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Comparing and Validating Dried Blood Spots with Plasma for Cytokine Assessments in Environmental Exposure Settings

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ABSTRACT

Comparing and Validating Dried Blood Spots with Plasma for Cytokine Assessments in Environmental Exposure Settings

By Yuchen Qian

Introduction: Traffic air pollution has been linked with many adverse health effects including asthma and acute systemic inflammation. Measuring biomarker levels has been widely utilized in environmental research settings to examine systemic inflammation after exposure to traffic air pollutants. However, the traditional venipuncture sampling method is invasive and requires clinical settings for sample collection, which limits its use among research studies in large population. A new sampling method, Dried Blood Spots (DBS), has been introduced to be a promising alternative to venipuncture, while few studies addressed its application in environmental research settings. This study is aimed to compare and validate DBS with plasma for cytokine assessments in environmental exposure settings.

Methods: Each subject in the study conducted a highway commute and was randomly and equally assigned a surface street commute or indoor clinic exposure as a comparison. DBS and plasma samples were collected at the same time from two separate time points (baseline and 8 hours after commute) for each commute and cytokine (IL-1β, IL-6, IL-8 and TNF-α) concentrations were analyzed using the same technology. Correlation analysis was conducted to examine the association between cytokine concentrations in the two matrices. Mixed effect linear regression modeling was then used to further assess influence from subject factors including health status, gender, race, age and BMI, while controlling for within-subject correlation due to the repeated measure structure of the dataset.

Results: IL-8 showed significant linear associations between DBS and plasma samples, where associations involving the other three measured cytokines were not significant. In asthmatic subjects, exclusively, significant, positive correlations were shown in the association between TNF-α in DBS and plasma. For non-asthmatics subjects, IL-6 in the two matrices was also significantly correlated**.**

Conclusion: DBS may be a promising alternative to plasma in measuring IL-8 concentrations in environmental research settings. To obtain absolute concentrations for concentration agreement analysis, validation tests should be performed for each cytokine on each matrix to yield standard sample handling and treatment process for the study.

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1. Introduction

As the automobile industry has quickly developed for over than a century, its impact on the environment and human health has also increased. While individuals benefit from the convenience of driving, the public has been burdened with the costs from motor vehicles. Private motor vehicles are the most substantial source of traffic-related air pollution (Batterman et al. 2014), exerting a substantial impact on corresponding ambient air pollution concentrations and human exposures. Emission from motor vehicles comprise a complex mixture of numerous pollutants, including particulate matter (PM), carbon monoxide (CO), hydrocarbons (HC), nitrogen oxides (NO_x), and substances collectively referred to as mobile source air toxics (MSATs), such as benzene and lead (HEI 2010). Numerous studies have shown exposure to traffic-related air pollutants to be linked with a range of adverse health effects including asthma (Chen et al. 2015; Loxham 2015; Klumper et al. 2015; Sarnat et al. 2012), respiratory and systemic inflammation (Loxham 2015; Armijos et al. 2015), chronic obstructive pulmonary disease (COPD) (Mannucci et al. 2015), cardiovascular diseases (CVD) (Beelen et al. 2014) and lung cancer (HEI 2010). Human exposure to traffic-related air pollutants can occur in various microenvironments, including in-vehicle, near-roadway, and as part of a background urban pollution mixture.

Time spent in these microenvironments results in personal exposures that may lead to both chronic and acute health response. Studies have shown that the populations potentially at greatest exposure to traffic pollutants are people who spend a considerable amount of time in traffic such as daily commuters (Strak et al. 2010). A 2009 U.S. Census Bureau report indicated workers in the U.S. spend 25 minutes on a one-way daily commute to work (McKenzie et al. 2011). According to data from U.S. travel surveys, in 2009

commuters spent 76 minutes per day in vehicles on average, with commuting times steadily increasing from 1990 to 2001 and decreasing slightly from 2001 to 2009 (Santos et al. 2011).

Although gaps remain in identifying the specific biological pathways involved in traffic induced health response, there is a growing consensus among health effects researchers on the importance of acute and chronic inflammatory processes in air pollution related etiologies (Brook et al. 2004). Increases in concentrations of several cytokines in particular, including IL-6, IL-8 and TNF-α, in response to short-term exposures to traffic-related air pollution have supported the role of inflammatory process disease exacerbation and development (Siponen et al. 2015; Kubesch et al. 2015; Hajat et al. 2015). Despite these findings from largely observational studies, there are still numerous questions concerning the specific biological pathways that are most affected by exposure to traffic-related pollution, and whether these pathways differ or are modified by the baseline health status of an exposed individual.

Biomarkers have been widely used in various research setting in different areas of epidemiology, to examine health effects associated with a range of environmental exposures. In environmental epidemiology, biomarkers can serve as indicative of biological processes or biomarkers of response, or as exposure biomarkers, indicative of metabolic traces of a chemical, its metabolite, or a reaction bi-product (de Oliveira et al. 2014). Cytokines, specifically, play critical regulatory roles in inflammation and serves as response biomarkers in humans (Skogstrand et al. 2005; Keustermans et al. 2013). Among the biomarkers that have been used for assessing air pollution exposures and effects in human, cytokines including interleukins 1β, 6, 8 (IL-1β, IL-6 and IL-8) and tumor necrosis factor (TNF)- α, have been measured to reflect general systemic inflammation occurring following exposures to traffic pollution. (de Oliveira et al. 2014; Siponen et al. 2015; Kubesch et al. 2015; Hajat et

al. 2015; Klumper et al. 2015). To date, cytokines collected as part of these studies have been quantitated primarily through plasma, obtained through moderately-invasive blood draws. Although effective indicators of systemic inflammation, there are numerous limitations to collecting blood for cytokine characterization including requirement of phlebotomist, method invasiveness, difficulties involving the handling and separation of serum and plasma, and large space required for storing blood samples.

