheimer's Disease: Sex Difference and Biosocial Mechanisms*" (R01AG057800) (Yang and Aiello) and [2] "*Health Disparities: Inflammatory Response, Immune Function, and Environmental (In)Congruence*" (R21HD095448) (Hargrove and Gaydosh). This document summarizes the rationale, equipment, protocol, assays, internal quality control, data cleaning, and external quality control for each one of the seven measures. 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.

Wave V measures of inflammation were based on venous blood collected via phlebotomy. In contrast, Wave IV measures of inflammation and immune function were based on assays of archived dried blood spots (DBS) collected by trained and certified field interviewers (FIs), as previously described (Whitsel et al., 2012a). Briefly, capillary whole blood was collected via finger prick, desiccated *in situ*, then shipped to the DBS laboratory and archived. Beginning in September 2019, archived DBS were assayed for the following measures of inflammation and immune function:

- 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] (EU/ml)
- Herpes Simplex Virus Type 1 [HSV-1] (index units)
- Helicobacter pylori [H. pylori] (EU/ml)

2. General Overview of Data Collection

A Blaise computer-assisted interview (CAI) program guided trained and certified FIs through the blood spot collection process. Help screens with step-by-step measurement instructions were accessible within the program. Each FI also carried a Job Aids Booklet that served as a quick reference guide to study protocols.

Respondents were free to decline any or all measurements and specimen collections while participating in other components of the interview. In the Wave IV data set, any measures that were missing due to unique circumstances at correctional facilities were coded as legitimate skips.

Some measurement protocols were revised in the period between the Wave IV Pretest (conducted in 2007) and the Main Study (conducted in 2008). Where the Pretest and Main Study data collection protocols differed significantly, this report documents the key differences between them. Pretest cases in the Wave IV data set are flagged for identification.

3. Capillary Whole Blood Collection

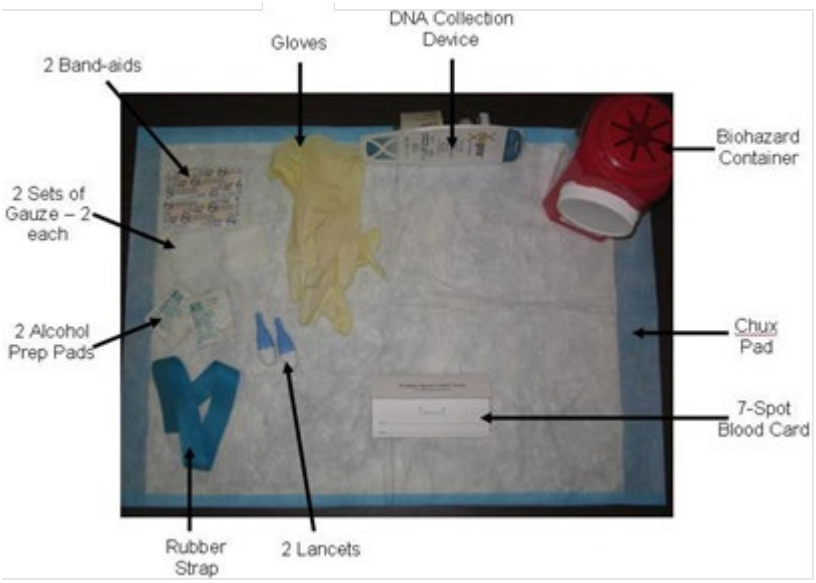
3.1 Rationale

Capillary whole blood was initially collected to provide Add Health with the biological specimens necessary to assay and interpret a pre-specified panel of metabolic, inflammatory, and immune biomarkers used in Wave IV. It also was collected to establish a dried capillary whole blood spot archive capable of supporting future assays and ancillary studies like those described herein.

3.2 Equipment

Equipment included sterile lancets, rubber strap, alcohol prep pads, gauze, Band-aid type adhesive dressings, gloves, biohazard container, Chux-type absorbent underpad, and a seven-spot capillary whole blood collection card (Whatman 903® *Protein Saver*, Whatman Inc., Piscataway, NJ) to which a stabilizing, buffered preservative had been pre-applied to Spot #1 (*Exhibit 1*).

Exhibit 1. Visit Supply and Blood Collection Kits



3.3 Protocol

3.3.1 Main Study

During the preceding anthropometric data collection procedure, all female respondents were asked specifically whether they had a prior mastectomy and, if so, on which side. If there were contraindications to using the right hand for capillary whole blood collection, the left hand was used. If there were contraindications on both hands, capillary whole blood was not collected.

FIs collected capillary whole blood from the respondent's middle or ring finger, unless one of the following contraindications was present:

- open sores, wounds, gauze dressings or rashes;
- casts, splints or shunts;
- intravenous (IV) catheters or other attached medical devices;
- swelling, withering or paralysis; or
- finger on same side as prior mastectomy.

FIs prepared the work surface for capillary whole blood collection and donned gloves. The help screen on the computer laptop directed FIs to refer to the Job Aids Booklet for the Fainting Protocol. FIs selected a finger for the procedure, cleaned it with the alcohol prep pad, and let it fully dry. While the finger was drying, FIs asked respondents to hang the selected finger below

their waist while applying the rubber strap to the midpoint of the upper ipsilateral arm. After placing the rubber strap, respondents started a timer on the laptop computer designed to sound an audible cue after three minutes to prompt removal of the rubber strap. FIs placed the clean finger against the work surface and firmly placed a sterile lancet against it to prick the fingertip, slightly lateral of center. FIs firmly wiped away the first drop of capillary whole blood with gauze, applying pressure to the base of (but not milking) the finger to facilitate flow. FIs were trained to allow a large droplet to accumulate before dropping it onto the first circle of the seven-spot capillary whole blood collection card and to do the same for the remaining six circles from left to right, all without allowing the fingertip to touch the card (*Exhibit 2*).

Exhibit 2. Collection of Capillary Whole Blood

When seven capillary whole blood spots were successfully collected (or blood droplet formation ceased), FIs wiped off remaining blood with gauze, instructed respondents to firmly apply the gauze to the finger for at least two minutes, and then applied a band aid to it. FIs collecting fewer than five spots less than 80% full from a single prick requested respondents' permission to repeat the capillary whole blood collection procedure on a second finger from the contralateral hand. FIs asked respondents to discard used capillary whole blood collection equipment in their own trash receptacle (except for lancets which were discarded in the biohazard container). FIs discarded them in the biohazard container when interviews were conducted in public locations.

FIs bar code labeled each capillary whole blood spot collection card with the corresponding respondent biospecimen ID and then air dried it for three hours.

3.3.2 Blood Processing and Shipment

After collection, FIs packaged each card with a desiccant pack and shipped it in a FedEx Priority Overnight envelope to the University of Washington Department of Laboratory Medicine (UW Lab Med, Mark H. Wener, M.D., Director, Seattle, WA) for assay.

UW Lab Med received the FedEx Priority Overnight envelopes containing a single dried blood spot collection card and desiccant pack. They scanned the FedEx tracking number and bar code-labeled card into a database in the order of receipt. They also keyed the receipt date, number of dried blood spots per card (0-7), number of adequate blood spots per card defined by blood filling ≥ 80% of the target area (0-7), comments on dried blood spot quality, and condition of the

desiccant pack alongside the biospecimen ID. They grouped the cards (≤ 25 per group), sealed the groups in Ziploc bags with desiccant packs, and stored them at -70°C until processing.

