PERIPHERAL BLOOD MARKERS OF CENTRAL NERVOUS SYSTEM EFFECTS FOLLOWING CONTROLLED HUMAN EXPOSURE TO DIESEL EXHAUST

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Abstract

Epidemiological and animal studies suggest that exposure to airborne pollutants may negatively impact the central nervous system (CNS). It is thought that traffic related air pollution (TRAP), and other forms of combustion-derived pollutants, may induce a maladaptive activation of the CNS immune system, however, the exact pathway is not understood. Animal models and epidemiological studies have inherent limitations including potential interspecies differences and residual confounding. Given this, the aim of this research is to examine effects of TRAP on the CNS using a controlled human exposure.

27 healthy adults were exposed to two conditions: filtered air (FA) and diesel exhaust (DE) (300μg $PM_{2.5}/m^3$) for 120 minutes, in a double-blinded crossover study with exposures separated by four-weeks. Prior to and at 0, 3, and 24 hours following exposure, serum and plasma were collected and analyzed for inflammatory cytokines IL-6 and TNF-α, the astrocytic protein S100b, the neuronal cytoplasmic enzyme neuron specific enolase (NSE), and brain derived neurotrophic factor (BDNF). The hypothesis was that IL-6, TNF-α, S100b and NSE would increase and BDNF would decrease following DE exposure. Changes in levels of biomarkers were assessed using a paired t-test to compare the change from baseline at each post-exposure timepoint following DE or FA exposure. A linear mixed effects model was build including exposure and timepoint as covariates, and subject ID as a random effect. Age and gender were examined as potential effect-modifying variables.

At no time-point following exposure to DE was a significant increase from baseline seen for IL-6, TNF-α, S100b or NSE, or decrease for BDNF, relative to FA exposure. The linear mixed effects model revealed indication of diurnal behavior for S100B, NSE and BDNF; however, no significant exposure-time-point interaction, suggesting the biomarkers were not affected by DE exposure. These results indicate that short-term exposure to DE amongst young, healthy adults does not acutely affect levels of the measured biomarkers. This study does not disprove a relationship between air pollution and adverse CNS effects and suggests a need to examine the effects of TRAP on the brain using in chronic exposure models or more sensitive CNS endpoints.

Preface

The overarching study, of which this thesis was a part, was designed by Dr. Christopher Carlsten and PhD candidate Jason Curran and funded by Health Canada. All human exposures were conducted at Vancouver General Hospital in the Air Pollution Exposure Laboratory. This study was approved by the ethical review board of the University of British Columbia (#H12-03025), Vancouver Costal Health Ethics Board (# V12-03025) and Health Canada's Research Ethics Board. The thesis content is the original work of the author, Rachel Cliff, and was conducted with the supervision of M.Sc. supervisor Dr. Christopher Carlsten, and thesis committee members Dr. Michael Brauer and Dr. Howard Feldman, all from the University of British Columbia, Faculty of Medicine.

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List of Abbreviations

AM	Arithmetic mean
APEL	Air Pollution Exposure Laboratory
BBB	Blood brain barrier
BC	Black carbon
BDNF	Brain-derived neurotrophic factor
CANTAB	Cambridge Neuropsychologial Test Automated Battery
CNS	Central nervous system
CSF	Cerebral spinal fluid
CO	Carbon monoxide
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DALYs	Disability-adjusted life-years
DE	Diesel exhaust
DEPM	Diesel exhaust particulate matter
EAPOC	Effects of Air Pollution on Cognition Study
EC	Elemental carbon
ELISA	Enzyme-linked immunosorbent assay
FA	Filtered air
fMRI	Functional neuroimaging
GE	Gasoline engine exhaust
GFAP	Glial fibrillary acidic protein
GM	Geometric mean
HEPA	High-efficiency particulate air
HC	Hydrocarbons
HO	Heme oxygenase
IL	Interleukin
kW	Kilo-watt
LOD	Limit of detection
LTA	Lipoteichoic acid
mg/m^3	milligrams per cubic meter
MMSE	Mini-Mental State Examination
Mn	manganese
ng/mL	nanograms per milliliter
NIOSH	National Institute for Occupational Safety and Health
NO_x	Nitrogen oxides
NO_2	Nitrogen dioxide
NSE	Neuron specific enolase
OC	Organic carbon
OD	optical density
OR	Odds ratio
O_2	Oxygen
PAH	Polyaromatic hydrocarbons

pg/mL	picograms per millilitre
PPB	Parts per billion
PPM	Parts per million
PM	Particulate matter
$PM_{2.5}$	Fine particulate matter; particulate matter with a mass median aerodynamic diameter less than 2.5 micrometers
PM_{10}	Inhalable particulate matter; particulate matter with a mass median aerodynamic diameter less than 10 micrometers
PNC	Particle number concentration
ROS	Reactive oxygen species
SD	Standard deviation
SMPS	Scanning Mobility Particle Sizer
SO_x	Sulfur oxides
TBI	Traumatic brain injury
TEOM	Tapered Element Oscillating Microbalance
TNF-α	Tumour necrosis factor-alpha
TRAP	Traffic related air pollution
TVOC	Total volatile organic compounds
TWA	8-hour time weighted average
UFPM	Ultrafine particulate matter
µg	micrograms
$\mu g/m^3$	micrograms per cubic meter
USEPA	US Environmental Protection Agency
WHO	World Health Organizations
^{13}C	Radioactively labeled carbon

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Dedication

This thesis is dedicated to my family; who celebrate my successes, offer support through my challenges, and continually inspire me to achieve my best.

Chapter 1: Introduction

1.1 Overview and Historical Context

Throughout the mid 1900's several extreme air pollution events occurred in western countries, which drew widespread attention, for the first time, to the potential risks of such exposures. The first of these occurred in December of 1930 in Liege, Belgium when stable atmospheric conditions allowed for industrial air pollution from steel mills, coke ovens, smelters and other sources to accumulate in the city. Over the course of two days, 60 people died; more than 10 times the typical mortality rate in the area at that time (1). Later, in 1948, the small town of Donora, Pennsylvania, encountered a similar air pollution event with comparable health consequences. Unusual atmospheric conditions caused emissions from local coke ovens, coal-fired furnaces, and metal works industries to settle in the valley town and 20 people died within a single week: a rate six times the normal mortality rate (1). Finally, what is considered the most well-known air pollution event of the 20th century occurred in London, England from December 5th to December 9th 1952 - an event that became known as the "London Fog". A thick smog, resulting from various industrial processes and coal-burning in homes, power plants, and factories settled in the city as a result of stagnant air conditions. Air pollution levels were approximately five to nineteen times above current regulatory environmental standards (1). It is estimated that the London Fog episode was responsible for causing 4,000 excess deaths in the short-term, and 12,000 additional mortalities in the subsequent months (1,2). These international episodes, particularly the London Fog, sparked an area of epidemiological research focusing on the health impacts of environmental air pollution (1-3).

Today, the negative cardiovascular and respiratory health effects of exposure to combustion related air pollution are well documented and generally accepted (3). That said, the scientific community has yet to determine a level of particulate matter (PM) exposure at which no adverse health effects can be detected on a population level (4). In modern times, common

sources of combustion related air pollution are from personal and industrial vehicles, and the product of this is often described as traffic related air pollution (TRAP). TRAP itself is a complex mixture of airborne compounds including carbon dioxide (CO_2), carbon monoxide (CO), hydrocarbons (HC), nitrogen oxides (NO_x), aldehydes, and PM (5). Although regulations have been developed to control industrial and personal vehicle emissions, worldwide exposure to high levels of anthropogenic and natural air pollution continues to be a public health problem. For example, it is estimated that 89% of the world's population resides in an area where the World Health Organization's (WHO) Air Quality Guideline for fine PM (particulate matter with an aerodynamic diameter less than 2.5 μm) is exceeded (6). The 2013 Global Burden of Disease study estimated that environmental PM contributed to 3 million premature deaths and 70 million disability-adjusted life-years, worldwide, in 2013 and in 2016 this estimate was increased to 2.9 million excess deaths (7,8).

In addition to the known cardiovascular and respiratory health effects of TRAP exposure, recent epidemiological and animal evidence suggests that air pollution may also negatively impact the central nervous system (CNS) (5). Indeed, various epidemiological studies have shown that exposure to air pollutants is correlated with delayed cognitive development in children and impaired cognitive function in the elderly. Adults residing in areas with higher TRAP levels have been noted to have poorer cognitive function and faster rates of cognitive decline. Such a relationship has been demonstrated in several studies examining populations of both men and women across a spectrum of specific age ranges, all above 55 years old (9-13). In children, a correlation between environmental black carbon and lower cognitive function has been observed (14) and an association between nitrogen dioxide (NO_2) exposure in schools and lower neurobehavioral scores has also been documented (15). This evidence suggests that air pollution has potential to impact the CNS at various stages of life, with individuals being particularly susceptible during neurodevelopment and aging.

Although the exact mechanisms by which air pollution may impact the CNS are not understood, the pathway is generally hypothesized to involve a maladaptive activation of the innate immune system, spanning four general mechanisms as follows: 1) It is relatively well established that inhalation of PM activates pro-inflammatory cytokines in macrophages, causing a release of various inflammatory responses and oxidative stress (16-18). It is then thought that this inflammatory effect may be transferred to the CNS, leading to the activation of further inflammatory pathways and oxidative stress within the brain; 2) There may be direct entry of PM into the CNS. Ultra-fine PM (<0.1 µm) (UFPM) may transverse alveolar epithelial cell membranes and then be carried by erythrocytes to extra-pulmonary organs, including the boundary with the brain, where they may impair the integrity of the blood brain barrier (BBB) and enter the CNS (18,19); 3) UFPM may bypass the BBB and enter the CNS through direct translocation across the olfactory epithelium (19,20). Alternatively, it has been suggested that air pollutants, including PM, may stimulate vaso-vagal reflexes or respiratory tract irritant receptors in the airways (21-23). The particles may therefore impact afferent autonomic nervous system (ANS) fibers and induces ANS dysfunction (21-24). The impact of air pollutants on the brain may therefore occur through the feedback of the altered ANS control, or be related to a pathway downstream of the pollution-induced cardiovascular effects (5,22). Regardless of the exact pathogenic mechanism, one of the popular overarching hypotheses is that PM exposure is associated with CNS inflammation, which may become clinically relevant with repeated exposures or during susceptible periods of brain development (18,25). In conclusion, it is possible that the peripheral immune response to air pollution is transferred to the CNS, activating inflammatory cascades and microglia – the innate immune cells of the brain and, additionally, that microglial cells respond to UFPM that reaches the CNS (18).

Neurocognitive diseases place significant burden on patients, their families, and public health systems, but the role of environmental risk factors is poorly understood. A recent report estimated that dementia costs US $818 billion, annually, due to societal and economic factors, and the psychological burden on family members of patients is also significant (26). Research to identify and address modifiable risk factors is an important public health priority (27) and

determining if exposure to air pollution increases risk of cognitive decline is an important, and relatively unexplored, area of research. Presently, findings from both epidemiological and animal research suggest a relationship between combustion-derived air pollutant exposure and adverse CNS outcomes. However, there is insufficient plausibility to strongly prove this relationship. Given this, further research, including controlled human exposure studies, are needed.

1.2 Objectives and Methodology

This study was designed to determine the acute effects of a TRAP exposure on blood biomarkers of systemic inflammation and CNS effects (5). 27 human volunteers were each exposed for 120 minutes, on two different occasions, to DE at a concentration of 300μg/m^3 $PM_{2.5}$ or filtered air (FA) in a double-blinded, crossover design. A four-week washout period was allowed between exposures and exposure conditions were randomized and counter-balanced. Various cognitive parameters were assessed by another student on the project and this thesis work is specific to the measurement of biomarkers before and after each exposure. Serum and plasma were collected at four time points before and after each exposure (immediately before and after, and three and twenty-four hours after) and the levels of five specific biomarkers were determined in these samples. The biomarkers were specifically selected with the targeted aim of enhancing our understanding of the effects of TRAP on the CNS. Plasma was analyzed for interleukin-6 (IL-6) and serum for tumour necrosis factor-alpha (TNF-α), as markers of systemic inflammation. Potential insults to the CNS were determined by analyzing serum levels of S100B, an astrocytic protein that has been recorded to increase in the serum following CNS insult (28,29) and a proposed marker of BBB permeability (28,30), and neuron specific enolase (NSE), a marker thought to increased as a result of neuronal death (29,31) and potential marker of neuroinflammation (32). For these four biomarkers, the hypothesis was that levels would increase following DE exposure relative to FA. Finally, brain-derived neurotrophic factor (BDNF), a molecule that is thought to protect neurons and encourage their growth and differentiation, was measured in serum (33,34). We used these biomarkers as a non-invasive approach to assess

the effects of a controlled air pollution exposure on the human brain, with the overarching aim of determining if, and to what extent, an acute TRAP exposure can impact the CNS.

The objectives of this study were to assess:

(1) The presence of an inflammatory effect of diesel exhaust (DE) exposure on the human subjects, characterized by an increase in the inflammatory cytokines IL-6 and TNF-α immediately and three hours following diesel exhaust exposure.

(2) An increase in serum levels of the protein S100B (a marker for CNS effects and a proposed marker for BBB permeability), three and twenty-four hours after exposure.

(3) An increase in serum levels of the CNS-specific protein NSE, three and twenty-four hours after exposure, and/or by showing a decrease in serum BDNF levels immediately following exposure.

1.3 Contribution to Literature

While the negative cardiovascular and respiratory health effects of TRAP are well documented, new evidence suggests that these exposures may impact the CNS. The potential of TRAP to impact the CNS at any capacity is of obvious concern to the general public, who are environmentally exposed to air pollution, as well as to the substantial number of workers who are experience high levels of TRAP exposures at their place of work.

Due to the emerging nature of the concept that combustion related air pollution may impact the brain, there remain many unanswered questions. Both animal models and epidemiological studies have inherent limitations including potential interspecies difference and the issue of residual confounding. Previous *in vivo* animal experiments and post-mortem analyses studies have had the ability to analyze brain tissue; an option clearly not available for a model involving living human volunteers. Here, blood collection is a feasible and relatively non-invasive alternative. There is potential for circulating proteins to provide indication of the biological mechanism behind the effect of DE on the CNS. The work in this thesis represents a sub-section of a larger study termed the "Effects of Air Pollution On Cognition" (EAPOC) study, in which other cognitive tests are also examined with the similar goal of showing if, and to what extent, DE exposures (at a level representative of high-ambient environmental and occupational conditions) can impact cognitive function. The use of blood markers in this study provide a unique opportunity to complement the cognitive tests and give indication of a systemic and/or CNS-specific response, offering mechanistic information to inform current and future observations.

Chapter 2: Literature Review

2.1 An Overview of Diesel Engine Exhaust

In 1892 the diesel engine was first patented by Rudolf Diesel. It quickly rose to popularity owing to its improved fuel efficiency compared to other forms of engines available at the time; a major advantage of diesel-powered vehicles still to this day (35). In modern times, diesel engines are used to power machinery such as personal vehicles, trucks, buses, agricultural equipment, locomotives, and ships (35). While it remains true that these engines offer greater fuel economy and durability than gasoline-powered engines, they emit more PM for the same workload (35) and, it is additionally concerning that diesel exhaust particulate matter (DEPM) have mutagenic and carcinogenic properties. Indeed, long-term inhalation of DE has been linked to increased lung cancer mortality in over 35 epidemiological studies (36). In addition, chronic, and acute cardiovascular and respiratory health outcomes have been associated with DE and other TRAP exposure, and DE is known to cause irritation of the eyes and upper respiratory tract. Diesel-powered vehicles are important in industries worldwide and, as a result, exposures to their exhaust is also common; in BC alone, it is estimated that approximately 108,000 people were exposed to diesel fumes in their workplace on an annual basis (37).

2.1.1 Diesel Exhaust Components

Complete combustion of diesel fuel would produce emissions of water and CO_2 (38), but in real world operation, diesel engine exhaust is in fact a complex mixture of hundreds of pollutants (35). Potential constituents of diesel exhaust (DE) include gases – such as oxygen (O_2), CO_2, CO, NO_x, sulfur oxides (SO_x), low molecular weight hydrocarbons (i.e. benzene, formaldehyde, acetaldehyde, 1,3-butadiene, polyaromatic hydrocarbons (PAHs) and nitro-PAHs), water vapour, and a range of PM (35,39,40). Primary DEPM is formed as a result of incomplete combustion of diesel fuel and is commonly referred to as “diesel soot” and consists primarily of an agglomerated elemental carbon (EC) core and ash, surrounded by adsorbed ash, organic

compounds and small amounts of sulfate, nitrate, metals and other trace elements and metals (35,39,41,42). In addition, secondary pollutants, such as ozone, can also be generated in the atmosphere as DE ages (35).

Of the components within DE, PM and NO_x are generally considered the most relevant compounds to human health (43), however, many of the other constituents also have environmental and health consequences (35). For example, CO_2 is a major contributor to global warming; NO_x and SO_x can initiate acid rain; NO_x, hydrocarbons, and aldehydes are all ozone precursors. CO is highly toxic to humans as it binds to hemoglobin 250 times more strongly than oxygen – potentially leading to oxygen deprivation – that said, levels of CO in DE emissions are generally too diluted in environmental settings to cause an acute hazard (44). Hydrocarbons, NO_x and SO_x are irritants of the eyes, upper respiratory tract and skin, and additionally, some aldehydes and hydrocarbons released in DE are considered carcinogenic (35,44). As will be discussed in more detail below, the toxicological potential for DEPM is based partially on the size of the PM and its structure. As a pollutant, DE contains less CO, and more NO_x, aldehydes and PM, than does gasoline engine exhaust (GE) (38).

Ambient PM is usually classified into fractions on the basis of its size: respirable PM is defined as PM with an aerodynamic diameter <10 μm (PM_{10}); coarse particles are those with a diameter between 2.5 and 10 μm ($PM_{2.5-10}$); fine PM, another subset of PM_{10}, consists of all particles with an aerodynamic diameter <2.5 μm; and finally, UFPM are the smallest size fraction of PM and consist of those with a diameter < 0.1 μm ($PM_{0.1}$) (40). The elemental core of DEPM has a high capacity for adsorbing various and potentially hazardous compounds such as organic material from unburned fuel, engine, lubricating oil, and other molecules found in the environment (35). By mass, approximately ≥90% of DEPM is $PM_{2.5}$, and 1-20% is UFPM (35,40). Although the UFPM fraction contributes little to the overall mass of DEPM, by number, the majority of DEPM is ultrafine (35). The small size of DEPM enables a high percentage of the particles to pass through the upper respiratory tract and readily deposit deep into lungs; while PM_{10} typically lands in locations within the upper respiratory tract, $PM_{2.5}$ penetrates deeper and

approximately 83% of fine PM deposits in the lower respiratory tract, including the alveoli (38,45). Another factor contributing to its toxicity is that DEPM has a large surface area per mass, allowing for high contact with the adsorbed compounds on its surface (35). This enhanced surface area-to-mass ratio and associated elevated toxicity is of greatest concern in particular for the ultra-fine ($PM_{0.1}$) fraction of DE (44). Finally, it has been proposed that UFPM may translocated across the alveolar wall, pass into systemic circulation, and reach extra-pulmonary tissue where other toxicological health impacts may additionally occur (44).

2.1.2 Diesel Engine technologies

As diesel engines operates through the following general mechanism: fuel is injected into air which has been compressed to a high pressure and temperature, thereby igniting it and releasing the stored chemical energy. The resulting combustion gases power the engine's piston and, afterwards, are released into the atmosphere as waste exhaust (35). Emissions of diesel engines will vary significantly in terms of chemical composition and PM size depending on operating conditions and engine factors such as: (1) whether the engine is light-duty or heavy duty (i.e. powering an automotive truck, car or small or large industrial equipment), (2) "on-road" or "off-road" (3) the engine age, (4) whether it is two- or four-stroke and (5) the fuel formulation being used (35).