In 1960s, Dried Blood Spot (DBS) sampling was introduced as a promising tool in screening infants for phenylketonuria (Guthrie et al. 1963). Over five decades, DBS sampling has broadened and is widely used as an alternative to venipuncture sampling in both clinical and research environments, (Wilhelm et al. 2013; Pennings et al. 2014; Olshan 2007) and has included cytokine endpoints such as CRP (McDade et al. 2004) and IL-6 (Miller et al. 2012). The advantages of using DBS in place of venipuncture blood draws include that it serves as a relatively painless and non-invasive collection procedure that can be conducted in non-clinical environments (McDade et al. 2007). Typical DBS sampling employs free-flowing drops of blood from a finger prick collected on cotton or cellulose based filter paper. Dried DBS samples can then be stored in freezers for lengthy periods before analysis and testing. DBS sampling is also simple to conduct using non-clinical staff, has convenient handling and transport protocol, and have been shown to be stable over weeks or years at -20°C (Mei et al. 2001).

However, since the vast majority of standard laboratory protocols and established clinical cut-points require serum or plasma, the validation of extraction protocols has been a critical concern among applications of DBS sampling method as an alternative to venipuncture (McDade et al. 2007; Guthrie et al. 1963). Moreover, few environmental exposure studies, have assessed whether cytokines measured in DBS serve as a suitable

alternative for examining acute inflammatory response associate with exposure to traffic pollutants.

The current analysis was conducted using concurrent DBS and plasma samples analyzed for IL-1β, IL-6, IL-8 and TNF-α to directly compare their absolute levels and variability in each of the matrices. The samples were originally collected as part of a panel-based exposure study, the Atlanta Commuters Exposure study (ACE), which was designed to examine acute cardiovascular and respiratory response in healthy and asthmatic subjects following either scripted car commute during rush hour, or a controlled exposure scenario (Sarnat et al. 2014). The objective of this analysis was to assess whether cytokine levels in DBS were similar to those measured in plasma, and how changes in DBS cytokine levels following these exposure scenarios compared with corresponding changes in the plasma cytokine concentrations. Results from this analysis are potentially beneficial in validating DBS as a biomarker matrix, and demonstrating its suitability for use in similar environmental epidemiological settings. If valid, DBS represents as a relatively easy, non-invasive approach for marking changes in a biologically-relevant inflammatory endpoint for use in environmental exposure and epidemiologic applications.

2. Methods

In order to investigate concordance between cytokines collected in DBS and plasma, we analyzed samples from the Atlanta Commuter Exposure (ACE) study. The ACE study was originally conducted to examine in-vehicle exposures among daily car commuters and corresponding acute cardiorespiratory response in a panel of asthmatic and non-asthmatic commuter participants in the metropolitan Atlanta area (Sarnat et al. 2014). The second phase of the ACE study, ACE-2, from which the current samples were collected was a

controlled randomized cross-over study, focused on comparing the effects between exposure to highway commute and that to surface street commute or indoor clinic control among young adults.

In ACE-2, 60 subjects were recruited in total, of which 30 were healthy adults and 30 were non-severe asthmatic adults. All of the subjects participated in a highway commute and were randomly and equally assigned to a surface street commute or indoor clinic exposure as a low-pollutant exposure comparison. Subjects used their personal vehicles to conduct the commutes. Each commute last about two hours which started and ended at the environmental health laboratory at the Rollins School of Public Health of Emory University. Routes were similar among commutes and were designed include heavily used commuting roadways with both gasoline and diesel engine vehicles (Sarnat et al. 2014).

2.1 Subject Demographics

Each subject completed a baseline questionnaire that provided demographic information and initial risk factor assessment prior to each commute. In addition to age, ethnicity, height and weight, we included other factors related to both exposure and health, such as proximity of subject residences to major roadways, potential exposures to indoor or outdoor pollution events, and recent health status.

2.2 Biomarker Measurements or Cytokines Measurements

We conducted cytokine measurements using two sampling matrices – DBS and plasma. For baseline (i.e., pre-exposure) measurements, DBS and whole blood samples were collected from the subject, approximately 30 minutes prior to each commute or clean, non-highway commute exposure, by a trained field technician and phlebotomist at our laboratory facilities at Emory University. DBS samples were also collected immediately following the

commute (~9:00 AM), and at hourly intervals for three hours after commutes (~10:00 AM, ~11:00 AM, ~12:00 PM), as well as 8 hours post commute (~5:00 PM). In addition to the pre-exposure whole blood draw, an additional blood draw was conducted for each subject was conducted approximately 8 hours post exposure (~5:00 PM). Thus, the comparisons between DBS and plasma included in the current analysis were taken from the time periods where overlapping method sampling was conducted, namely baseline and at the 8 hour post exposure measurement period.

Whole blood samples were collected in EDTA-treated vacuum tubes, cooled on ice and imminently centrifuged for 15 minutes after the collection. Following centrifugation, as a preparation for cytokines tests, we immediately transferred and aliquoted the liquid component (plasma) into clean individual one-milliliter polypropylene tubes using Pasteur pipettes. All aliquots were labeled by subject ID and stored in the -80 °C freezer in our environmental health laboratory before further analysis.

For DBS sampling, the participant's finger was cleaned with alcohol swab, and then pricked with a sterile, disposable lancet. The first drop of blood was wiped away. Free-flowing drops (approximately 100μl in total) were collected in a capillary tube and transferred to a piece of filter paper (Whatman 903TM Protein Saver Card), designed specifically for the collection. The samples were left out to dry during the study day (7am-6pm) and then stored in plastic bags at -80 °C. At the end of the study (lasting approximately eight months) the samples were shipped, as a single batch, on dry ice to laboratory facilities at the US Environmental Protection Agency (EPA) in Research Triangle Park, NC, for analysis.

For blood extraction from DBS, a 6mm (in diameter) punch was first cut from the center of each blood spot, with Harris Uni-Core punch. Each punch of the sample were

ensured to be saturated with blood all the way to the diameter to avoid DBS appearing uneven, or not at least 6mm in diameter. Then each punch was placed in a well of 96-well plate and 200 μl of PBS (0.5% Tween 20) was added to each well for blood extraction. The plate was shaken overnight at 4°C using a rotary plate shaker at approximately 400 rpm. Afterwards, plate was stored at -80°C until further analysis.

