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Linking physiological parameters to perturbations in the human exposome: Environmental exposures modify blood pressure and lung function via inflammatory cytokine pathway

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Abstract

Human biomonitoring is an indispensable tool for evaluating the systemic effects derived from external stressors including environmental pollutants, chemicals from consumer products, and pharmaceuticals. The aim of this study was to explore consequences of environmental exposures to diesel exhaust (DE) and ozone (O₃) and ultimately to interpret these parameters from the perspective of *in vitro* to *in vivo* extrapolation. In particular, the objective was to use cytokine expression at the cellular level as a biomarker for physiological systemic responses such as blood pressure and lung function at the systemic level. The values obtained could ultimately link *in vivo* behavior to simpler *in vitro* experiments where cytokines are a measured parameter. Human exposures to combinations of DE and O₃ and the response correlations between forced exhaled volume in 1 second (FEV₁), forced vital capacity (FVC), systolic and diastolic blood pressure (SBP and DBP, respectively), and 10 inflammatory cytokines in blood (interleukins 1 β , 2, 4, 5, 8, 10, 12p70 and 13, IFN- γ , and TNF- α) were determined in 15 healthy human volunteers. Results across all exposures revealed that certain individuals displayed greater inflammatory responses compared to the group and, generally, there was more between-person variation in the responses. Evidence indicates that individuals are more stable within themselves and are more likely to exhibit responses independent of one another. Data suggest that *in vitro* findings may ultimately be implemented to elucidate underlying adverse outcome pathways (AOP) for linking highthroughput toxicity tests to physiological *in vivo* responses. Further, this investigation supports assessing subjects based upon individual responses as a complement to standard longitudinal (pre vs. post) intervention grouping strategies. Ultimately, it may become possible to predict a physiological (systemic) response based upon cellular-level (*in vitro*) observations.

Declaration of interest

The authors declare they have no competing financial interests.

Introduction

Overview

In the short term, many human adverse health effects originating from environmental chemicals and stressors exposures may be subtle, but small non-significant biological changes might accrue over a lifetime of insults and overwhelm cellular, tissue, and DNA repair functions resulting in development of various human disease types including cancer, and autoimmune, neurological, and cardiovascular disorders. This concept was developed over the past decade within the construct of the human exposome proposed by Christopher Wild (Wild, 2005). More recently, Stephen Rappaport and Martyn Smith proposed that "... 70 to 90% of disease risks are probably due to differences in environments," essentially implicating the genetic interaction with the environment (G x E) as the causative agent (Rappaport & Smith, 2010).

Because the human exposome is now defined in the broadest sense as the totality of all exogenous chemicals, metabolites, and endogenous biomolecules in the human system, this is more of a philosophical construct than a well-defined measurable entity found in blood, breath, urine, and other human media (Athersuch, 2012; Pleil, 2012a; Pleil & Stiegel, 2013; Rappaport et al., 2014; Wallace et al., 2016). The exposome is complex and constantly changing that no single analytical platform, biological fluid, or time point can fully capture the extent of the chemical constituency; however, the exposome is considered the ultimate path toward understanding human disease (Lioy & Rappaport, 2011; Rappaport et al., 2014).

Certain strata of the chemical space have now been identified as valuable indicators of exposure and effect. Turner et al. (2014) noted that one such group consists of small messenger proteins of the cytokine/chemokine class. Cytokines have been studied as markers of inflammatory response resulting from external stressors and chronic lowlevel inflammation associated with various adverse health outcomes (Feghali & Wright, 1997; Van Eeden et al., 2001). As such, these chemicals have been exploited both for *in vivo* and *in vitro* toxicity investigations using different immunochemistry platforms (Chaturvedi et al., 2015; Pleil et al., 2015a). The overall hypothesis has been to develop adverse outcome pathways (AOP) at the systemic level, reflecting the totality of *in vivo* systems biology, and subsequently utilize *in vitro* tests, such as those that generate cytokines, as indicator probes of adverse effects for suspect chemical compounds (Angrish et al., 2016; Edwards et al., 2016; Groh & Tollefsen, 2015; Vinken, 2013). Termed "highthroughput toxicity screening" (HTTS), this is a newer approach for prioritizing manufactured and commercialized chemicals for potential risk without time-consuming and expensive animal testing (Dix et al., 2007; Wetmore et al., 2012). This technology is now implemented at U.S. Environmental Protection Agency as Toxicity Forecasting or "ToxCast" and described in detail by Richard et al. (2016). To date, HTTS is (1) relegated to testing series of single molecular pathways, (2) not yet fully developed to mimic the totality of the systemic response, (3) has difficulty with some pharmaceutical classes and metabolic assessments, and (4) cannot predict physiological changes (Pleil et al., 2012b; Shah & Greene, 2014; Silva et al., 2015; Thomas et al., 2012).

Previously, different aspects of cytokine response were examined at the *in vivo* level (both human and rat) to this end and are now being developed as analogous cellular *in vitro* tests to establish predictive models of toxicity using AOP and diagnostic probe molecules (Angrish et al., 2015; Pleil et al., 2015b). Thus, the objective of those studies was to develop a secondary link from *in vivo* findings that look to predict physiological responses to exogenous exposures utilizing stratified exposome testing data of representative suites of cytokines. Consequently, previously developed exposure and cytokine data were interpreted within the context of perturbations of cardiopulmonary function, specifically blood pressure and lung function. As a secondary experiment, the glutathione S-transferase M1 (GSTM1) polymorphism was measured to assess potential confounding from detoxification pathways (Kasthurinaidu et al., 2015). The ultimate goal is to provide a mechanistic link between specific exposures and cytokine expression and parameterize this with respect to physiological outcomes that may not be tested *in vitro*.