Diesel engines are popular in Europe due to their better fuel economy and, in part, as a result of strong diesel-favouring tax incentives. Additionally, worldwide sales of diesel engines have risen significantly over the past decade also due to their higher fuel economy and durability (40). Since 2005, progressively stricter diesel truck and car emission standards – mostly concerning CO, hydrocarbons, NO_x PM and sulfur – have been implemented across North America, Europe and Japan (40,46). Significant advances in ultra-low-sulfur diesel fuel and engine technology – involving electronic controls, oxidation catalysts and diesel particulate filters – have been developed to meet the newest emission standards (40,47). Although the exhaust of the newest DE engines emit less NO_x and PM for the same workload, the technologies have caused

the average size of DEPM to be smaller and, therefore, the exhaust typically contains more PM by particle number (48).

2.1.3 Workplace and Environmental Exposure Levels

As diesel engines are more efficient and durable than their gasoline counterparts they are frequently used to power machinery in transportation, mining, construction, agriculture and various manufacturing industries (49). In Canada it is estimated that 879,000 Canadians are exposed to DE in their workplace, annually, with the most significant exposures occurring with truck drivers and heavy equipment operators. Occupations of greatest risk for on-road and off-road DE exposure include bus, truck, subway and professional drivers, bus garage workers, tool-booth and parking garage attendants, forklift operators, firefighters, lumberjacks, traffic controllers and car mechanics and workers in railroad, marine, mining and forestry industries (50).

Assessing exposure to DE is challenging due to its various components and complex nature; most regulation agencies have exposure limits for the specific gaseous constituents and little, if any, guidelines surrounding DEPM. For example, although WorkSafeBC has exposure limits for gases such as CO, NO_2 and SO_2, there are no regulations for DEPM, specifically. Respirable dust (PM with aerodynamic diameter ≤10µm) is limited to 3mg/m^3 for an 8-hour time weighted average (TWA), but this does not address DE-specific PM (51). The US Mining Safety and Health Administration is one of the few agencies to set a DEPM limit, which is 160µg/m^3 for an 8-hour TWA in underground mines (52). Further complicating this issue, there are a variety of methods that may be used in studies to assess exposure to DEPM. National Institute for Occupational Safety and Health (NIOSH) has published the NIOSH 5040 method which is used to determine the specific mass of organic carbon (OC) and EC in a given volume of air by a thermal optical analysis technique (53). Some studies quantify EC to assess DEPM, whereas others simply examine PM_{10} or $PM_{2.5}$, which may be derived from diesel engines, other combustion sources, or

non-combustion-derived dusts. Regardless, these exposure methods have been used to estimate exposures to DEPM in various environments and workplaces.

In 2009, Pronk *et al* performed a literature review examining estimated DEPM exposures across several occupational settings. The geometric mean (GM) of EC exposures ranged from 0.9 – 19 μg/m^3 across track and bus drivers working in America, Estonia and Sweden. Respirable PM exposures were higher than the EC exposures, with GMs ranging from 20 μg/m^3 to 580 μg/m^3. For vehicle mechanics, GMs for EC exposure ranged from below the limit of detection (<LOD) to 38 μg/m^3 and from 118 to 1020 μg/m^3 for respirable PM. The aerometric means (AM) of firefighter exposures in studies across America and United Kingdom ranged from <LOD to 40 μg/m^3. Workers in the train industry had GM of exposure to EC ranging from 4 to 16 μg/m^3 and construction workers from 8 to 163 μg/m^3 **(54)**. It has been estimated in the America that workplace DEPM exposures to railroad workers range from 39 to 191 μg/m^3, 7 to 98 μg/m^3 for public transit workers and 5 to 65 μg/m^3 for mechanics and dock workers **(35)**. Importantly, several occupational studies have noted that underground miners have high potential for DEPM exposure: one group has observed levels up to 1,280 μg/m^3 being noted **(35)** and Pronk *et al* found that the GM of EC exposures in studies of underground miners in the US, United Kingdom, Sweden and Estonia ranged from 66 to 202 μg/m^3 and the AMs from 66 to 637 μg/m^3 **(54)**.

The US Environmental Protection Agency (USEPA) has regulatory standards for environmental levels of $PM_{2.5}$ as follows: 24-hour limit: 35 μg/m^3 and an annual mean limit: 12 μg/m^3 and the 24-hour limit for PM_{10} is 150 μg/m^3 **(55)**. The current WHO guidelines are comparable for $PM_{2.5}$: 25 μg/m^3 and 10 μg/m^3 for the 24-hour average limit and annual mean, respectively **(56)**. Despite the regulations, it is not uncommon for levels of $PM_{2.5}$ to greatly exceed these guidelines in environmental settings. For example, in American cities, average $PM_{2.5}$ levels typically range from 5 to 50 μg/m^3 over a 24-hour average period and peaks can be up to 100 μg/m^3 **(23)**. Due to its larger mass, exposure to respiratory PM (PM_{10}) will be greater by mass than $PM_{2.5}$ exposures, and environmental PM_{10} levels in the United States range from 10

to 100 μg/m^3 over a 24-hour average period, with peaks as high as 300 μg/m^3 (23). Air pollutant levels are generally comparable between American and Canada cities of similar sizes (42); however, exposure to air pollution and PM can be strikingly higher in the developing world and East Asia. It is estimated that 99% of the population in Southern and Eastern Asia live in an area where the WHO Annual Air Quality Guideline for $PM_{2.5}$ is exceeded (6). In 2005, the annual concentration of $PM_{2.5}$ in Beijing was a staggering 58 μg/m^3 (6) and the issue has continued to worsen. As an example starting in January 2013, staggeringly extreme air pollution events occurred throughout Northern regions in China (57). During January 2013 hourly maximums of $PM_{2.5}$ in Beijing and the city of Shijiazhuang were 1000 ug/m^3 with highest daily means being recorded of 351 μg/m^3 and 502 μg/m^3, respectively (57,58). In one particular region of Beijing the daily mean throughout January 2013 was an alarming 211.13 μg/m^3. Some rural areas of Northern China similarly experience air pollution events, and elevations in $PM_{2.5}$ was statistically associated with increased rates of mortality in these regions (57). These, and other alarming air pollution episodes and prolonged periods of poor air quality in South East Asia has gained increasing media attention and public outcry over the past years.

2.2 Known Health Effects of Traffic Related Air Pollution Exposure

Many toxicological and epidemiological studies have considered the potential health outcomes associated with air pollution exposures in the ambient environment and workplaces. While the overarching principles of environmental and occupational studies are similar, there remain some intrinsic differences between these types exposures. Compared to the ambient environment, diesel exhaust exposures in workplaces can be relatively predictable. For example, if a worker is known to operate a diesel powered engine, one would anticipate that DEPM would make up a significant portion of their $PM_{2.5}$ exposure. In contrast, environmental exposure to DE will generally occur in combination with a wide variety of other combustion-related pollutants. In 1988 it was estimated that DEPM made up 23% of anthropogenic combustion-related $PM_{2.5}$, and only 6% when naturally occurring environmental sources were also considered, although the contribution from DEPM is likely higher in urban settings (35). A more recent publication

estimated that motor vehicles contribute about 2.1% of total worldwide $PM_{2.5}$ (40). In controlled human exposures volunteers can be exposed to a known form of pollutant and PM source (for example, DE), however, the issue of the general public and workers being exposed to a mixture of air pollutants is a consideration in both occupational and environmental exposure assessments. For example, a truck driver who operates a diesel engine, or an individual who lives on a major roadway, may be exposed to DE in addition to other air pollutants. Furthermore, exposure levels may vary depending on meteorological conditions and other factors. Research in this area generally uses exposures to specific combustion-related by-products, distance from a major roadway or an occupational task-based or geographical matrix as surrogate estimates of TRAP exposures.

Epidemiological research has found that long-term exposure to TRAP, and in particular PM, is associated with premature death due to cardiovascular and respiratory health outcomes such as ischemic heart disease, dysrhythmias and cardiac arrest (45). Similarly, there is an association between short-term increases in PM – as determined by local monitoring networks – and acute day-to-day mortality counts due to cardiovascular or respiratory events (59). Exposure to TRAP is also attributed to adverse health outcomes that can impact quality of life including increased prevalence of asthma, allergic respiratory disease and slowed lung development in children, reduced lung function in adults and children, increased lower respiratory infections and exacerbation of asthma, and chronic obstructive pulmonary disease (40,60-62). Research in human and animal models have provided biological plausibility behind this relationship; ambient PM has been shown to induce pulmonary and systemic inflammation, leading to downstream oxidative stress, endothelial dysfunction and leukocyte and platelet activation. Particularly in susceptible individuals, this toxicological pathway gives plausibility to the observed cardiovascular outcomes (40).

2.3 Effect of Air Pollution on the Brain

2.3.1 Epidemiological and Human Environmental Studies

Although the health effects of air pollution – such as TRAP, PM, and other form of combustion-derived pollutants – are well established with regard to cardiovascular and respiratory endpoints, epidemiological researchers recently began examining the association of such exposures with adverse cognitive outcomes. This relationship has been seen in both children (suggesting air pollution can impact cognitive development) and the elderly (indicating air pollution can accelerate cognitive decline). Given the growing and aging nature of the worldwide population, the neurotoxicological potential of air pollution is of obvious concern to public health.

In neurologically developed adults and the elderly, residential exposure to various forms of air pollutants has been to be associated with accelerate cognitive decline, even after potential confounding variables were taken into account. Several studies of these have noted relationships with surrogate TRAP or PM exposures, specifically. In 2011, a study involving 680 men aged 71 (±7) in the Boston area found that a doubling of environmental black carbon (BC) was significantly associated with lower Mini-Mental State Examination (MMSE) score (9). Another Boston-based study, examining adults 65 years old and older (average age 78yrs (±5)) found that proximity of residence to a major roadway was significantly associated with risk of having a low MMSE in individuals with college education and in those less than 77 years old. Exposure to BC was also associated with low MMSE score, but not statistically significantly so (10). An American cohort of women aged 70 to 81 years olds showed an association between $PM_{2.5}$ and PM_{10} exposure and faster cognitive decline over a two-year period (11). In 2014, Ailshire and Clarke considered a population of 780 individuals over the age of 55 and found those with a high residential exposure to $PM_{2.5}$ had significantly greater rate of errors in cognitive function testing. The relationship was most notable in adults 85 years and older (12). Tonne *et al* issued cognitive tests of reasoning, memory and phonemic and semantic fluency to a group of people

approximately 61 years old, on two occasions, separated by a five-year interval. They found that $PM_{2.5}$ was associated with faster cognitive decline between these two tests (13). Not all studies have observed a positive association, however. For example, Loop and colleagues examined exposure to $PM_{2.5}$ in a group of Americans with an average age of 64 years. $PM_{2.5}$ was not associated with increased odds of cognitive impairment, when factors such as temperature, season and incident stroke were accounted for (63).

Other researchers have found a significant relationship between adverse cognitive outcomes and exposure to air pollutants ; however, the finding was not robust when PM alone was considered. For example, American adults (37.4 ± 10.9 years) participated in a study that estimated ambient PM_{10} and ozone, at their home address, and various measures of CNS function (simple reaction time, coding ability, attention and short-term memory). PM_{10} exposure was initially associated with reduced brain function but not when socioeconomic status and other potential confounders were controlled for (64). However, there was a positive association between ozone exposure and lower age-standardized cognitive decline (64). In 2009, a cross sectional study examining German women aged 68-79 years considered PM_{10} exposure and residential distance to a major roadway as measures of TRAP exposure. Distance from residential address to the next busy road was associated with higher rates of mild cognitive impairment; however, no relationship with PM_{10} was seen (65).

Aside from cognitive function, other epidemiological studies have found indication of a correlation been ambient TRAP exposure and altered brain structure. Brain volume was reported to be highest in women age 71-89 who were exposed to the lowest quartile range of $PM_{2.5}$ in an American study (66). A 2015 study also found that a 2 $\mu g/m^3$ increase in $PM_{2.5}$ was significantly associated with 0.32% lower total cerebral brain volume and significantly higher odds of covert brain infarcts (odds ratio (OR): 1.46) (67). Alzheimer's and Parkinson's disease-like neuropathology has also been reported in post-mortem analysis of people who resided in Mexico City – and therefore were exposed to high levels of air pollution during their life time – relative to age-matched control individuals who lived in a comparable, but less polluted, city

(25,68-70). For example, increased levels of Aβ42 and hyper-phosphorylated tau were seen in young adults (mean age 18.3 and 21.8 for exposed and controls, respectively) (70), and elevated levels of α-synuclein was observed in young adults, aged 25.1 ± 1.5 (25). Finally, $PM_{2.5}$ levels have recently been associated with increased rates of first-time hospitalizations for dementia, Alzheimer's disease and Parkinson's disease (71).

While the research above generally refers to the issue of cognitive decline or changes in cognitive function in fully functioning adults; evidence also suggests air pollution may be able to impact cognitive development. Although several studies have noted such a link, only a few examples are briefly listed here. A study conducted in Quanzhou, China, considered the cognitive function of children attending one of two primary schools. Ambient air pollution (NO_2) was associated with a significant decline in six of nine cognitive assessment tests performed, even after controlling for potential confounding factors (15). Similarly, researchers observed an association between residential BC and poor verbal, and nonverbal intelligence, and memory in ten year-old children (14). An association between autism and proximity of maternal residence from a major roadway during the third trimester has also been noted (72,73). Combined, these findings indicate that during critical periods, exposure to air pollution may impact neuro-development. Epidemiological evidence suggests that air pollution has potential to impact the CNS at various stages in life, with individuals being particularly susceptible during neurodevelopment and aging.

From this literature review it is clear that the cognitive outcomes shown to be associated with air pollution, TRAP and/or PM exposures are highly variable. Clinical effects, ranging from poor neurological development in children, to behavioural changes, depressive symptoms and faster cognitive decline in adults, to structural and pathological changes in the brain in post-mortem analysis have been noted in observational studies. It is reasonable to assume that the pathways involved in such outcomes are not necessarily identical. Similarly, the issue of confounding variables cannot be ruled out; while epidemiological studies are useful for observing long-term effects following real-world exposures, they are subject to potential

confounding which may be difficult to account for. For example, individuals of lower socio-economic status may be more likely to reside close to major roadways or sources of industrial pollutants, making them more vulnerable to the health effects of air pollution as they have a greater risk for more frequent and higher levels of exposures (23). Additionally, poor socio-demographic factors may be correlated with lower education and be associated with adverse cognitive endpoints; a classic confounding variable example. As another example, residing in close proximity to a major roadway also exposes inhabitants to traffic noise, which may also impact cognitive function. There is limited research exploring the area of the combined effect of traffic related noise and air pollution exposure on cognitive outcomes (74). The issue of causality makes it difficult to attribute the observed adverse cognitive outcomes to air pollution exposures, from epidemiological studies alone (65). However, regardless of the intrinsic limitations of epidemiological research, considerable and relatively consistent evidence suggests that TRAP and PM air pollution can impact the CNS.

2.3.2 Proposed Mechanisms

Although the exact mechanism by which air pollution may impact the CNS is not fully understood, the pathway is thought to involve a maladaptive activation of the innate immune system. It is first important to briefly discuss some aspects of the structure and cells present within the brain. The capillaries of the CNS are surrounded by the BBB, an "impermeable" structure that covers most regions of the brain and spinal cord and is responsible for controlling blood-CNS exchange. When intact, this barrier tightly regulates the influx and efflux of various molecules to maintain brain homeostasis and prevent toxins and pathogens from reaching the organ (5,28,75,76). The endothelial cells of the BBB differ from those in the rest of the body in that they lack fenestrations and have more extensive tight junctions and less pinocytic vesicular transport (18,75,77,78). Activation or damage of the cellular components of the BBB can lead to BBB dysfunction and increased permeability, which is associated with some neurological diseases (73,79).

Sufficient inhalation of PM can induce a low grade inflammatory response in the lungs as a result of interactions between inhaled particles, alveolar macrophages, and airway epithelial cells. This can cause a release of pro-inflammatory mediators such as TNFα and IL-6 into systemic circulation. These cytokines can be transported across the BBB where they may directly induce apoptosis in neurons and/or activate microglia cells and trigger inflammatory cascades (16,25,75,78,80). Alternatively, some of the inflammatory molecules released following exposure to air pollution (such as TNFα and IL-6) may be able to impair the BBB through release of endothelial NO and trigger the signalling of inflammatory cascades in the brain through this pathway (25,78).

Alternatively, it is possible that inhaled nanoparticles can penetrate cellular membranes, transverse through the lung, and be carried through systemic circulation to extra-pulmonary organs, including the brain (16,19,20,81,82). Such particles may be able to directly affect the integrity of the BBB, and for example, nanoparticles injected into rat arteries were able to disrupt and cross the BBB (83,84); however, this was only noted in cases of high concentrations of charged NPs and, as the particles were injected, doesn't necessarily compare to an environmental exposure where PM is inhaled chronically at a lower dose (83). Other studies involving similar models have failed to consistently demonstrate that UFPs can cross the BBB (19). It is notable, however, that in an *in vitro* model, isolated rat brain capillaries were exposed to DEPM. The particles dose-dependently increased production of ROS, leading to a decrease in expression of tight junction proteins and altered expression of transporter proteins (76). Although it is challenging to compare *in vitro* models to the biology of an intact, living organism, these findings show that if DEPM does reach the brain in sufficient quantities, the particles may be capable of altering BBB function. PM is also known to absorb compounds from ambient air such as PAHs, metals and lipopolysaccharides, which themselves may be able to alter the BBB (18). Furthermore, if the BBB were to be disrupted by circulating cytokines released through air pollution exposures, it also seems more feasible that UFP may be able to enter the CNS. In fact, PM – although from an unknown source – was identified in the brains of individuals who lived in Mexico City during their life-time (25). Furthermore, post-mortem analysis of the brains of

humans exposed to air pollution showed cerebral vasculature endothelial damage, indicating alteration of BBB function (85). BBB dysfunction is associated with many CNS diseases, including dementia, epilepsy, trauma and multiple sclerosis. While it is difficult to determine if BBB disturbance is the pathogenic factor itself, or as a result of the disease, such dysfunction could contribute to CNS pathology (28,78).

Alternatively, some have proposed that airborne pollutants may gain access to the CNS through a nose-to-brain mechanism (19,20,86). The olfactory bulb is a collection of specialized neurons that project to the olfactory epithelium lining the nasal cavity. These neurons connect to the olfactory nerve which passes through the skull. During inhalation, it is possible that airborne toxicants, including UFPM, may deposit on the olfactory epithelium and enter the brain through translocation across these neurons, thereby bypassing the protective BBB (19,86).

Indeed, various ultrafine metals undergo olfactory transport when intranasally instilled onto the olfactory epithelium (20). More relevant to an ambient inhalation exposure; olfactory transport of UF manganese (Mn) particles was demonstrated in rats exposed to such particles with either both nostrils open or the right nostril occluded. Various inflammatory markers were elevated in the olfactory bulb as was glial fibrillary acidic protein (GFAP), a marker of astrocyte activity, and this was only observed in the left olfactory bulb in rats with the right nostril blocked (87). Another study observed a similar "asymmetrical" delivery of Mn in rats, with either both nostrils patent or one occluded, exposed to radioactively labeled manganese phosphate aerosol (20). It is possible translocation across olfactory nerves is influenced by the type chemical species, and importantly, olfactory transport of carbon nanoparticles – which are typically poorly-soluble, and more representative of TRAP derived PM – was demonstrated by Oberdorster and colleagues, who exposed rats to airborne radioactively labeled carbon (^{13}C) and found that ^{13}C particle accumulation in the olfactory bulb (19). This olfactory entry pathway is additionally interesting in that olfactory dysfunction and loss of smell commonly occurs in the early stages of both Alzheimer's and Parkinson's disease (5).

It can be difficult to directly compare findings from animal models to humans, due in part to interspecies and physiological differences. For example, rodents may be more susceptible to olfactory transport as they are obligate nasal breathers (19). Additionally, the relative surface area of the nasal olfactory mucosa is much smaller in humans (5%) than it is in rodents (50%) (20). That said, there is support of such a pathway in environmental research: post-mortem analysis showed that children and adults who resided in Mexico City, and died of non-CNS related causes, had up-regulation of various inflammatory in the olfactory bulbs relative to control individuals who resided in less polluted environments (25,79). This provides support to the olfactory entry hypothesis in a model specific to humans.