Electrochemiluminescence (ECL) tests were conducted on plasma and extracted DBS samples using the Mesoscale Discovery MESO QuickPlex SQ 120. Each sample was diluted 2-fold in the diluent supplied with the kit and specified in the company protocol (MSD 2014). For the assay, each well received 25μl sample (extracted DBS samples or plasma) mixed with 25μl diluent. The following analytes were characterized: IL-1β, IL-6, IL-8 and TNF-α. Plasma samples were measured in duplicates in our lab, while DBS extractions were measured in singlets at the US EPA. Experiments were performed on two continuous days in our environmental health laboratory by the same trained research assistant to avoid inconsistency.

2.3 Data Analysis

The means, standard deviations and coefficient of variations were calculated from duplicates for each plasma sample and from singlets for DBS, and are shown in Table 2 and Figure 1.Correlation analysis was initially performed to examine linear association between cytokine concentrations in the two matrices. Mixed effect linear regression modeling was then used to further assess influence from subject factors including health status, gender, race, age and BMI, while controlling for within-subject correlation due to the repeated measure structure of the dataset. For these analyses, a generalized form of the mixed effects models can be expressed as:

$$DBS = \alpha + \beta PL + \gamma_1 x_1 + \gamma_2 x_2 + \partial_3 x_1 PL + \partial_4 x_2 PL + b_i + \epsilon \quad (1)$$

where '*DBS'* is the concentration of a specific cytokine in DBS; '*PL*' is the concentration of a specific cytokine in plasma; '*x*' is a potential predictive factor, such as health status or gender that may also predict a cytokine concentration in DBS; β represents the degree to which DBS changes with changes in PL given the other factors included in the model; γ's represent the degree to which DBS changes with changes in the other potential predictive factors, given PL; 'δ' is interaction effect between '*DBS*' and 'x'; 'b_i' is 'the subject-specific random effect; and 'ϵ' is the random error term.

3. Results

In total, we analyzed 136 observations of paired DBS and plasma samples, from 48 subjects participating in this study. Thirty of the 48 subjects included in the analysis participated twice during the study; once where a scripted highway commute was conducted and once for a controlled exposure scenario. In total, there were 39 paired samples from highway commutes, 21 commutes along a surface street, and 18 exposures conducted in controlled (i.e., 'clean') indoor environments (Table 1). Participant ages ranged from 18 to 39 years, with a median age of 26. Female subjects consisted of 48% of the study population. Both asthmatic and non-asthmatic subjects were recruited for this protocol, with samples used in the analysis distributed approximately evenly by baseline health status. In addition, based on chi-square tests for categorical variables (gender and race) and t-test for numeric variable (age), no significant differences were present in either gender distribution or proportion of Caucasians between the two health status groups, but the asthmatics were

slightly younger ($p<0.05$) though age ranges overlapped considerably, and all were young adults.

Primary descriptive analyses were conducted for the cytokine concentrations in both DBS and plasma samples, with and without stratification by health status (Table 2). Due to lack of detection associated with samples found in concentrations below analytical detection limits, 63 DBS samples had reported concentrations of zero pg/ml for IL-1β (46% of total), and 17 plasma samples had zero concentrations for IL-6 (13% of total). Given that the same platform was used for both plasma and DBS, and that lower limits of detection (LLODs) vary slightly between plates even at the same site, the median LLODs reported for each kit in the Mesoscale company literature (MSD 2014) were used. For these observations with zero pg/ml in concentrations, the values were corrected and expressed as $^{1}/_{\sqrt{2}}$the median LLODs (Skogstrand et al. 2008; Hornung et al. 1990) (median LLOD for IL-1β: 0.04pg/ml; median LLOD for IL-6: 0.06 pg/ml). Levels of all other cytokines, both in DBS and plasma, were all found to be above their respective limits of detection and are presented as quantitated from the analyses.

As expected, the distribution of most the cytokines in both matrices was right-skewed, with the exception of TNF-α in plasma (which was approximately normally distributed). Natural logarithm transformation was performed to normalize distributions for subsequent correlation analysis and statistical modeling. Normality was examined again after natural logarithm transformation, and concentration distributions were approximately normal for all cytokines, with or without stratification by health status. Generally, cytokine concentrations were equivalent by health status except for IL-1β in DBS, where asthmatic participants were shown to have elevated IL-1β concentrations compared to healthy subjects

(2.733 pg/ml for asthmatic group; 0.737 pg/ml for healthy group). This result was not seen in corresponding IL-1β plasma concentrations in asthmatics.

In extracted DBS samples, measured concentrations were higher for IL-1β, but lower for IL-6 and TNF-α, and equivalent for IL-8, compared to plasma. This was likely due at least in part to differences in the extraction and analysis protocol between the two methods. According to the protocol we used, plasma samples were measured in 2-fold dilution and absolute concentrations were calculated and output from the instrument's curve fitting software. For DBS, in contrast, the calculated concentrations output by instrument's curve fitting software were those in extraction buffer instead of the actual blood or plasma equivalent of the DBS. Specifically, each DBS punch contains approximately 6μl of serum (McDade et al. 2007) and was extracted in 200μl PBS buffer. Consequently, there was a 33.3 fold pre-dilution of DBS compared to plasma before measurements were conducted. The absolute concentration calculation for DBS would be based on the 33.3-fold pre-dilution assumption, which thus yields substantially higher concentrations in DBS samples for all cytokines.

