

***Documentation of Interleukin-6 (IL-6)
Assays from Dried Blood Spots
2014 and 2016***

User Guide

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Introduction

This document describes the collection of Interleukin-6 (IL-6) in 2014 and 2016 Assays from Dried Blood Spots. This was the last time DBS based markers were collected in HRS. DBS have been collected from approximately half the sample in each year beginning in 2006.

Detailed descriptions of the procedures for collection and assay of DBS based data as well as VBS blood-based markers beginning in 2016 are available on the HRS website.

Documentation for the 2014 data are provided in “Documentation of Biomarkers in the 2014 Health and Retirement Study.” 2017. Eileen Crimmins, Jessica Faul, Jung Ki Kim and David Weir. https://hrs.isr.umich.edu/sites/default/files/biblio/Biomarker%202014_Dec2017.pdf

Additional documentation for the 2014 data are provided in “HEALTH AND RETIREMENT STUDY Sensitive Health Data Blood-Based Biomarkers 2014 Health and Retirement Study Data Description and Usage.” Version 1.0, December 2017.

https://hrsdata.isr.umich.edu/sites/default/files/documentation/data-descriptions/Biomarker2014DD_0.pdf

Blood-Based Biomarkers in the HRS

HRS began to collect DBS blood-based biomarkers on half the sample in 2006, and the other half of the sample provided DBS biomarker data in 2008. The first group was asked for blood samples again in 2010 and 2014; the second group gave repeat samples in 2012 and 2016. From 2006 through 2012, the dried blood spot (DBS) samples were assayed for 5 biomarkers.

In 2014 a 6th was added, IL-6, which is a cytokine indicator of inflammation. The biomarkers available now in the 2014 release and for 2016 include the following:

1. Total cholesterol (TC) an indicator of lipid levels
2. High Density Lipoprotein cholesterol (HDL), an indicator of lipid levels
3. Glycosylated hemoglobin (HbA1c) – an indicator of glycemic control over the past 2-3 months
4. C-reactive protein (CRP), a general marker of systemic inflammation
5. Cystatin C, an indicator of kidney functioning
6. IL-6, a cytokine indicator of inflammation

Laboratories

A series of labs have been used over the years to assay HRS DBS. However, in 2012, 2014 and 2016 the University of Washington did all assays.

University of Washington Department of Medicine Dried Blood Spot Laboratory

Immunology Division, Department of Laboratory Medicine

Director: Mark H. Wener, MD, wener@u.washington.edu

Project Director: Alan Potter, Ph.D., apotter@uw.edu

Procedures

Sample. The blood tests were intended for all of those who were available for the EFTF interview. Special informed consent was acquired for the blood acquisition process.

Consent Rate. The blood spot consent rate in 2014 was 90.68%. The completion rate, conditional on consent, was 99.64%. The overall completion rate was 90.36%. The blood spot consent rate in 2016 was 86.7%. The completion rate, conditional on consent, was 98.9%. The overall completion rate was 85.7%.

Collection. DBS samples were collected halfway through the interviews (Section I) by trained interviewers by filling up to ten circles with blood droplets across two Whatman blood spot cards. DBS cards were placed in a specially-designed cardboard box allowing airflow on all sides for a minimum of two hours of drying time prior to shipment. The loaded cardboard boxes were placed in placed in foil pouches with desiccant.

Shipping. In 2016, interviewers mailed the DBS cards directly to the Department of Laboratory Medicine at the University of Washington in Seattle for assay. On receipt, the lab staff in Washington coded the quality and characteristics of the DBS cards. DBS cards were stored at -70°C prior to and after analysis.

Assays

The Health and Retirement Study has used dried blood spots (DBS) to produce data on IL-6 in 2014 and 2016. DBS assays were done at the University of Washington for both years; however, a different reagent was used for the assays at the two dates resulting in data that are not comparable without significant adjustment.

In this document we describe the data from the 2014 and 2016 DBS assays and the process used by the lab for making 2016 values equivalent to 2014 and then for making plasma equivalent values for both of the assays.

2014 DBS (N=6131)

In the original data, there are 6,131 cases with valid values and HRS IDs. The lowest data value was 0.20 and the highest data value was 19.99. Values lower than 0.20 (N=1,207) were coded as Out Of Range-Low; and values higher than 19.99 (N=6) were coded as OOR-High. We coded OOR-High as 20.001 and coded OOR-Low as 0.19 to estimate means.

Mean DBS value (pg/mL)

N	Mean	Minimum	Maximum
6131	0.52 (SD=1.31)	0.19	20.001

2016 DBS (N=4908)

In 2016, there were 4,908 valid DBS samples with an HRS IDs. There were 139 cases that are lower than the detection limit (<0.329). The 2014 and 2016 assays differ in how the lower limit of detection was coded by the lab. For example, while IL-6 lower than 0.329 (N=139) was categorized as OOR-Low in 2016, the lower values are preserved in the data. This is in contrast to 2014, when

cases whose IL-6 was lower than the detection limit were coded as OOR-Low without values. In order to maintain consistent coding for lower and higher values in our analysis, we assigned 0.328 for those below the lower level of detection in 2016. Values from eight samples above the assay upper limit were topcoded at 20 and coded as >20.0 in the lab. In our analysis, in order to maintain consistent coding for higher values with 2014, we assigned 20.001 for those above upper limit.