The pathways discussed are by no means mutually exclusive, and it is possible that a combination of them occurs. Regardless of whether particles themselves enter the CNS (through systemic circulation or olfactory transport) or systemic inflammation is transferred to the CNS, it is thought that various components of TRAP and combustion-derived air pollution are capable of eliciting a CNS immune response. Microglia, the innate immune cells of the brain, release pro-inflammatory cytokines and reactive oxygen species (ROS) when they encounter a foreign stimulus (5,88). While this immune pathway is critical to protect the CNS against pathogens, an excessive inflammatory response can be harmful to CNS cells and is thought to be involved in the pathology of various neurodegenerative diseases (5,18). It is possible that the peripheral immune response to air pollution is transferred to the CNS, activating inflammatory cascades and microglia, and that microglial cells respond to UFPM that potentially reaches the CNS (18). In an *in vitro* model, DEPM was shown to activate microglial cells, inducing the release of reactive oxygen species (ROS), microglial NADPH, and the death of dopaminergic neurons in a dose-dependent fashion (89). Similarly, Sama *et al* exposed a mice microglia cell line to concentrated ambient $PM_{2.5}$ collected in New York City and noted a stimulation and upregulation of various inflammatory pathway genes associated with inflammatory pathways and the release of TNFα and IL-6 (88).

The cytokines and ROS released by microglia are critical to immune function as they recruit macrophages and further microglial cells to sites of infection and injury. To prevent unnecessary cellular damage, these mediators have a short half-life, but if chronically activated, may eventually induce toxicity (77). While microglial are critical in protecting the brain, excessive and prolonged activation, termed "microgliosis", can be detrimental to the organ (5,90). In microgliosis, cytokines and ROS are chronically released from microglial cells and issue direct insult to neurons. This progressive loss of CNS cells leads to the accumulation of extracellular debris, cytotoxic substances, inflammatory factors, and ROS in the brain; a self-perpetuating neurotoxicity which further contributes to the activation and recruitment of microglia (90,91). Regardless of the exact pathogenic mechanism, the overarching hypothesis is that chronic PM exposure is associated with low grade CNS inflammatory activation, which may become clinically relevant with repeated exposures or during vulnerable periods of brain development (16,18,25).

2.3.3 Research in *in vitro* Animal Models

The ability for air pollution, specifically PM inhalation, to illicit CNS inflammation and adverse effects has been demonstrated in controlled studies using *in vivo* rodent models. Mice were exposed to concentrated airborne $PM_{2.5}$ and UFPM collected from a Los Angeles freeway for five days a week, for two weeks, at intervals of four hours a day. Upon sacrifice, the brains of the exposed mice had elevated levels of the cytokines IL-1α, TNFα and the transcription factor NF-κB compared to control mice (77). Rats exposed to DE at a level of 173 $\mu g/m^3$ total PM mass for six hours a day, five days a week, for four weeks had significantly elevated levels of the cytokines TNFα and IL-1α. Additionally, the rats showed a non-significant increase in the expression of mRNA for TNF-α and TNF-receptor subtype I, and activation of the transcription factors NF-κB and AP-1 in some brain regions (92).

Similarly, in 2011 two studies were published by the same researchers, in which rats were sub-chronically exposed to DE. In one of these experiments rats that were exposed to DE, at one of two doses (2.0 or 0.5 mg/m^3 $PM_{2.5}$), for four weeks showed dose-dependent elevations in

interleukin (IL)-6 and nitrated protein, in whole brain, and elevated levels of the cytokines TNF-α, IL-1β, and IL-6 in specific brain regions. It was observed that the midbrain was most substantially affected and, interestingly, this region also has the highest number of microglial cells; providing support to the theory that microgliosis is a critical aspect of the pathway by which air pollution impacts the CNS (93). In the second study, rats were exposed to DE at various concentrations for six months. Similar finding were observed; inflammatory proteins were dose-dependently elevated in the brain, with the midbrain being the most sensitive, and the cerebellum – which has fewer microglia – being relatively unaffected. Furthermore, the mice exposed to the highest dose (992 μg/m^3 PM) showed elevated levels of Aβ42 in the frontal lobe and α synuclein in the midbrain, and mice exposed to the two highest doses (992 and 311 μg/m^3 PM) had elevated levels of phosphorylated tau (pS199) in the temporal and frontal lobe. These findings all suggest pathological outcomes to the brain as a result of DE exposure; Aβ42 deposition and hyperphosphorlyation of Tau (and Tau [pS199]) in the brain has been associated with diseases such as Alzheimer's disease and frontotemporal dementia; and high levels of α synuclein has been seen in Parkinson's disease (94). This combined research involving controlled animal exposures suggests that chronic CNS inflammation can be induced by exposure to air pollution. Interestingly, microglia over-activation is seen in autistic children and is considered central to the pathology of neurodegenerative diseases, such as AD and PD (5). Examining an acute exposure, Van Berlo *et al* found that mice exposed to concentrated DE for two hours via nose-only inhalation showed significantly elevated levels of heme oxygenase-1 (HO-1) and cyclooxygenase-2 (COX-2) in the cortex and cerebellum. The authors suspected an olfactory pathway was critical to the observed CNS inflammation, as analysis of lung tissue did not reveal significant elevation in inflammatory cytokines whereas, HO-1 was elevated in the olfactory bulb. It is however noteworthy that these authors used a very high exposure of 1900 μg/m^3 DEPM (95).

In addition to examining brain pathology, some groups have also considered changes in mouse cognitive function prior to sacrifice. Female mice exposed to a high dose of nano-particle rich DE (average PM diameter = 0.025 μm) had impaired object recognition, changes in the expression of genes related to glutamate transmission, and a tendency towards microglia

activation in the hippocampus (96). Additionally, mice exposed to $PM_{2.5}$ at a level of 94.38 μg/m^3 for six hours per day, and five days a week over nine months showed impaired spatial learning relative to control mice. Increased pro-inflammatory cytokine expression and altered neuronal morphology in the hippocampus – characterized by reduced apical spine density, decreased apical dendritic length, and reduced cell complexity – was also noted (97). In another study, mice exposed to ambient PM collected from a Los Angeles city freeway showed a decrease in glutamate receptor sub-unit GluA1 protein in the hippocampus, a region of the brain responsible for memory and learning. Glial cells in the brains of the exposed mice were also activated (98). Finally, Win Shwe *et al* exposed mice to nanoparticle-rich DE for four weeks at a rate of five hours a day, five times a week. Some animals were additionally exposed to DE in parallel with the *Staphylococcus aureus* cell wall component lipoteichoic acid (LTA) to further stimulate an immune response. They noted that the mice exposed to both compounds had significantly slower Morris Water Maze task times, indicating poor spatial learning. These mice similarly had higher expression of IL-1β and TNFα mRNA in the hippocampus (96). In addition to the previously presented epidemiological evidence, there is considerable research in studies involving rats and mice exposed to high levels of PM that suggested that air pollution exposures can induce inflammation in the CNS and impact cognitive function.

2.4 Inflammatory Cytokines

2.4.1 Inflammatory Cytokines and Air Pollution

Sufficient inhalation of PM can induce a low grade inflammatory response in the lungs as a result of interactions between inhaled particles, alveolar macrophages, and airway epithelial cells (99,100). Indeed, *in vitro* research has shown that alveolar macrophages incubated with ambient urban PM release cytokines such as TNF-α and IL-6 (99). The induction of pulmonary inflammation can potentially lead to the downstream release of pro-inflammatory mediators into systemic circulation (23,77,99). Indeed, exposure to PM is known induce pulmonary inflammation, and some studies have measured a systemic inflammatory response following

exposure to combustion related air pollutants (23,77). A study of six European cities found a positive correlation between systemic IL-6 and acute changes in regional levels of total particle number concentration (PNC) (101). There has also been an observed correlation noted between various inflammatory cytokines and elevated environmental levels of O_3, NO_2, SO_2 and $PM_{2.5}$ (102) and PM_{10} (103). Similarly, researchers observed elevated markers of inflammation (including the cytokines IL-6 and TNF-α) in humans exposed to PM_{10} from regional forest fires (99), firefighters within four hours of a fire-fighting shift (104), workers following an eight-hour shift in tunnel construction (105), and in volunteer research subjects exposed to controlled levels of DE (106).

2.4.2 Inflammatory Cytokines in the Brain

Neurons and glial cells within the CNS produce and respond to inflammatory cytokines which are naturally present in low levels in the healthy brain and immune cells and inflammatory molecules produced by CNS cells play a role in promoting plasticity-related structural changes, and participate in the modulating of the brain (107). CNS-released TNF-α and IL-6 are required for many normal functions of the organ, including learning and memory consolidation – particularly with regard to processes involving the hippocampus – but when excess inflammation occurs such cytokines may induce behavior changes and adversely affect learning, memory, and cognitive function (107). There is growing evidence to suggest that inflammation of the brain is involved in the development of neurological decline and diseases (108). Some of the many processes to occur during aging involve various modifications of the immune system, including a general increase in proinflammatory cytokines and chronic low grade inflammatory state (109). Such changes can be more profound in patients suffering from neurodegenerative disorders; although the role is not entirely clear, inflammation appears to contribute to the pathological features and symptoms of diseases such as Alzheimer's (108,109).

Although it has been suggested that air pollution exposures may directly, or indirectly, induce an immune or inflammatory response in the CNS, brain tissue cannot be examined for levels of inflammatory cytokines in an acute, human inhalation study, as has been seen in research involving animal models. Alternatively, systemic inflammation can be measured to provide indications of an intermediate step and provide biological plausibility behind the effects of air pollution on the brain. Although less specific to the CNS, this approach is relatively non-invasive and readily feasible. While it was historically believed that there was a distinct separation between the brain and peripheral immune systems, it has become apparent that there are interactions and communications between circulating cytokines and the CNS (108,110,111).

In some cases, peripheral cytokines are believed to directly influence the CNS; in response to peripheral cytokines, endothelial cells of the BBB are known to produce inflammatory molecules (109). A well-known example of interaction between the peripheral immune system and the brain is seen in "sickness behavior", where peripheral cytokines induce CNS disturbances during illness (109). Administration of systemic inflammation in animal and human models has been shown to acutely impact mood states, learning and cognitive function. For example, it is thought that pro-inflammatory responses released by the peripheral immune system in response to illness may directly or indirectly be responsible for 'sickness behavior' – a condition used to describe the behavioral changes associated with illness which include malaise, lethargy, inactivity and impaired cognition. Although the specific effect of each cytokine on brain function is not completely understood on a molecular level it does appear that communication between the peripheral immune system and the CNS occurs (112,113).

Additionally, in the case of chronic health outcomes, there is increasing evidence suggests that even moderate systemic inflammation can impact neurological function and may serve as a biomarker for CNS pathology (114), the association between elevated CNS inflammation and a detectable peripheral inflammatory response remains controversial (115).

Neurocognitive diseases such as Alzheimer's disease are associated with CNS inflammation and patients with the disease are known to have elevated brain levels of pro-inflammatory cytokines and CRP within the brain. Furthermore, it is conceptually believed that peripheral cytokines can influence the onset of cognitive decline and AD, however, the association with a peripheral inflammatory response is less consistent (109). Higher levels of inflammatory cytokines TNF-α and IL-6 in systemic circulation has been associated with faster cognitive decline and elevated rates of dementia in some epidemiological studies (116). Similarly, an inverse correlation between plasma IL-6 and hippocampal volume has been noted in middle-aged adults (109). Another group noted an association between elevated levels of cytokines, including TNF-α, and rates of cognitive decline in mild to severe Alzheimer's disease patients over 6 months (109). However, the evidence between AD and systemic inflammation is otherwise limited and controversial and other researchers examining a similar relationship found no such associations (108,109). Given the occurrence of TNF-α and IL-6 in both air pollution literature and studies of cognitive function, it seems reasonable to examine both cytokines in peripheral blood following exposure to DE.

2.5 S100B as a Central Nervous System Biomarker

A biomarker is a characteristic protein that can be measured and evaluated as an indicator of normal biological function or of a pathogenic process (117). When selecting a brain-specific marker which can be detectable in systemic circulation, the presence of the BBB creates certain challenges as this barrier tightly regulates the influx and efflux of molecules between the brain and blood. That said, there are some proteins which are detectable in the blood of healthy adults and are considered CNS biomarkers. One of these is S100B, which is part of a family of calcium binding proteins and is expressed by astrocytes (118,119). In low concentrations, S100B acts as a growth and/or differentiation factor for neurons and glia cells and regulates dopaminergic and glutamatergic synaptic functions (119,120). However, at higher concentrations the protein induces the expression of nitric oxide synthase and other pro-inflammatory cytokines, leading to neuronal dysfunction and apoptosis (120,121). S100B is normally present in

the cerebral spinal fluid (CSF) and, in healthy individuals, can also be found in peripheral circulation at about 1/3 the concentration found in the CSF (30,122). The brain specificity of the protein has been questioned as white and brown fat, skin, skeletal muscle and malignant melanoma cells have also been shown to contribute to circulating S100B (123-126). However, it has been demonstrated that these extracranial sources do not substantially alter S100B serum levels, unless a traumatic bodily injury has occurred (123,125,126).

If found in peripheral blood at sufficient concentrations, S100B is considered a marker of CNS damage and BBB disruption (28,30,117,120,122,127). Indeed, elevated serum S100B is seen in a variety of neuropathological conditions including traumatic brain injury (TBI), psychiatric disorders, severe CNS events such as stroke or brain ischemia, neurodegenerative disease, and post-surgery delirium (118-122,128-130). Following TBI, serum S100B levels correlate with the size of the area of the infarction and can predict the chance of patient survival and good neurological outcome (117,123,125). The is also biomarker is considered an indicator of susceptibility for schizophrenia and elevated risk of suicide in adolescents with mood disorders (120). S100B has been reported to increase in healthy individuals hiking at high altitude and, therefore, exposed to mild hypoxia (131).

Although there is significant evidence to suggest that S100B is a marker of CNS distress, the exact mechanism that causes levels to increase in the serum following CNS insult is not fully understood. Some have hypothesized that an increase in serum S100B is due to an elevated expression of the protein in astrocytes (132). It is important to be aware of the issue of causation; S100B may be increased as a therapeutic, healing response to brain damage rather than through a pathological process (132). Alternatively, some scientists believe that S100B can additionally serve as a marker of damage to the BBB in that, if the BBB were to be disrupted, S100B protein may flow from the CSF into peripheral circulation. Kapural and colleagues examined the expression of S100B and NSE – a marker of neurological damage – in patients undergoing chemotherapy, following medically-induced BBB disruption by use of the drug

mannitol. Serum S100B increased significantly with mannitol infusion; whereas NSE levels remained unchanged, suggesting that S100B will increase in circulation in the absence of neurological damage, if BBB permeability increases (30). Other researchers noted an association between serum S100B, peripheral mediators of oxidative stress, integrity of the BBB, and severity of neurological complications in children with bacterial meningitis (126). Failure of the BBB is known to contribute to the pathology of many neurological disorders, and this may explain why peripheral S100B is elevated in some chronic CNS diseases and acute CNS events. As previously discussed, it has been hypothesized that exposure to PM may affect BBB function. Of additional interest, S100B release into serum has been correlated with elevated serum levels of the inflammatory cytokines IL-6 and interleukin-8 (127). It is noteworthy that chronic exposure to air pollution has been linked to CNS diseases that are also associated with elevated serum S100B (5). Since S100B appears to be CNS-specific and increases in serum immediately following CNS insult, it is an appealing endpoint for effects of acute inhalation of DE on the brain.

2.6 NSE as a Central Nervous System Biomarker

NSE is a glycolytic enzyme located in the cytoplasm of neurons and neuroendocrine cells (31,133). The protein can be measured in systemic circulation at some level, but because NSE is primarily located in neuron cells and not readily secreted, an increase in blood levels is believed to be an indicator neuronal death (29,31,127). NSE is also found in small concentrations in erythrocytes and platelets and, therefore, levels of the molecule may appear elevated in hemolysed serum samples (32,128). Assuming no hemolysis has occurred, an increase in serum or CSF NSE is thought to indicate neuronal damage and is observed in cases of significant brain trauma such as stroke (134), traumatic head injury, hypoxic brain damage (32) or brain ischemia due to acute cardiac arrest (31,121). This suggests that brain ischemia and neuron death can cause NSE to be released into systemic circulation. Additionally, serum NSE may be related to neuroinflammation, as serum levels were shown to be correlated with IL-6 in patients with severe traumatic brain injury (32).

2.7 BDNF as a Neuro-Protective Factor

BDNF is a neurotrophic factor which signals neurons to grow and differentiate, encourages their survival and is considered a neural analogue for long-term memory formation, retention, and recall memory (33,34,135,136). BDNF may be useful in the treatment and prevention of various neurodegenerative diseases (137). Serum BDNF levels are lower in patients with CNS diseases including depression and panic disorder (33). Additionally, attenuated expression of BDNF mRNA in the hippocampus is considered a pathogenic factor in Alzheimer's disease and depression (137). The molecule is detectable in circulating blood and, as BDNF can cross the BBB freely, peripheral levels are thought to reflect CNS concentration (33). Notably, BDNF is released following strenuous exercise in many animals, including humans, and the protein has been attributed to the beneficial effects of exercise on long term CNS function (33,34,138). Indeed, forced exercise has been shown to increase BDNF protein mRNA in the hippocampus of rats (138-140).

Despite its name "brain-derived" neurotrophic factor, BDNF is not solely produced by cells of the CNS; vascular endothelial cells, peripheral blood mononuclear cells, eosinophils, vascular smooth muscle cells, pituitary gland, and salivary gland cells all produce BNDF (33,137). The origin of exercised-induced elevations in circulating BDNF is of particular debate, and findings from animal, human and *in vitro* studies have disagreed as to whether this increase is as a result of CNS-derived BDNF or from contracting muscles (33). However, most recent research supports the hypothesis that exercise-induced BDNF is in fact derived from the brain in humans (141). Furthermore, the exact cellular-source of exercise-induced BDNF is arguably irrelevant as the molecule can easily cross the BBB and therefore, peripheral concentration of circulating BDNF are thought to be representative of brain levels (33,141-144).

BDNF has been considered in air pollution research and, in a crossover study, Bos and colleagues examined serum BDNF following cycling in two environmental conditions: (1) on a

polluted roadway, where subjects were exposed to TRAP or (2) in a clean room with minimal PM and air pollutants. Cycling in the presence of air pollution attenuated exercise-induced BDNF release and the authors hypothesized that air pollution-induced systemic inflammation may have been responsible (145). Interestingly, systemic infection and inflammation has been associated with decreased capacity of hippocampal transcription of BDNF (136). A study by Wu and colleagues found that one week following peripheral LPS injection, BDNF expression was decreased in the substantia nigra of sedentary mice, relative to those injected with saline (34). Similarly, *e. coli*-challenged rats had a reduction of BDNF in the hippocampus (144). Indeed, CNS oxidative stress has been linked to a decrease in BDNF expression (34), as has inflammation (136). The potential for TRAP inhalation to lower BDNF expression could hypothetically produce downstream neurological consequences given BDNF's neuroprotective functions.

2.8 Study Rationale

It has been well established that exposure to air pollution can result in harmful effects to the cardiovascular and respiratory systems, particularly in those with already compromised health. As a result, air pollution is a substantial contributor to human morbidity and mortality worldwide (5). In addition to these well known health consequences, emerging evidence suggests combustion-derived air pollutants may also impact the brain. Neurocognitive diseases place significant burden on patients, their families, and public health systems, but the role of environmental risk factors in relation to disease development is poorly understood. Dementia is a chronic and progressive syndrome in which cognitive functions, including memory, executive function, language, and praxis decline and interfere with everyday functions (27,146,147). It is most prevalent in later life, with a doubling in prevalence every five years after 65 years of age (147). In 2015 it was estimated that 46.8 million people globally were living with dementia (26). With aging demographics globally, expert predict that these numbers will increase dramatically in the next two to three decades (27,146,147).

Research to identify unappreciated modifiable risk factors, such as air pollution, for neurodegenerative disease is an important public health priority (27,147). To provide critical plausibility for observational and animal-based studies, more research – including human exposure studies – is warranted. Accordingly, we studied human volunteers exposed to either DE or FA, in a controlled setting and used circulating blood as a quick, and minimally-invasive alternative to directly sampling the CNS. Generalized neurological inflammation has been hypothesized to be central to the potential neurotoxicological effects of PM exposure (18,90,91), given this, the cytokines IL-6 and TNFα were measured as a proxy for such inflammation. Additionally, S100B, NSE and BDNF were used as biomarkers which, if found to increase, or decrease, in sufficient, relative quantities following acute DE exposure, would provide indication of the biological mechanism regarding the effect of air pollution on the CNS. The blood biomarker panel therefore considers the following:

(1) IL-6 and TNF-α, markers of systemic inflammation, to provide support for the "inflammatory hypothesis" following DE exposure.