To examine the degree of linear association between cytokines measured in DBS and plasma collected from traditional venipuncture methods, scatterplots are presented (Figure 1) and correlation analysis was performed for each cytokine, both with and without stratification by health status (Table 3). Overall, IL-8 showed positive, significant correlations between the two sampling methods. These associations were robust to health status (Pearson correlation coefficients (p-value): overall = 0.38 ($p < 0.05$), asthmatic = 0.38 ($p < 0.05$) and healthy = 0.39 ($p < 0.05$)). In asthmatic subjects, exclusively, significant, positive correlations were shown in the association between TNF-α in DBS and plasma

(Pearson correlation coefficient = 0.23 ($p < 0.05$). For non-asthmatics subjects, IL-6 in the two matrices was also significantly correlated (Pearson correlation coefficient = 0.33 ($p < 0.05$)).

To further examine the strength of linear association between the DBS and plasma samples, while controlling for the lack of within-subject independence in the data, we also conducted mixed effect regression analyses. Mixed effect models enabled us to model subject as a random effect and also provided us with a chance to examine whether additional factors, such as health status and gender, modified the strength of the association between the two cytokine quantification methods.

Similar to the results from the initial correlation analysis, IL-8 showed significant linear associations between DBS and plasma samples, where associations involving the other three measured cytokines were not significant. The mixed effect models also indicated that no other selected independent factors, including health status, gender, race, age and BMI were significantly predictive of IL-6 or IL-8 concentrations. For IL-1β, health status was shown to be strongly predictive of corresponding DBS concentrations. As noted above, IL-1β levels were higher in asthmatic subjects as compared to those without asthma. Race and gender were also independently predictive of IL-1β, with higher levels shown in males and non-Caucasians. For TNF-α, race and gender were shown to be significant predictors for concentration individually and within models including other factors. No significant interactions were detected for each cytokine in models examining modification by health status, gender, race, age and BMI.

4. Discussion

Findings from the current analysis provide promising indications concerning the utility of DBS as a means of characterizing some cytokine levels a panel-based urban air pollution epidemiologic study. The robust linear correlations for IL-8, IL-6 (healthy) and TNF-α (asthmatic) between DBS and those cytokines quantitated in plasma, using a reference method, suggest that DBS may have the ability to capture temporal trends in theses cytokines and may serve as a potential alternative to venipuncture sampling.

During the past 50 years, approaches for analyzing a range of biomarkers in DBS has proliferated rapidly. Today, DBS has become a widely utilized matrix used to characterize biomarkers in large population-based studies, both in the U.S. and globally (McDade et al. 2007). As an alternative to venipuncture sampling and other invasive methods, research has focused on validating DBS analyses as accurate and sensitive indicators of biomarker concentration and variability (Adam et al. 2000; Brindle et al. 2010; Keustermans et al. 2013; McDade 2014; McDade et al. 2004; McDade et al. 2007; Miller et al. 2012; Skogstrand 2012; Skogstrand et al. 2008; Skogstrand et al. 2005; Wilhelm et al. 2013; Zheng et al. 2015). Validation of DBS as a suitable matrix has centered around the role that various factors play in affecting concordance between results derived from using this method compared to other matrices. These factors include sample collection, handling, and treatment in various protocols (Skogstrand 2012; Keustermans et al. 2013; Skogstrand et al. 2008).

Among the limited number of studies conducted to compare DBS and blood-based matrices (Wilhelm et al. 2013; Brindle et al. 2010; Sarafoglou et al. 2011; Skogstrand 2012), two studies, in particular, compared IL-6 (Miller et al. 2012) and CRP (McDade et al. 2004; Brindle et al. 2010) concentrations in both serum and DBS with findings showing strong

overall agreement between the cytokine levels in each of the two matrices. Specifically, for IL-6, Miller et al. (2012) developed and validated a protocol for DBS with low IL-6 concentrations. In order to ensure validation, especially for samples with low concentrations, 8 calibrators were spotted on filter paper cards, which were the same ones used to obtain DBS, with concentrations range 0 pg/ml to 25 pg/ml. Both the McDade et al. (2004) and Brindle et al. (2010) studies compared CRP concentrations in three matrices (serum, plasma and DBS), and strong agreements were found among these matrices; DBS stability was examined under different storage conditions (time and temparature) and CRP was found to be less stable in DBS than previously shown in other studies.

Importantly, these previous studies were conducted in non-environmental health study settings, following protocols not designed to explicitly examine inflammation in response to exposure to an environmental stressor. Moreover, to date there have been no prior studies validating the use of DBS of a surrogate of systemic cytokine levels and variation following exposures to urban air pollution.

A goal of this thesis is to compare cytokine levels in plasma versus DBS in the same individuals. Since plasma is derived from venous circulation, but DBS is derived from capillary blood, we do not necessarily expect these values to be exactly the same, and some cytokines may give better correlations between plasma and DBS than others.

In assessing correlations between methods, among the four cytokines we compared, IL-8 was the only endpoint that showed strong patterns of linear association in DBS and plasma. Strength of association was robust to the inclusion of other factors in the model. In the published literature, no previous studies have reported similar associations with IL-8, although the results should not be completely unexpected. A possible explanation may be

that among the four cytokines we chose to examine, IL-8 was unique in that its observed concentrations occurred in the middle of the detection range for both methods, which might play the most important role in the strong association.

Association for the other three cytokines we measured were not significant between DBS and plasma. Although IL-6 has been shown to demonstrate good correlation between DBS and serum in a previous study (Miller et al. 2012), we were not able to replicate this finding in the current analysis.

Our measured concentrations in plasma were consistent with the expected values. For all cytokines, concentration medians for plasma (Table 2) were comparable to those provided by an in-house validation conducted by the Mesoscale company from 27 EDTA-plasma samples (MSD 2014). Specifically, the reported median levels in the in-house validation were 0.20 pg/ml for IL-1β, 0.29 pg/ml for IL-6, 0.52 pg/ml for IL-8 and 0.74 pg/ml for TNF-α. DBS had higher "absolute" calculated concentrations than plasma. Skogstrand et al. (2008) study has shown similar results of elevated concentration in DBS, compared to plasma and serum, for IL-1β, IL-6 and IL-8.