Mean DBS value (pg/mL)			
N	Mean	Minimum	Maximum
4908	0.74 (SD=1.10)	0.328	20.001

This figure compares the distributions of the 2014 DBS IL-6 and 2016 DBS IL-6 for the data provided by the lab with the adjustments described above. The difference in the distributions reflects the difference in the assays. The vertical bar represents the lower level of detection and where the first cases appear.

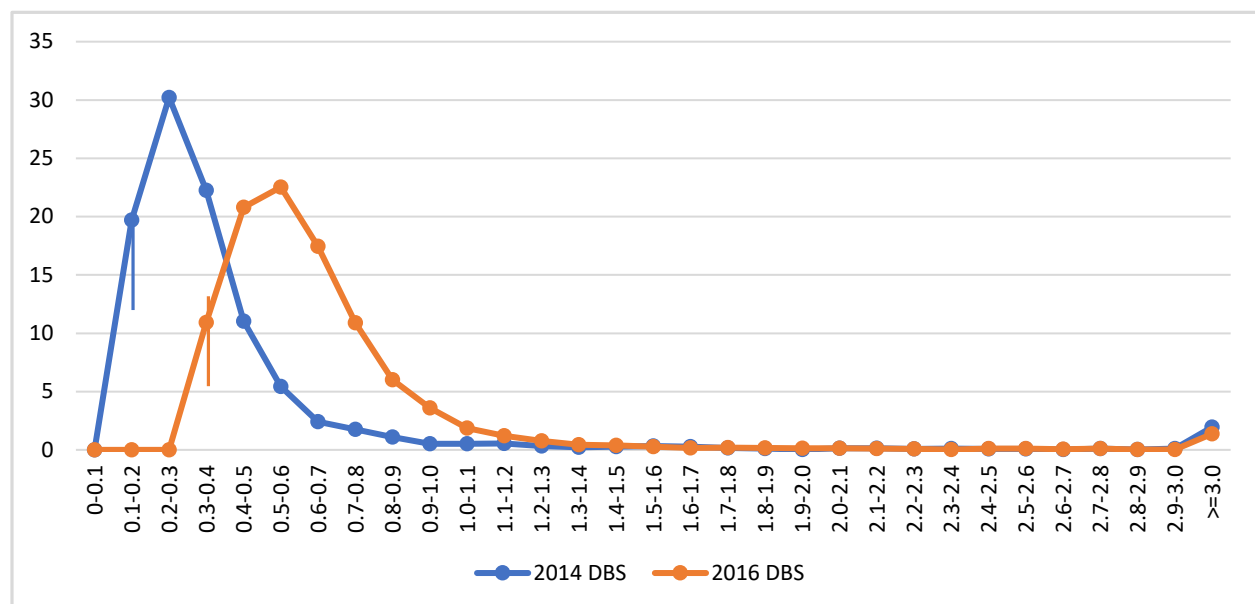


Figure 1 Distribution of 2014 and 2016 DBS IL-6

Making the two years equivalent

The University of Washington Laboratory reran a set of 2014 samples, which had been frozen using the 2016 assay to produce a 2014 equivalent value for the 2016 assay.