(2) S100B, a biomarker of CNS distressed and proposed marker of BBB disruption (28,30,117).

(3) NSE, a serum biomarker for neuronal death and CNS pathology.

(4) BDNF, a neuro-protective factor which has been found to be adversely effected by air pollution exposure in exercising individuals (135,138). Hypothetically, alterations in blood levels of BDNF following DE exposure may provide biological plausibility behind the adverse neurological outcomes associated with air pollution.

Exposures to TRAP, combustion-derived PM, and other forms of air pollution are common in an environmental settings and in workplaces worldwide. It is already known that

such exposures place significant burden on human health through cardiovascular and respiratory outcomes. The potential for air pollution to additionally harm the brain is of great and obvious concern. By understanding the environmental risk factors which contribute to various CNS diseases we can target interventions to minimize their occurrence and possibly delay the development of progressive neurological diseases, such as dementia, Alzheimer's and related illnesses. This research, represents a critical research incentive and exploratory study in which the potential for DE (as a surrogate model for TRAP exposure) to impact the human CNS is examined.

Chapter 3: Methods

3.1 Hypotheses

Hypothesis 1*:* the concentration of the inflammatory cytokines IL-6 and TNF-α, will increase from baseline following DE exposure, relative to FA.

Hypothesis 2: the concentration of S100B in peripheral blood will increase from baseline following DE exposure, relative to FA, with levels peaking at 3hr or 24hr post exposure.

Hypothesis 3: the concentration of NSE in peripheral blood will increase from baseline following DE exposure, relative to FA, with levels peaking at 3hr or 24hr post exposure.

Hypothesis 4: the concentration of BDNF in peripheral blood will decrease from baseline immediately or 3hr post DE exposure, relative to FA.

3.2 The Overarching Study: Effects of Air Pollution on Cognition

The work presented in this thesis is the sub-section of a larger research project termed the "Effects of Air Pollution on Cognition" (EAPOC) Study, and pertains to the analysis of CNS-specific blood markers before and after exposure to DE (note that the other cognitive endpoints examined represent the work of PhD candidate Jason Curran, and are discussed in more detail in Appendix I). The aim of the overall study was to examine the impact of DE exposure on various aspects of cognitive and CNS function. The controlled human exposures to DE were made possible through use of the Air Pollution Exposure Laboratory (APEL) **(148)** headed by principal investigator Dr. Christopher Carlsten.

This project is a blinded crossover study which considers two exposure conditions: DE and FA. A four-week washout period was placed between exposures, and the order of exposure conditions was randomized and counter-balanced. A critical aspect of the study design is that each subject serves as his/her own control, thereby eliminating bias due to confounding factors attributable to personal characteristics. Although the subtle changes in parameters indicative of cognitive function is critical to Jason Curran's project focus, it is difficult to discuss the methodology for this proposal without touching on some of his work. Figure 3.1 is a schematic which shows the overall EAPOC study outline starting with subject recruitment.

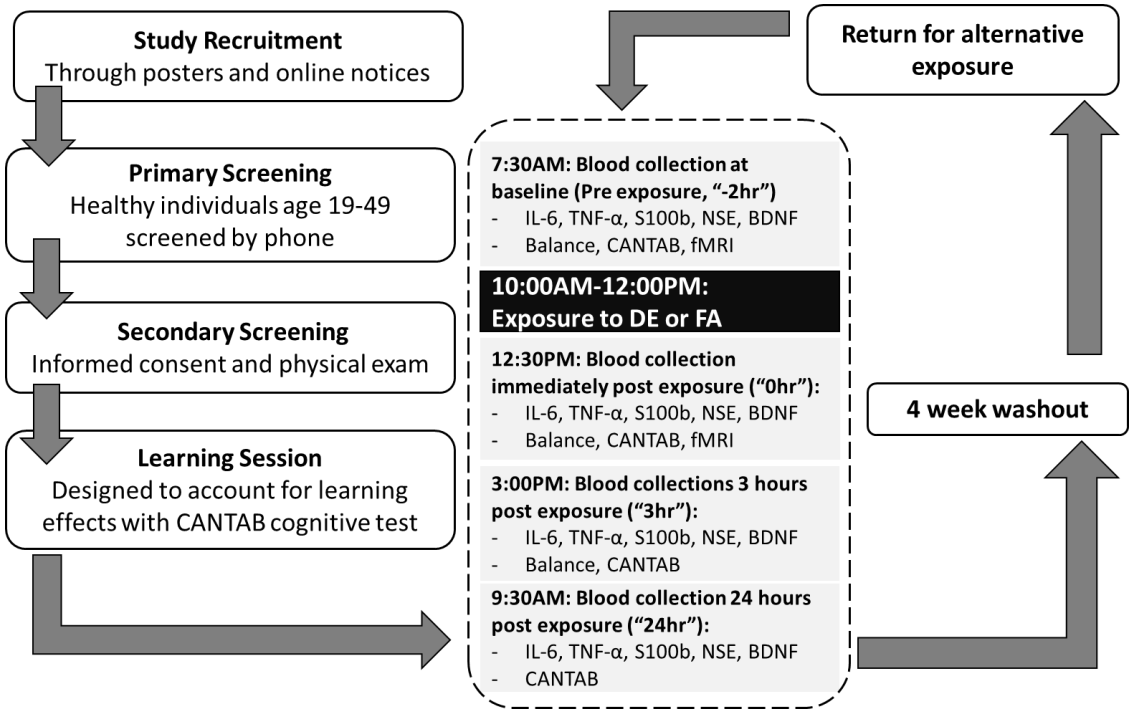

Figure 3.1: Schematic of the Overall Study Design. See Appendix I for more specific information on the CANTAB test, static balance assessment and fMRI.

3.3 Experiment Procedures

3.3.1 Subject Recruitment

Volunteer subjects were recruited through posters in the community, online notices, e-mail notifications to the Vancouver Costal Health Staff List-Serve and by contacting subjects who have previously been involved in research studies at APEL and requested to be re-contacted for future projects. The aim was to have a total of 30 subjects tested for the study.

Primary Screening: Subjects were assessed over the phone to determine suitability for the study given the following inclusion and exclusion criteria.

- Inclusion criteria: (1) between the ages of 19-49, (2) healthy, (3) non-smoker, (4) able to speak and read English proficiently. There was no gender or racial exclusion

- Exclusion criteria: (1) pregnancy or breastfeeding, (2) colour blindness, (3) claustrophobia, (4) presence of implanted metal that may interfere with the MRI, (5) presence of co-existing medical conditions or medications that may interfere with the study's protocol, (6) participation in another study that conflicts with this study's protocol.

Secondary Screening: Subjects were invited for a secondary screening during which a physical exam and the study protocol was explained in greater detail. Additionally, the subject underwent a training session for the CANTAB protocol. Lastly, the subjects were given the opportunity to ask any further questions. To conclude, subjects who agreed to participate in the study were required to give written and informed consent. The consent forms were approved by The University of British Columbia Clinical Research Ethics Board (#H12-03025), Vancouver Coastal Health Ethics Board (# V12-03025), and Health Canada's Research Ethics Board.

3.3.2 Exposures

Diesel exhaust at the APEL facility is provided by a Tier-3 compliant Yanmar YDG5500E generator with a max AC output of 4.6 kVA. The engine was run using commercially-available ultra-low sulfur diesel fuel (< 15 parts per million (ppm) sulfur) and, to mimic on-road conditions, was run with a 2.5 kW load after incremental increases of 500W up to 4000W by a Simplex Swift-E load bank operated at 240 Volts. The freshly produced DE was then filtered in a two-stage dilution with air, initially mixed at a ratio of approximately 9:1, and immediately thereafter secondarily diluted at a ratio of approximately 25:1, in order to achieve the target $PM_{2.5}$ concentration.

During the exposure days, the DE was allowed to enter a 1.22 m (wide) x 1.83 m (deep) by 2.14 m (high) exposure booth. For the control exposure to FA, room air is high-efficiency particulate air (HEPA)-filtered before entering the exposure chamber. During the exposure, in-booth $PM_{2.5}$ mass concentration was monitored at 10 minute intervals using a Tapered Element Oscillating Microbalance (TEOM) and total size-resolved particle number counts were measured using a Scanning Mobility Particle Sizer (SMPS). CO and NO_x levels were continuously modeled using Thermo Model 48C and Model 42C analyzers, respectively and total volatile organic compounds (TVOC), CO_2 and relative humidity and temperature were continuously monitored using a GrayWolf TG-503 probe (148).

Subjects were exposed to either FA or DE (300µg $PM_{2.5}$/m3) for 120min (148). This dose reflects high-ambient short-term exposures found in some global and occupational settings (54,149,150) and is in line with other human studies (106,151). During each exposure the participants cycled on a stationary bike at a light effort for two 15 minute periods to maintain a level of activity similar to what would be achieved during waking hours. Heart rate, oxygen saturation levels, and blood pressure were recorded at 20 minute intervals. Table 2 summarizes the exposure conditions during the study.

3.3.3 Blood Collection

Blood collection was performed by members of the research team who were certified venipuncturists, having taken and passed the BCIT Basic Venipuncture for Allied Health Professionals. On each blood draw one 10 ml serum vial, (BD, Mississauga, Ontario, Canada) and one 6 ml EDTA plasma vial (BD, Mississauga, Ontario, Canada) were collected from each subject. The timeline of a typical exposure day is shown in Table 3.1 below. As can be seen, blood was collected at timepoints, which correlated with the CANTAB battery. Note that from here forth, the term "immediately post-" blood collection refers to blood collection immediately following exposure whereas "3hr post-" and "24hr post-" refer to blood collection 3 hours and 24 hours following exposure, respectively.

Table 3.1: Timeline of typical exposure day

Time	Timepoint	Parameter
7:00 am	Baseline/pre-exposure	Blood collection
7:15 am	Baseline/pre-exposure	CANTAB and Balance assessment
8:20 am	Transport subject to Women's and Children's Hospital	
8:40 am	Baseline/pre-exposure	fMRI
9:40 am	Transport subject to Vancouver General Hospital and APEL	
10:00 am	2-hour exposure to DE or FA	
12:00 pm	Immediately post-exposure	Blood collection
12:15 pm	Immediately post-exposure	CANTAB and Balance assessment
1:20 pm	Transport subject to Women's and Children's Hospital	
1:40 pm	Post-exposure	fMRI
2:40 pm	Transport subject to Vancouver General Hospital and APEL	
3:00 pm	3hr post-exposure	Blood collection
3:15 pm	3hr post-exposure	CANTAB
3:45 pm	All blood is centrifuged, serum and plasma is collected, and stored at -80^{o}C	
FOLLOWING DAY		
10:00 am	24hr post-exposure	Blood collection
10:15 am	24hr post-exposure	CANTAB
11:00 am	All blood is centrifuged, serum and plasma is collected, and stored at -80^{o}C	

3.3.4 Other Aspects of the Study

For a detailed description of the other parameters examined in the greater study see Appendix I. Briefly, the Cambridge Neuropsychologial Test Automated Battery (CANTAB) was used to assess rapid visual processing (a task of continuous performance and visual sustained attention), delayed matching to sample (a task of forced choice recognition memory), reaction time, attention switching task (a measure of frontal lobe and executive function), special working memory, and mood state assessment. These tests were performed at baseline (pre-exposure) and immediately post-, 3hr post- and 24hr post- exposure. Balance was also assessed immediately prior and following the DE/FA exposure using the balance error scoring system, a technique frequently used in sports medicine. Finally, functional neuroimaging (fMRI) was performed at the Child and Family Research Imaging Facility at Women's and Children's hospital prior to and following exposures. During the scan the Sternberg test for working memory was performed.

A questionnaire was given to each subject the morning of an exposure or follow up day. Subjects were asked about their previous night sleep quality, caffeine intake, and whether any adverse or stressful events had occurred. Participants were asked to maintain a similar sleep schedule, diet, and caffeine intake on their first and second exposure days.

3.4 Laboratory methods

3.4.1 Blood Processing

Immediately after collection, blood vials were inverted as per the manufactures instructions, and placed in a +4^{o}C fridge within 10 minutes of the blood draw. The samples were left in this fridge until the end of the day when processing was possible. Following this, all blood was spun at 1500 rpm for 12 minutes and the supernatant was collected into appropriately labeled aliquot tubes. Each aliquot contained between 0.5 and 1.0 ml of supernatant and was stored in a -80^{o}C freezer. Relevant aliquots were removed from the -80^{o}C freezer the afternoon

before enzyme-linked immunosorbent assay (ELISA) analysis and allowed to thaw in the fridge overnight. Attempt was made to minimize freeze thaw cycles and no aliquots were allowed to be thawed from freeze more than two times.

3.4.2 ELISA Procedures

Commercial ELISA kits were used to analyze the levels of the biomarkers as per the manufactures instructions. To eliminate potential batch effects, all samples from a given subject were analyzed on the same plate (see Appendix II for the typical plate design). IL-6 was measured in EDTA plasma and TNF-α, S100B, NSE, and BDNF were measured in serum. IL-6 and TNF-α were analyzed using kits from R&D Systems (Minneapolis, MN, USA). S100B and NSE were analyzed using kits from Millipore (Billerica, MA, USA) and R&D (Minneapolis, MN, USA), respectively. For NSE, the results from samples which visually appeared haemolysed were removed as lysis of red blood cells can artificially elevate levels of this protein (31). BDNF was analyzed using kits from R&D (Minneapolis, MN, USA) and serum samples were diluted 20-fold as per the manufactures instructions.

A microplate reader was used to obtain the optical density (OD) at wavelengths of 490 and 690 nanometers for IL-6, 490 and 690 for TNF-α, 450 and 590 for S100B, and 540 and 570 for NSE and BDNF. MasterPlex Software was used to generate a four parameter logistic (4-PL) curve-fit to calculate ELISA results. Samples <LOD were assigned a value of half the sensitivity of the kit with sensitivity values as follows: IL-6 0.039 pg/ml, TNF-α 0.106 pg/ml, S100B 2.7 pg/ml, NSE 0.020 ng/ml, and BDNF 20 pg/ml. All samples were run in duplicates except TNF- α, which was run in singlicate. For those run in duplicates, if variability between duplicates was greater than 20% from the mean then all samples for this subject were re-run on the same plate.

3.5 Statistical Methods

Statistical analysis was performed using STATA 14 software (StataCorp LP, College Station, Texas, USA) and R Statistical Software. Figures were made using Graph Pad Prism 6 Software.

3.5.1 Subject Characteristics

Using STATA 14 software the mean, standard deviation and range of the age of all subjects, and males and females in groups, were determined. Age was calculated by considering exact birth date by day and first exposure day.

3.5.2 Descriptive Statistics of the Blood Markers

Descriptive statistics of the arithmetic mean, median, standard deviation (SD) and geometric standard deviation for each marker for all blood samples was determined using STATA 14 software. All data were also assessed for log-normality using STATA 14 software to produce histograms and normal quantile plots of the biomarkers. These parameters were used to assess if the data followed a normal distribution. The decision was made to log-transform IL-6 and BDNF prior to subsequent analysis (see Appendix III).

3.5.3 Unpaired and Paired T-Tests of Baseline Values and Delta Values from Baseline

Baseline values of the various biomarkers by gender were determined, and an unpaired t-test was used to determine if these values were statistically different between males and females prior to any exposure. Similarly, a paired t-test was used to determine if there was a difference between the baseline values (irrespective of gender) on FA and DE exposure days prior to any exposure. Both calculations were performed using STATA 14 software. Secondly, for

each biomarker the delta value from baseline following FA exposure (i.e. post-FA exposure value – baseline FA value) and the delta value following DE exposure (i.e. post-DE exposure value – baselined DE value) was calculated for each subject at each timepoint. Three paired t-tests per biomarker were performed using STATA 14 software to determine if the change in protein levels differed by exposure condition at each of the post-exposure timepoints. Finally, a calculation was done to determine the absolute number of subjects that experienced an increase (IL-6, TNF-α, S100B and NSE) or decrease (BDNF) in levels following exposure to DE, relative to FA. This value was expressed as both a fraction and a percentage.

3.5.4 Developing a linear mixed effect model

All remaining portions of the statistical analysis were performed using R software: the data was transformed into a 'long' format within excel and then uploaded into R and the packages 'lmerTest' and 'lsmeans' were used in the analysis. See appendix IV for the code used to develop the mixed effect model, using the biomarker IL-6 as an example. A model considering the interaction between exposure type and exposure day (first day or second, regardless of exposure), with Subject ID being treated as a random effect, was run for each biomarker to determine if a carryover effect was present. If so, this finding was examined further prior to any subsequent analysis. Assuming no interaction was seen, the analysis continued to the next steps.

Effect modification was next tested for gender and age at first exposure by determining if these variables significantly interacted with exposure condition. If so, an interaction term for these variables would be included in the final model. Additionally, effect modification to determine if an interaction existed between exposure condition and timepoint was similarly tested. This indicates whether change of the biomarker over the course of the experiment (i.e. from baseline to the various post-exposure timepoints) was altered by DE (relative to FA). If none

of these interaction terms yielded a statistically significant effect ($p < 0.05$) then they were not included in the final model.

The variables gender, age at first exposure, exposure condition, exposure day and timepoint were then included in the subsequent model as fixed effects, and any statistically significant interactions found in the previous steps were re-introduced to the model as well. If these variables had a significant ($p < 0.05$) effect on the outcome, then they were included in the final model. However, as exposure and timepoint` were the main effects of interest they remained in the model regardless of their statistical significance. Similarly, the interaction existed between exposure condition and timepoint was tested to indicate if change of the biomarker over the course of the experiment (i.e. from baseline to the various post-exposure timepoints) was altered by DE (relative to FA). If the interaction yielded no significant ($p < 0.05$) effect on the outcome variable then the interaction term was removed and only condition and timepoint were included in the final model. Once this final model was developed, the effect of the exposure condition and timepoint on the outcome of interest was assessed using a pairwise least-squares means *post hoc* test with a Tukey correction for multiple comparison.

Chapter 4: Results

In total, 28 subjects were recruited and completed the entire study protocol. Of these, one subject fainted during the initial blood draw on the first exposure day. The decision was made to discontinue all further blood draws, but have the subject continue through other aspects of EAPOC. Therefore, 27 EAPOC subjects had their blood drawn for the endpoints in this thesis. There were occasions when the venipuncturist was unable to draw blood at a single post-exposure timepoint. As a result, some post-exposure timepoints are missing, as detailed discussed below.

4.1 Exposure Characteristics

Table 4.1 shows the average exposure conditions on FA and DE exposure days.

Table 4.1: Diesel exhaust and filtered air exposure characteristics from the average results of the DE and FA runs in this study.

	Filtered Air		Diesel Exhaust	
	Mean	Standard Deviation	Mean	Standard Deviation
Temperature (°C)	26.6	0.6	26.4	1.2
Relative humidity (%)	32.1	8.5	35.5	7.5
TVOC (ppb)	124.5	103.0	1425	364.5
CO_2 (ppm)	794.1	109.0	2098	353.5
CO (ppm)	0.7	0.9	11.5	2.85
NO (ppb)	26.7	34.6	7778	2211
NO_2 (ppb)	51.9	59.8	283.1	238.7
NO_x (ppb)	64.7	55.1	8062	2331
$PM_{2.5}$ (μg/m^3)	2.4	7.1	289.6	58.0

**TVOC: Total volatile organic compounds, CO_2: carbon dioxide, CO: carbon monoxide, NO: nitrogen oxide, NO_2: nitrogen dioxide, NO_x: nitrogen oxides, $PM_{2.5}$: particulate matter of aerodynamic diameter <2.5μm, ppb: parts per billion, ppm: parts per million*

On average, there were 34 days between the first and second exposure, with the shortest difference being 23 days and the longest 86 days. Table 4.2 shows the typical time of

each blood draw and time relative to exposure time. This information is presented for all exposures (n = 54) and for FA and DE exposures separately.

Table 4.2: Time of blood draws.