For IL-1β and IL-6, the lack of correlation might be due to their low concentrations among healthy adults, which were often below or at the LLOD for most of their immunoassays. In our study, 63 DBS samples (46%) were below LLOD of IL-1β, and 17 plasma samples (13%) were below LLOD of IL-6, all yielding zero pg/ml in the concentration, which was not biologically plausible. It is worth noting that after stratification on health status, we did find significant results indicating stronger associations for other cytokines, most notably for TNF-α in the asthmatic cohort and IL-6 for the healthy cohort.

Due to its short history, no standard protocols currently exist for DBS handling and treatment, although evaluation has been conducted on various protocols (McDade et al. 2007). Additionally, handling protocols have been shown to be heavily dependent on the biomarkers being measured (McDade et al. 2007). Therefore, it is recommended that validation tests should be conducted before actual quantitation is done (Skogstrand 2012). Factors that can influence measurement results in DBS include hematocrit, storage temperature, punch size and numbers, extraction time and temperature, and number of duplicates (Skogstrand 2012; Keustermans et al. 2013; Skogstrand et al. 2008; Mei et al. 2001; Adam et al. 2000). All these factors should be taken into account in development and validation of assay protocols (McDade 2014). Specifically, good correlation results were reported when standards were constructed from dried spots to measure IL-6 (Miller et al. 2012), while in our study, for example, standards were constructed using calibrators from the kit directly without spotting these onto filter paper cards. In our handling and measuring process, a center punch was performed and only one punch was extracted in buffer for each sample, while other protocols use multiples punches from multiple spots to avoid random error in sample collection (Zheng et al. 2015). Additionally, each endpoint was measured in singlet, which will increase the possibility of including random errors in DBS concentrations. Consequently, the current analyses are comparing relative cytokine concentrations (in extraction buffer) to absolute concentrations in the plasma samples.

We selected cytokines that were used in previous studies to examine systematic inflammation. However, IL-1β and IL-6 had very low concentrations at or below LLODs resulting in difficulty in accurate comparison between the two matrices.

In conclusion, DBS could be a promising alternative to plasma in measuring IL-8 concentrations in environmental research settings. To obtain absolute concentrations for concentration agreement analysis, validation tests should be performed for each cytokine on each matrix to yield standard sample handling and treatment process for the study. The elevation of IL-1β among asthmatics found in DBS but not plasma suggests that the DBS spotting process may activate immune cells to release this cytokine, and that this process could be differentially regulated in asthmatic compared to healthy control subjects.

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Tables and Figures.

Table 1. Baseline characteristics of study population by health status

Characteristics	**All Participants (n=48)**	**Participants with asthma (n=25)**	**Participants without asthma (n=23)**	**P value[a]**
Microenvironment				
Highway commute	39	18	21	
Surface street commute	21	12	9	-
Clinic	18	10	8	
Female sex (%)	23 (48%)	14 (56%)	9 (39%)	0.2425
Age, years				
Median	26	23	27	0.0035
Range	(18-39)	(18-34)	(22-39)	
Caucasians (%)[b]	32 (67%)	14 (56%)	18 (78%)	0.1022

a p-values are for comparisons between participants with and without asthma; t-tests for continuous variables and chi-square tests for categorical tests

b Race was self-reported. The other categories include African-American, Asian, Hispanic, Biracial and Tri-racial.

Table 2. Descriptive statistics for cytokine concentrations in two matrices by health status. (All cytokine concentrations are expressed in pg/ml.)

Cytokines	N	Mean (STD)		Median		Range	
	All	DBS	Plasma	DBS	Plasma	DBS	Plasma
IL-1β	136	1.852 (3.038)	0.116 (0.152)	0.979	0.052	(0.048 - 27.631)	(0.000 - 0.908)
(Non-Asthmatic)	60	0.737 (0.874)	0.101 (0.145)	0.466	0.044	(0.085 - 5.263)	(0.003 - 0.908)
(Asthmatic)	76	2.733 (3.773)	0.127 (0.158)	1.705	0.060	(0.048 - 27.631)	(0.000 - 0.665)
IL-6	136	0.074 (0.125)	0.602 (0.569)	0.042	0.440	(0.003 - 1.160)	(0.013 - 3.489)
(Non-Asthmatic)	60	0.074 (0.157)	0.724 (0.719)	0.042	0.458	(0.003 - 1.160)	(0.033 - 3.489)
(Asthmatic)	76	0.074 (0.092)	0.506 (0.394)	0.042	0.433	(0.003 - 0.451)	(0.013 - 2.396)
IL-8	136	4.857 (3.280)	3.281 (2.499)	4.304	2.913	(0.172 - 35.549)	(0.740 - 26.203)
(Non-Asthmatic)	60	4.551 (2.258)	3.339 (1.644)	4.033	3.081	(0.917 - 13.187)	(0.840 - 9.502)
(Asthmatic)	76	5.098 (3.901)	3.235 (3.019)	4.621	2.827	(0.172 - 35.549)	(0.740 - 26.203)
TNF-α	136	0.082 (0.061)	2.080 (0.734)	0.071	1.901	(0.002 - 0.376)	(0.928 - 4.134)
(Non-Asthmatic)	60	0.072 (0.049)	2.216 (0.772)	0.062	2.088	(0.002 - 0.280)	(0.928 - 4.134)
(Asthmatic)	76	0.090 (0.069)	1.974 (0.689)	0.078	1.786	(0.007 - 0.376)	(1.026 - 3.837)

Table 3. Pearson correlation coefficients (p-value) among cytokines, measured in DBS and plasma, by health status

Cytokines	All (n=136)	Asthmatic (n=76)	Non-Asthmatic (n=60)
IL-1β	-0.01 (0.95)	0.00 (0.97)	-0.13 (0.34)
IL-6	0.07 (0.44)	-0.10 (0.39)	0.33 (<0.05)
IL-8	0.38 (<0.05)	0.38 (<0.05)	0.39 (<0.05)
TNF-α	0.01 (0.94)	0.23 (<0.05)	-0.14 (0.29)