Environmental diesel exhaust and ozone exposures

Associations between outdoor air pollution and adverse human health effects have been studied extensively, with special attention on particulate matter (PM), especially fine PM less than 2.5 μm , and gas phase pollutants due to their (mostly) anthropogenic origination and health impacts (2014; Brook et al., 2009; Costa et al., 2014; De Oliveira et al., 2014; Dong et al., 2013; Fariss et al., 2013; Holgate et al., 2003). Diesel exhaust (DE) emissions are one of the most prevalent contributors to fine PM pollution, and data showed that DE exposure induces or exacerbates respiratory irritation, cardiovascular physiological changes, lung inflammation, and a host of other pulmonary-related adverse health effects (Holgate et al., 2003; Madden et al., 2014; Tornqvist et al., 2007). Ozone (O_3) is one of the most studied gas phase pollutants given the environmental prevalence, known reactivity within the body, and relation to a variety of adverse pulmonary and cardiovascular health effects (Barath et al., 2013; Devlin et al., 2012; Kim et al., 2011; Tank et al., 2011). Epidemiological studies demonstrated associations between DE and O_3 exposures with disease progression and potential increased mortality rates (Morishita et al., 2015; Shan et al., 2014).

While findings demonstrate a relationship between DE and O_3 exposures and adverse health effects, fewer studies attempted to clarify biological pathways and mechanisms attributed to exposure leading to observed health effect(s) using *in vivo* approaches. One of the restrictions to assessing such links from exposure to adverse health effect is that typical spot-measures (snapshots in time) are difficult to interpret with respect to long-term risk; at best, these can only be estimated using external data (Pleil & Sobus, 2016). Similarly, the reverse of reconstructing exposures from preclinical (health) markers is equally difficult (Tan et al., 2012). These issues are addressed in part by use of scripted exposure studies, either case-control, or chambers.

It has been proposed that an inflammatory response is a key-initiating event that creates a cascade of events toward adverse health effects (Bautista et al., 2005; Bhagat & Vallance, 1997; Margretardottir et al., 2009). Th1 and Th2 cytokines were studied as biomarkers of inflammation, and expression of specific cytokines may be indicative of an immune response to an exogenous stressor. As such, controlled inhalation exposures to either DE or

O₃ were employed to investigate inflammatory cytokine responses and resultant changes in health (Ghanem & Movahed, 2007; Xu et al., 2013). Lung function changes are the most studied health effect, but exacerbation of existing pulmonary conditions such as asthma and chronic obstructive pulmonary disorder (COPD), changes in heart rate variability (HRV) reflecting inherent self-regulatory capacity, and variations in systolic and diastolic blood pressure (SBP and DBP) were also examined (Alexis et al., 2009; Devlin et al., 2012; Tornqvist et al., 2007).

Morishita et al. (2015) recently showed an association between environmental exposures and modifications in SBP in 32 healthy human volunteers following a controlled 2-hour exposure to concentrated ambient coarse PM_{2.5-10} (particles between 2.5 and 10 µm aerodynamic diameter). Holgate et al. (2003) and Tornqvist et al. (2007) did not determine blood pressure changes but demonstrated relationships between DE exposure and inflammation. Barath et al. (2013) investigated controlled O₃ exposures (at 300ppb × 75min) in 36 healthy young men and found no marked associations between the exposure and modifications in either SBP or DBP. In contrast, Alexis et al. (2009), Devlin et al. (2012), and Barker et al. (2015) found that O₃ exposures induced resulting inflammatory responses. Madden et al. (2014) established a relationship between DE and O₃ through the use of a controlled DE+O₃ co-exposure chamber study demonstrating an interactive effect on lung function changes including loss of forced expiratory volume following combined exposure versus individual exposure to DE or O₃. Finally, Stiegel et al. (2016) noted a similar relationship between DE +O₃ co-exposure and inflammatory responses.

Synergistic interactions

The previously cited studies (Madden et al., 2014; Stiegel et al., 2016) demonstrated individual relationships between DE, O₃, and DE+O₃ exposures, and lung function modifications and blood pressure (BP) changes, yet no apparent attempts were undertaken to investigate connections between measured outcome variables of inflammation and health measures, especially for combined DE+O₃ exposures. The DE+O₃ exposures are relevant to a “real-world” environmental exposure given the fact that an environmental exposure almost always is a mixture of contaminants. This study investigated correlations between inflammation biomarkers, lung function, and BP, with a highly controlled exposure chamber study. The ultimate goal was to provide new insight into the relationship between exposure and physiological parameters through linkage of cytokine expression.

Methods

Study design

The study population and exposure scenario is described in Madden et al. (2014) (ClinicalTrials.gov # NCT01874834). All participants were nonsmoking, healthy young adult volunteers that were required to exercise for 4, 15-minute increments over a 2-hour time frame on a recumbent bike. Volunteers also had to be willing to discontinue use of non-steroidal anti-inflammatory drugs, vitamin C, and vitamin E, prior to the exposure study. Table 1 characterizes the 15 healthy human volunteers that participated in the study, including GSTM1 status (genetic encoding of detoxification enzymes). Briefly, the majority

of the participants (11/15) are males, 9/15 of all subjects are GSTM1+ (where the expected value is approximately 50% (Kasthurinaidu et al., 2015), and mean female BMI was greater than average male BMI.

This study was designed as a random-crossover double-blind study with 4 exposure arms, with each arm being separated by approximately 2 weeks. Volunteers were exposed in an environmentally controlled exposure chamber at the US EPA Human Studies Facility in Chapel Hill, NC to filtered air, DE (300 µg/m³), O₃ (0.3 ppm), or a combination of DE and O₃ for 2 hours (IRB Study #: 09–1344). These exposures were subtle from an odor perspective, and the subjects were distracted by their scripted activities so that they did not generally recognize which exposure arm they were experiencing.