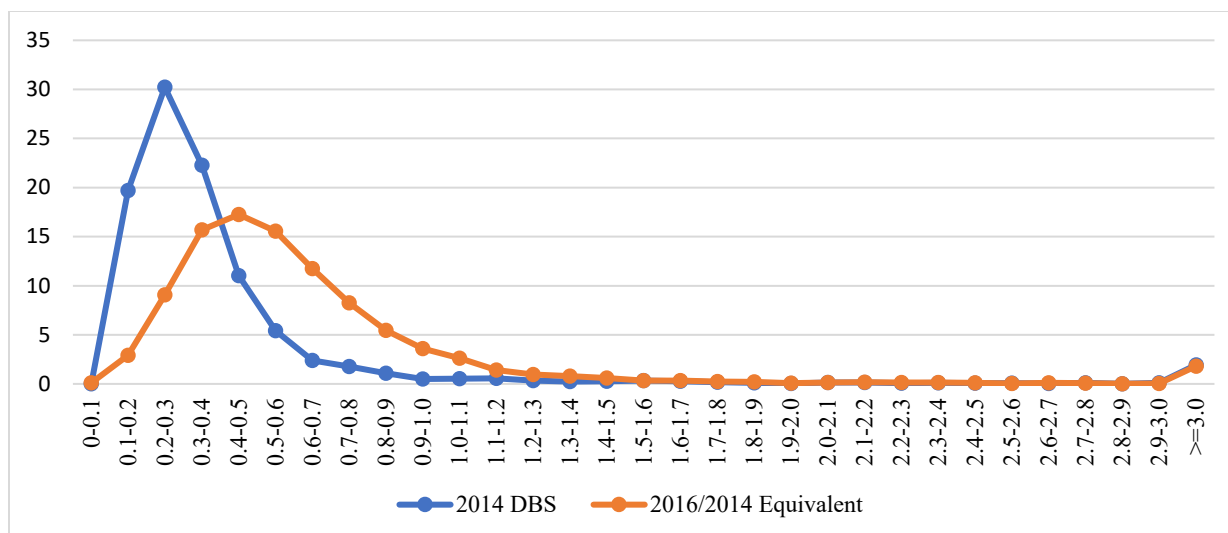


Figure 2 2014 DBS and 2016/2014 Equivalent IL-6

Making the 2016 DBS and 2016/2014 equivalent values

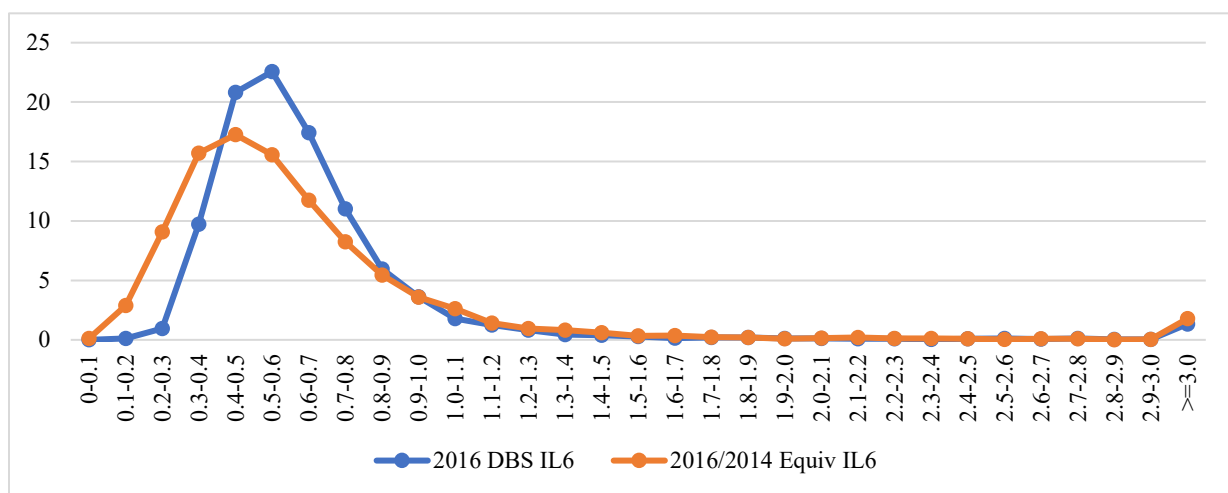


Figure 3 2016 DBS and 2016/2014 Equivalent IL-6

Because DBS values are so different from values found using serum or plasma, the laboratory provided plasma equivalent values for 2014 based on their comparison of the DBS assays with plasma values.

The following equation was used to create plasma equivalent values for the 2016 DBS IL-6 values.

$$2016 \text{ Plasma Equivalent IL-6 values} = 8.8569 \times (2016/2014 \text{ Equivalent IL-6 values}) - 0.9418$$

2014 PE and 2016/2014 Equivalent PE

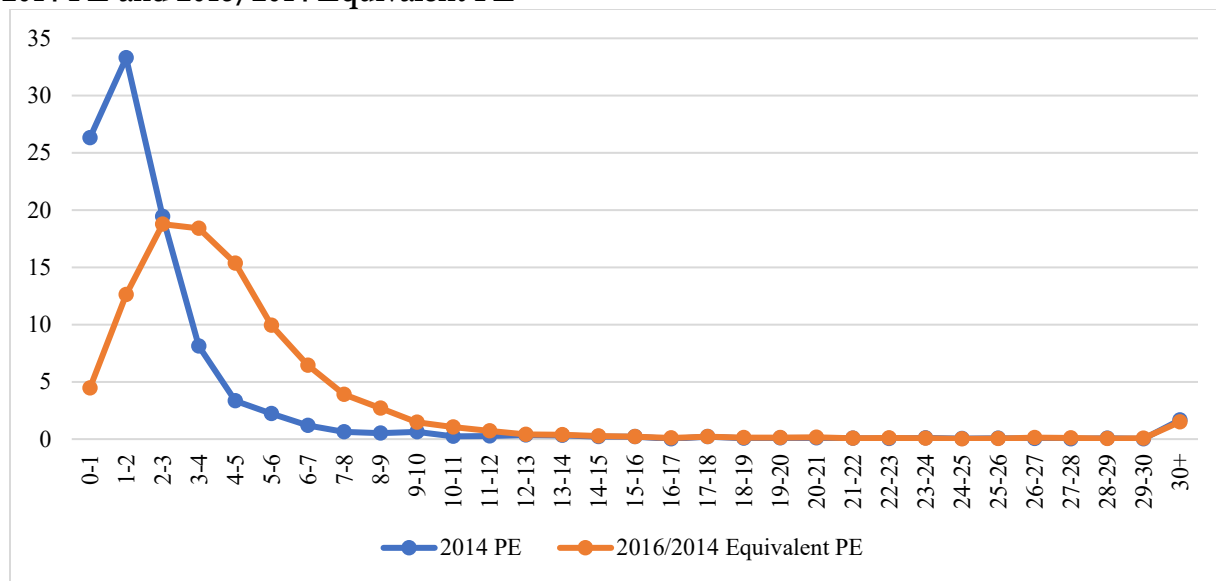


Figure 4 2014 PE and 2016/2014 PE IL-6

Values after these two conversions are shown below along with the original values.