Figure 1. Correlation scatter plots between DBS and plasma among cytokines

A. IL-1β

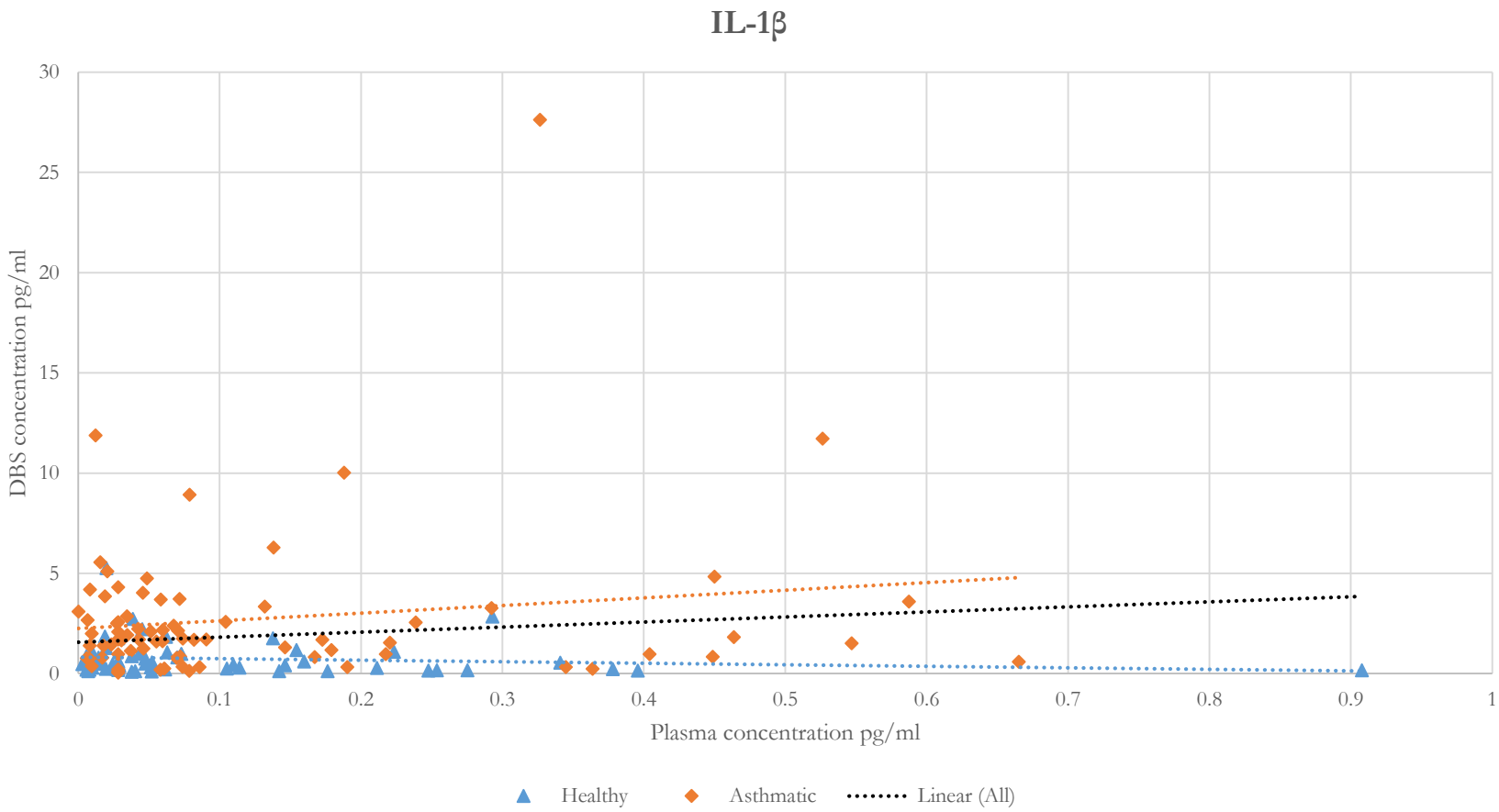

Figure 1. Correlation scatter plots between DBS and plasma among cytokines

B. IL-6

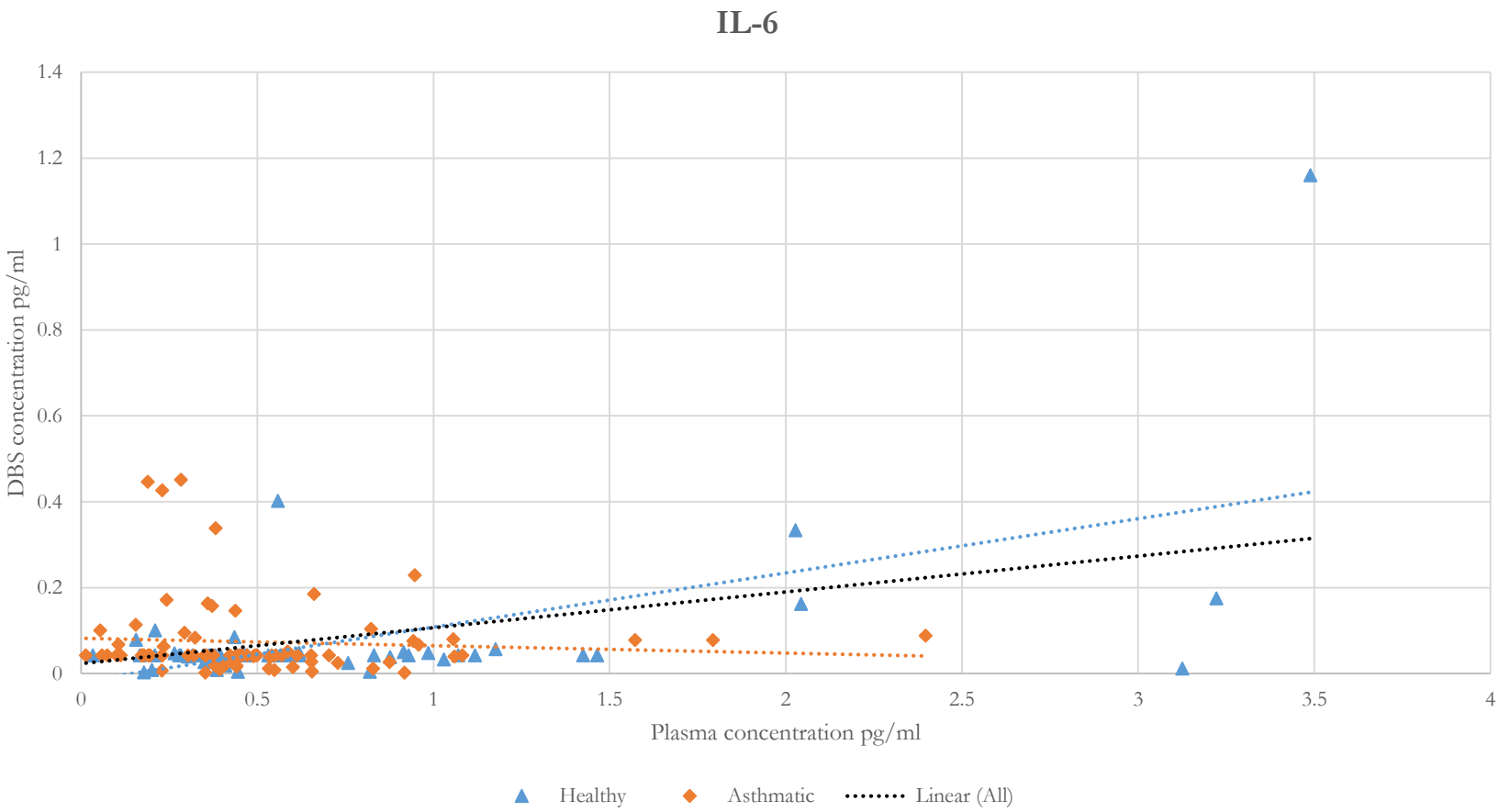

Figure 1. Correlation scatter plots between DBS and plasma among cytokines

C. IL-8

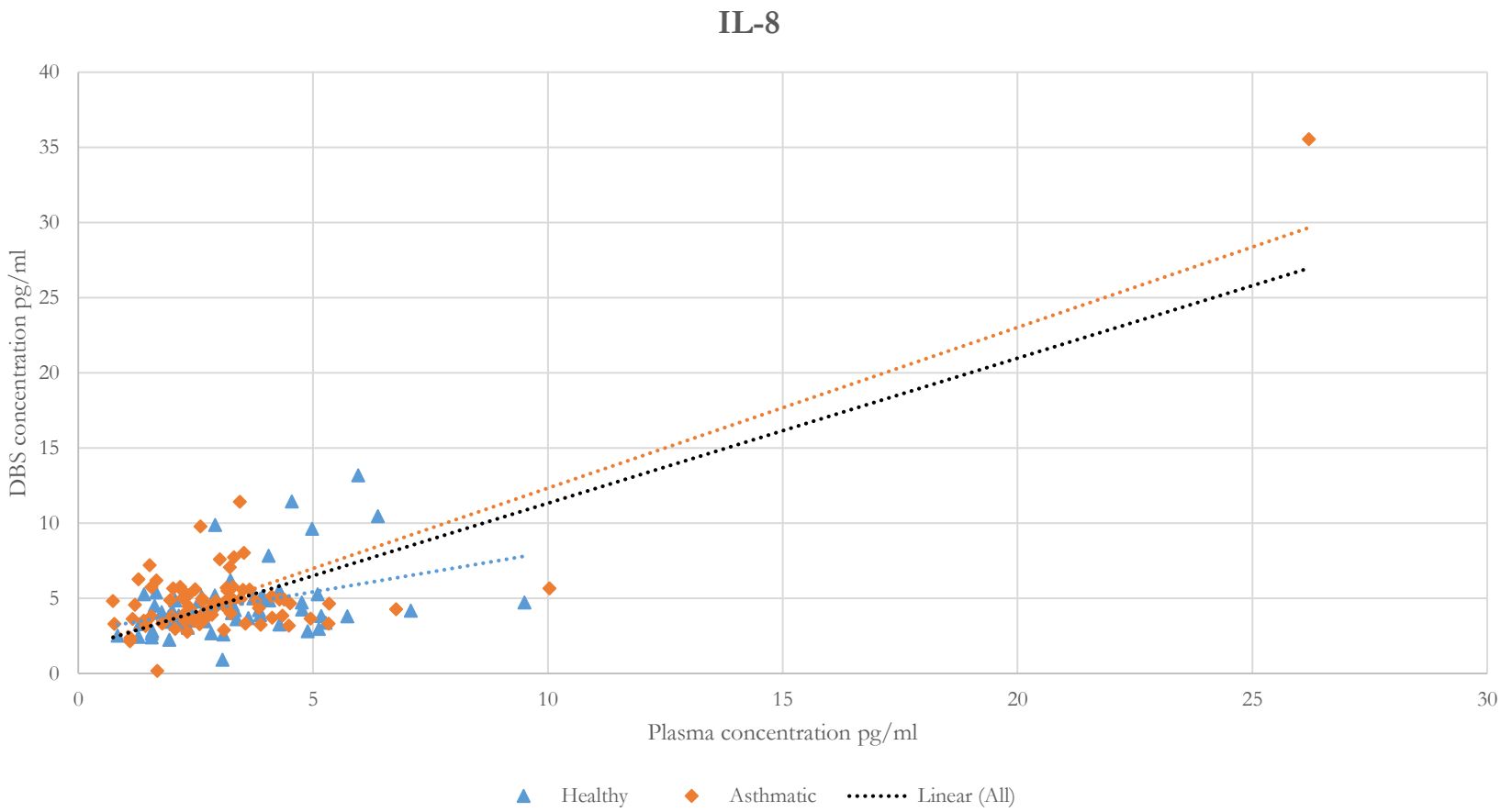

Figure 1. Correlation scatter plots between DBS and plasma among cytokines

D. TNF-α

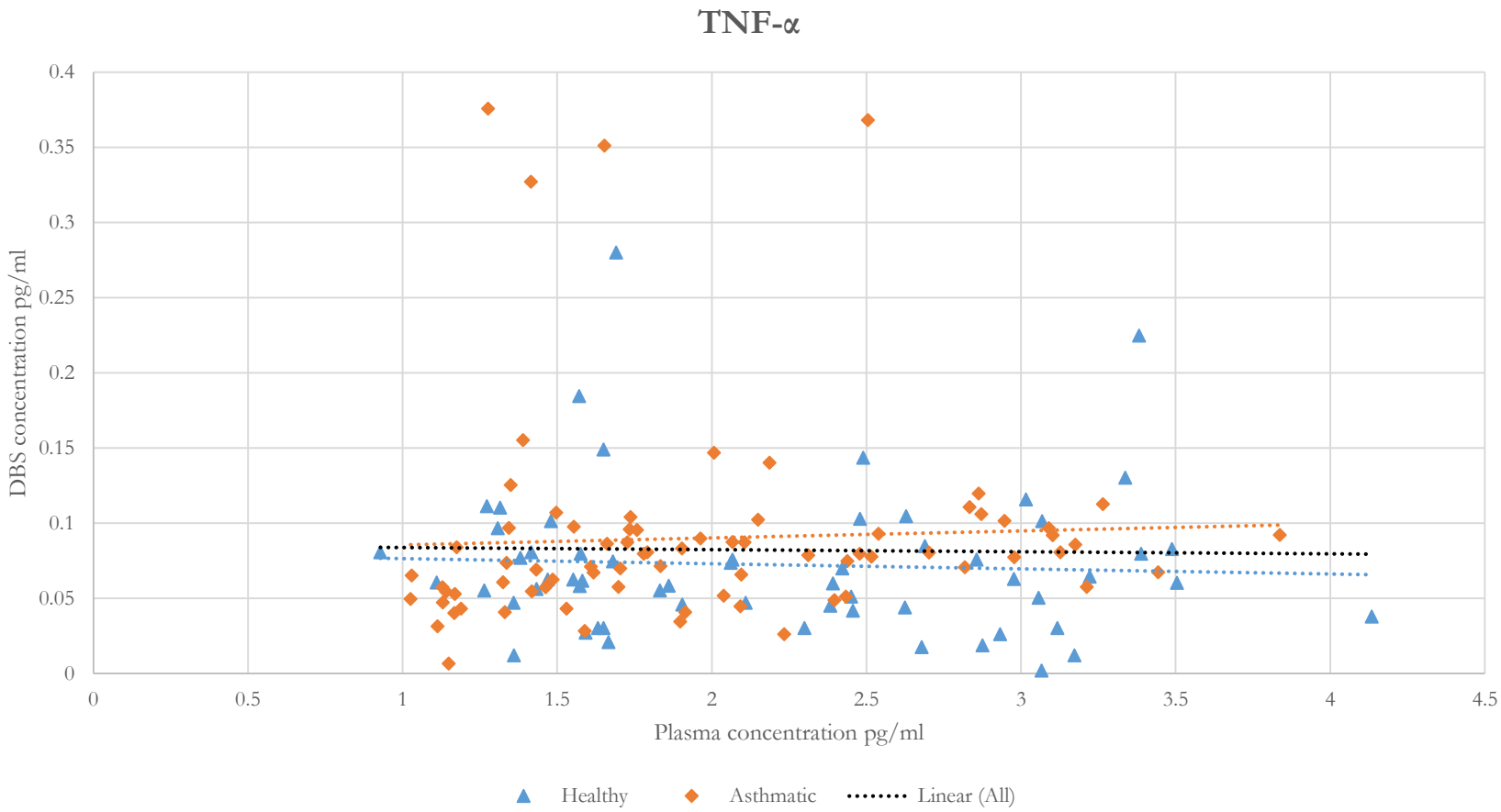

Table 4. Linear mixed effect models among cytokines examining strength of linear association between levels quantitated in DBS and plasma.