	Time of day (24hr clock, range)	Time (hours:minutes) relative to exposure start (range)
	Baseline	
All exposures	7:36 (6:17, 10:23)	-02:39 (-00:08, -03:32)
FA	7:31 (7:01, 10:15)	-02:42 (-00:15, -03:21)
DE	7:40 (6:17, 10:23)	-02:59 (-00:08, -03:32)
	Immediately post-exposure	
All exposures	12:31 (11:22, 16:05)	00:16 (00:05, 01:09)
FA	12:31 (11:55, 16:05)	00:17 (00:06, 01:09)
DE	12:31 (11:22, 14:10)	00:15 (00:05, 01:05)
	3hr post-exposure	
All exposures	15:08 (13:56, 18:20)	02:53 (02:25, 03:44)
FA	15:13 (14:25, 18:20)	02:58 (02:28, 03:44)
DE	15:04 (13:56, 16:35)	02:48 (02:25, 03:21)
	24hr post-exposure (following day)	
All exposures	9:21 (7:00, 12:25)	21:05 (19:00, 23:19)
FA	9:21 (7:00, 12:25)	21:07 (19:00, 22:55)
DE	9:20 (7:00, 12:15)	21:04 (19:04, 23:19)

4.2 Study Participants and Number of Blood Draws

Subject characteristics are shown in Table 4.3.

Table 4.3: Subject characteristics

	Gender	N	Mean	Minimum and maximum
All subjects in the study	Male + Female	27	29.0	21.9, 49.7
	Male	14	29.1	21.9, 36.1
	Female	13	29.0	21.9, 49.7
Subjects with complete blood draws	Male + Female	23	28.1	21.9, 36.1
	Male	13	30.3	22.1, 49.7
	Female	10	29.5	21.9, 49.7
Subjects with one or more blood draws missing	Male + Female	4	29.0	21.9, 31.7
	Male	1	31.7	Not applicable
	Female	3	26.2	21.8, 28.8

Due to an error, the testing order was not properly balanced. 10 subjects received DE first, whereas 17 received FA first. Those conducting the experiment were not aware of this poor counter-balance until after the data was analyzed. There were no significant differences the group who received DE first and those who received FA first (Table 4.4).

Table 4.4: Characteristics of subjects in each order-grouping and unpaired t-test result to assess differences between groups.

Order Type	Characteristic	N	Mean ± SD	Median	Lower and Upper Quartiles	Minimum and Maximum
DE first	Women : Men	6 : 4				
FA first		8 : 9				
		Unpaired t-test result		P = 0.534		
DE first	Age, yr at first exposure	10	31.12 ± 8.52	30	23, 35	21, 49
FA first		17	27.79 ± 5.32	27	22, 29	21, 38
		Unpaired t-test result		P = 0.222		

4.3 Descriptive Statistics of Blood Markers

Once all the data from the ELISA analysis was collected the descriptive statistics (such as the AM, GM, SD and genomic SD) were calculated. It is important to note that in some cases, specific samples were removed or not analyzed for particular biomarkers: (1) plate design: one subject's data could not be fit onto the first five ELISA plates. This subject was a female, aged 22, who had blood drawn on the pre-exposure and post-exposure timepoints only. Measuring the marker would have resulted in an entirely new plate being used to measure only these four samples from one subject and, for cost reasons, this was not done for S100B or NSE. (2) For unexplainable reasons, the TNF-α values for one subject were substantially higher than the other values in seven of the eight samples. The values were 4.12, 4.48, 4.63, 4.63, 5.51, 5.57 and 6.01 pg/ml whereas the expected range of the kit was between 0.550-2.816 pg/ml. All other TNFα values of the remaining samples fell within this range. Furthermore, the subject's baseline value before DE exposure did fall within the expected range (0.73 pg/mL); because of this significant bias may have been introduced to the findings. As it could not be explained why the TNFα values

for seven of this subject's samples varied so greatly from the remaining samples, the decision was made to remove this subject from the TNFα analysis. (3) For NSE, the results from samples which visually appeared hemolysed were removed as lysis of red blood cells can artificially elevate levels of this protein (31). To remove potential bias, the "matching" sample from this subject (same timepoint, other exposure day) was removed as well. This applied to five samples (three at baseline, one at 3hr post-exposure, and one at 24hr post-exposure) and four subjects (three males, one female; ages 21, 27, 34 and 36) and the values of these samples were 4.18 ng/ml, 5.29 ng/ml, 6.07 ng/ml, and 13.04 ng/ml.

In total, this resulted in the following number of samples at each timepoint on each exposure day.

- IL-6: baseline n=27, post-exposure n=26, 3hr post-exposure n=25, 24hr post-exposure n=24.
- TNFα: baseline n=26, post-exposure n=25, 3hr post-exposure n=24, 24hr post-exposure n=23.
- S100B: baseline n=26, post-exposure n=25, 3hr post-exposure n=25, 24 hr post-exposure n=24.
- NSE: baseline n=23, post-exposure n=25, 3hr post-exposure n=24, 24hr post-exposure n=23.
- BDNF: baseline n=27, post-exposure n=26, 3hr post-exposure n=25, 24hr post-exposure n=24

R&D, the manufactures of the ELISA kits for all biomarkers except S100B, presented approximate expected ranges in biological samples of the kits developed from samples of healthy volunteers. These ranges were: IL-6 in plasma 0.428 – 8.87 pg/mL, TNF-α in serum 0.550 – 2.816 pg/mL, NSE in serum 1.85 – 4.14 ng/mL and BDNF in serum 6186 – 42580 pg/mL. As presented in Table 4.5, the majority of samples were within these ranges.

Table 4.5. Distribution of the values of each measured biomarker.

Biomarker	n=	Arithmetic Mean	Geometric Mean	Standard Deviation	Lower and Upper Quartiles	N <LOD	Range*
IL-6 (pg/ml)	204	1.02	0.78	0.92	0.48, 1.22	0	0.16, 6.76
TNFα (pg/ml)	196	1.01	0.90	0.38	0.75, 1.31	3	0.05, 1.88
S100B (pg/ml)	200	16.96	14.29	9.16	9.92, 21.76	3	1.37, 48.62
NSE (ng/ml)	190	2.61	2.44	0.86	2.09, 2.98	1	0.01, 6.86
BDNF (pg/ml)	204	27779	25505	11026	19119, 35348	0	6315, 56923

* LOD values of kits were IL-6 0.039 pg/ml, TNFα 0.106 pg/ml, S100B 2.7 pg/ml, NSE 0.02 ng/ml and BDNF 20 pg/ml; a value of ½LOD was assigned to each biomarker that was <LOD

Histograms and normal quantile plots of serum or plasma biomarkers were produced using STATA 14 software to examine normality or log-normality of the distribution, as well as the AM, GM, standard deviation (SD) and median values. In order to determine if the variables should be transformed, the Shapiro-Wilk test for normality was performed on both the untransformed and log-transformed variables. Whether or not variables were log-transformed was based on a combination of visualization of the histograms and comparisons of the descriptive statistics and goodness of fit tests. In a right-skewed distribution the median falls to the left of the geometric mean (median < GM). There was strong evidence that IL-6 was log-distributed and these results were therefore log-transformed. There was no evidence to support log-transforming TNF-α, S100B, and NSE. BDNF was not normally distributed and log-transformation slightly improved the normal fit, although not perfectly so. The decision was made to log-transform BDNF.

4.4 Baseline Values, Distributions at Each Time Point and Delta Values from Baseline

Baseline values by gender, and by exposure condition (i.e. baseline on FA exposure day versus baseline on DE exposure day) are shown in Table 4.6. There was no significant difference at baseline between males and females for any of the markers and, similarly, the baseline values were the same on the morning of the DE exposure and the morning of the FA exposure.

Table 4.6: Baseline values by gender, exposure day and exposure condition and unpaired t-test result to determine if the respective values differed.

	Male (mean +/- SD)	Female (mean +/- SD)	Unpaired t-test
IL-6	0.88 +/- 0.76	1.13 +/- 0.84	0.11
TNF-α	1.12 +/- 0.35	0.95 +/- 0.50	0.16
S100B	19.93 +/- 9.66	17.72 +/-8.97	0.40
NSE	3.05 +/- 1.08	3.27 +/- 2.37	0.66
BDNF	28547 +/- 11697	29977 +/- 9568	0.63
	DE baseline	FA baseline	Paired t-test
IL-6	0.97 +/- 0.75	1.03 +/- 0.86	0.84
TNF-alpha	1.08 +/- 0.38	1.00 +/- 0.48	0.30
S100B	18.35 +/- 9.37	18.26 +/- 7.41	0.94
NSE	2.61 +/- 0.86	3.01 +/- 1.15	0.12
BDNF	28567 +/- 10254	29904 +/- 11185	0.38

Table 4.7 shows the descriptive statistics (average and standard deviation) of the biomarkers at each timepoint on each exposure day (FA or DE). Secondly, Table 4.8 illustrates the mean change and standard deviation of the change in levels of each marker from baseline at all three post-exposure timepoints (post, 3hr, 24hr). A calculation was also performed to determine how many subjects had an increase (IL-6, TNF-α, S100B, and NSE) or decrease (BDNF) in levels following exposure to DE, relative to what was experienced following exposure to FA. This value was expressed as both a fraction and a percentage. Finally, a paired t-test was preformed to compare the delta-values from baseline at each timepoint for FA and DE. All this information is displayed on Table 4.8 and Figures 4.1,4.2 and 4.3. No clear, consistent trend of an increase or decrease in levels of each marker following DE exposure, relative to baseline and the FA exposure, was observed and the statistical testing revealed no significant differences between each timepoint (p-value range 0.269 – 0.923). Furthermore, these calculations did not control for the fact that multiple comparisons were made due to several paired t-tests being performed. Statistical significance is determined by a p-value of less than or equal to 0.05. When this occurs ($p < 0.05$) the statistician rejects the null hypothesis and, in this case, the interpretation would be that the two values (DE vs. FA deltas) are statistically unique from on another. However, a p-value of 0.05 only suggests statistical significance at the "95% confidence level", which means

that 1 out of every 20 times the positive result occurs due solely to chance. This is a concern when multiple comparisons are performed, as it increases the 1 in 20 likelihood of a positive result occurring due to random error or chance. There are statistical methods which can be done to "correct for multiple comparisons", however, as none of the p-values were <0.05 it was unnecessary to account for the issue that a positive finding may have occurred by chance.

A noted trend was that NSE levels decreased at the 24hr post-exposure timepoint relative to baseline, regardless of exposure condition. However, the average decrease was respectively less substantial following exposure to DE (-0.23 (±0.79) ng/ml) relative to FA (-0.59 (±1.14) ng/ml). This trend was in the direction of the initial hypothesis however it was not statistically significant ($p = 0.269$). Similarly, a trend in the direction of the initial hypothesis was seen for BDNF levels immediately following exposure. Following exposure to FA, on average, BDNF levels did not change, however, a decrease in BDNF levels were, on average, seen following exposure to DE. Again, this trend was not statistically significant ($p = 0.312$). In both cases, it is important to note that only approximately half of the subjects showed a trend in the direction of the hypothesis. Furthermore, these results from the T-tests were not corrected for multiple comparisons, and with such a correction, the p-values would be expected to become less significant.

Table 4.7: Concentration (mean, ±SD) of blood markers at each timepoint before and after exposure.

Marker	Filtered Air				Diesel Exhaust			
	Baseline	Post	3hr post	24hr post	Baseline	Post	3hr post	24hr post
IL-6 (pg/ml)*	1.03 (±0.87)	1.18 (±1.00)	1.15 (±1.35)	1.04 (±0.94)	0.97 (±0.75)	1.07 (±0.96)	0.93 (±0.83)	0.85 (±0.48)
TNF-α (pg/ml)	1.00 (±0.48)	0.94 (±0.37)	0.93 (±0.34)	0.97 (±0.44)	1.08 (±0.38)	1.08 (±0.37)	1.04 (±0.30)	1.08 (±0.38)
S100B (pg/ml)	19.27 (±8.35)	15.55 (±8.18)	17.18 (±12.20)	15.48 (±9.23)	17.69 (±9.35)	15.68 (±8.37)	15.98 (±8.26)	16.33 (±7.90)
NSE (ng/ml)#	3.09 (±1.15)	3.06 (±1.11)	2.41 (±0.86)	2.49 (±0.52)	2.68 (±0.86)	2.58 (±0.62)	2.48 (±0.89)	2.45 (±0.81)
BDNF (pg/ml)	29900 (±11190)	30070 (±11760)	26690 (±11390)	27960 (±13500)	28570 (±10250)	26650 (±9050)	25420 (±9260)	26650 (±12010)

**IL-6 measured in EDTA plasma, all other markers measured in serum*

+<LOD assigned as 1.35, which is half of the sensitivity of the kit

#<LOD assigned as 0.01, which is half of the sensitivity of the kit

Table 4.8: Average change (±SD) in concentration of blood markers at each timepoint, relative to baseline, following exposure and the number and percentage of subjects who showed a trend in the direction of the study hypothesis (increase or decrease) following DE exposure relative to FA.

Time point	Filtered Air	Diesel Exhaust	Number and percentage of subjects with a greater increase/decrease* following DE exposure relative to FA	Paired T-test
		IL-6 (pg/ml)		
Post	0.17 (±0.54)	0.17 (±0.61)	12/26 (46%)	0.854
3hr post	0.17 (±0.85)	0.039 (±0.36)	11/25 (44%)	0.461
24hr post	0.00 (±0.33)	-0.06 (±0.47)	14/24 (58%)	0.923
		TNF-α (pg/ml)		
Post	0.00 (±0.44)	-0.01 (±0.33)	13/25 (52%)	0.574
3hr post	-0.01 (±0.45)	-0.06 (±0.38)	13/24. 54%	0.889
24hr post	0.02 (±0.49)	-0.01 (±0.40)	14/23 (61%)	0.752
		S100B (pg/ml)		
Post	-3.24 (±6.01)	-2.42 (±5.34)	15/25 (60%)	0.540
3hr post	-2.18 (±10.02)	-3.56 (±4.78)	10/25 (40%	0.517
24hr post	-3.10 (±7.05)	-1.31 (±5.81)	15/24 (63%	0.372
		NSE (ng/ml)		
Post	0.11 (±0.85)	-0.01 (±0.68)	8/22 (36%)	0.660
3hr post	-0.65 (±1.62)	-0.35 (±0.77)	14/21 (67%)	0.421
24hr post	-0.59 (±1.14)	-0.23 (±0.79)	11/22 (50%)	0.269
		BDNF (pg/ml)		
Post	5.2 (±5185.9)	-2166.2 (±7585.5)	14/26 (54%)	0.312
3hr post	-4131.1 (±10759.5)	-3338.4 (±7541.4)	13/25 (52%)	0.591
24hr post	-2611.1 (±7265.5)	-2094.9 (±7633.1)	12/24 (50%)	0.624

**IL-6, TNF-α,* S100B *and NSE all refer to an increase in levels following DE exposure, relative to FA, whereas BDNF refers to a respective decrease*

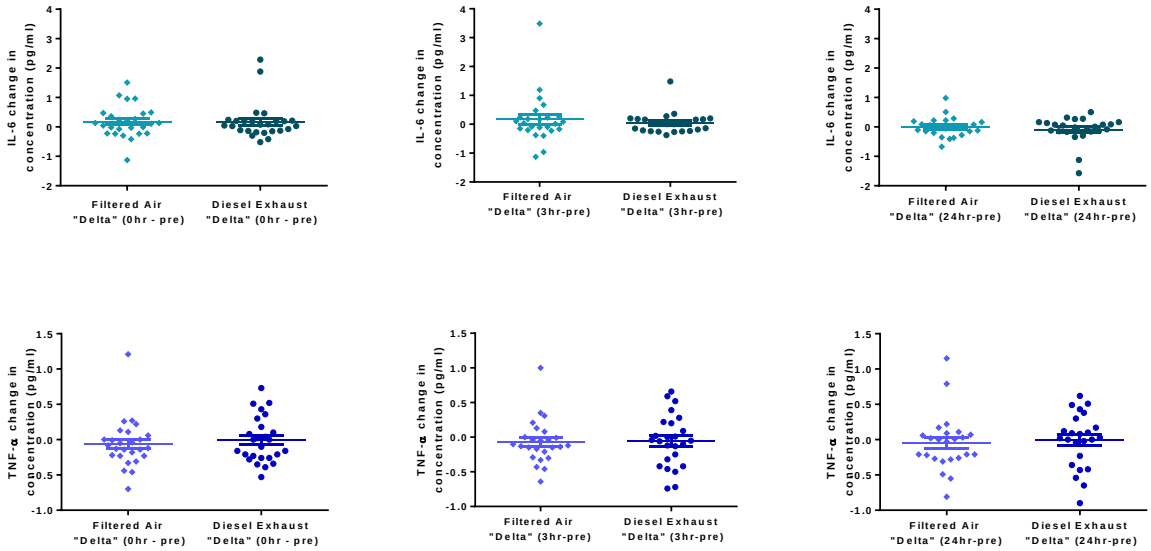

Figure 4.1: Delta values for the inflammatory cytokines IL-6 (pg/ml) in EDTA plasma samples (FIRST ROW) and TNF-α (pg/ml) in serum samples (SECOND ROW). Each scatterplot depicts the change in baseline levels at a single time point, with dots representing an individual subject (FA light dots, DE dark dots) and the mean± SEM shown. Top left: IL-6 at 0h post exposure (n=26); top middle: IL-6 at 3h post exposure (n=25); top right: IL-6 at 24h post exposure (n=24). Bottom left: TNF-α at 0h post exposure (n=25); bottom middle: TNF-α at 3hr post exposure (n=24); bottom right: TNF-α at 24hr post exposure (n=23).

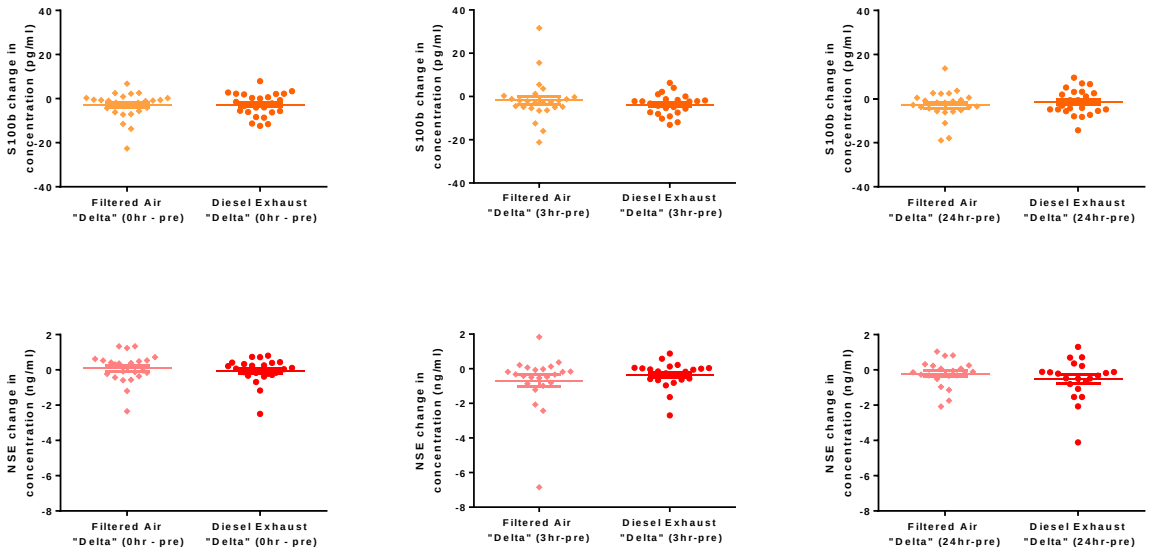

Figure 4.2: Delta values for the inflammatory cytokines S100B (pg/ml) (FIRST ROW) and NSE (ng/ml) in serum samples (SECOND ROW). Each scatterplot depicts the change in baseline levels at a single time point, with dots representing an individual subject (FA light dots, DE dark dots) and the mean± SEM shown. Top left: S100B at 0h post exposure (n=25); top middle: S100B at 3h post exposure (n=25); top right: S100B 24h post exposure (n=24). Bottom left: NSE at 0h post exposure (n=22); bottom middle: NSE at 3hr post exposure (n=21); bottom right: NSE at 24hr post exposure (n=20).