Whole blood was collected before and directly after each exposure arm in 10ml EDTA Vacutainer® (Product Number: 368589, Becton, Dickinson and Company, Franklin Lakes, New Jersey) collection tubes, centrifuged, aliquoted, and frozen at –80°C until analysis. The plasma fraction from whole blood samples were analyzed using Human Th1/Th2 10-plex Ultra-Sensitive Kits and a Meso Scale Discovery (MSD) SECTOR Imager 2400 (Meso Scale Discovery, Gaithersburg, MD) for the following cytokines: interleukins (IL) 1β, 2, 4, 5, 8, 10, 12p70 and 13, IFN-γ, and TNF-α. GSTM1 status was determined from isolated peripheral white blood cell DNA using QIAamp DNA real-time polymerase chain reaction Mini Kits (Qiagen Inc., Valencia, CA) (Gilliland et al., 2004; Kim et al., 2011). Forced exhaled volume in 1 second (FEV₁) and forced vital capacity (FVC) were measured using a 10.2 L dry seal digital spirometer (SensorMedics, Model #1022, Palm Springs, CA) (Madden et al., 2014). Blood pressure (BP) was measured with an Ultraview 2700 system (Spacelabs Healthcare, Snoqualmie WA).

Statistical analysis

Lung function and BP measurements as well as cytokine concentrations reported and analyzed represent preexposure and immediate postexposure values on Day 1 of the exposure regimen, and not any follow up measurements. For each exposure arm, data were tested for normality using Shapiro-Wilks tests (PROC UNIVARIATE, SAS statistical software package version 9.3, SAS institute, Cary, NC) and confirmed using QQ-plot observations (Pleil 2016a). FEV₁, FVC, and SBP/DBP measurements were normally distributed. Right-skewed cytokine data was natural log-transformed before statistical analysis to accommodate assumptions of normality. Preexposure to postexposure cytokine concentration differences, lung function changes, and BP changes were investigated using paired Student t-tests (Madden et al., 2014; Stevens et al., 2014; Stiegel et al., 2016). A *p*-value <0.05 was considered statistically significant.

Linear mixed-effect models (PROC MIXED, SAS) and restricted maximum likelihood estimates of variance components were used to calculate estimated intra-class correlation coefficients ($ICC = \hat{\rho}_h$) for each cytokine/vital measurement and each exposure using the

following equation (Rappaport & Kupper, 2008): $\hat{\rho}_h = \frac{\hat{\sigma}_{bY_h}^2}{\hat{\sigma}_{bY_h}^2 + \hat{\sigma}_{wY_h}^2}$, where *h* = biological

measurement (cytokine, FEV₁, etc.) $\hat{\sigma}_{bY}^2$ = estimated total between-person variance, and $\hat{\sigma}_{wY}^2$ = estimated total within-person variance. Linear mixed-effect models were also used to examine exposure-response differences by GSTM1 genotype.

Post/preexposure ratios for each exposure arm and biological measurement were calculated. These ratios were used for Spearman correlation coefficient calculations (PROC COR, SAS) and for graphical comparisons examining pre-to-post percentage changes for any statistically significant correlations. GraphPad (GraphPad Prism version 6.0, GraphPad Software, La Jolla, CA) and Microsoft Excel (Microsoft Excel for Mac 2011, Microsoft, Redmond, WA) were used for graphical representations.

Results

Summary statistics

Table 2 summarizes BP and lung function measurements preexposure and immediately postexposure from the four exposure arms stratified for GSTM1 genotype (Madden et al., 2014; Stevens et al., 2014). The descriptive statistics are shown as a reference for the exposure-related health endpoints used in the cytokine comparison. Across all exposures, the preexposure (pre) BP measurements ranged from a group minimum of 98/58 mm Hg to a maximum of 138/91 mm Hg, with a mean of 117/72.6 mm Hg. The group minimum postexposure (post) BP (89/51 mm Hg) across all exposures was less than the group minimum pre (98/58 mm Hg), while the group post maximum (137/95 mm Hg) was similar to the group pre (138/91 mm Hg). The pre FEV₁ and FVC lung function measures ranged from 3–5.94 ($\mu = 4.32$) to 3.5–7.14 ($\mu = 5.32$). Both post FEV₁ and post FVC measurements exhibited a wider range of responses than premeasures varying (min. to max.) from 1.84–5.89 ($\mu = 4.1$) for FEV₁ and from 2.94–7.17 ($\mu = 5.06$) for FVC. There were no statistically significant alterations in any of the measurements for the control or DE exposure arm. Results from O₃ exposure showed significant decreases in both lung function measurements following exposure, but there were no marked changes in either the SBP or DBP. The DE +O₃ co-exposure arm displayed significant pre to post reduction for SBP, FEV₁, and FVC. For all exposures, the relative responses were not markedly different from one another when stratified by GSTM1 genotype.

As described in more detail in Stiegel et al. (2016), but summarized here, Table 3 illustrates significant results from cytokine analysis following the three exposure arms (DE, O₃, and DE+O₃) and control. All significant results show that exposures decrease cytokine response. IL-5 and IFN- γ demonstrated a significant fall following the clean air exposure, and this trend appeared in both DE and DE+O₃ suggesting that this “effect” might be actually associated with exercise activity rather than the exposures.

The average percentage change from baseline for IFN- γ exhibited a diminishing tendency from –14.1% for clean air arm to –17.2% and –24.4% for DE and DE+O₃ exposures, respectively. However, a subsequent analysis compared post/pre relationships for IFN- γ across clean air, DE, and DE+O₃ arms and found no significant differences between clean air and either DE or DE+O₃ exposures. TNF- α , a Th1 pro-inflammatory cytokine, displayed

a significant decrease in expression following DE and DE+O₃ exposures, with mean reduction of -11.9% and -16.7%. IL-12p70, also a Th1 pro-inflammatory cytokine, showed a significant decrease following DE+O₃ exposure arm, averaging -14.4%.