Immediately before processing, they warmed cards to room temperature (23°C) and re-scanned the bar code-labeled card into the database. The cards were punched for all assays except hemoglobin A1c (HbA1c), returned to the freezers, then shipped frozen to the Carolina Population Center (CPC, University of North Carolina, Chapel Hill, NC) for permanent archival at -80°C in the Add Health biospecimen freezers. At the CPC, Spot #1 on each card was removed, re-bundled (≤ 25 per group), sealed in plastic bags with desiccant, and shipped frozen by next day air to FlexSite Diagnostics, Inc. (Robert A. Ray, Ph.D., Director, Palm City, FL) for HbA1c assay.

3.3.3 Pretest Methodological Variations

During the Pretest, respondents chose the middle or ring finger and FIs were directed not to use the thumb, index finger or fifth digit/little finger for capillary whole blood collection. FIs also collected up to ten capillary whole blood spots: three on a BIOSAFE Blood Collection Card for HbA1c and Cholesterol Panel (BIOSAFE Laboratories, Inc., Chicago, IL) and seven more on a Whatman 903® Protein Saver, (Whatman Inc., Piscataway, NJ). The BIOSAFE card was made of Whatman 903® filter paper (Whatman International, Dassel, Germany) with a top layer of TELFA (Kendall Healthcare Products, Mansfield, MA) to minimize the effects of blood spot layering and inadvertent touching of cards with respondent fingertips (Tyrrell, 1999; Maggiore, 2002; Bui et al, 2002a; Bui et al, 2002b; Grzeda et al, 2002). A stabilizing borate buffered preservative was pre-applied by BIOSAFE to the area of the BIOSAFE card designated for HbA1c assay and dried. As in the main study, the Whatman 903® Protein Saver card was shipped to UW Lab Med for assay.

3.3.4 Selection and Shipment of Samples for Additional Assays

DBS cards of respondents targeted by the two ancillary studies were sampled from the complete set of archived DBS cards. Because both studies planned to rely on DBS cards, use the same external laboratory, and assay some of the same analytes, a single set of cards was pulled from the archive and sent to the Laboratory for Human Biology Research (Northwestern University, Thomas McDade, Director, Chicago, IL), as described below.

Study #1 (R01AG057800) (Yang and Aiello) targeted a random sample of 5000 respondent DBS cards for assay of CMV, HSV-1, H. Pylori, and IL-6 to match the biosample participants in Wave V. This study was approved first. Later, a second study (Study #2 (R21HD095448) (Hargrove and Gaydosh)) proposed to add several of the same biomarkers and additional cytokines. Specifically, they targeted 1600 respondent DBS cards for assay of CMV, IL-6, and newly added IL-8, IL-10, and TNF-α, including those of all sibling pairs in Wave IV and a random sample of approximately 700 non-Hispanic white, non-Hispanic black, and Hispanic respondents.

Given those specifications, Add Health generated a pull list of 5021 respondent DBS cards with enough spots to run all the assays. To that set, Add Health interleaved a second (masked) DBS card from 22 of the 5021 Add Health respondents in a race / ethnicity-and sex-stratified random sample among whom blood had been collected twice, one-two weeks apart (see section 5.1). Add Health also interleaved 13 DBS cards from the same non-participant.

Thereafter, the 5021 + 22 + 13 = 5056 DBS cards were pulled from -80°C Add Health biospecimen freezers and immediately placed into large cardboard boxes containing approximately 20 cold packs previously frozen at -80°C. The boxes were sealed and sent overnight to the McDade lab by FedEx in two shipments. Upon receipt the following day, the DBS cards were unpackaged and immediately transferred to -30°C freezers. Of the total number of 5056 cards, 2 were non-viable upon receipt. The 22 masked cards and the 13 additional non-participant cards are not included in the final dissemination file. *All told, then, the released data set contains an N of 5,019.*

4. Assays and Internal Quality Control

4.1 Rationale

Ancillary Study #1 (R01AG057800) (Yang and Aiello) and #2 (R21HD095448) (Hargove and Gaydosh) hypothesized that chronic stress is associated with inflammation, compromised immune function, and ultimately, cardiovascular and neurological disease. To help address their hypotheses, the ancillary studies therefore assayed four cytokine concentrations as measures of inflammation (IL-6; IL-8; IL-10; TNF-α) and three antibody titers as measures of immune function (i.e. CMV; HSV-1; *H. pylori*).

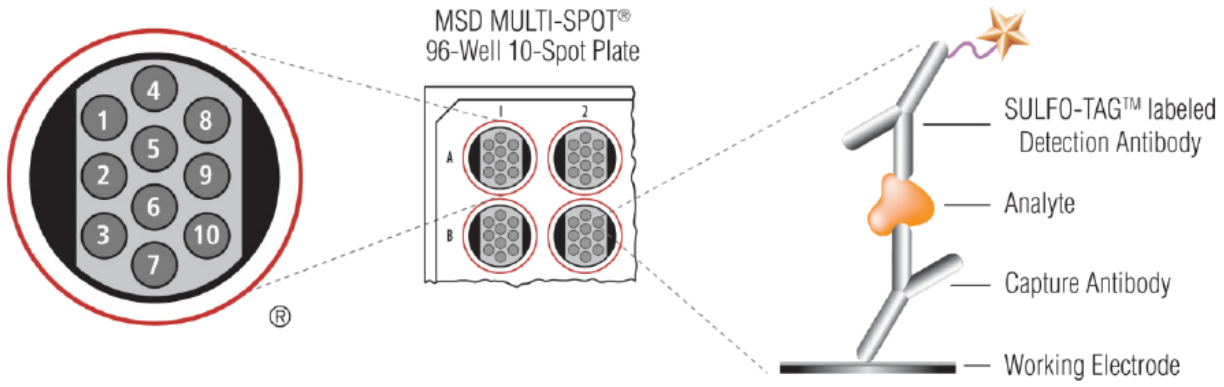
4.2 Cytokine Assays [H4IL6, H4IL8, H4IL10, H4TNFA]

4.2.1 Assay Principle

Samples were analyzed for 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; K151AOH) and read using an MSD Sector Imager 2400 (Meso Scale Diagnostics, Rockville, MD) (McDade et al., 2020). This platform allowed for the simultaneous quantification of multiple targets 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 DBS assay method was based on modifications to the V-PLEX kit designed for quantification of cytokines in cell culture, plasma, serum, and urine. 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 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 (Exhibit 3). 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 3. VPLEX Cytokine assay Scheme



4.2.2 Kit Calibration Preparation

DBS calibration material was made as follows (McDade et al., 2020, McDade, 2014). Washed red blood cells were obtained by adding equal volumes of normal saline (0.86 g NaCl/100 ml H_2O) and packed red blood cells (Valley Biomedical, #HP1002) to 14 ml borosilicate glass tubes (VWR, #47729-576), mixing gently, and centrifuging at 1500 x g for 15 minutes. Saline and any remaining buffy coat were removed with a pipet, and the process was repeated for a total of three washes. Kit calibrator was reconstituted in dilution buffer (phosphate-buffered saline with 4.0% (40 g/l) bovine serum albumin (BSA, Millipore Sigma 291025GM) and serially diluted 1:4 in dilution buffer four times, and then 1:2 twice. Neat dilution buffer was used as the zero calibrator. Starting concentrations were as follows: IL-6, 767.0 pg/ml; IL-8, 584.0 pg/ml; IL-10, 355.0 pg/ml; and TNF-α, 340.0 pg/ml. Each diluted calibrator was then mixed with an equal volume of washed red blood cells and whole blood calibration material was applied to labeled filter paper cards (Whatman 903 Protein Saver Card, #10534612) in 70 ul aliquots using a pipette (Rainin pipet lite LTS L100). Cards were allowed to dry overnight at room temperature, and then stored at -30C in gas impermeable bags with desiccant (61161, VWR, Radnor, PA).