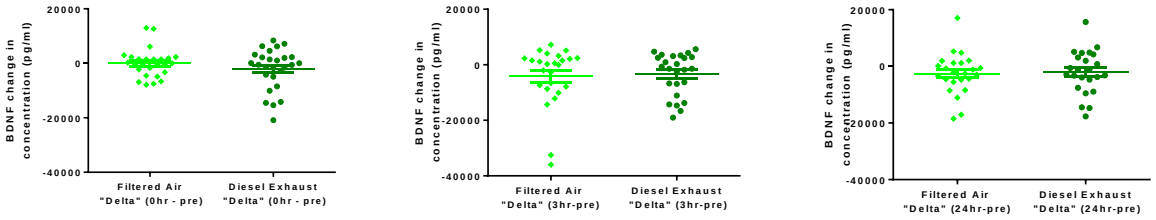

Figure 4.3: Delta values for the inflammatory cytokines BDNF (pg/ml) in EDTA plasma samples. Each scatterplot depicts the change in baseline levels at a single time point, with dots representing an individual subject (FA light dots, DE dark dots) and the mean± SEM shown. Left: BDNF at 0h post exposure (n=26); middle: BDNF at 3h post exposure (n=25); right: BDNF 24h post exposure (n=24).

4.5 Linear Mixed Effect Models

There was no significant carryover effect observed for Il-6 (p = 0.41), S100B (p = 0.68), NSE (p = 0.07), or BDNF (p = 0.28). A significant carryover was seen for TNF-α (p = 0.01) and this finding is discussed in more detail below in section 4.5.5.

4.5.1 Inclusion of demographic variables and interaction terms

The interaction between exposure and gender, age and time was analyzed and is shown below in Table 4.9. There was no effect modification by gender or age of these variables and these terms were not included in the overall model. In no case was an interaction between exposure and timepoint noted, however, as the interaction effect between exposure condition and timepoint was the primary outcome of interest for each biomarker, the timepoint and exposure condition interaction was examined in the final model.

Table 4.9: Mixed-effects model testing for the interaction of gender, age, and timepoint with exposure condition with the various biomarkers as dependent variables. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Interaction	Log(il-6) n=27	TNF-α n=26	S100B n=26	NSE n=26	Log (BDNF) n= 27
	ANOVA p-value				
Exposure & timepoint	0.580	0.887	0.790	0.137	0.662
Exposure & gender	0.897	0.219	0.358	0.860	0.888
Exposure & age	0.064	0.112	0.248	0.521	0.643

The initial fixed effect model considered exposure condition (DE or FA), timepoint (baseline, post-, 3hr-, and 24hr- after exposure), age at first exposure, gender (male or female), and exposure day (day one or day two, regardless of exposure condition) with the random effect of subject ID included to determine if these values independently impacted the levels of biomarkers. The P-values of these fixed variables are shown in Table 4.10. For the inflammatory cytokines IL-6 and TNF-α, with the exception of exposure day for TNF-α, none of the variables had a significant effect on the levels of either inflammatory cytokines. As a result, these variables were not included in the final mixed-effect model.

For the neurologically specific biomarkers S100B and NSE, it was found that exposure condition had a significant effect on NSE levels ($p = 0.046$) and that timepoint had a significant effect on S100B and NSE levels ($p = 0.036$, $p = 0.001$, respectively). All remaining variables did not have a significant effect on the levels of either S100B or NSE, and these variables were not included in the final mixed-effect models. Finally, it was found that timepoint had a significant effect on BDNF levels ($p = 0.002$) and that all remaining variables did not have a significant effect on the levels of BDNF, and were therefore not included in the final mixed-effect model.

Table 4.10: Mixed-effects model testing for significance of gender, age, timepoints, exposure condition, and exposure day in predicting each inflammatory markers as dependent variables. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Fixed Overall Effect	Log(il-6) n=27	TNF-α n=26	S100B n=26	NSE n=26	Log(BDNF) n=27
	ANOVA p-value				
Exposure condition	0.387	0.055	0.430	0.046*	0.413
Timepoint	0.511	0.412	0.036*	0.001**	0.002**
Age	0.052	0.662	0.950	0.987	0.739
Gender	0.195	0.180	0.823	0.185	0.532
Exposure day	0.090	0.0002**	0.067	0.916	0.073

4.5.2 Final Mixed Effect Models Considering Effect of Exposure, Time, and the Interaction Between Exposure and Time – Inflammatory Cytokines

For IL-6, the final mixed effect model considered exposure condition and timepoint only, as none of the other variables were found to have a significant effect on IL-6 concentration. Exposure condition and timepoint, and the interaction between exposure and timepoint were initially included as these were the main variables of interest in the experiment. However, once it was confirmed that the exposure and timepoint interaction did not significantly impact biomarker levels (table 4.11), then the interaction term was removed and only exposure and timepoint were included in the final model with subject ID acting as a random variable. For TNF-α the final mixed effect model considered exposure condition, timepoint and exposure day only. The effect estimates from the final mixed-effects models are shown in Table 4.12.

Table 4.11: Final mixed-effects model testing for the effect of exposure condition, timepoint, and the interaction between these two terms with the two inflammatory markers as dependent variables, or, if run, the estimates for the second mixed effects model including only timepoint and exposure condition. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Interaction or Overall Fixed Effect	Log(il-6) n=27	TNF-α n=26
	ANOVA p-value	
Exposure Condition	0.150	0.0583
Timepoint	0.216	0.708
Exposure & timepoint	0.904	0.927
Exposure & exposure day	-	0.007**
Final Mixed Effect Model Fixed Effects		
Exposure Condition	0.153	0.056
Timepoint	0.210	0.696
Exposure day	-	<0.001**

Post hoc least-squares means analysis was performed on the final mixed effect models, including only exposure condition and timepoint. This analysis showed that IL-6 was not significantly altered by exposure condition or timepoint of the blood draw. Serum TNF- α showed a significant carry over effect, suggesting that the effect of the first exposure impacted the second exposure. This is described in more detail in Section 4.5.5. The results from the *post hoc* least-squares means analysis showed that the levels of TNF-α were higher on the second day of exposure than the first, with a corresponding effect estimate of 0.144 pg/ml.

Table 4.12: Least-squares means pairwise comparisons for exposure and timepoint and, in the case of TNF-α, exposure day for the two inflammatory cytokines. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are denoted with a **.

Contrast	Plasma log(IL-6) n=27		Serum TNF-α n=26	
	Effect (log(pg/mL))	p-value	Effect (pg/mL)	p-value
Post-Pre	0.131	0.323	-0.033	0.918
3hr-pre	0.025	0.988	-0.061	0.637
3hr-post	-0.106	0.537	-0.029	0.948
24hr-pre	-0.023	0.991	-0.030	0.941
24hr-post	-0.155	0.212	0.003	1.00
24hr-3hr	-0.049	0.930	0.031	0.936
DE – FA	-0.079	0.153	0.073	0.056
Day2 – Day1	-	-	0.144	0.0002*

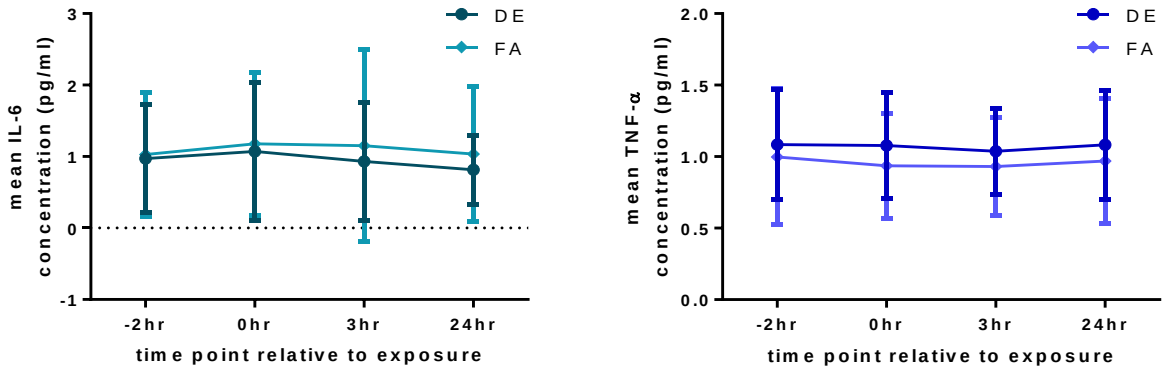

Figure 4.4: Levels of EDTA plasma IL-6 and serum TNF-α from baseline (-2 hours) and immediately post (0 hours), 3, and 24 hours after. Dots (DE) and diamonds (FA) represent mean levels and error bars represent standard deviation. The p-values from the mixed-effects model for IL-6 are 0.210 and 0.153 and for TNF-α are 0.696 and 0.056 for timepoint and exposure condition, respectively.

4.5.3 Final Mixed Effect Models Considering Effect of Exposure, Time, and the Interaction Between Exposure and Time – S100B and NSE

For S100B and NSE the final mixed effect models considered exposure condition and timepoint only; none of the other variables were found to have a significant effect on the

concentration of these biomarkers. Exposure condition and timepoint, and the interaction between exposure and timepoint were initially included, as these were the main variables of interest in the experiment. However, once it was confirmed that the exposure and timepoint interaction was not statistically significant (Table 4.13), then the interaction term was removed and only exposure and timepoint were included in the final model with Subject ID acting as a random variable. The effect estimates from the final mixed-effects models are shown in Table 4.14.

Table 4.13: Final mixed-effects model testing for the effect of exposure condition, timepoint, and the interaction between these two terms with S100B and NSE as dependent variables, or, if run, the estimates for the second mixed effects model including only timepoint and exposure condition. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Interaction or Overall Fixed Effect	S100B n=26	NSE n=26
	ANOVA p-value	
Exposure Condition	0.762	0.040*
Timepoint	0.017*	0.001**
Exposure & Timepoint	0.653	0.313
Final Mixed Effect Model Fixed Effects		
Exposure Condition	0.775	0.0374*
Timepoint	0.017*	0.001**

Post hoc least-squares means analysis showed that S100B levels changed over time, however, not with exposure. The results from the *post hoc* least-squares means analysis showed that the levels of S100B were significantly lower at the immediate post-exposure and 3hr post-exposure timepoints relative to baseline, with corresponding effect estimates of -2.84 pg/mL ($p = 0.027$) and -2.72 pg/mL ($p = 0.038$), respectively. It is important to note that although the levels did change with time throughout the exposure day, there was no significant effect of exposure on S100B levels. Similarly, there appeared to be no exposure-time interaction; indicating that the effect of time on S100B levels were the same irrespective of whether the subject was exposed to DE or FA.

Post hoc least-squares means analysis showed that NSE levels changed over time, and was affected by exposure. Results from the analysis showed that the levels of NSE were significantly lower at the 3hr post-exposure relative to baseline, and at the 3hr and 24hr post exposure timepoints relative to immediately post-exposure with corresponding effect estimates of -0.466 ng/mL (p = 0.008), -0.485 ng/mL (p = 0.004), and -0.373 ng/mL (p = 0.045), respectively. NSE concentration was significantly lower on DE exposure days relative to FA exposure days with a corresponding effect estimate of -0.210 ng/mL (p = 0.037). I It is important to note that this difference was observed throughout the entire exposure day, including baseline, and that there was no exposure-time interaction. This suggests that levels of NSE were lower throughout the DE exposure day, even at baseline, and that exposure had no effect on how levels of NSE changed before and after exposure.

Table 4.14: Least-squares means pairwise comparisons for exposure and timepoint for S100B and NSE. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are denoted with a **.

Contrast	Serum S100B n=26		Serum NSE n=23	
	Effect (pg/mL)	p-value	Effect (ng/mL)	p-value
Post-Pre	-2.84	0.027*	0.019	0.999
3hr-Pre	-2.72	0.038*	-0.466	0.008*
3hr-Post	0.121	0.999	-0.485	0.004*
24hr-Pre	-2.26	0.124	-0.354	0.075
24hr-Post	0.579	0.943	-0.373	0.045*
24hr-3hr	0.458	0.971	0.112	0.866
DE – FA	0.206	0.775	-0.210	0.037*

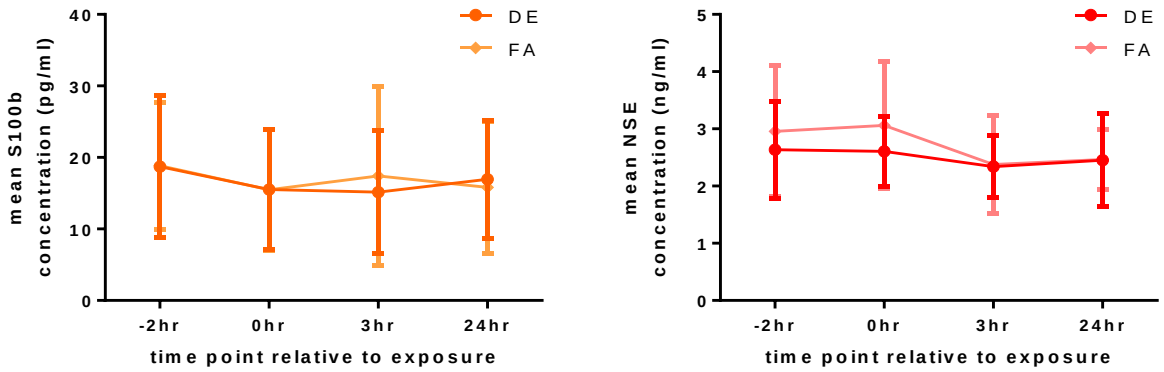

Figure 4.5: Levels of serum S100B and NSE from baseline (-2 hours) and immediately post (0 hours), 3 and 24 hours after. Dots (DE) and diamonds (FA) represent mean levels and error bars represent standard deviation. The p-values from the mixed-effects model for S100B are 0.017* and 0.775 and for NSE are 0.001** and 0.0374 for timepoint and exposure condition. (Note: if included in the model, the p-value for the interaction term between the exposure condition and timepoint was 0.313 for NSE.)

4.5.4 Final Mixed Effect Models Considering Effect of Exposure, Time, and the Interaction Between Exposure and Time – BDNF

For BDNF the final mixed effect models considered exposure condition and timepoint only, as none of the other variables were found to have a significant effect on the concentration of these biomarkers. Exposure condition and timepoint, and the interaction between exposure and timepoint were initially included as these were the main variables of interest in the experiment. However, once it was confirmed that the exposure and timepoint interaction was not statistically significant (Table 4.15), then the interaction time was removed and only exposure and timepoint were included in the final model with subject ID acting as a random variable. The effect estimates from the final mixed-effects models are shown in Table 4.16.

Table 4.15: Final mixed-effects model testing for the effect of exposure condition, blood draw, and the interaction between these two terms with BDNF as the dependent variable, and the estimates for the second mixed effects model including only timepoint and exposure condition. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Interaction or Overall Fixed Effect	Log(BDNF) n=27 ANOVA p-value
Exposure Condition	0.171
Timepoint	0.011*
Exposure & Timepoint	0.833
Final Mixed Effect Model Fixed Effects	
Exposure Condition	0.162
Timepoint	0.011*

Post hoc least-squares means analysis showed that BNDF levels changed over time, however, not with exposure. Results from the analysis showed that the levels of BDNF were significantly lower at the 3hr post-exposure timepoint relative to baseline, with a corresponding effect estimate of -0.129 log(pg/mL) ($p = 0.038$). It is important to note that although the levels did change with time throughout the exposure day, there was no significant effect of exposure on BNDF levels. Similarly, there was no exposure-time interaction, indicating that the effect of time on BDNF levels were the same whether the subject was exposed to DE or FA.

Table 4.16: Least-squares means pairwise comparisons for exposure and timepoint for BDNF. Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are denoted with a **.

Contrast	Serum log(BDNF) n=27	
	Effect (log(pg/mL))	p-value
Post-Pre	-0.025	0.954
3hr-Pre	-0.129	0.038*
3hr-Post	-0.104	0.140
24hr-Pre	-0.125	0.052
24hr-Post	-0.100	0.171
24hr-3hr	0.004	1.00
DE – FA	-0.047	0.162

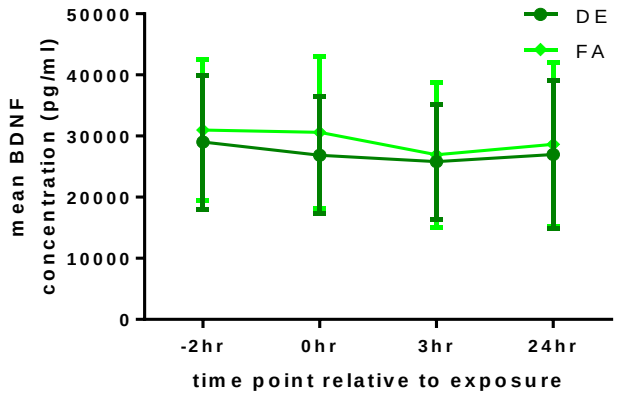

Figure 4.6: Levels of serum BDNF from baseline (-2 hours) and immediately post (0 hours), 3, and 24 hours after. Dots (DE) and diamonds (FA) represent mean levels and error bars represent standard deviation. The p-values from the mixed-effects model for BDNF are 0.011* and 0.162 for timepoint exposure condition, respectively.

4.5.5 Order Effect: TNF-α

It was found in the initial mixed effect model that exposure condition and exposure day had a significant interaction on TNF-α, suggesting that a carryover effect may have occurred from the first exposure to the second. Figure 4.7 shows the mean levels of TNF-α at each timepoint on DE and FA exposure days and suggests that those exposed to FA first showed a greater increase in TNF-α with DE, relative to those who first were exposed to DE, but this trend

was not significant (Table 4.17). As there is little biological plausibility to a carryover effect specific to TNF-α (a short-acting cytokine), we suspect that the order effect suggested was due to chance.

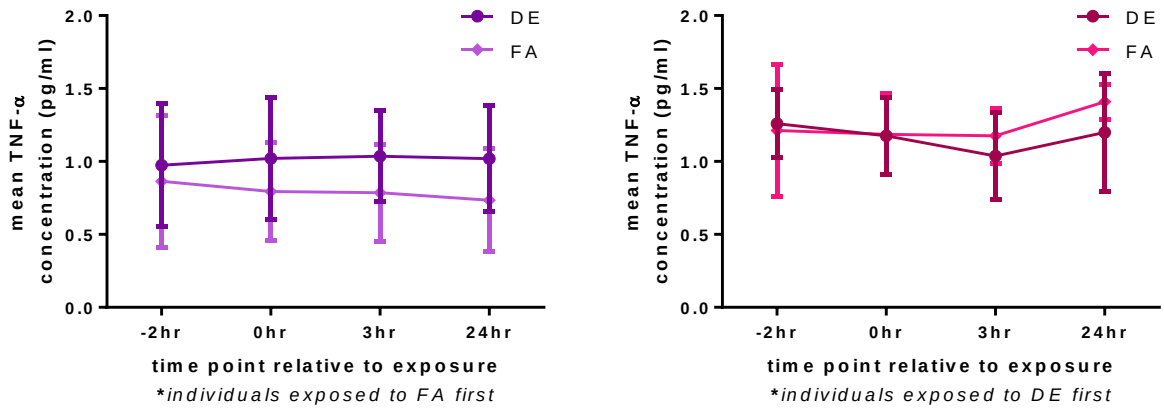

Figure 4.7: Levels of TNF-α at baseline (-2 hours), and immediately post (0 hours), 3 and 24 hours after exposure, considering order. Left side (purple) shows subjects who were exposed to FA first (n=16 (at -2hr), n=16 (at 0hr), n=15 (at 3hr), n=15 (at 24hr)) and right side (fuchsia) shows subjects who were exposed to DE first (n=10 (at -2hr), n=9 (at 0hr), n=9 (at 3hr), n=8 (at 24hr)).

Table 4.17: Mixed-effects model testing for significance of timepoints, exposure condition (FA or DE) and timepoint – exposure condition interaction for TNF-α examining those who received FA first (group 1) and those who received DE first (group 2) separately. (n = the number of subjects in each data set). Significant p-values ($p < 0.05$) are denoted with a * and p-values ($p < 0.01$) are with a **.