Figure 1 illustrates the post-to-pre measurement ratios for SBP, DPP, FEV₁, FVC, and 7 inflammatory cytokines for DE+O₃ exposure (3/10 cytokines demonstrated no significant pre-to-post alteration for any of the four exposure arms and thus were omitted). This figure shows that SBP, FEV₁, FVC, IL-4, IL-5, IL-12p70, IFN- γ , and TNF- α displayed median postexposure reduction, IL-2 and IL-8 showed median increases, and DBP showed no marked change following the DE+O₃ exposure. Figure 1 also illustrates that the three Th1 cytokines with significant decrease in levels (IL-12p70, IFN- γ , and TNF- α) are suppressed to a greater extent than the significant decrease as evidenced by the one Th2 (IL-5) cytokine. IL-2 and IL-8 demonstrated the greatest range in the post/pre ratio, and generally there was more variation for cytokine ratios compared to the four health measurements.

Correlations

Table 4 displays the estimated intra-class correlation coefficients ($\hat{\rho}_h$) for the respective exposures. Here, observations for the clean air arm represent the distribution of variance for respective physiological measures and cytokines for unexposed subjects. Across all exposures, SBP results show that DE and DE+O₃ arms migrate from greater between-people variance toward more within-person variance. This shift toward more within-person variance demonstrates that the response to exposure(s) is seemingly independent of the individual. In addition, across all exposures, lung function data demonstrated that the majority of the variance is between-person (average $\hat{\rho}_h = 0.852$ and 0.932 for FEV₁ and FVC, respectively). These clean air observations illustrate that the majority of the measurements display more between-person variance than within-person variance. Under no external pressure (exposure) these findings indicate the majority of variance is dependent on the individual (i.e., the individual responses in the group are different from one another). The DE results show ICC estimates that are similar to the clean air arm, except SBP that exhibits more within-person variance and IL-8 displays more between-person variance. The O₃ exposure is also similar to filtered air exposure with IL-4 being the one exception. The DE+O₃ exposure observations exhibit the most changes when compared to the clean air arm. The $\hat{\rho}_h$ for SBP is solely within-person, FEV₁ trends toward a split of the variance within-person and between-person, and IL-4 switches to more between-person variance.

Results from the ICC estimates indicate that variation in responses to exposures deserves investigation on the individual level. Table 5 describes the number of individuals where arbitrarily a >10% increase or >10% decrease was selected in individual response as the breakpoint for the respective exposure versus the individual response during the clean air arm. Generally, the majority of measurements are “low” compared to “high.” The DE exposure results displayed no marked lung function measurements where individuals are outside the $\pm 10\%$ window, while the O₃ findings showed approximately one-third of the participants exhibited more than a 10% decrease compared to respective clean air response, and approximately two-thirds of participants in the DE+O₃ exposure exhibited a 10% decrease. There were fewer subjects in the DE, O₃ and DE+O₃ exposures that displayed

SBP or DBP measurements outside the $\pm 10\%$ window compared to the number of subjects outside this window for lung function measurements, demonstrating that a slightly lower response was detected on the individual level following these exposures (Figure 5). The cytokines have wide variations in the individual responses to the exposures, as evidenced by the fact that the majority of the participants are either higher or lower than their respective clean air arm responses, across all exposures.

Spearman correlation results for post/preexposure responses between the 7 cytokines, BP measurements, and lung function observations for each exposure arm are displayed in Table 6. Results investigating within-cytokine or within-physiological measurement correlations are not presented in Table 6. There were no significant correlations between SBP and DBP or between any of the lung function measures and BP measurements for the DE, O₃, and DE + O₃ exposure arms. There were significant positive relationships between FEV₁ and FVC for the O₃ and DE+O₃ exposures. The three exposure arms displayed a noteworthy difference between the two DE-associated exposures and O₃. The DE exposure displayed a negative association between DBP and IL-12p70, and DE+O₃ displayed negative correlations between SBP and both IL-8 and IFN- γ . The three Th1 cytokines (IL-8, IL-12p70, and IFN- γ) solely and negatively were associated with the BP measurements. Conversely, no marked correlations between any Th1 cytokines and BP measurements were noted during the O₃ exposure, but a significant negative association between a Th2 cytokine (IL-5) and DBP was detected. O₃ exposure also exerted positive correlations with both lung function measurements and IFN- γ .

Figure 2 displays the relationship between the postexposure and preexposure ratios following the DE+O₃ exposure for SBP and IL-8. Here, there are two important results to note. One, when preexposure SBP is greater than postexposure SBP, the inverse is true for cytokine concentration where preexposure IL-8 concentration is less than the post IL-8 levels and vice versa at the other end of the distribution, in 14 of 15 subjects. Two, there is a centralization of four of the GSTM1– subjects around 1,1 with nine GSTM1+ subjects showing more variation in their overall responses (seen at the extremes of the blood pressure/IL-8 comparison).

Figure 3 is similar to Figure 2 in that it presents the same type of negative correlation for DE + O₃ exposure and SBP, but in this case with IFN- γ . The association between SBP and IFN- γ is stronger, and more tightly grouped, than SBP/IL-8 comparison. The majority of SBP/IFN- γ comparisons are less than, or very close, to having both a SBP exposure ratio less than “1” and an IFN- γ exposure ratio less than “1,” indicating that postexposure measurements are lower than preexposure determinations. The GSTM1– subjects seemed to exhibit more of a 1:1 relationship between SBP and IFN- γ compared to GSTM1+ subjects, but any difference in trend is difficult to ascertain given the low number of GSTM1– participants.

Comparisons of percentage change from baseline

Figures 4 and 5 investigated group changes from baseline for O₃ and DE+O₃ exposures. The cytokines and vital measurements in these figures were selected based upon the significant correlations in the respective exposure. Figure 4 illustrates a mean decrease for each of the

five parameters that ranged from -4.27% to -10.3% . The cytokines displayed more variation in response to O_3 exposure compared to DBP, FEV_1 , or FVC. The stratification of determined responses into GSTM1-/+ shows that in every instance, except for DBP, the mean percentage change following exposure for the GSTM1+ participants is greater than that for GSTM1- participants. There were no significant differences between these two genotypes; however, GSTM1+ participants were close to having significantly different responses when compared to GSTM1- participants for FEV_1 and $IFN-\gamma$.