4.2.3 Assay Protocol

DBS calibrators, controls, and samples were eluted overnight in a Millipore MultiScreen HTS Filter Plate (Millipore, MSHVN4510). Three x 5 mm disks were removed from each spot using a pneumatic hole punch (Analytical Sales & Services, Inc, DBS-1) and placed in each well. Barcode IDs on each sample were scanned immediately prior to punching. All determinations were done in duplicate, for a total of 6 disks per sample. Samples were eluted by adding 50 µl buffer (phosphate buffered saline, 0.1% Tween-20) to each well, making sure all disks were submerged. Plates were covered, placed on a non-wicking surface, and incubated overnight at 4°C (McDade et al., 2020).

The next day, the MSD assay plate was washed three times with wash buffer (300 µl/well phosphate buffered saline, 0.05% Tween-20). The filter plate was removed from the refrigerator, stacked on top of the MSD assay plate, and centrifuged for 2 minutes at 1,900 x g (Eppendorf 5804, A-2-DWP) to separate filter paper disks and transfer eluate to wells pre-coated with capture antibodies. The MSD assay plate was incubated while shaking (~700 rpm) at room temperature for two hours. The plate was washed as before and 25µl of detection antibody solution was added to each well, followed by incubation while shaking (~700 rpm) for two hours at room temperature. The plate was washed as before, 150 µl of diluted read buffer (1:2 with deionized water) was added to each well and the plate was immediately analyzed on an MSD Sector Imager 2400. A 4-parameter logistic curve was generated to fit the signal and calibrator concentrations, and the concentrations of unknowns were calculated based on the standard curve for each analyte. Data output from each assay were exported from the Mesoscale Diagnostics reader software to an Excel template where results from each well were matched to the scanned barcode IDs.

The lower level of detection (LLOD) was defined as the lowest concentration of cytokine that can be confidently distinguished from zero. Ten replicates of the DBS zero calibrator were included in a single run, and the LLOD was calculated as the cytokine concentration (pg/ml) corresponding to the value three standard deviations above the mean value for the 10 replicates. The LLOD of each cytokine is listed in Exhibit 4.

Exhibit 4. VPLEX KIT Dynamic Reporting Range

Analyte	LLOD (pg/ml)
IL-6	0.4
IL-8	0.2
IL-10	0.4
TNF-α	0.9

4.2.4 Internal Quality Control

All assays were run by the same technician, using a single lot number of reagents from MSD and the same set of DBS calibrators and controls that were manufactured at the beginning of the project. Samples, calibrators, and controls were run in duplicate, and calibrators and controls were included in each 96-well plate. Following each assay, the lab inspected the calibration curve for fit, range and level of signal, and detection limits. Control samples with low, middle, and high cytokine concentrations were used to estimate inter-assay variability as a coefficient of variation (%CV), i.e. 100 x (standard deviation ÷ mean), to identify individual plates with values shifted higher or lower than expected, and to identify drift in results over time. Analysis of control values across all runs yielded the following inter-assay variability (Exhibit 5).

Exhibit 5. Inter-Assay Variability of Control Sample Data

	IL-6 (pg/ml)			IL-8 (pg/ml)			IL-10 (pg/ml)			TNF-α (pg/ml)		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Mean	2.29	13.29	65.21	1.76	9.93	49.73	1.35	15.89	86.73	0.99	6.00	30.22
SD	0.34	1.26	5.04	0.36	3.04	3.51	0.33	1.62	11.04	0.40	0.78	2.04
%CV	14.66	9.45	7.74	20.49	30.65*	7.05	24.24	10.21	12.72	40.30	12.94	6.75

** After excluding one outlier, 11.82 (see text).*

Higher variability is often seen at the low end of the assay range, and for these assays, a CV<15% is generally considered acceptable. Following the initial analysis of all samples, those samples with a high coefficient of variation (CV ≥ 15) across all four analytes were flagged, and those with sufficient sample volume were reanalyzed. The low control for TNF-α is lower than the other cytokines, which likely accounts for the higher CV while the mid and high controls indicate lower variability. For IL-8, the high CV for the mid-level control was driven by one outlier value. When this value was removed, the %CV was 11.82.

No plates were identified with consistently elevated or consistently reduced control values. Therefore, it was concluded that there were no consistent shifts in values for subsets of samples in particular plates. Control values were also plotted over time to inspect for evidence of drift. There was no consistent pattern of increase or decrease in any of the control samples over time for each of the cytokines.

4.2.5 Relationship Between Plasma- and DBS-Based Concentrations

Since the Meso Scale VPLEX kit was originally designed to be run on serum or plasma, and the two ancillary studies were based on DBS, matched plasma and DBS from 28 non-Add Health

subjects (demographic attributes unavailable) were assayed to examine the relationship between plasma and DBS-based concentrations estimated using the same VPLEX kit.

Venous blood was drawn into EDTA-coated vacutainer tubes from the antecubital fossa with a butterfly needle. Immediately following collection, five drops of whole blood (60 ul each) were transferred by pipet to filter paper (Whatman #903, GE Healthcare, Pascataway, NJ), allowed to dry overnight, and then stacked and stored at -30C. EDTA-venous blood was centrifuged for 20 minutes at 1800 x g, and aliquots of plasma were stored in cryovials at -80°C.

Samples were analyzed using the DBS protocol as described above, with the same calibration material and lot number of reagents used with Add Health DBS. Plasma was analyzed per kit instructions provided by MSD. Passing-Bablok regression models (*plasma concentration = intercept + slope x DBS concentration*) and bivariate correlations were used to evaluate patterns of statistical association across the samples (Passing et al., 1983, Stöckl et al., 1998). Difference plots were inspected for evidence of bias or inconsistent variability across the range of measurement (Bland et al., 1986, Bland et al., 1999). DBS values were converted to plasma equivalents using equations generated by Passing-Bablok regression before difference plotting and estimating the concordance correlation of absolute agreement. Parameters from Passing-Bablok regression were as follows (Exhibit 6).

Exhibit 6. Passing-Bablok Regression Parameters

Cytokine	Intercept	95% CI	Slope	95% CI	Concordance correlation
IL-6 (pg/ml)	0.01	-0.05, 0.11	0.78	0.66, 0.89	0.97
IL-8 (pg/ml)	1.05	-1.35, 3.29	0.03	-0.00, 0.06	0.25
IL-10 (pg/ml)	-0.03	-0.16, 0.07	0.63	0.44, 0.84	0.77
TNF-α (pg/ml)	0.18	-0.44, 0.75	0.49	0.29, 0.68	0.79