Overall Effect	Group 1 (FA first) TNF-α n=16	Group 2 (DE first) TNF-α n=10
	ANOVA p-value	
Exposure Condition	0.458	0.219
Timepoint	0.073	0.286
Exposure*Timepoint	0.171	0.459

4.6 Conclusions to Results

Hypothesis 1: The concentration of the inflammatory cytokines IL-6 and TNF-α, will increase from baseline following DE exposure, relative to FA.

- Conclusion: No significant exposure and timepoint interaction was noted for either IL-6 or TNF-α. No effect of timepoint or exposure condition, independently was noted. Levels of IL-6 and TNF-α were not affected by time of blood draw, or by whether a research subject was exposed to FA or DE.

Hypothesis 2: The concentrations of S100B and NSE in peripheral blood will increase from baseline following DE exposure, relative to FA, with levels peaking at 3hr or 24hr post exposure.

- Conclusion (S100B): No significant exposure and timepoint interaction was observed for S100B. A significant effect of time was observed, suggesting that levels of these biomarkers fluctuated through the day. As no effect of exposure condition, or interaction between exposure condition and timepoint was noted for S100B this suggests that the changes in levels of S100B throughout the exposure day were not impacted by whether a research subject was exposed to FA or DE.
- Conclusion (NSE): No significant exposure and timepoint interaction was observed for NSE. A significant effect of time was observed, suggesting that levels of these biomarkers fluctuated through the day. Additionally, significant effect of exposure condition was noted, suggesting levels (at all timepoints, including baseline) were affected by whether a subject was exposed to FA or DE. As no interaction between exposure condition and timepoint were seen it was concluded that the behavior of NSE levels through the day was not affected by whether a research subject was exposed to FA or DE.

Hypothesis 3: Concentration of BDNF in peripheral blood will decrease from baseline immediately or 3hr post DE exposure, relative to FA.

- Conclusion: No significant exposure and timepoint interaction was noted for BDNF. A significant effect of time was observed, suggesting that levels of these biomarkers fluctuated through the day. As no effect of exposure condition, or interaction between exposure condition and timepoint was noted for BDNF this suggests that the changes in levels of BDNF throughout the exposure day were not impacted by whether a research subject was exposed to FA or DE.

Chapter 5.0: Discussion

5.1 Overview

This study is the first of its kind to investigate the circulating levels of inflammatory cytokines and the CNS specific markers – S100B, NSE, and BDNF – in a controlled, blinded, human exposure to FA and DE. The hypothesis that a 2hr DE exposure at 300μg/m^3 $PM_{2.5}$ would increase levels of IL-6, TNF-α, S100B and NSE and decrease BDNF, was tested, with the aim of furthering the understanding of the acute effects of TRAP on these CNS. These biomarkers were selected for specific purposes: the cytokines IL-6 and TNF-α to determine if a systemic inflammatory response occurred following exposures as inflammation and immune activation is hypothesized to be a central to the pathway of the effect of air pollution on the brain. Secondly, serum S100B and NSE were selected in order to assess the acute effects of DE on brain pathology; these proteins are amongst the few that are considered CNS specific and measurable in human circulation. Finally, serum BDNF was measured as the protein is a neuroprotective factor, detectable in serum, that has been noted to decrease in a specific human model, following TRAP exposure. The exposure did not induce changes in levels of these biomarkers, and therefore, the results suggest that a 2hr exposure to DE does not acutely induce insults to the CNS in the examined model. However, when we consider the limitations of this study, including the relatively small sample size of young, healthy adults, the low sensitivity of the biomarkers considered and the exploratory nature of their selection, the hypotheses were not definitely tested, and therefore, the results do not conclusively suggest that an acute exposure to DE cannot impact the brain.

5.2 Levels of Biomarkers in Subjects

5.2.1 Levels of Inflammatory Cytokines IL-6 and TNF-α

With the exception of one subject, who had significantly outlying values for seven of their eight TNF-α values, levels of the inflammatory makers IL-6 in EDTA plasma and TNF-α in serum were within the expected ranges of the kit. For IL-6 the mean plasma value was 1.02 pg/ml (range: 0.16 – 6.76 pg/ml). R&D, the makers of the ELISA kit used, reported a mean concentration of 1.49 pg/ml (range: 0.428 – 8.87 pg/ml) on 35 EDTA plasma samples from healthy volunteers. In this study, the mean TNF-α concentration was 1.01 pg/ml (range: <LOD (1.5% of samples) – 1.88 pg/ml), excluding the outlier values, and R&D reported a mean concentration of 1.206 (range: 0.550 – 2.816 pg/ml) when TNF-α levels were assessed from 33 healthy subjects using this kit. The ranges found in this study were comparable to these values.

5.2.2 Levels of Neurologically Specific Biomarkers, S100b and NSE

Although Milipore, the makers of the S100B ELISA kit used, did not present an expected serum range, a brief literature search was performed to determine the typical level of this biomarker in healthy individuals (typically control subjects in clinical studies). A limitation to this is that the levels measured are likely to vary depending on the kits used, and there is some debate as to the exact "normal range" of S100B, but that said, the values reported in the literature were generally in line with what was seen in this study. One group noted 495 healthy controls had a medium serum value of 10 pg/ml, and 97.5% of the values were below 130 pg/ml (152). Another group found higher levels of serum S100B in healthy volunteers: 50 pg/ml with an interquartile range of 30 to 60 pg/ml. However, this variation may have been a result of a different kit being used (153). Regardless, the medium S100B value in the EAPOC study was 14.29 pg/ml, and the maximal value was 48.62 pg/ml, comparable to what has been previously reported in healthy individuals (152).

R&D reported that the serum samples of 15 volunteers had a mean NSE level of 3.02 ng/ml (range 1.85 – 4.14 ng/ml). The EAPOC study noted a comparable mean NSE value of 2.44 ng/ml (range: 0.89 – 6.86 ng/mL, excluding the one <LOD sample). Prior to removal of haemolysed samples, the mean NSE value was 2.75 ng/ml, with a maximal value of 13.04 pg/ml. This was expected as NSE is carried within red blood cells, and hemolysis will release the molecule into serum causing artificially elevated levels (31). These outlying NSE possibly occurred as a result of poor venipuncture technique as the technicians who drew blood were relatively inexperienced.

In the linear mixed effect model, time had a significant effect on S100B and NSE levels ($p = 0.037$ and $p = 0.001$ respectively, in the final models). This result was not associated with exposure condition, suggesting this relationship between timepoint of blood draw and predicted level of S100B or NSE followed the same trend regardless of whether the subjects were exposed to FA or DE. S100B levels were significantly lower at the immediately post-exposure timepoint (occurring at 12:31PM, on average) (effect size: -2.84 pg/mL, p-value 0.027) and at the 3hr post-exposure timepoint (occurring at 3:08PM, on average) (effect size = -2.72 pg/mL, p-value 0.038), than at baseline (occurring at 7:36AM, on average). There was no difference in S100B levels between the immediately post- and 3hr post-exposure timepoints (p-value 0.999). S100B levels across the study day were statistically impacted by the blood draw timepoint, a trend potentially explained by a diurnal effect.

To accommodate for the research subject's schedule, the timing of the 24hr post-exposure blood draw showed the widest variation of any timepoint: ranging from 7:00AM to 12:25PM, a 5hr and 25min difference. In contrast, the baseline, and immediately post- and 3hr-post exposure timepoint showed a maximum range of 4hr and 6min, 4hr and 43min, and 4hr and 24min, respectively. As with the immediately post- and 3hr post-exposure timepoints, there was a trend that levels of S100B were lower at the 24hr post-exposure timepoint than at baseline (effect size: -2.26). This trend was not statically significant ($p = 0.124$), possibly due to the wide

variation in terms of actual time that the 24hr post-exposure blood was drawn. This further supports the hypothesis that S100B followed a diurnal cycle, regardless of exposure condition, with levels being highest in the morning (7:30AM) and decreasing later in the day as the 24hr post-exposure blood draw occurred at a later time, the following day, than the baseline measurement. This observation cannot be confirmed as no peer reviewed research was found which examined the diurnal behaviour of S100B in serum.

Similar to S100B, NSE levels appeared to change throughout the exposure day, irrespective of whether a subject was exposed to DE or FA. Levels of NSE were comparable between the immediately post-exposure timepoint and baseline timepoint with an effect size of 0.016 ng/ml (p-value = 0.999) from baseline to immediately post-exposure. However, the levels were significantly lower at the 3hr post-exposure timepoint than at baseline, and immediately post-exposure, with an effect size of -0.466 pg/ml (p-value = 0.008), and -0.485 pg/ml (p-value = 0.004), respectively. The 24hr exposure blood draw varied significantly relative to the other timepoints but, generally occurred the following day at 9:21AM (range: 7:00AM – 12:15PM), a range comparable to the baseline and immediately post-exposure timepoints. If one assumes a diurnal effect occurs with NSE then levels at the 24hr post-exposure timepoint should hypothetically be comparable to those at baseline and immediately post-exposure (as was seen with S100B). In contrast, the NSE values at the 24hr post-exposure timepoint showed a trend of being lower than at baseline (effect size: -0.354 ng/ml, p-value = 0.075) and were significantly lower than immediately post-exposure (effect size: -0.373 ng/ml, p-value = 0.045). In contrast, NSE values were similar at 24hr post- and the 3hr post-exposure (effect size 0.112 ng/ml, p-value = 0.866).

An alternative theory to a diurnal effect occurring is that NSE levels were affected by sample processing. Due to staff restraints, blood samples were processed by an EAPOC technician at the end of the exposure day. Although the biological samples were moved to the fridge immediately following blood draw, they were not spun until the end of the study day. This occurred at approximately 4:00 pm on the exposure day, and an hour after blood draw on the

follow-up day. This meant that baseline samples were in the fridge the longest, followed by the immediately post-exposure samples, prior to processing. In contrast, the 3hr post- and 24hr post-exposure blood were typically centrifuged an hour after collection. This was not ideal, as the instruction manual for the NSE kit recommended that serum samples be allowed to clot for 30 minutes prior to centrifugation. Although the manufactures do not provide an upper limit to this time, the baseline and immediately post-exposure serum samples were processed, on average, 8.5 and 3.5 hours following blood draw; substantially longer than 30 minutes. Although no peer-reviewed literature can be found to address this hypothesis, it is possible that NSE was released from red blood cells in the unspun samples resulting in higher levels in the baseline and immediately-post exposure samples. Similarly, no peer-reviewed research was found which considers the diurnal behavior of NSE.

5.2.3 Levels of BDNF

As with the other biomarkers, levels of BDNF in serum were comparable to what was expected. R&D reports an average expected concentration of 27793 pg/ml (range: 6186 – 42580 pg/ml), well in line with the samples in this study (average concentration of 27779 pg/ml, range: 6315 – 56923 pg/ml). In the final mixed effect model, timepoint of blood draw was a significant predictor of BDNF levels ($p = 0.002$). When both FA and DE exposures were considered, BDNF levels were comparable between baseline and immediately post-exposure (effect size: -0.025 log(pg/ml), p-value = 0.954). However, BDNF showed a significant decrease at the 3hr post-exposure timepoint relative to baseline (effect size: -0.104 log(pg/ml), p-value = 0.038). There were no other significant differences between each timepoint, although there was a borderline significant trend that 24hr post-exposure values of BDNF were lower than the pre-exposure values (effect size: -0.100 log(pg/ml), p-value = 0.052).

Unlike NSE and S100B, several studies have been conducted which evaluate BDNF levels over multiple timepoints, and interestingly, some reported a diurnal trend. In plasma, BDNF levels in men were found to be highest in the morning (8am) and continued to lower at the

subsequent blood draw timepoints (12pm, 4pm, 8pm), reaching a minimal value at the end of the day (12am) (154). Plasma BDNF levels show a similar diurnal rhythm in women, although this trend was less prominent at some stages of the menstrual cycle (155). A similar pattern seen in serum samples; BDNF levels were highest at 8am, lower at the subsequent blood draw timepoints (2pm and 8pm), and reached a minimal value at 1:30am (156). The results from this study suggest a comparable trend of BDNF levels in serum: concentration was highest at the first blood draw, were lower (but not significantly so) at the second (12:30pm), and were lowest in the latest blood draw of the exposure day (3:00pm). However, the 24hr post-exposure timepoint did not follow this expected trend in that levels were not comparable to the baseline and immediately post-exposure timepoints, despite the sample being collected at the same time.

As with NSE, sample handling may have impacted BDNF levels. In 2008, Skogstrand and colleagues examined levels of various biomarkers, including BDNF, in serum, plasma, and dried blood. The authors noted that serum BDNF levels significantly increased if samples sat at 4^{o}C for 4 hours prior to centrifugation, and that levels increased further after 24 hours (157). As has been discussed, in the EAPOC study, baseline samples were typical processed 8.5 hours after blood draw, immediately post-exposure samples were processed 3.5 hours after blood draw, and 3hr post- and 24hr post-exposure samples were processed one hour after blood draw. Given this, it would be expected that baseline levels would be substantially higher than 3hr post- and 24hr post-exposure samples and, slightly higher than the immediately-post exposure timepoint: this was precisely what was observed. In conclusion, the literature supports two separate hypotheses to explain why BDNF levels varied over the exposure day: it is possible that diurnal effects impacted BDNF levels, or that sample handling was the causing factor. Interestingly, the same group also examined IL-6 and TNF-α in blood samples following various handling protocol ands noted that levels of IL-6 in plasma and TNF-α in serum increased significantly if samples were left at room temperature for over 4 hours, however, no changes occurred up to 48 hours after blood draw if samples were placed at 4^{o}C (157). This suggests that sample handling was not an issue for the inflammatory cytokines and supports our observation that time of blood draw had no effect on the levels of these inflammatory markers.

5.3 Study Hypothesis

5.3.1 Inflammatory Markers

Previous studies have demonstrated that exposure to various forms of combustion-derived air pollutants can induce a systemic inflammatory response. The pathway by which TRAP and related air pollutants may impact the brain is thought to involve maladaptive inflammation, oxidative stress and immune responses in the CNS, and systemic inflammation may be intermediate step in this process. In this work, plasma and serum levels of the cytokines IL-6 and TNF-α were measured following exposure to DE, relative to FA, for the following reasons: (1) These cytokines are among the pro-inflammatory molecules released following exposure to PM. By some argument, the examination of these markers was to serve as a positive control, however, as was discussed in Chapter 2, these cytokines are not consistently released following controlled human exposure and therefore, the examination of these cytokines does not represent a "perfect" positive control exploration **(158)**. (2) In the overarching EAPOC study, behavioral cognitive outcomes were tested in addition to the CNS specific blood makers. Given the potential intermediate role inflammation plays on the effects of PM on the CNS, it seemed reasonable to measure the inflammatory response in EAPOC subjects following DE exposure. An eventual goal of the overall study could be to correlate an inflammatory response with the other cognitive outcomes examined in the overarching EAPOC project. Despite these two reasons, it is important to note the major limitation that CNS specific inflammation could not be tested without access to CSF.

The approach to analyze markers of inflammation (IL-6 and TNF-α) in systemic circulation is an indirect method and, therefore, the findings presented here do not necessarily represent levels of CNS inflammation. Measurement of inflammation in the CSF would have, hypothetically, been a more direct indication of the inflammatory state of the CNS following DE exposure. Regardless, these mediators were selected as, in systemic circulation, they have been known to be capable of increasing BBB permeability, crossing the barrier and inducing neuro-

inflammatory responses (25,79,159). Additionally, elevated levels of IL-6 and TNF-α have been associated with cognitive decline and increased incidence of neurocognitive diseases in several cross-sectional and prospective studies (107,160), although this evidence has not always been consistent (108,109). In this study, there was no impact, up to 24 hours after exposure to DE, on circulating levels of IL-6 or TNF-α. This finding was not entirely unexpected as, while air pollution has been demonstrated to induced systemic inflammation and levels of these cytokines, the evidence has been inconsistent. Several studies considering environmental exposures have found an association between elevated levels of various forms of PM and increases in circulating concentrations of IL-6 and TNF-α (99,101,103-105) whereas controlled human exposure studies have yielded conflicting results, with some finding a positive association (106), and others not (151,161).

There are methodological differences between environmental assessment studies and controlled human exposures with each type of research having its own strengths and limitations. Firstly, it is less challenging to study exposure-responses (such as biological markers) in a large group of subjects in environmental studies, increasing study power and the likelihood of a statistically significant outcome. In contrast, a large study size can be expensive and time consuming with the controlled human exposure design. Similarly, epidemiological studies can more easily and safely select a population with increased susceptibility to the health effects of air pollution. Susceptibility has been defined as "a heightened risk for a particular [event] to occur compared with the general population at the same concentration of exposure" (23). It is thought that individuals with pre-existing conditions, such as cardiovascular risk factors, coronary artery disease, heart failure, chronic lung disease, metabolic syndromes (i.e. diabetes, obesity), and the very young and elderly, are more likely to be impacted by the health, and inflammatory effects of air pollution exposures (23,59). For example, Ruckerl and colleagues found a positive association between plasma IL-6, fibrinogen and CRP, and PNC of PM_{10} levels 12-17hr before blood draw. These authors conducted their research on a large population base (n = 1,003), and selected participants who had previously survived a myocardial infarction (MI) and therefore may have been more sensitive to the air pollution episode (101). Similarly, a study conducted in

Los Angeles, California, noted a positive association between elevated IL-6, TNF-α, and CRP with environmental levels of various indicators of combustion-related air pollutants when considering a sample size of 60 elderly subjects, who suffered from coronary heart disease (162). A Swiss study also found an increase in environmental PM_{10} was associated with higher levels of IL-6 and TNF-α in systemic circulation; however, once again, a significant population base of 6183 adults was studied (103). Furthermore, while controlled human exposures typically last one to two hours in durations due to safety and practical reasons, environmental studies can consider longer duration exposures: Delfino and colleagues noted that CRP and IL-6 levels were positively correlated with past-9 day levels of combustion related air pollutants (including $PM_{2.5}$) (163).

A considerable benefit of controlled exposure studies is that the air pollution exposures can be closely controlled and therefore predictable. A dose that is high, but still acutely safe, can be selected and implemented. Importantly, if an appropriate control, such as FA is used, any observed health outcomes can be directly attributed to the air pollution exposure. In contrast, it can be challenging to accurately characterize the air pollutant components and their concentrations in environmental studies and the issue of causality and confounders are hard to completely account for. While this is a clear advantage of the controlled human study design – such as the one presented here – the singular nature of the freshly generated DE exposure used may not completely represent exposures that occur in the ambient environment and, in this case, may not have been sufficient to induce an inflammatory response. The diesel engine in the exposure chamber produces exhaust typical of current engines, however, it is freshly generated, leaving minimal chance for it to become oxidized in the environment. As a result, the particles may not be capable of inducing a strong inflammatory effect in systemic circulation. Indeed, it has been noted that ozone can increase the potential for DE to initiate airway inflammation (164). There are environmental studies which observe an increase in inflammatory cytokines, such as IL-6 and TNF-α, in correlation with a short-term increase in PM or other combustion related compounds using a smaller sample size ($n < 50$) and healthy individuals (99,103,159). Such responses may occur as a result of exposure to particles, which have a different make up or toxicological potential than the DEPM generated in the APEL facility.

Additionally, there are a variety of methods that can be used to assess inflammation. In this study, levels of IL-6 in plasma, and TNF-α in serum, were utilized. Despite this, other markers, including CRP and counts of various immune and blood cells, to represent a cellular pro-inflammatory response, have also been considered with varied results (23). Recently, subtler physiological responses, such as epigenetic modifications, and changes in gene expression, have been analyzed following exposure to air pollution (23). Only serum and plasma were collected in the EAPOC study and therefore, cellular counts and epigenetic assessment could not be performed on the samples.

Given that blood was drawn at three times over the 24-hour period following exposure it seems unlikely that an acute inflammatory response was "missed" by this method. The null findings pertaining to a systemic inflammatory response may have occurred for a variety of reasons, including inadequate duration of exposure, a lack of sensitivity in the biomarkers used to assess systemic inflammation, or an issue that the PM within the freshly generated DE was not capable of eliciting such a response. The basis of assessing systemic inflammation in the EAPOC study was to determine if a systemic inflammatory pathway was an intermediate step in the effects of air pollution on the brain. Other pathways have been proposed, including the hypothesis that the PM component of TRAP directly activates the CNS immune system, entering the brain through translocation across the olfactory bulb, or by translocating across pulmonary tissue into systemic circulation to reach, and cross, the BBB.