Figure 5 demonstrates that there are mean decreases of -5.35% for SBP, -24.4% for $IFN-\gamma$, and a mean increase of $+6.40\%$ for IL-8 compared to baseline “pre” samples. Similar to O_3 exposure, the two cytokines displayed more variation in response compared to BP measurements. The DE+ O_3 exposure results also exhibited similar responses after stratification into GSTM1-/+ genotypes; the mean percentage alteration following exposure for the GSTM1+ participants is greater than that for GSTM1- participants. Finally, while there were no significant differences between the two genotypes for any of the three measurements, GSTM1+ participants were close to displaying a significantly different response compared to the GSTM1- participants for the change in IL-8 concentration following exposure.

Discussion

This study examined associations between controlled acute exposures to DE, O_3 , and a DE+ O_3 combination, inflammatory responses, lung function changes, and variations in BP. Previously, investigators demonstrated that filtered clean air or DE exposure exerted no marked influence on FEV_1 , FVC, SBP systolic, or DBP (Madden et al., 2014; Stevens et al., 2014). These studies also showed O_3 -derived lung function changes as well as DE+ O_3 -induced lung function decrements and decreases in SBP (Madden et al., 2014; Stevens et al., 2014). Stiegel et al. (2016) noted reduction in Th1-derived cytokines following DE, O_3 , and DE+ O_3 exposures, with DE+ O_3 exposure exhibiting the largest inflammatory response compared to other exposures. Here, interest lies in examining how the respective exposures influenced exposure response outcome measurements on an individual or group level and how these responses are correlated.

Group-level response

The relationship between pulmonary function and BP has been examined extensively, mostly with respect to hypertension and potential adverse effects on lung function (Brook et al., 2009; Chae et al., 2001). Data demonstrate mixed results, with various studies showing no marked relationship between lung function and BP and some epidemiological studies reporting inverse relationships between lung function and BP (Wu et al., 1998). The correlation results from this present investigation indicate that there is an independent relationship between lung function and the BP findings and that this independent relationship is not markedly influenced by any of the exposures. While there was a significant reduction in the lung function determinants and DBP following O_3 exposure, and significant fall in lung function measures and SBP following DE+ O_3 exposures, not all participants responded in the same manner or to the same degree where subjects that started

with high values within the group did not necessarily end up in the same relative position and those that started low did not necessarily end up low. The initial hypotheses were that both BP and lung function were more stable within individuals than between subjects. If this were the case, it would be illustrated in the results by more between-person variation.

However, the variance for individual SBP responses illustrates that DE and DE+O₃ exposures displayed more within-person variance than expected. This same trend was not seen for clean air or O₃ exposures. The SBP observations for clean air and O₃ arms demonstrate that individual responses are relatively independent of overall group trends, thus leading to the conclusion that O₃ is influencing the manner in which specific individuals respond to the exposure; the overall response for DE and DE+O₃ exposures is, however, more of a “group” than individual response. On the other hand, the intra-class correlations for lung function measurements showed that responses were more stable within the individual even when considering exposure conditions. The stability of lung function observations may be attributed to the fact that individual responses are more closely related to meta-data categories such as BMI, gender, and age that subsequently lead to less variation in individual response as compared to group response. Finally, the shift toward more between-person variance for IL-4 measurements in O₃ and DE+O₃ exposures demonstrates that O₃ potentially exerts more of an effect on group as a whole than the individual.

Correlations between inflammatory response and physiological measurements were wholly dependent on specific exposure. Previously, investigators found that increases in BP are directly correlated with elevation in levels of inflammatory markers (Mirhafez et al., 2014; Schnabel et al., 2011). The majority of these studies examined the relationship between BP and inflammatory markers on a case-control or cross-sectional basis, irrespective of an exposure, and thus looked primarily at groups that already exhibited “high” or “low” blood pressure (Bautista et al., 2005; Ghanem & Movahed, 2007; Margretardottir et al., 2009). The primary conclusions were that hypertension and inflammation are correlated, but there were no indications that either inflammation or BP initiated a response for the other respective measurement. The volunteers for this study were healthy, displaying a mean BP of 117/72.6 mm Hg, and thus would not be considered hypertensive or hypotensive. Preexposure lung function data from this study were also in the expected range for the given age, height, weight, race, and gender of the respective participants. As such, any relationships between inflammatory markers, BP, and lung function should be indicative of a response to the exposure.

These results demonstrated a large difference between Spearman correlation findings for DE+O₃ and O₃ exposures. The DE+O₃ showed no significant associations with either of the lung function measurements, while for O₃ exposure, strong positive correlations were noted between the cytokine response and both FEV₁ and FVC. On the other hand, DE+O₃ exposure displayed stronger negative relationships with SBP measurements. IL-8 and IFN- γ , the two cytokines in the DE+O₃ exposure that were strongly correlated with SBP, are Th1-derived cytokines that are known to elicit phagocyte-dependent immune responses (Chung & Barnes, 1999). IL-5, the one cytokine in the O₃ exposure that exhibited a significant association with DBP, is a Th2-derived cytokine, and Th2 cytokines are usually responsible for phagocyte-independent immune responses (Chung & Barnes, 1999). Given

there was no particulate during O₃ exposure, the assumption is that DE particulate component of the DE+O₃ exposure is modulating the expression of the Th1 cytokines and BP, and the correlation findings show that this relationship is inversely related. While previous research independently found exposure-related changes of Th1 cytokines and SBP following particles and O₃ exposures (Hoffman et al. 2012; Moldoveanu et al., 2009), evidence indicates that these two observations are correlated but not predictive or causative, of one another, that is, they could be behaving independently from each other (Stiegel et al., 2016). To our knowledge, this is the first controlled human-based exposure study that demonstrated significant relationships between fall in SBP and increases in IL-8 and IFN- γ following DE+O₃ exposure.