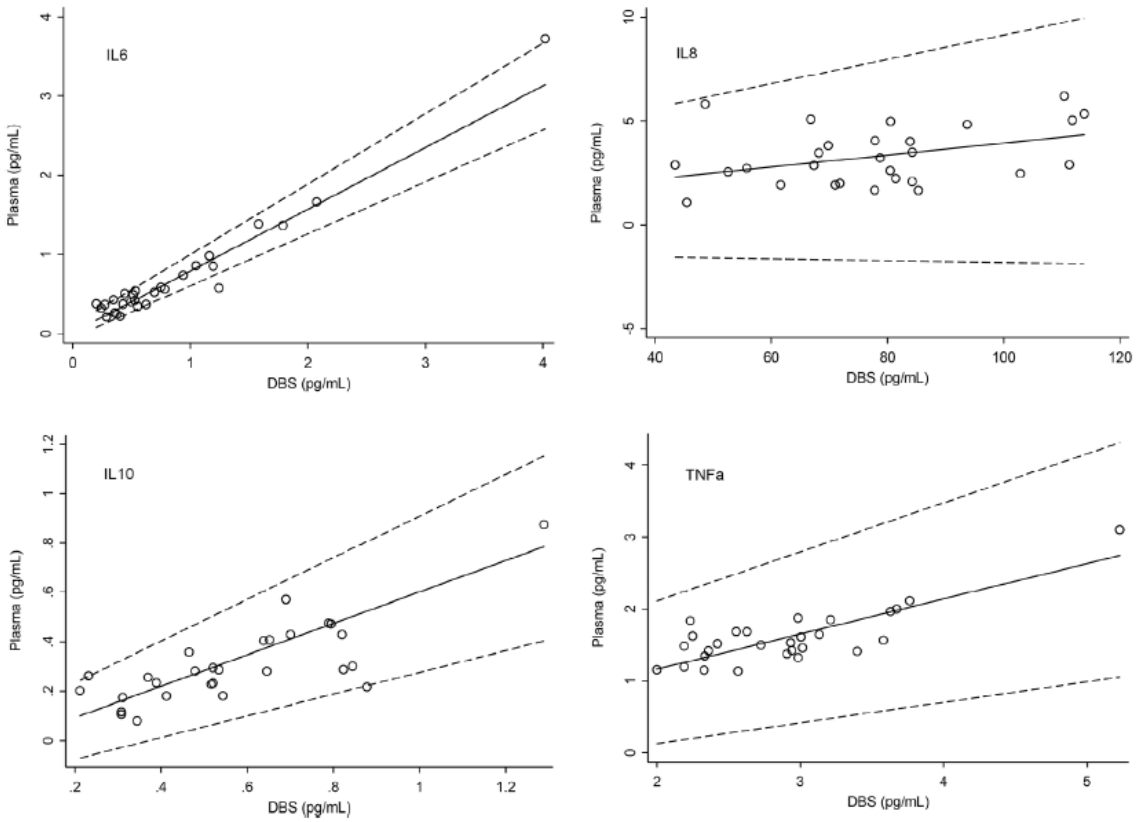
CI = Confidence Interval

The intercept and slope measured the systematic and proportional differences between the plasma- and DBS-based concentrations. Their 95% confidence intervals (CIs) were used to test the hypotheses that the intercept = 0 and slope = 1. Across cytokines, the intercept CI uniformly contained 0, but the slope CI was uniformly lower than and excluded 1, reflecting the fact that in general, DBS-based concentrations were higher than plasma-based concentrations, with disproportionately higher plasma concentrations at the higher end of the measurement range.

For IL-6, the pattern of association was linear across the measurement range (Exhibit 7), with very high correlation between DBS and plasma values and relatively tight 95% limits of agreement in the difference plot (-0.29 to 0.34 pg/ml). Results were similar for IL-10 and TNF-α indicating a strong pattern of agreement in results, although correlations of values were lower than IL-6.

Results for IL-8 were qualitatively different, with low correlation between DBS and plasma concentrations and high 95% limits of agreement (-2.55, 2.59 pg/ml).

Exhibit 7: Scatterplot and Passing-Bablok Regression Line (95% Confidence Interval)



Based on the results of this analysis—particularly the higher inter-assay variability of IL-8 reported above—analyses of DBS-based cytokine concentrations should proceed with caution. A key consideration here is the fact that red blood cells—which are present in DBS, but not serum or plasma, serve as reservoirs for cytokines/chemokines in circulation, e.g. IL-8 (Darbonne et al., 1991, Karsten et al., 2018). Although they may play important immunoregulatory roles in vivo, the standard is therefore to quantify them in serum/plasma. Moreover, the validity of the capillary whole blood DBS-based cytokine concentrations was only addressed within the 28 non-Add Health subjects introduced above. Finally, the range of DBS-based cytokine concentrations was much narrower in this small group of 24 subjects (IL-6: 0.2 – 4.0 pg/ml; IL-8: 43.4 – 113.8 pg/ml; IL-10: 0.2 - 1.3 pg/ml; TNF-α: 2.0 – 5.2 pg/ml) than in Add Health Wave IV (IL-6: 0 – 850 pg/ml; IL-8: 18 – 27,096 pg/ml; IL-10: 0 – 379 pg/ml; TNF-α: 0 – 590 pg/ml).

An important implication for users of these data is that DBS-based IL-8 concentrations should not be converted to plasma-equivalent concentrations based on the regression equation above given the low correlation and a strong biological basis for expecting different concentrations across sample types. Similarly, DBS-based IL-6, IL-10, and TNF-α concentrations should not be extrapolated to plasma-equivalent concentrations outside the observed range of DBS-based concentrations in the

28 non-Add Health subjects. All other conversions should acknowledge the limitations associated with the critical reliance of the regression parameters used to effect conversions on a small, non-representative group of non-Add-Health subjects with a narrow range of cytokine concentrations.

4.3 Antibody Titer Assays [H4CMV, H4HSV, H4HPYL]

4.3.1 Cytomegalovirus (CMV)

4.3.1.1 Assay Principle

Samples were analyzed for anti-CMV IgG antibodies using an enzyme-linked immunosorbent assay (ELISA) (Diamedix, 720-320, Miami Lakes, FL) as previously described (Dowd et al., 2011). In this assay, diluted samples are incubated with CMV antigen bound to the solid surface of a microtiter well. If IgG antibodies against CMV are present in the samples they will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. Substrate is then added and incubated. In the presence of bound enzyme, the substrate is converted to an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to CMV antigen present in the sample.

4.3.1.2 Kit Calibration and Preparation

Calibrators were provided with known concentrations of CMV antibody reported in ELISA units: 0, 10, and 160 EU/ml. Absorbance values from the calibrators were used to generate a point-to-point fit, from which CMV antibody concentrations were calculated based on each sample's absorbance value (Gen 5 3.09, BioTek).

With the exception of DBS controls, all materials for each assay were provided by the kit manufacturer. In order to evaluate comparability in DBS results across plates, negative and positive DBS control samples were manufactured as follows: For the negative DBS control, whole venous blood was collected from a negative donor and immediately transferred to filter paper cards (Whatman 903 Protein Saver Card, #10534612) in 50 µl aliquots using a pipette (Eppendorf Repeater E3). Cards were allowed to dry overnight at room temperature, and then stored at -30°C in gas impermeable bags with desiccant (61161, VWR, Radnor, PA). Control material was punched and eluted prior to each assay along with other samples. For the positive DBS control, the negative DBS control was eluted in 250 µl sample diluent and then spiked with 150 µl high

calibrator (160 EU/ml). After mixing, the positive DBS control was transferred to the assay plate along with other samples, controls, and calibrators.

4.3.1.3 Assay Protocol

The day before an assay was to be performed, DBS samples were removed from the freezer and one 3.2-mm diameter disc was removed using a pneumatic hole punch (Analytical Sales & Services, Inc, DBS-1) and placed in each well. Barcode IDs on each sample were scanned immediately prior to punching. 250 µl of phosphate buffer sample diluent (supplied with the kit) was added, and the sample was covered and incubated overnight at 4°C. The following day, 100 µl of whole blood eluate was transferred in duplicate to microtiter plate wells pre-coated with purified CMV antigen. Three levels of anti-CMV antibody in stabilized human sera were also added as calibrators, along with three serum kit controls and two DBS controls. Following a wash step, anti-human IgG conjugated with horseradish peroxidase was added to bind to the antibody-antigen complex. Excess conjugate was washed away and a chromogenic substrate was added to catalyze color development directly proportional to the amount of anti-CMV IgG antibody in the sample. Absorbance at 450 nm was obtained with a plate reader spectrophotometer (BioTek ELx 808, Winooski, VT) (Dowd et al., 2011).