Variable	N	Mean (SD)	Min	Max
2014				
2014 DBS IL-6	3,489	0.50 (SD=1.30)	0.19	20.001
Plasma Equivalent 2014 DBS IL-6	3,489	3.45 (SD=10.74)	0.81	171.59
2016 VBS	3,489	6.31 (SD=9.45)	0.42	68.001
2016				
2016 DBS IL-6	2,764	0.75 (SD=1.10)	0.16	20.001
2016/2014 DBS Equivalent IL-6	2,764	0.75 (SD=1.37)	0.00	23.78
2016/2014 Plasma Equivalent IL-6	2,764	5.67 (SD=12.10)	0.00	209.68
2016 VBS	2,764	6.33 (SD=9.07)	0.67	68.001

2016 VBS (N=9091)

IL-6 was also measured in serum in 2016 by an enzyme-linked immunosorbent assay (ELISA) technique using the Human Interleukin 6 Simple Plex Assay on the ELLA System from Protein Simple (San Jose, CA). The manufacturer inter-assay CV is 8.3% at a concentration of 41.5 pg/mL and 7.1% at a concentration of 1800 pg/mL.

In the 2016 VBS, there were 9,091 IL-6 cases with a biomarker weight.

	N	Mean (SD)	Min	Max
Original	9091	8.89 (71.96)	0.42	3372.21
Top 1%ile recoded as 68.001	9091	6.20 (8.76)	0.42	68.001

There are 7 cases that have high values, of over 1000 (1310.15, 1658.39, 2262.98, 2605.04, 2624.77, 3039.89, 3372.21). The top 1%ile is ≥ 68 (N=90). For comparing these assay values with the

plasma equivalents from DBS, we top-coded high values over 68 as 68.001. When this is done the new mean is 6.20 (SD=8.76).

	Deciles for 2014 IL6 and 2016 VBS			Deciles for 2016 IL6 and 2016 VBS			
	VBS	2014 DBS	2014 Plasma Equivalent	VBS	2016 DBS	2016/2014 DBS Equivalent	2016/2014 Plasma Equivalent
1	0.42-1.80	0.19	0.81	0.67-1.82	0.16-0.40	0.00-0.29	0.00-1.66
2	1.81-2.28			1.83-2.30	0.41-0.45	0.30-0.36	1.67-2.25
3	2.29-2.70	0.20-0.22	0.82-1.08	2.31-2.73	0.45-0.50	0.36-0.42	2.26-2.74
4	2.71-3.17	0.23-0.25	1.09-1.32	2.74-3.22	0.50-0.54	0.42-0.47	2.76-3.24
5	3.18-3.73	0.26-0.28	1.33-1.61	3.23-3.82	0.54-0.58	0.47-0.54	3.25-3.81
6	3.74-4.46	0.29-0.32	1.62-1.94	3.83-4.53	0.59-0.63	0.54-0.60	3.82-4.35
7	4.47-5.43	0.33-0.36	1.95-2.33	4.54-5.51	0.63-0.69	0.60-0.68	4.36-5.10
8	5.44-6.98	0.37-0.43	2.34-2.93	5.52-7.29	0.69-0.78	0.68-0.80	5.11-6.10
9	7.00-10.78	0.44-0.60	2.94-4.38	7.31-11.10	0.78-0.94	0.80-1.02	6.11-8.11
10	10.79-68.001	0.61-20.001	4.41-171.59	11.44-68.001	0.95-20.001	1.03-23.78	8.14-209.68

Figure 5 Deciles for 2014 IL6 and 2016 VBS

	2014 DBS	2014 Plasma Equivalent	2016 DBS	2016/2014 DBS Equivalent	2016/2014 Plasma Equivalent
2016 VBS	0.2536 <.0001	0.2701 <.001	0.2493 <.0001	0.2597 <.0001	0.2597 <.0001