A lack of significant correlations between any of the inflammatory cytokines and lung function determinants is also important to note given the significant reduction in FEV₁, FVC, and four different cytokines following DE+O₃ exposure. The initial assumption, given those exposure-related results and findings showing strong links between inflammation and lung function, was that there would be a significant correlation between one or more of the cytokines and lung function parameters. However, this was not the case. There are a few hypothetical reasons why a correlation was absent. As previously discussed in Stiegel et al. (2016), the DE+O₃ combination is potentially suppressing phagocytic immune responses. The O₃, or rise in NO₂, is either inhibiting macrophage from phagocytizing the particulate matter or limiting the recruitment or differentiation of macrophages in the epithelial lining of the lung tissue (Saito et al., 2002; Sawyer et al., 2010; Yang et al., 2008). There is an indication in humans from a controlled exposure study that O₃-induced lung function decrements were not associated with lung levels of several cytokines (with the exception of IL6) and neutrophilia as sampled by lavage (Hazucha et al., 1996), suggesting different mechanisms of response for lung inflammation and physiological endpoints. The results in the present study illustrate that this inhibition is correlated with an elevation in BP, and there is potentially another mechanism more closely related than the measured cytokines that is driving lung function observations. The significant exposure related responses for the cytokines may still be associated with lung function; however, the time dependent nature of the exposure in relation to measurement(s) and biological sampling most likely also played a part in the lack of correlations; volunteers performed lung function testing, blood pressure measurements, and blood draws at relatively the same time. Lastly, other inflammation related cytokines (not measured in the present) and other mediator classes, for example, lipids, may show more reliable correlations for the associations examined in this investigation.

The GSTM1– polymorphism has been associated with inflammatory sensitivity to O₃ exposure and, potentially in combination with other genotypes, decreased lung function (Alexis et al., 2009; Bergamaschi et al., 2001), and enhanced production of cytokines and neutrophilia in response to DE particles instilled intranasally (Gilliland et al., 2004; Yang et al., 2008). As such, the significant correlations noted in this study were examined by GSTM1 genotype, and while there were no significant differences or changes in the associations after stratification, the findings are noteworthy. In general, the GSTM1– group displayed less of an exposure-related response for all correlated health determinants and cytokines, but this group also displayed greater variation around the respective response. The

increased variation and low number of volunteers with the GSTM1– polymorphism may lead to lack of a significant difference between the two groups. Given more GSTM1– participants, the variation would most likely trend toward the respective means, and the current non-significant differences between the GSTM1+ and GSTM1– groups might become significant.

Individual-level responses

The group-level results are, for the most part, equivocal with respect to relationships between cytokines and physiological responses, but the question remains as to the basis for this. This study explored commonalities at the individual level for high responders. From the observations discussed above, and summarized in Table 6, the top 5 responding subjects were selected from each arm of the experiment based upon pre to postexposure percentage change for SBP, DBP, and FEV1. The rationale is that certain individuals may be at greater physiological risk from these exposures and that this may be signaled by cytokine response. The clean air exposure should serve to illustrate the exercise component of the responses, whereas DE, O₃, and DE+O₃ exposures are expected to interact with this response. For comparison, this experiment was also repeated for the five lowest responders. Adverse responses were defined as a decrease in FEV1 and increases in SBP and DBP. Some arguments may be made that reduction in BP might also signify an adverse event and thus was also investigated. Data were interpreted separately for each of the exposure arms. The following qualitative observations may be made regarding cytokine behavior with respect to exposure:

Clean air: Putative exercise effect

FEV1 change was relatively unaffected—only one individual decreased (less than 1%), and the rest remained stable or increased slightly up to 6%. Cytokines were essentially unaffected, except INF- γ and TNF- α that both demonstrated numerical rising trends for those individuals who exhibited a positive change in FEV1.

Systolic blood pressure change was symmetric about zero with the individual extremes ranging from a –7% to +16%. Only IL-4 demonstrated a significant alteration, trending positive with a positive change in SBP.

Diastolic blood pressure change has a wide range; the two extreme individuals displayed a low of –32% and a high of +26%. Cytokines alterations, however, were completely scattered with no observable trend.

DE exposure

FEV-1 was altered due to DE exposure ranging from –3.3% to +5.5%. IL-8 trended linearly with FEV-1, from the lower group at –7% to the higher group at +10%, whereas IL-12 showed an inverse trend from +12% to –14% (barring one outlier).

Systolic blood pressure change was also symmetric about zero with individual extremes ranging from a –13% to +10%; however, cytokines trends were completely not correlated with SBP.

Diastolic blood pressure change exhibited a wide range from a low of –31% to a high of +18%. Only IL-12 showed any relationship with an inverse trend from a mean of +38% to –11%.

O₃ exposure

FEV-1 demonstrated a prevalent fall for all subjects ranging from –28% to approximately 0%, indicating that O₃ uniformly diminished pulmonary function. INF- γ showed a strong correlation with decreased lung function with a mean reduction of 37% for the five subjects with the most decrement in FEV-1. TNF- α exhibited a similar trend with the exception of one outlier subject.

Systolic blood pressure change varied symmetrically from –14% to +8%. Cytokines trends were completely not associated with SBP.

Diastolic blood pressure change exhibited a wide range from a low of –31% to a high of +12%. A decreasing trend correlated to drop in DBP around –20% overall was noted in IL-5, IL12p70, INF- γ , TNF- α .

DE $\pm$ O₃ exposure

FEV-1 showed a prevalent fall for all subjects ranging from –45% to –1.3%, indicating that DE+O₃ also uniformly diminished pulmonary function. IL-2 elevation by approximately 30% (plus one outlier at 400%) for the most affected subjects was found. IL-8 demonstrated a definite anti-correlation trend with pulmonary function; five subjects with the greatest decrement in FEV-1 displayed a pre-to-post rise in IL-8 of approximately 28% (with one removed outlier). The remaining cytokines are equivocal.

Systolic blood pressure change varied symmetrically from –23% to +14%. IL-8 demonstrated a strong correlation with SBP for five subjects that exhibited a reduction, IL-8 increases by +34%; and for five subjects with SBP elevation, the IL-8 expression dropped by approximately –22%. The remaining cytokines are equivocal.