4.3.1.4 Internal Quality Control

Analyses of samples was performed from 1/28/21 to 4/8/21. All assays were implemented by the same technician, using a single lot number of kits from the manufacturer, and the same set of DBS controls that were manufactured at the beginning of the project. Samples, calibrators, and controls were run in duplicate, and calibrators and controls were included on each 96-well plate. Following each assay, the calibration curve was inspected for fit, range and level of signal, and detection limits. Kit controls were inspected to confirm that values were within expected manufacturer ranges. In addition, kit and DBS control samples were used to calculate inter-assay variability as a coefficient of variation (%,CV)), i.e. 100 x (standard deviation ÷ mean), to identify individual plates with values shifted higher or lower than expected, and to identify drift in results over time. The negative controls were not included in these calculations—they were used only to confirm the absence of false positive results.

All calibration curves were acceptable in terms of fit, range, and detection limits. Analysis of control values across all runs indicates that inter-assay variability was as follows (Exhibit 8):

Exhibit 8. CMV Inter-assay Control Values

	Kit Low (EU/ml)	Kit High (EU/ml)	Positive DBS (EU/ml)
Mean	25.9	102.1	92.6
SD	2.1	5.4	4.9
%CV	8.1	5.3	5.3

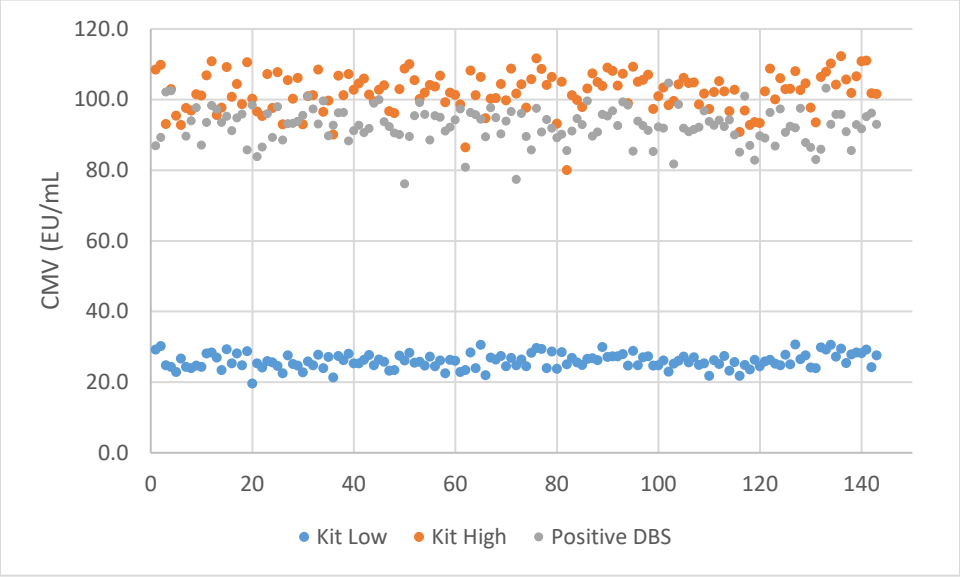
Per Diamedix, acceptable values are as follows: negative control, <5; Low control, 18-35; High control, 80-120.

No out-of-range values were identified for any of the kit controls on any of the assays and no plates were identified with consistently elevated or consistently reduced control values. Therefore, it appears that there were no consistent shifts in values for subsets of samples on particular plates.

Following the initial analysis of all samples, samples with CMV values above the lowest seropositivity cut-off (35 EU/mL) were identified for rerun if the coefficient of variation for sample duplicates was greater than 10%.

Control values were plotted over the duration of the sample testing to measure evidence of drift (Exhibit 9). Based on this data, it was concluded that there were no consistent patterns of increase or decrease in any of the control samples over time.

Exhibit 9. CMV Plot of Control Values Across Assays



4.3.1.5 Relationship Between Plasma- and DBS-Based Concentrations

Agreement between DBS and serum results was previously evaluated in a set of matched samples collected from 75 non-Add Health subjects (demographic attributes unavailable) for the purpose of assay validation (Dowd et al., 2011). Samples were analyzed using the protocol as described above. Correlation between blood spot and serum concentrations among positive serum samples was linear and high (Pearson correlation R=0.96.

Validation samples were also used to evaluate concordance in seropositivity determinations and to establish seropositivity cut-off values for DBS samples (Dowd et al., 2011). Exhibit 10 provides a range of cut-off values that can be applied to the DBS results, albeit recognizing that in general, sensitivity and specificity often differ markedly across populations.

Exhibit 10. DBS Seropositivity Sensitivity and Specificity

Cut-Off (EU)	Sensitivity* (%)	Specificity# (%)
35	100	80.9
46	92.9	93.6
48.5	85.7	95.7
51.5	82.1	97.9
56	82.1	100.0

**Sensitivity = % of positive serum samples testing positive with DBS*
#Specificity = % of negative serum samples testing negative with DBS

According to Dowd et al. (2011), "For the purposes of measuring continuous CMV antibody level in middle-aged and older populations where the majority of individuals are already seropositive, precise ascertainment of dichotomous seropositivity is less crucial and investigators may choose a lower, more lenient cut-off to reduce the number of false-negative individuals. For investigators more concerned with seropositivity as an outcome itself, a higher, more conservative cut-off can be used to help reduce the number of false-positives."

4.3.2 HSV-1

4.3.2.1 Assay Principle

Samples were analyzed for IgG antibodies against herpes simplex virus type 1 (HSV-1), using an enzyme immunoassay protocol modified and validated in-house for use with DBS. The protocol was based on a commercially available enzyme immunoassay kit for the quantification of HSV-1 IgG antibodies (Focus HerpesSelect 1 ELISA IgG ELISA Kit, #EL0910G). In this assay, polystyrene microwells were coated with recombinant gG-1 antigen. Diluted serum samples and

controls were incubated in the wells to allow specific antibody present in the samples to react with the antigen. Nonspecific reactants were removed by washing and peroxidase-conjugated anti-human IgG was added and reacted with specific IgG. Excess conjugate was removed by washing. Enzyme substrate and chromogen were added, and the color was allowed to develop. After adding the Stop Reagent, the resultant color change was quantified by a spectrophotometric reading of optical density (OD). Sample optical density readings were compared with reference cut-off OD readings to determine results (Focus Diagnostics, Cypress, CA) reported in index units i.e., the optical density divided by the mean of the cut-off calibrator absorbance.

4.3.2.2 Kit Calibration and Preparation

With the exception of DBS controls, all materials for each assay were provided by the kit manufacturer. In order to evaluate comparability in DBS results across plates, whole venous blood was collected from a positive donor. This blood was diluted to create a range of control material and then transferred to filter paper cards (Whatman 903 Protein Saver Card, #10534612) using a pipette (Eppendorf Repeater E3). Cards were allowed to dry overnight at room temperature, and then stored at -30°C in gas impermeable bags with desiccant (61161, VWR, Radnor, PA). Control materials were punched and eluted prior to each assay along with other samples following identical procedures.

Two levels of positive controls, as well as a negative control, and a cut-off calibrator were supplied by the test kit. All specimens, controls and calibrators were diluted 1:101 using a kit supplied sample diluent prior to the assay procedures.