Figure 6 Correlation

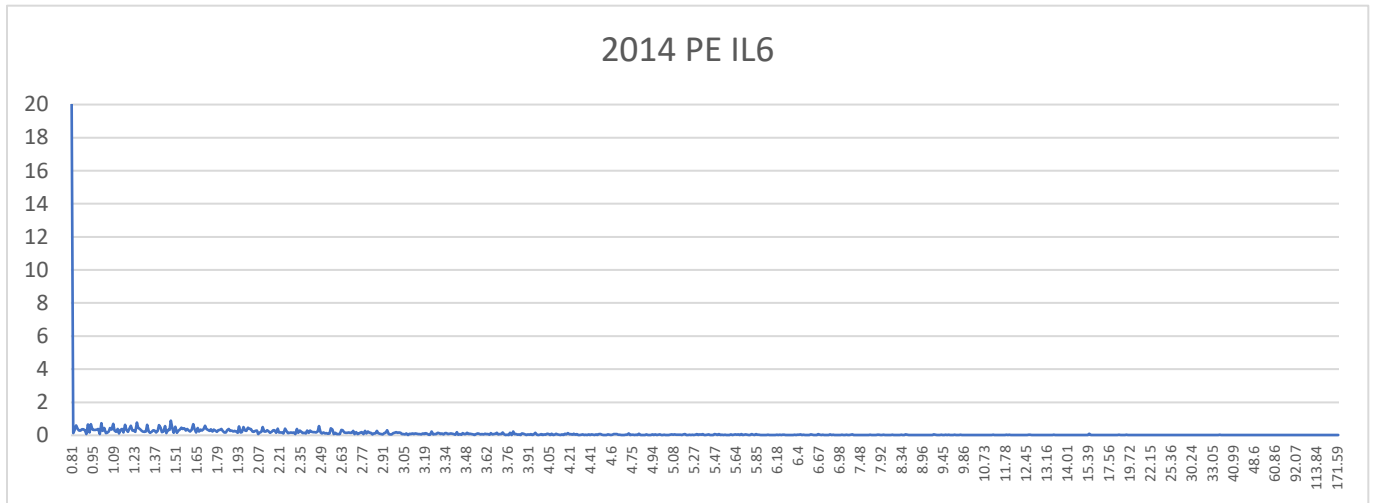


Figure 7 Frequency distribution of recorded values

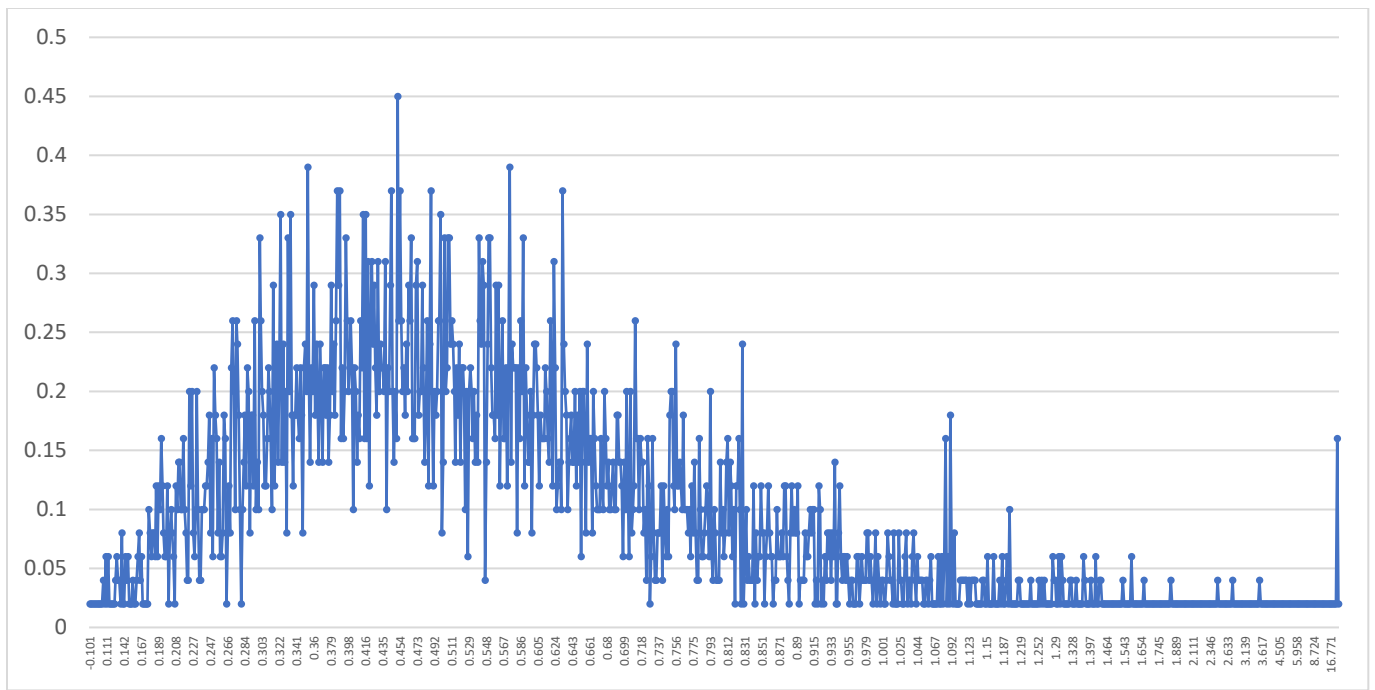


Figure 8 2016/2014 DBS Equivalent IL6

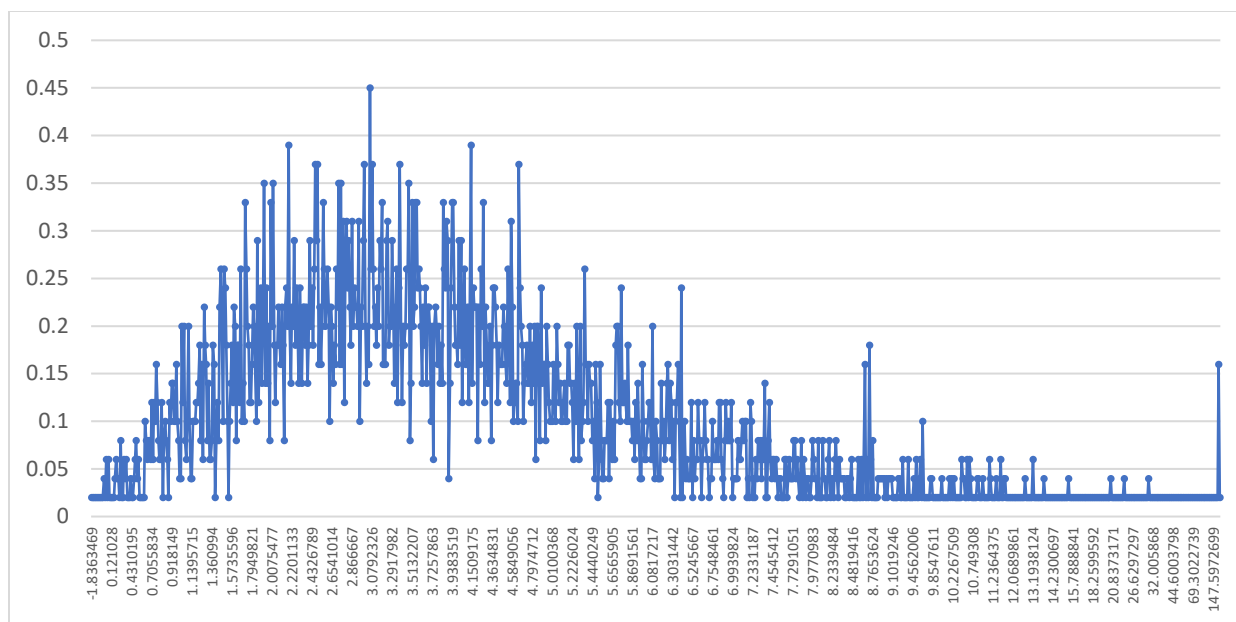


Figure 9 2016/2014 PE IL6

DBS Interleukin 6 Assay – 2014

The DBS interleukin-6 (**IL6**) assay was performed by ELISA optimized to accommodate the limited microliter volume available from a DBS sample. Principal reagents were the R&D Systems Human IL-6 Quantikine HS (high sensitivity) ELISA Kit obtained from Fisher Scientific, Houston, TX. Performance of the ELISA has been standardized to the NIBSC/WHO (UK National Institute for Biological Standards and Control /World Health Organization) 1st International Standard for IL-6 (89/548).

Performance Parameters

Accuracy

DBS assay results were verified by comparing IL6 concentrations obtained from 983 DBS samples analyzed by the DBS microtiter plate ELISA versus DBS-matched plasma samples analyzed by the conventional microtiter plate ELISA. The correlation coefficient of the linear regression comparison was $R^2 = 0.95$. The linear regression equation, used to convert DBS direct IL6 concentrations into DBS plasma-equivalent (P-E) IL6 concentrations, was: DBS P-E IL6, mg/L = $-0.63 + 11.62 \times \text{DBS direct IL6, mg/L}$.

Precision:

Variability (CV) from DBS low IL6 QC, DBS moderate IL6 QC and DBS high IL6 QC samples assayed in duplicate 94 times over 2 months was:

Sample	Mean IL6, pg/mL	Standard Deviation	Intra-Assay CV	Inter-Assay CV
DBS low IL6 QC	0.17	0.05	24.1%	27.4%
DBS moderate IL6 QC	0.38	0.08	6.4%	20.3%
DBS high IL6 QC	0.97	0.14	8.5%	14.0%

Analytical Measurement Range:

The LLOD of the DBS microtiter plate assay was 0.07pg/mL DBS direct IL6 concentration. The DBS P-E IL6 concentration of this LLOD was plasma IL6 0.14pg/mL. Any sample with a DBS

direct IL6 concentration of less than 0.07pg/mL would be given a DBS P-E IL6 0.14pg/mL fill value. The DBS direct IL6 concentration measurement range was 0.07pg/mL to 10pg/mL.

Description of 2014 Blood-Based Biomarker Data

Assay Values from Dried Blood Spots vs. Whole Blood

Biomarker values based on DBS vary across assays and laboratories and may be quite different from the more conventionally used whole blood assays; moreover, many analysts want to make comparisons to such standard assays. Therefore, we compare our results to those from a similarly aged nationally representative sample with conventional assays: the Midlife in the United States Study (MIDUS). We have also constructed and released a variable for each assay, which we call a MIDUS equivalent value. **We recommend the equivalent assay values for analytic use.** These variables were constructed by assuming that the distribution of the DBS assays is similar to that in MIDUS; we determine the value of both assays at each percentile; and then transform the DBS assays into the MIDUS scale.