IL-1β					
Dependent Variable	**Independent Variables**	**No.**	**Co-efficient**	**p-value**	**Intercept**
DBS	**Plasma**	136	-0.043	0.475	-0.273
DBS	**Plasma**	136	-0.052	0.372	0.251
	Health Status*		1.162	<0.05	
DBS	**Plasma**	136	-0.061	0.292	0.546
	Health Status*		1.257	<0.05	
	Gender*		-0.584	0.008	
DBS	**Plasma**	136	-0.048	0.404	0.062
	Health Status*		1.066	<0.05	
	Race*		0.505	0.036	
DBS	**Plasma**	136	-0.057	0.322	0.360
	Health Status*		1.169	<0.05	
	Gender*		-0.527	0.015	
	Race*		0.417	0.073	
IL-6					
Dependent Variable	**Independent Variables**	**No.**	**Co-efficient**	**p-value**	**Intercept**
DBS	**Plasma**	136	0.106	0.805	-3.119
IL-8					
Dependent Variable	**Independent Variables**	**No.**	**Co-efficient**	**p-value**	**Intercept**
DBS	**Plasma**	136	0.334	<0.05	1.118
TNF-α					
Dependent Variable	**Independent Variables**	**No.**	**Co-efficient**	**p-value**	**Intercept**
DBS	**Plasma**	136	-0.004	0.979	-2.701
DBS	**Plasma**		-0.004	0.975	-2.548
	Gender*		-0.324	0.019	
DBS	**Plasma**	136	0.002	0.990	-2.832
	Race*		0.411	0.006	
DBS	**Plasma**		0.001	0.996	-2.686
	Gender*		-0.286	0.032	
	Race*		0.376	0.010	

*** Reference groups for modeling were the healthy, males and Caucasians, respectively**