4.3.2.3 Assay Protocol

The day before an assay was to be performed, study samples and two DBS controls were removed from the freezer and two 3.2-mm diameter discs were removed from each using a manual hole punch (McGill Handheld Gem Punch, 1/8 inch, #MCG401CR) and placed into a single well of a deep well non-binding plate (Beckman Coulter, #267006) with 250 µl of kit supplied sample diluent. Barcode IDs on each sample were scanned immediately prior to punching. The sample was covered and incubated overnight at 4°C. The following day, whole blood eluates were transferred in duplicate from the deep well plate to microtiter plate wells pre-coated with recombinant HSV-1 IgG ab antigen. A blank, cut-off calibrator, and kit controls were also added. Following a wash step, anti-human IgG conjugated with horseradish peroxidase was added to bind to the antibody-antigen complex. Excess conjugate was washed away, and chromogenic substrate was added to catalyze color development directly proportional to the amount of anti-HSV-1 IgG antibody in the sample. The reaction was stopped with 1 M sulfuric acid and absorbance at 450 nm was obtained with a plate reader spectrophotometer (BioTek ELx 808, Winooski, VT).

4.3.2.4 Internal Quality Control

Analysis of samples began on 3/31/22, with the last set of samples run on 6/22/22. All assays were implemented by the same technician (except 4 runs due to technician illness), using a single lot number of kits from the manufacturer, and the same set of DBS controls that were manufactured at the beginning of the project. Samples and controls were run in duplicate, the cut-off calibrator was run in duplicate three times and averaged, and the calibrators and controls were included on each 96 well plate. Following each assay, results were inspected for range of cut-off calibrator, level of signal, and kit controls were inspected to confirm that values fell in the expected ranges per the kit manufacturer. In addition, kit and DBS control samples were used to calculate inter-assay variability (% coefficient of variation, CV; SD/mean), to identify individual plates with values shifted higher or lower than expected, and to identify drift in results over time. The negative kit control was not included in these calculations—it was used only to confirm the absence of false positive results. The lower level of detection for this assay = 0.002 index units.

Based on data from the quality control analysis one run was identified as requiring re-analysis. In this run the low positive control was outside of the specified manufacturer's performance parameters. A rerun resulted in a plate with acceptable control levels. Additionally, following the initial analysis of all samples, individual samples with values above the negative cutoff (index units ≥ 1.20) were identified for rerun if their coefficient of variation for duplicates was greater than 10%. Those samples that were flagged and had sufficient sample volume were rerun.

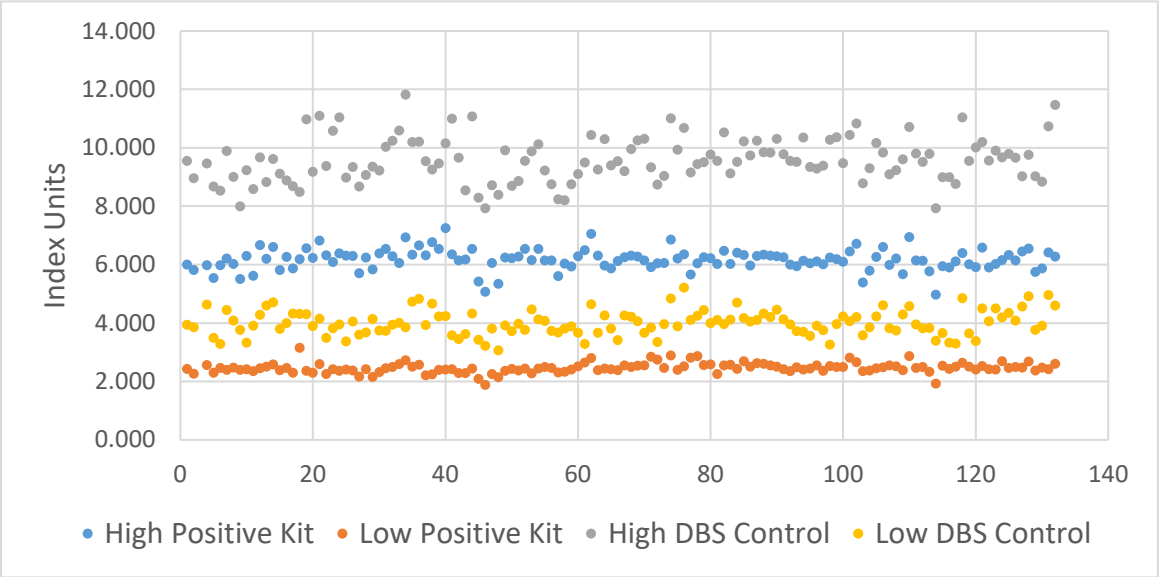
Analysis of control values across all runs indicates that inter-assay variability was as follows:

Exhibit 11. Table of HSV-1 Controls (index units) Across Assays

	High Positive (Kit)	Low Positive (Kit)	High DBS	Low DBS
Mean	6.2	2.5	9.6	4.0
SD	0.4	0.2	0.8	0.4
%CV	5.8	7.1	8.0	10.5

Control values over time were inspected for evidence of drift. There were no consistent patterns of increase or decrease in any of the control samples over time.

Exhibit 12. Plot of HSV-1 Controls (index units) Across Assays



From this, it was concluded that the results were comparable across all runs with acceptably low levels of inter-assay variability.

4.3.2.5 Relationship Between Plasma- and DBS- Based Concentrations

Agreement between DBS and plasma results was evaluated in a set of matched samples, collected from N=39 individuals (demographic attributes unavailable) for the purpose of assay validation. Samples were analyzed using the protocol as described above, with the same calibration materials and lot number of reagents used with Add Health samples. Correlation between blood spot and plasma values was linear and high (Lin's concordance correlation coefficient = 0.97). Seropositivity of plasma samples was determined according to test kit specifications. The corresponding seropositivity cut-offs for DBS samples (in index units) were determined based on the matched plasma seropositivity results and listed in Exhibit 13, albeit recognizing that in general, sensitivity and specificity often differ markedly across populations.

Exhibit 13. HSV-1 Seropositivity (index units)

Index Units	Result
> 1.43	seropositive
1.20 ≤ x ≤ 1.43	equivocal
< 1.20	seronegative

4.3.3 Helicobacter pylori (H. pylori)

4.3.3.1 Assay Principle

Samples were analyzed for H. pylori using an ELISA for the quantitative determination of IgG antibodies to H. pylori in human serum (Abnova, KA0220, Taipei City, Taiwan). In summary, purified *H. pylori* antigen was coated on the surface of microwells. Diluted serum was added to the wells, and the *H.pylori* IgG-specific antibody, if present, bound to the antigen. All unbound materials were washed away. Enzyme conjugate was added, which bound to the antibody-antigen complex. Excess enzyme conjugate was washed off and a solution of TMB Reagent was added. The enzyme conjugate catalytic reaction was stopped at a specific time, and the intensity of the color generated was proportional to the amount of IgG-specific antibody in the sample. The results were read by a microwell reader compared in a parallel manner with calibrator and controls (Helicobacter Pylori IgG ESLISA Kit Instructions).

4.3.3.2 Kit Preparation, Calibration, and Protocol

With the exception of DBS controls, all materials for each assay were provided by the kit manufacturer. Calibration standards of 0, 6.25, 12.5, 25, 50 and 100 U/ml were used to form the calibration curve, along with a negative control sample (<6.25 EU/ml), and a positive control sample (>100 EU/ml). The day before an assay was to be performed, DBS samples were removed from the freezer and duplicate 3.2-mm diameter discs were removed using a pneumatic hole punch (Analytical Sales & Services, Inc, DBS-1) and placed in microtiter wells of a 96 well filter plate. Barcode IDs on each sample were scanned immediately prior to punching. 100 µl of sample diluent (supplied with the kit) was added, and the sample was covered and incubated overnight at 4°C. On the day of the assay, the six standards, plus the positive and negative controls were diluted 1:40 in sample diluent and mixed thoroughly.