Comparison of the HRS DBS values and those from venous blood assays is described in detail in a report, “Results from the Health and Retirement Study Biomarker Validation Project.” 2013. Crimmins, E., Kim, J.K., McCreath, H., Seeman, T.; Validation of Blood-based Assays using Dried Blood Spots for use in Large Population Studies. 2014. Crimmins, E., Kim, J.K., McCreath, H., Faul, J., Weir, D., Seeman, T. *Biodemography and Social Biology*, 60: 38-48; and the HRS Documentation for the 2006 and 2008 blood-based assays.

These sources make it clear that different lab assays and procedures result in different assay values. As mentioned above and described more below, the HRS solution to the problem of different assays is to produce an Equivalent Value using the distribution in a study which uses conventional assays.

Constructing MIDUS Equivalent Values

The equivalent values make the assay levels for the HRS data based on DBS similar to the level in MIDUS where values are based on conventional assays while the variability in the HRS sample is preserved.

IL-6 from conventional assays is not available for another national sample in this age range. For this reason we use the MIDUS sample to produce our equivalent value. The MIDUS sample used for comparison is from the MIDUS II project 4 (2004-2009) which includes a biomarker subsample MIDUS (N=1,255). We use those ages 50 and over to match with the age of HRS, which leads to the final sample of 880 for our use.

Our approach is to first calculate the values of the assays corresponding to (weighted) 100 percentiles in HRS and in MIDUS. Since the MIDUS sample we use is not nationally representative, we assign 1 for the weight variable in our calculation. For HRS, we use the biomarker weights (OBIOWGTR). [To facilitate construction of percentiles when values are discrete and have many individuals scored at the same value, we first add a very small random number to each observed value, create the (weighted) percentiles based on the altered values, and then take the mean of the actual assay values at each percentile]. For IL-6, we use the 2004-2009 MIDUS data. We then regress the HRS value on the MIDUS value to create an equation that can be used to convert HRS values into MIDUS Equivalent values.

The following equation was applied to create MIDUS equivalent variables of biomarkers.

$$OIL6_ADJ = 1.960717 + OIL6 * 2.068127$$

	N	Mean	SD	Min	Max
HRS 2014 assay					
IL-6 (pg/mL) - OIL6	4587	0.58	1.28	0	19.48
MIDUS					
IL-6 (pg/mL)	880	3.15	3.11	0.16	23.00
HRS 2014 MIDUS Equivalent Value					
IL-6 (pg/mL) - OIL6_ADJ	4587	3.15	2.65	1.96	42.25

Sample Weights

Separate sample weights exist for the biomarker sub-sample in each wave. These weights, OBIOWGTR for 2014, can be found in the Cross-Wave Tracker File and in the biomarker data file for each collection wave. The biomarker sample weight is the product of the HRS core sampling weight and a non-response adjustment factor. The HRS sampling weight from the concurrent interview was used as the base weight. The nonresponse adjustment factor was obtained from a propensity model predicting the probability of completing the biomarker portion of the EFTF interview among those selected and eligible to participate. The propensity model was estimated by logistic regression and weighted by the 7 base weight. Predictor variables included age, sex, race/ethnicity, education, coupleness, self-rated health, number of physical limitations and report of a chronic health condition (i.e., diabetes, use of diabetes medications, hypertension, heart conditions, myocardial infarction, angina, congestive heart failure or stroke). Predictor variables were taken from the current interview. The inverse of the fitted probability of completion formed the non-response adjustment factor. Finally, the weights were poststratified to closely match the HRS sample composition by age, gender, and race.