Diastolic blood pressure change exhibited a low of –20% to a high of +14%. Cytokines trends were completely not related with DBP.

Summary of individual level results

This semi-quantitative investigation of results at the individual subject level suggested potential explanations for the often-ambiguous findings in case-control or longitudinal studies of inflammatory markers. It is of interest that for DE+O₃ co-exposure scenario shown in Figure 1, only TNF- α demonstrated an obvious trend for the composited cytokine data. However, at the individual level, it was found that IL-8 increases for the five subjects with the greatest decrease in FEV-1 and that IL-2 reduction was marked for those same subjects. For DE-only exposure, IL12 elevation was a mean of 38% for five -subjects with the greatest loss of DBP. For the O₃ exposure, INF- γ and TNF- α were lowered significantly for the five subjects with greatest loss of lung function. These results indicate that cytokines response may be useful at the individual level in ways that are masked in broader group interpretations. Certainly, this is based upon only 15 subjects and so cannot be generalized,

but it is intriguing that certain cytokines may become specific markers for physiological changes in certain individuals.

Future investigations

Although standard *in vivo* toxicity testing generally relies on analyses of blood and urine media, more recently exhaled breath samples have become an important option due to ease of access (non-invasive collection) and overall simplified logistics in that breath (1) can be self-collected, (2) does not generate potentially infectious waste, and (3) does not require medical personnel or privacy (Pleil, 2016b). Further, methods are now available to assess toxicological response at the cellular level all the way to crowds of subjects expanding the realm of biological media beyond usual fluids (Pleil, 2016c; Williams & Pleil, 2016). In addition, exhaled breath is not restricted to only the gas phase; both exhaled breath condensate (EBC) and exhaled aerosols can be collected and assayed for proteins, bacteria, polar organic compounds, cytokines, and airway pH (Hunt, 2002; Montuschi, 2007). There is precedence that air pollution such as O₃ exposure induced changes in soluble mediators in EBC as demonstrated by Madden et al. (1997). For this study, human plasma samples were utilized; however, parallel analyses were also developed using EBC samples, and these will be featured in future investigations. Further, from a clinical and critical care perspective, EBC is a preferred medium for monitoring ongoing inflammatory status of mechanically ventilated hospital patients as it is accessible non-invasively (Boshuizen et al., 2015; Vaschetto et al., 2015). As cytokine data from EBC samples are already developed, this application will allow us to develop a greater dynamic range of inflammatory markers as biological specimens from ventilated patients that may reflect a wider range of health status.

Conclusions

This study showed that expression of plasma IFN γ following O₃ exposure demonstrates a significant positive correlation between both lung function measures, suggesting that O₃-related modifications in FEV₁ and FVC may be mechanistically related to expression of IFN- γ . Data also show that controlled acute exposure to DE+O₃ induced SBP and lung function changes, but these responses are independent of one another. Plasma inflammatory cytokine responses following DE+O₃ exposure were not correlated with lung function but are associated with alterations in SBP.

This is the first controlled human exposure study that demonstrated significant negative correlations between inflammation in blood and SBP following DE+O₃ exposure. These results suggest that the two Th1-derived cytokines (IL-8 and IFN- γ) might be facilitating alterations in BP, but further investigation into this relationship is warranted. The findings also indicate that there is a significant reduction in SBP following DE+O₃, but that individuals responded differently from one another. GSTM1 genotype appears to exert a minimal differential effect on the responses, but this analysis was underpowered and would require more study participants to fully clarify these differences. Finally, one may conclude that there may be more value for investigating subsets of individuals beyond the standard longitudinal grouping process. This concept is still unproven. However, such approaches might be developed with follow-on experiments targeted at discerning linkages between

changes in lung or cardiac function and inflammatory marker expression, regardless of initiating events such as environmental exposures.

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Figure 1. Post/Pre DE+O₃ Exposure Ratios for Blood Pressure, FEV₁, FVC, and Th1/Th2 Inflammatory Cytokines. The boxes display median, upper 75%, and lower 25% of the range of post/preexposure ratios while the bars display the upper 97.5% and the lower 2.5%; a “*” indicates statistical significance.

Figure 2.

Systolic Blood Pressure Post/Preexposure Ratio versus IL-8 Post/Preexposure Ratio for the DE+O₃ Exposure. The “post” concentration for IL-8 increases as the “post” systolic blood pressure measurement decreases.

Figure 3. Systolic Blood Pressure Post/Pre Exposure Ratio versus IFN- γ Post/Pre Exposure Ratio for the DE+O₃ Exposure. The “post” concentration for IFN- γ increases as the “post” systolic blood pressure measurement decreases.

Figure 4. Percent Change from “Pre” Measurement following O₃ Exposure; shows the mean and standard error of the mean (SEM) for the percent change (“post” vs. “pre”) following the O₃ exposure articulated by GSTM1 status.

Figure 5. Percent Change from “Pre” Measurement following DE+O₃ Exposure; shows the mean and standard error of the mean (SEM) for the percent change (“post” vs. “pre”) following the DE +O₃ exposure.

Table 1

Demographics of Study Participants.

Gender	Age ^a	BMI ^b	GSTM1 ^c /TH
Male (<i>n</i> = 11)	27.3 (24.4–30.5)	26.5 (24.7–28.3)	6+/4–/1nd
Female (<i>n</i> = 4)	26.2 (22.9–29.9)	30.7 (21.7–39.7)	3+/1–

^aGeometric mean (GM) and 95% confidence interval of the GM.^bMean (μ) and 95% confidence interval of the μ .^cGSTM1 genotype status (+ = positive, – = null, nd = no determination).