In order to evaluate comparability in DBS results across plates, negative and positive DBS control samples were manufactured as follows: For the negative DBS control, whole venous blood was collected from a negative donor and immediately transferred to filter paper cards using a pipette. Cards were allowed to dry overnight at room temperature, and then stored at -30°C in gas impermeable bags with desiccant. Control material was punched and eluted prior to each assay along with other samples. For the positive DBS control, the negative DBS control was eluted in 100 ul sample diluent and then spiked with 50 ul of the high kit control. After mixing, 100ul of the positive DBS control was transferred to the filter plate and centrifuged into the assay plate along with other samples, controls, and calibrators.

The filter plate containing the eluted serum was placed on top of the ELISA plate and centrifuged at 3,600 rpm for two minutes to transfer the eluted DBS samples into the assay plate pre-coated

with purified H. pylori antigen. Next, 100 µl of standards, controls, and sample diluent (as a blank) were dispensed into the assay plate and it was incubated for 30 minutes at room temperature. All plates were then washed four times in wash buffer and once in distilled water in an automated plate washing machine. 100 µl of enzyme conjugate was added to each well and the plate was incubated for 30 minutes at room temperature. The plates were then washed repeating the procedure listed above. 100 µl of TMB solution was then added to each well to develop color. The assay plates were incubated for 20 minutes at room temperature, and then 100 µl of stop solution was added to all wells. Absorbance at 450 nm was obtained with a plate reader spectrophotometer (BioTek ELx 808, Winooski, VT). Absorbance values from the calibrators were used to generate a best fit four parameter logistic curve, from which H. pylori antibody concentrations were calculated based on each sample's absorbance value

4.3.3.4 Internal Quality Control

Analysis of samples began on 6/25/21, with the last set of samples run on 10/27/21. All assays were implemented by the same technician, using a single lot number of kits from the manufacturer, and the same set of DBS controls that were manufactured at the beginning of the project. Samples, calibrators, and controls were run in duplicate, and calibrators and controls were included on each 96 well plate. Following each assay. The calibration curve was inspected for fit, range and level of signal, and detection limits. Kit controls were inspected to confirm that values fell in the expected ranges, per the kit manufacturer. In addition, kit and DBS control samples were used to calculate inter-assay variability (% coefficient of variation, CV; SD/mean), to identify individual plates with values shifted higher or lower than expected, and to identify drift in results over time. The negative kit control was not included in these calculations—it was used only to confirm the absence of false positive results. The lower limit of detection for this assay was 0.507 EU/mL.

Following the completed initial analysis of all samples, the results were reviewed for reruns for samples above the lowest equivocal range cut-off (12.2 EU/mL – see section 4.3.3.5) and within range of the assay. Samples with the highest coefficient of variation for sample duplicates were identified and those with a CV > 18.5 and sufficient sample volume were re-analyzed.

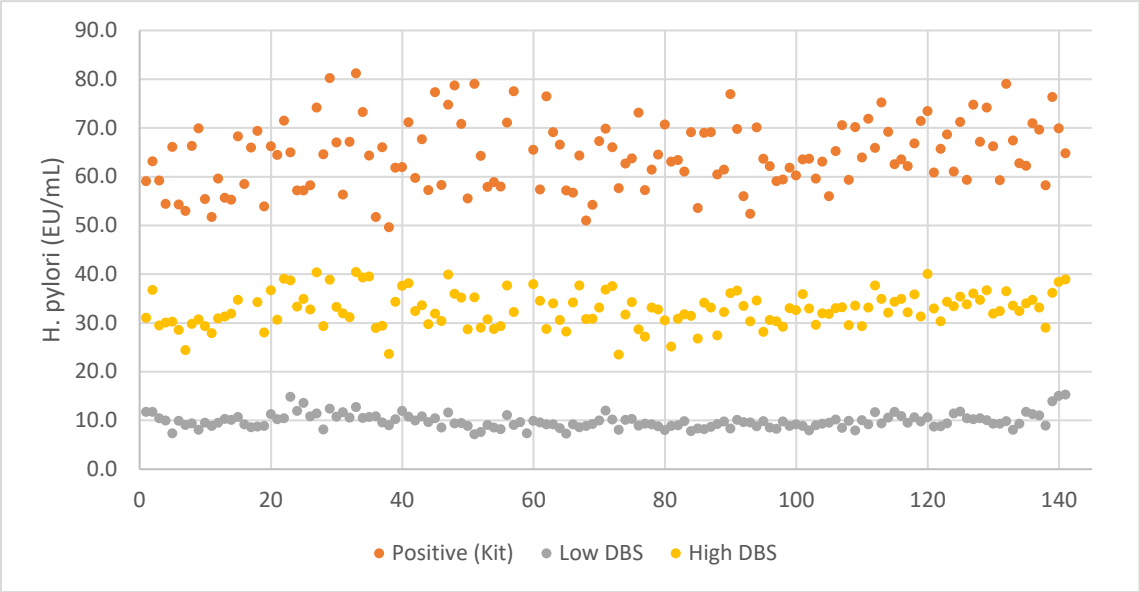
All calibration curves throughout the assay were acceptable in terms of fit, range, and detection limits. Analysis of control values across all runs indicates that inter-assay variability was as follows:

Exhibit 14. H. Pylori Inter-Assay Control Values

	Kit Low (EU/ml)	Kit High (EU/ml)	High DBS (EU/ml)
Mean	64.4	9.8	32.8
SD	7.0	1.5	3.6
%CV	10.9	15.0	10.9

Control values were plotted over time to inspect for evidence of drift. There was no consistent pattern of increase or decrease in any of the control samples over time.

Exhibit 15. H. Pylori Plot of Control Values Across Assays



From this data, it was concluded that the results are comparable across all runs with acceptably low levels of inter-assay variability. We further conclude that no samples need to be re-assayed due to technical errors or shifts in values produced by any particular plates.

4.3.3.5 Relationship Between Plasma- and DBS-Based Concentrations

Agreement between DBS and serum results was evaluated in a set of matched samples, collected from N=37 individuals (demographic attributes unavailable) for the purpose of assay validation. Samples were analyzed using the protocol as described above, with the same calibration material and lot number of reagents used with Add Health samples. Correlation between blood spot and serum values among positive serum samples was linear and high (Lin's concordance correlation coefficient = 0.95).

Seropositivity of serum samples was determined according to test kit specifications, with > 20 U/ml = seropositive. The corresponding seropositivity cut-off for DBS samples determined based on the matched serum seropositivity results was 13.2 EU/ml, (95% confidence interval = 12.2-15.3), albeit recognizing that in general, sensitivity and specificity often differ markedly across populations.

5. External Quality Control

5.1 Reliability

5.1.1 Cytokine Assays

Within a race / ethnicity- and sex-stratified random sample of (n = 22) Add Health respondents among whom capillary whole blood was collected twice, one-two weeks apart, intra-individual reliability of IL-6, IL-8, IL-10 and TNF-α concentrations was estimated as an intra-class correlation coefficient (95% CI) before and after (1) natural log transformation; (2) natural log transformation and exclusion of concentrations identified using an extreme, studentized deviate multiple outlier detection procedure; and (3) decile ranking (Exhibit 16). The estimates suggest that the dried capillary whole blood spots collected at Add Health Wave IV yield lower reliability measures of untransformed IL-6, IL-8, IL-10 and TNF-α concentrations than venous blood collected at Add Health Wave V, but their reliability can be modestly increased by transformation, outlier exclusion, and by decile ranking, in particular. Decile ranking and / or other tools designed to incorporate measurement error in exposure-outcome models should therefore be considered when working with these data.