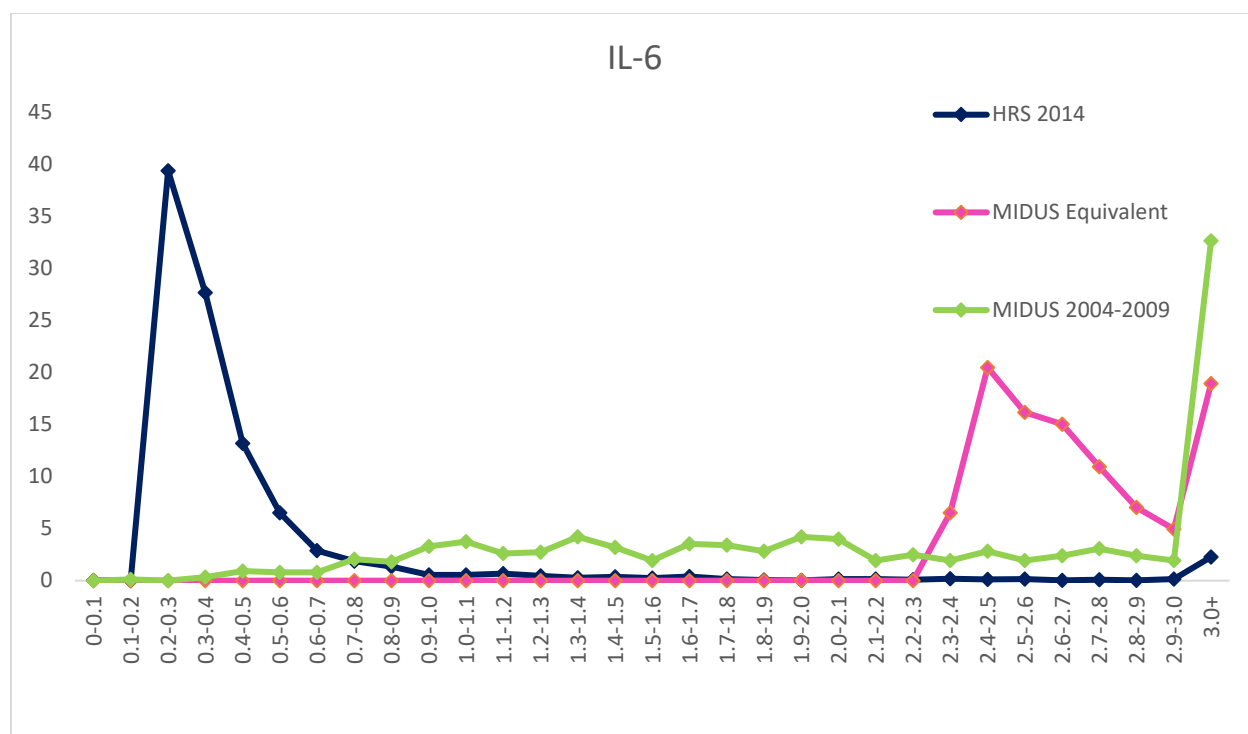


Figure 10 Frequency Distribution of Biomarkers from HRS assay, MIDUS equivalent, and MIDUS values

Description of Assays:

This material is derived from technical reports provided Alan Potter who directs the HRS Laboratory work at the University of Washington

Dried blood spot (DBS) quality control (QC) samples, DBS assay calibrators and DBS study samples are sealed in Ziploc bags with desiccant packs and stored in -70°C freezers (Revco Ultima Plus, Thermo Fisher Scientific, Pittsburg, PA) at UW Lab Med (University of Washington Department of Laboratory Medicine, Seattle, WA). Prior to processing, DBS are warmed to room temperature (RT). A single 3.2mm (1/8in) diameter disc is punched from each DBS sample for each assay (four discs for the IL-6 assay) into a deep-96 well microtiter plate well (Greiner Bio-One, Monroe, North Carolina) by a BSD700 Semi-Automated Dried Sample Puncher (BSD Robotics, Brisbane, QLD, Australia). Microtiter plates are then either immediately assayed or are sealed (CapMat, Greiner Bio-One) and stored at -70°C . Frozen microtiter plates are warmed to RT prior to assaying.

The DBS high-sensitivity **interleukin 6** (IL-6) Assay run at UW Lab Med is a sandwich ELISA. IL-6 Assay Diluent (R&D Systems, Minneapolis, MN) is added to the four DBS discs in each plate well and the plate is then sealed, gently shaken for 1hr on a Delfia Plateshake microplate shaker (PerkinElmer) and held for 16hr at 4°C to elute IL-6. Each well of the ELISA plate is pre-coated with an anti-IL-6 mAb that binds IL-6 in the elution solution (solid phase immobilization). The ELISA plate is gently shaken at RT for 45min and then washed 6X with 400 μl Wash Buffer (R&D Systems). Conjugate Reagent (R&D Systems) containing anti-IL-6 Ab coupled to phosphatase (enzyme-linked antibody) is then added to each well and the plate gently shaken at RT for 2hr resulting in IL-6 being sandwiched between the solid phase and enzyme-linked Ab. The plate is washed 6X with 400 μl Wash Buffer. Substrate Solution (R&D Systems) containing iodonitrotetrazolium Violet (INT-violet) is added to each well, the plate held for 1hr at RT,

Amplifier Solution (R&D Systems) containing NADPH is added to each well and the plate held for 30min at RT; NADPH, reduced by the phosphatase, reacts with INT-violet and causes the solution to develop a blue color. The reaction is stopped by addition of Stop Solution (R&D Systems). The IL-6 concentration is directly proportional to the OD of the solution; absorbance at 490nm excitation is measured on a Synergy HT microtiter plate reader (BioTek). A 4-parameter calibration curve is constructed by plotting the OD values of the calibrators against the assigned IL-6 concentrations (Gen 5 Software, BioTek). The calibration curve is used to convert the OD value of each sample into a DBS direct IL-6 concentration. Acceptability of the assay is determined by comparing the IL-6 concentrations of the QC samples with the established values.

DBS IL-6 Assay Calibrators (R&D Systems) were serially diluted with Assay Diluent to the desired final concentrations. Three DBS QC samples were constructed by UW Lab Med from a separate pool of human plasma, either undiluted (high IL-6 concentration QC sample) or diluted with assay buffer (medium IL-6 concentration QC sample and low IL-6 concentration QC sample). Each QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto Whatman No. 903 filter paper (GE Healthcare) and dried for 4hr at RT. The IL-6 concentration of each QC sample solution was determined by ELISA.

The IL-6 assay LLOD is 0.205pg/ml, within-assay CV is 12.9% and between-assay CV is 23.2%. The IL-6 concentrations of 246 DBS samples analyzed by the DBS assay correlated with the IL-6 concentrations of paired plasma samples (Pearson R = 0.91) and were linearly related (DBS assay IL-6 value = 0.166 + plasma-equivalent IL-6 value X 0.106).