Table 2

Baseline and Postexposure Blood Pressure and Lung Function Measurements^{*}

		Exposure									
		Air		Diesel		Ozone		DE+O ₃			
		Pre	Post	Pre	Post	Pre	Post	Pre	Post		
Systolic Blood Pressure ^a	Total	117 (1.99)	119 (2.54)	118 (1.50)	117 (2.76)	115 (1.88)	114 (2.31)	118 (2.23)	111 (2.47)		
	GSTM1+	113 (2.62)	115 (3.60)	115 (1.75)	116 (4.37)	113 (2.82)	112 (2.85)	116 (2.44)	109 (3.21)		
	GSTM1-	122 (1.20)	124 (2.58)	122 (2.80)	116 (2.89)	118 (2.29)	120 (3.79)	123 (4.46)	116 (4.41)		
Diastolic Blood Pressure ^a	Total	73.3 (1.80)	72.1 (3.12)	73.9 (1.52)	72.1 (2.27)	70.2 (1.87)	66.7 (2.00)	72.9 (1.93)	70.7 (2.02)		
	GSTM1+	72.4 (2.09)	69.7 (3.38)	74.0 (2.45)	71.7 (1.84)	69.7 (2.46)	66.1 (1.65)	72.1 (2.04)	67.7 (2.01)		
	GSTM1-	76.8 (3.26)	76.2 (7.36)	74.4 (1.29)	71.6 (6.31)	72.6 (3.28)	67.2 (5.60)	76.2 (4.14)	77.2 (3.56)		
FEV ₁	Total	4.35 (0.236)	4.40 (0.227)	4.37 (0.243)	4.41 (0.247)	4.39 (0.252)	4.00 (0.265)	4.37 (0.246)	3.60 (0.253)		
	GSTM1+	4.21 (0.315)	4.27 (0.296)	4.24 (0.319)	4.29 (0.326)	4.25 (0.324)	3.74 (0.337)	4.24 (0.314)	3.41 (0.378)		
	GSTM1-	4.45 (0.439)	4.49 (0.439)	4.49 (0.472)	4.45 (0.464)	4.46 (0.488)	4.29 (0.475)	4.46 (0.491)	3.77 (0.321)		
FVC	Total	5.35 (0.310)	5.37 (0.305)	5.32 (0.313)	5.33 (0.307)	5.36 (0.313)	4.90 (0.304)	5.27 (0.334)	4.64 (0.340)		
	GSTM1+	5.14 (0.370)	5.18 (0.354)	5.13 (0.365)	5.11 (0.340)	5.18 (0.373)	4.67 (0.367)	5.02 (0.392)	4.20 (0.348)		
	GSTM1-	5.52 (0.663)	5.53 (0.673)	5.48 (0.696)	5.50 (0.699)	5.43 (0.667)	5.06 (0.607)	5.48 (0.707)	5.14 (0.721)		

^{*} data from Madden et al. (2014), Stevens et al. (2014), mean (SEM), "Total": $n = 15$, "GSTM1+": $n = 9$, "GSTM1-": $n = 5$, FEV₁ = forced exhaled volume in 1 second (L/1 sec), FVC = forced vital capacity(L),

^a mm Hg.

Table 3

Summary of Statistically Significant Day 1 Pre- to Postexposure Results for Selected Cytokines—all postresults were significantly lower than prerresults*.

Clean air	DE	O ₃	DE+O ₃
IL-5 [−15.8%(0.0110)]	IL-5 [−14.5%(0.02)]	IL-2 [−27.2%(0.006)]	IL-5 [−12.1%(0.05)]
IFN- γ [−14.1%(0.035)]	IFN- γ [−17.2%(0.05)]		IL-12p70 [−14.4%(0.002)]
	TNF- α [−11.9%(<0.0001)]		IFN- γ [−24.4%(0.003)]
			TNF- α [−16.7%(0.0003)]

Table 4Estimated Intraclass Correlation Coefficients ($\hat{\rho}_h$) for the Four Exposures.*

	Clean Air	DE	O ₃	DE+O ₃
Systolic BP	0.730	0.427	0.644	0.136
Diastolic BP	0.387	0.187	0.229	0.435
FEV1	0.927	0.994	0.869	0.620
FVC	0.999	0.996	0.896	0.835
IL-2	0.780	0.716	0.871	0.640
IL-4	0.413	0.563	0.983	0.826
IL-5	0.843	0.722	0.913	0.744
IL-8	0.139	0.843	0.429	0.271
IL-12p70	0.895	0.633	0.950	0.889
IFN- γ	0.982	0.934	0.924	0.754
TNF- α	0.902	0.913	0.744	0.683

* $\hat{\rho}_h$ ranges from 0 to 1, where “0” is only within-person variance, “0.5” is an equal division between within and between person variance, and “1” is only between-person variance.

Table 5

Individual Responses by Exposure in Comparison to the Clean Air Arm *

	DE		O ₃		DE+ O ₃	
	Low	High	Low	High	Low	High
FEV ₁	0	0	6	0	11	0
FVC	0	0	6	0	8	0
SBP	3	1	1	1	6	0
DBP	1	2	4	3	6	2
IL-2	9	3	10	2	6	4
IL-4	6	7	2	9	3	8
IL-5	4	6	5	4	7	6
IL-8	3	5	4	6	5	9
IL-12	10	5	9	2	10	3
IFN- γ	7	6	5	7	6	5
TNF- α	6	0	8	3	10	1

* the number in a respective box is the number of participants, out of the total ($n = 15$), that had a postexposure to preexposure ratio (Post/Pre) that was 10% lower (‘‘Low’’) or 10% higher (‘‘High’’) than the same post/pre response for that individual and that measurement for the ‘‘clean air’’ arm.

Table 6

Statistically Significant Correlations between Blood Pressure, FEV₁, FVC, and Cytokines, by Exposure.

	Exposure			
	Clean air	DE	O ₃	DE+O ₃
Systolic BP	-	-	-	IL-8 [-0.675(0.0058)] IFN- γ [-0.729(0.002)]
Diastolic BP	-	IL-12p70 [-0.529(0.045)]	IL-5 [-0.546(0.038)]	-
FEV ₁	-	-	IFN- γ [0.843(<0.0001)]	-
FVC	-	-	IFN- γ [0.711(0.003)]	-