Exhibit 16. Reliability of IL-6, IL-8, IL-10 and TNF-α

Measure (pg/ml)	Untransformed [N: ICC (95%CI)]	Log-Transformed [N: ICC (95%CI)]	Log-Transformed & Outliers Excluded [N: ICC (95%CI)]	Decile-Ranked [N: ICC (95%CI)]
IL-6	22: 0.20 (-0.20, 0.61)	22: 0.42 (0.08, 0.77)	21: 0.53 (0.22, 0.84)	22: 0.45 (0.11, 0.78)
IL-8	22: 0.39 (0.02, 0.74)	22: 0.44 (0.10, 0.78)	20: 0.47 (0.12, 0.81)	22: 0.54 (0.24, 0.84)
IL-10	20: 0.46 (0.12, 0.81)	20: 0.34 (-0.05,0.73)	20: 0.34 (-0.05,0.73)	20: 0.43 (0.06, 0.79)
TNF-α	22: 0.25 (-0.15, 0.64)	22: 0.28 (-0.11,0.67)	22: 0.28 (-0.11,0.67)	22: 0.27 (-0.12, 0.66)

5.1.2 Antibody Titer Assays

Within a race / ethnicity- and sex-stratified random sample of (n=22) Add Health respondents among whom capillary whole blood was collected twice, one-two weeks apart, intra-individual reliability of CMV, H.pylori and HSV concentrations were estimated as an intra-class correlation coefficient (95% CI) (Exhibit 17). The estimates suggest that the whole blood collected at Add Health Wave IV yields highly reliable CMV, H.pylori and HSV concentrations.

Exhibit 17. Reliability of CMV, H.pylori and HSV

Measure	N	ICC	95% CI
CMV	21	0.99	(0.98, 1.00)
H. pylori	18	0.99	(0.97, 1.00)
HSV	21	0.98	(0.97, 1.00)

5.2 Drift

As noted in Section 3.3.4, Add Health interleaved 13 DBS cards from the same non-participant for assays of IL-6, IL-8, IL-10, TNF-α and CMV concentrations by laboratory staff, who were masked to the origin of the cards. Summary statistics across assays are provided below (Exhibit 18). Because assays of the 13 DBS cards were run over 3-6 months, seasonal variation and drift were graphically examined by plotting assayed concentrations versus assay date. Concentration-time slopes did not differ substantially from the null (Exhibit 19).

Exhibit 18. Summary Statistics for Assayed Concentrations

Measure	N	Mean	SD
IL-6 (pg/ml)	10	0.52	0.20
IL-8 (pg/ml)	13	71.10	11.56
IL-10 (pg/ml)	8	0.28	0.21
TNF-α (pg/ml)	12	1.91	0.49
CMV (EU/ml)	13	7.10	2.06
H.pylori (EU/ml)	13	8.90	1.79
HSV (index units)	7	0.45	0.10

Exhibit 19. Concentration-Time Slopes (95% Confidence Intervals)

Measure	N	Slope (95% CI)
IL-6 (pg/ml)	10	0.013 (-0.03, 0.06)
IL-8 (pg/ml)	13	0.824 (-1.13, 2.78)
IL-10 (pg/ml)	8	0.016 (-0.07, 0.03)
TNF-α (pg/ml)	12	0.024 (-0.07, 0.12)
CMV (EU/ml)	13	-0.106 (-0.50, 0.28)
H.pylori (EU/ml)	13	0.127 (-0.20, 0.46)
HSV (index units)	7	-0.039 (-0.09, 0.01)

6. Constructed Measures

6.1 Concentration Type Variables

All assay concentrations were categorized based on their corresponding LODs and extrapolation beyond them, then flagged as tabulated below (Exhibits 20-26). 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 (Shoari & Dubé, 2018)

Exhibit 20. Flag Indicating the IL-6 Concentration Type [H4IL6FL]

Code	Source of Result
1	IL-6 concentration is below the lower LOD (< 0.4 pg/ml) – missing
2	IL-6 concentration is below the lower LOD (< 0.4 pg/ml), but extrapolated
3	IL-6 concentration is within the LODs (0.4 – 849.59 pg/ml)
9	IL-6 concentration is missing – quantity not sufficient

Exhibit 21. Flag Indicating the IL-8 Concentration Type [H4IL8FL]

Code	Source of Result
1	IL-8 concentration is below the lower LOD (< 0.2 pg/ml) – missing
3	IL-8 concentration is within the LODs (0.2 – 27096.23 pg/ml)
9	IL-8 concentration is missing – quantity not sufficient

Exhibit 22. Flag Indicating the IL-10 Concentration Type [H4IL10FL]

Code	Source of Result
1	IL-10 concentration is below the lower LOD (< 0.4 pg/ml) – missing
2	IL-10 concentration is below the lower LOD (< 0.4 pg/ml), but extrapolated
3	IL-10 concentration is within the LODs (0.4 – 378.95 pg/ml)
9	IL-10 concentration is missing – quantity not sufficient

Exhibit 23. Flag Indicating the TNF-α Concentration Type [H4TNFAFL]

Code	Source of Result
1	TNF-α concentration is below the lower LOD (< 0.9 pg/ml) – missing
2	TNF-α concentration is below the lower LOD (< 0.9 pg/ml), but extrapolated

3	TNF-α concentration is within the LODs (0.9 – 590.14 pg/ml)
9	TNF-α concentration is missing – quantity not sufficient

Exhibit 24. Flag Indicating the CMV Concentration Type [H4CMVFL]

Code	Source of Result
3	CMV concentration is within the LODs (1.50 – 159.62 EU/ml)
5	CMV concentration is above the upper LOD (> 160.00 EU/ml) – missing

Exhibit 25. Flag Indicating the H.pylori Concentration Type [H4HPYLFL]

Code	Source of Result
3	H.pylori concentration is within the LODs (0.782 – 124.88 EU/ml)
5	H.pylori concentration is above the upper LOD (> 125.00 EU/ml) – missing

Exhibit 26. Flag Indicating the HSV Concentration Type [H4HSVFL]

Code	Source of Result
3	HSV concentration is within the LODs (-0.011* – 15.66)
9	HSV concentration is missing – quantity not sufficient

*Negative indicates that the o**ptical density was** slightly **< that in the blank (HSV = 0) sample**

7. The Inflammation and Immune Function Data File (binflam4.xpt)

7.1. Structure

The structure of the disseminated inflammation and immune function data file is flat. This means that it is a respondent-level data file, where each respondent has one and only one record. The respondent's identifying number (the AID variable) will appear in the data file only once.

7.2. Contents

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

Variable Name	Variable Description
AID	Respondent Identifier
H4IL6	IL-6 assay result (pg/ml)
H4IL6FL	Flag indicating IL-6 concentration type
H4IL8	IL-8 assay result (pg/ml)
H4IL8FL	Flag indicating IL-8 concentration type
H4IL10	IL-10 assay result (pg/ml)
H4IL10FL	Flag indicating IL-10 concentration type
H4TNFA	TNF-α assay result (pg/ml)
H4TNFAFL	Flag indicating TNF-α concentration type
H4CMV	CMV assay result (EU/ml)
H4CMVFL	Flag indicating CMV concentration type
H4HPYL	H. pylori assay result (EU/ml)
H4HPYLFL	Flag indicating H. pylori concentration type
H4HSV	HSV assay result (index units)
H4HSVFL	Flag indicating HSV 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 here and above. Their existence suggests that the measures of inflammation and immune function described in Sections 1-6 may not be readily comparable from wave to wave without standardization. Caution should therefore be exercised when leveraging repeated measures of inflammation and immune function 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 (IL-6; IL-8; IL-10; TNF-α) and the potential pitfall otherwise associated with equating values in their original units (pg/ml) across waves should be carefully considered before using these data.

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