A separate part of the EAPOC study involves assessment of various cognitive function outcomes. If DE exposure is found to induce effects in the other cognitive outcomes, it would seem unlikely that an inflammatory response was an intermediate step in this pathway as no systemic inflammatory response was observed in the subjects. However, it is important to note that the hypothesis that PM directly reaches and impacts the CNS, or the concept of an autonomic nervous system imbalance in response to DE exposure, were not directly tested by this study design. Furthermore, once the examination of all cognitive parameters considered in

the overarching EAPOC study is complete, an interesting avenue to explore would be to assess the correlation between systemic inflammatory response (i.e. subjects who showed an increase in IL-6 or TNF-α) and those who show adverse cognitive effects.

5.3.2 Effect of DE Exposure on the Blood Brain Barrier

The hypothesis that acute DE exposure increases BBB permeability was tested by measuring levels of S100B following a 120min exposure to DE. S100B is a calcium binding protein, which is primarily located in astrocytes, but also detect in pituicytes, neuronal progenitor cells, maturing oligodendrocytes and some neural populations within the CNS, and can also be detected in human serum (28,165). Elevated serum or CSF S100B is a marker of CNS damage (28,29) and increased BBB permeability, even in the absence of neurological damage (28,30,165). The BBB typically prevents the free diffusion of S100B from the CNS to systemic circulation and therefore, the molecule will more readily reach the blood if the BBB is damaged (166). An increase in serum S100B has been noted following mannitol infusion – a drug known to increase BBB permeability (30,167).

There was no indication that DE exposure increased serum levels of S100B at any timepoint post exposure, relative to what was seen after exposure to FA. This suggests that a 2hr exposure to DE does not alter BBB permeability. *In vitro* experiments have shown that DEPM is capable of disrupting the BBB by inducing oxidative and pro-inflammatory pathways in microvascular endothelial cells of the BBB (76,168). In *in vivo* exposures it has been proposed that inhaled nanoparticles may penetrate alveolar cell walls and travel through systemic circulation to reach the brain (16,19,20,81,82). While there is some experimental evidence to support this hypothesis, when we consider the context of the single 2hr exposure examined here, it seems less likely that BBB disruption would occur. The number of UFPM that would have been inhaled, translocated into systemic circulation, and reach the BBB would be substantially lower than what is seen in the *in vitro* studies and *in vivo* animal models described in Chapter 2.

It is unlikely that inhaled UFPM directly reached and affected BBB function at a sufficient level to allow the PM to cross.

However, when we consider that environmental air pollution exposures are generally chronic by nature, it seems more probable that the direct effects of PM and the general effects of air pollution-induced inflammation, in combination, aggravate the BBB. It remains possible that the effects of air pollution on BBB permeability occur through a chronic mechanism, rather than the acute 2hr exposure examined in the EAPOC study. For example, post-mortem analysis of healthy individuals who resided in Mexico City showed that these individuals had cerebral vasculature endothelial damage and indication of altered BBB function (18,25,79). These individuals were exposed to high ambient air pollution throughout their lifetime. A similar finding was also seen in dogs that lived in Mexico City (79,85).

Alternatively, DE exposure could have induced BBB effects that were too minor to be notable through a change in serum S100B levels. The biomarker has been seen to increase after severe CNS insults such as stroke, global ischemia (121,128), and traumatic brain injuries (122,129). It is estimated that levels above 350pg/ml suggest brain damage, and levels above 500pg/ml indicate poor prognosis (29). Not surprisingly, a 2hr exposure to DE did not appear to cause significant CNS injuries and the highest serum value noted in the EAPOC subjects was 48.62pg/ml. There is, however, evidence that S100B can be a more sensitive marker of BBB opening in cases of less dramatic, but more prolonged, environmental challenges. Researchers noted increased S100B levels in seven healthy volunteers hiking to altitudes above 3500m and up to 4554m for a period of 3 days; this increase occurred within the first day of hiking. The authors proposed that this was due to increased BBB permeability in response to hypoxic conditions (131). In contrast, no increase in S100B was seen in volunteers who sat in hypobaric chambers at the equivalent altitude of 4600m for 18 hours (165). Combined, this evidence may suggest that S100B is inadequately sensitive to consistently increase in these relatively minor CNS events, but in some cases may be able to.

Additionally, it is important to note that the concept that air pollution can impact the CNS is a new and developing research area, and therefore, there remain many answered questions with regards to the time frame in which effects may occur. For example, if PM does indeed reach the BBB and cause disruption, at this point, there is no consensus on specifically how long this process would take. S100B is a marker that is quick to respond to insult, and has a half-life of two hours (165). In fact, S100B has been noted to elevate within 40 minutes following minor head trauma (153). Assuming the effects of air pollution on the BBB were to occur shortly after air pollution exposure, then S100B would have increased at a time within the first 24 hours of exposure. It remains possible, however unlikely, that an effect on the CNS occurred at a later timepoint than those examined by this study. Finally, there are many limitations to S100B as a biomarker and many researchers have questioned its exact function and effects and the reasons for its increase in the brain and serum following cerebral injuries such as TBI (132). With this in mind, it is important to consider that S100B does not represent an "absolute" biomarker for CNS effects, although the protein was selected as it was identified as the best candidate that is detectable in blood. In conclusion, despite limitations, the results from this study showed that S100B did not increase following DE exposure, suggesting that a 2hr exposure to DE does not alter BBB permeability in human subjects.

5.3.3 Effect of DE on the Central Nervous System

The final step in the proposed pathway is that exposure to TRAP will induce an inflammatory and immune response in the CNS, leading to eventual neurotoxic effects. If combustion related PM is capable of reaching the CNS (through translocation across the olfactory bulb, or the BBB) it is thought that the particles themselves may activate microglial cells, causing the release oxidative and inflammatory proteins, which may, in turn, damage neurons (90). Similarly, a systemic inflammatory effect induced by air pollution could transfer to the CNS to activate microglial cells (18). The particles themselves may also induce direct cellular toxicity to neurons: an *in vitro* study found that dopaminergic neurons underwent cell death when exposed directly to DE particles (89,169). Hypothetically, the damage of neuronal cells

could further activate microglial cells, leading to a self-perpetuating toxicity in the CNS (18). Indeed, using MRI, white matter lesions were observed in otherwise healthy children who resided in Mexico City at significantly higher rates than in age matched controls living in the less polluted Mexican city of Polotitlán. Similarly, post mortem studies of dogs who lived in these cities showed that the Mexico City animals displayed mild reactive astrocytosis, CNS inflammation and frontal lesions (25).

NSE is a glycolytic enzyme located in the cytoplasm of neurons and neuroendocrine cells, and found in low concentrations in serum (133). It is not actively secreted into circulation so an increase in serum levels is thought to indicate neuronal damage (29). In this research, NSE levels did not significantly increase following DE exposure, suggesting that CNS injury, if any occurred, was below the level of detection for the biomarker. Interestingly, the mixed effects model generated did suggest that exposure condition had a significant effect on NSE levels ($p = 0.040^*$). However, the effect of exposure was not significantly correlated with timepoint ($p = 0.137$), suggesting that the overall effect of exposure-type on NSE values showed the same trend and behavior regardless of timepoint and whether the measurement was taken before or after exposure (Table 4.13). The effect was that NSE levels were significantly higher (effect size: 0.210 ng/mL) on FA exposure days than compared to DE days.

Upon closer examination of the results (Figure 4.5), a trend was noted whereby NSE levels were higher at the baseline and immediately post-exposure timepoints on FA exposure days than on DE days. As will be explained below, this was likely a chance finding. Because the levels for FA and DE were comparable at the later timepoints (3hr post- and 24hr post-exposure) a trend appears in the delta-analysis to suggest that, relative to what occurred on FA exposure days, NSE levels relatively increased (or, specifically, “decreased less”) from baseline at 3hr post- and 24hr post-exposure. In both cases a decrease of NSE levels from baseline occurred, however, following DE exposure, NSE levels, on average, decreased less than following FA exposure. For all subjects, the mean difference between the NSE concentration at 3hr post-exposure and baseline level was -0.65 (±1.62) ng/ml following exposure to FA, and -0.35 (±0.77)

ng/ml following exposure to DE. The mean difference between NSE concentrations at 24hr post-exposure and baseline level was -0.59 (±1.14) ng/ml following exposure to FA and -0.23 (±0.79) ng/ml following exposure to DE. While this trend was in the direction of the study hypothesis, it is important to note that it was not significant, or even borderline so ($p = 0.421$ and $p = 0.269$ for the 3hr post- and 24hr post-exposure "delta" timepoints, respectively).

Given the lack of statistical significance in this trend, including the fact that exposure condition and timepoint were not correlated, the most likely conclusion is that the trend occurred due to random chance and experimental error. It is noteworthy that NSE levels were higher at baseline on FA exposure days than on DE exposure days (3.09 ng/ml and 2.68 ng/ml, respectively). Similarly, the standard deviation for NSE levels on the FA baseline days was greater than on DE days (Table 4.7). A possible explanation for this was that hemolysis of the blood samples occurred, by chance, on more FA exposure mornings than DE. Although serum samples that appeared visually pink were discarded, even very limited hemolysis can increase NSE levels (170). It is possible that a sample was not obviously hemolysed, but that lysis of some red blood cells caused elevated levels of NSE. In conclusion, despite the trend discussed, it was concluded that a 2hr exposure to DE did not induce levels of NSE in serum relative to exposure to FA.

Serum NSE increases following acute cerebral injuries such as hypoxic brain damage, traumatic brain injury (31) and CNS hypoxia following cardiac arrest and CO poisoning (171), suggesting that significant CNS damage is needed for changes in levels of this biomarker. Of important interest to this study, NSE levels may reflect posttraumatic inflammatory responses in the brain following TBI (172). However, NSE is typically examined in cases of CNS trauma that is much greater than the level of damage, if any, that what would occur following an acute DE exposure. The cut-off levels to indicate substantial neurological damage in cases of stroke and other significant CNS insults is reported to be greater than 12.0-12.6 ng/mL (32,128,170) although levels less than this have been reported to be correlated with adverse neurological effects (170). Not surprisingly, the levels of NSE reported in this study do not suggest substantial brain damage – a similar conclusion to what was noted for the S100B biomarker results.

After stroke, serum NSE has been noted to increase within 4 to 8 hours, however, maximal levels may not be reached until after 24 hours (although this may be due to a separate physiological aspect in process of traumatic brain injuries that is not applicable to potential TRAP-induced CNS effects) (133). As the latest blood draw in the study was 24hr post-exposure, although it remains possible that a later measurement may have been optima if the effects of DE on the CNS are delayed. Regardless, the results suggest that there is no neuronal injury after acute DE inhalation, at least at a level that is detectable using NSE as a biomarker.

5.3.4 Effect of DE on BDNF

BDNF is a neurotrophic factor, which signals neurons to grow and differentiate, encourages their survival, and promotes long-term memory formation (34,135). Serum BDNF levels are lower in patients with various cognitive and neuropsychiatric disorders. Attenuated BDNF mRNA expression in the hippocampus is considered a potential pathogenic factor in Alzheimer's disease and depression (33). Importantly, BDNF is released following strenuous exercise, and the protein has been attributed to the beneficial effects of exercise on cognitive function (34). In trained rats, a bout of forced exercise increased BDNF mRNA in the hippocampus (139,140). Similarly, in humans, serum levels of BDNF increase following exercise and it is believed this occurs through release from the CNS (141). The exact source of exercise-induce BDNF is physiologically irrelevant as BDNF can readily cross the BBB and peripheral levels therefore reflect BDNF brain levels (142-144). However, the fact that research in both human and animal models suggest that exercise-induced BDNF occurs from brain release is important as it allows for comparability between the two study designs.

In this research, serum BDNF levels did not significantly decrease following DE exposure, relative to FA. The mixed effects model generated for the BDNF biomarker revealed no significant effect of exposure condition ($p = 0.162$) on BDNF levels. Although there was a

significant effect of blood draw timepoint (p = 0.002), there was no association with timepoint and exposure condition, indicating that the overall effect of blood draw time on BDNF values showed the same behavior regardless of whether the exposure was to DE or FA. Upon closer examination of the results (Figure 4.6) a pattern is noted whereby average BDNF levels are similar from baseline to immediately post-exposure following FA exposure, but decrease from baseline immediately following exposure to DE. BDNF levels are comparable at all other post-exposure timepoints, regardless of exposure condition. For all subjects, the mean difference between the BDNF concentration between baseline level and immediately post-exposure was +5.2 (±5185.9) pg/ml following exposure to FA, and -2166.2 (±7585.5) pg/ml following exposure to DE. However, it is important to note that the trend was not significant, or even borderline so (p = 0.312), and was only observed in 14 of the 26 subjects (54% of the population). Furthermore, the statistical results for the paired t-tests did not consider the fact that multiple comparisons were made, which further decreases the confidence in the statistical results and increases the risk that the finding occurred by random chance. Even ignoring this issue, a post-hoc power calculation for the paired t-test estimated that a very large sample size of 194 subjects would provide 80% power to generate statistical significance at the 95% confidence level or, alternatively, that an effect size of an average decrease of 4999 pg/mL BDNF immediately post DE exposure from baseline, relative to what was observed following FA exposure, would have been needed for a statistically significant result to be noted (given the standard deviation of the data and the number of subjects (n=27) involved).

Disregarding this weak trend at the immediately post-exposure timepoint, there was no evidence to suggest that serum BDNF levels were lowered as a result of exposure to FA relative to DE. This finding is somewhat in contrast to previous findings. In a cross-over study, Bos and colleagues found that cycling in the presence of air pollution attenuated exercise-induced BDNF release; it was hypothesized that systemic inflammation was responsible **(145)**. The EAPOC subjects showed no decrease in BDNF following DE exposure, however, due to the lack of sufficient exercise in the study to stimulate BDNF (an intensity of above 50% of VO2 max) this work does not directly compare to the findings by Bos *et al*. The conclusion from this is that air

pollution exposure may, specifically, prevent exercise-induced BDNF release, but not otherwise decrease serum BDNF at regular activity levels. Alternatively, the Bos *et al* (2011) finding may have occurred due to confounding variables as a result of the study design. The FA and TRAP exposure scenarios in this study were not perfectly identical; the FA exposure took place in a quiet room with a clean air source, whereas, for the TRAP exposure, subjects cycled along a busy roadway (145). This may have created a more stressful situation which potentially introduced a source of bias as BDNF levels have been reported to be inversely correlated with cortisol levels (155,156).

Despite this statement, there is evidence in animal models to suggest that systemic inflammation or exposure to air pollution may lower BDNF levels in the brain, regardless of exercise status. For example, rats intraperitoneally injected with LPS had a reduction of BDNF in some brain regions, which appeared to be mediated by the induced systemic inflammation (34). In 2012, Bos *et al* published a study in which rats were separated into four experimental groups: (1) exposed to UFP air and forced to exercise, (2) exposed to UFP air at rest, (3) exposed to ambient air and forced to exercise and (4) exposed to ambient air at rest (control). Each exposure condition lasted for 90 minutes, and rats were sacrificed 24 hours after the experiment. As expected, hippocampal BDNF mRNA was significantly up-regulated ($p < 0.05$) following forced exercise in FA, relative to the control (rest at FA). In contrast, hippocampal BDNF mRNA expression showed no change when the rats exercised in air pollution. This further supports the hypothesis that air pollution exposure blunts exercise-induced BDNF expression. Interestingly, in addition to this, there was a borderline-significant trend ($p = 0.059$) that BDNF mRNA levels decreased when rats were exposed at rest to UFPM, relative to being at rest and exposed to FA (136). The latter finding suggests that BDNF levels may decrease in response to PM exposure, even in the absence of exercise. However, it is important to note that the finding may have occurred by chance, as it was not statistically significant, and that the exposure to PM was much higher than the 300 $\mu g/m^3$ $PM_{2.5}$ used in the EAPOC study (136). Furthermore, it is possible that, if a similar response is seen in the human brain, following exposure to UFPM, that the effect is not strong enough for it to be detectable in BDNF serum.

In the interest of following up on the work from Bos *et al* 2011, who examined serum BDNF levels following exercise and air pollution exposure, serum BDNF was also examined in this study. However, measuring BDNF in plasma may have been a more sensitive method to determine the effects of DE exposure on BDNF levels. BDNF can be found in two locations in systemic circulation – both in free floating plasma (a relatively minor amount) and stored in platelets (the majority). In serum collection, BDNF is released from platelets during the clotting process, but in contrast, plasma collection vials contain an anti-coagulant, so platelet clotting and therefore, release of BDNF, does not occur. As a result, concentrations of BDNF are typically 200-fold higher in serum than in plasmas (33). Furthermore, freshly generated BDNF remains in plasma for approximately one hour, whereas platelets can circulate for up to eleven days. Therefore, plasma level likely more sensitively reflect acute fulgurations in BDNF release compared to serum levels, which likely represent both short term fluctuations and long term storage (33). It is thought that the hippocampus is the main source of BDNF plasma, although this is not currently scientifically confirmed (156). Although plasma levels of BDNF have not been examined in a TRAP exposure model, Bhang and colleagues examined 45 smokers and 66 non-smokers. They noted that baseline plasma BDNF levels were lower in smokers than in non-smokers, and that plasma BDNF levels, in smokers, increased to comparable concentration as the non-smokers following a four-week period of smoking cessation (173). Although it is important to note that this study examined a large sample size, and considered a repetitive exposure model (daily smoking), future studies could consider examining BDNF levels in plasma following air pollution exposure. It is critical to also note that "platelet poor" plasma should be collected to avoid any contamination from BDNF stored in platelets; plasma samples must be spun down two times and for a longer duration than what was used in the blood processing in the EAPOC study (173-176).

In conclusion, there was no statistically significant evidence to suggest that BDNF levels were decreased following a 2hr exposure to DE at 300 $\mu g/m^3$. A trend in the direction of the hypothesis was seen immediately post exposure, however, this finding was not significant, and

only relevant to half of the subjects. Never-the-less, there is support in the literature to suggest that BDNF levels may be decreased following air pollution exposure as a result of the induced systemic inflammatory response. Given the null findings with regard to BDNF, it is also noteworthy that no inflammatory effect was seen in these subjects following DE exposure. It would be interesting to examine BDNF levels in a study in which an observable inflammatory response was noted following air pollution exposure. Future studies could consider measuring BDNF in platelet-poor plasma, or examining the effects of a controlled TRAP exposure on exercise-induced BDNF levels by involving sufficiently intense exercise to induce BDNF.

5.4 Risk Assessment and Strengths and Limitations

Air pollution exposure is a significant health issue for populations worldwide; exposures to PM and combustion-derived pollutants from TRAP and other sources are common in the workplace and environmentally. In this study, healthy adults were exposed to DE, as a model of TRAP, and various cognitive outcomes were assessed before and after. Serum and plasma biomarkers were selected in the interest of determining if such an exposure could impact the CNS in a human model, and to provide indication of the biological mechanism responsible.

This study is unique in that it is the first to investigate the circulating levels of inflammatory cytokines and the CNS specific markers – S100B, NSE, and BDNF – in a controlled, blinded, human exposure to FA and DE. The hypothesis that a 2hr DE exposure containing 300μg/m^3 $PM_{2.5}$ would increase levels of IL-6, TNFα, S100B and NSE and decrease BDNF levels was tested with the aim of furthering the understanding of the acute effects of TRAP on the CNS. Although the exposure did not induce significant changes in the levels of these biomarkers, the results do not disprove the relationship between chronic air pollution exposure and adverse cognitive effects; this is especially the case given this model considered only an acute exposure. While the results suggest that acute DE exposure does not acutely induce insults to the CNS, it is important to highlight the insensitivity of these biomarkers and it cannot be ruled out that subtle effects occurred in the CNS that are not detectable by serum S100B, NSE or BDNF. No comment

can be made regarding the other cognitive outcomes examined in this study as this portion of the data was being analyzed at the date of this writing.

A considerable strength of the study is the controlled, double blinded cross over design. Each subject acted as their own control, and many aspects in the study including sleep, caffeine consumption, and research protocol on each exposure day were closely regulated. Additionally, blood draws were preformed frequently throughout the day, and the scheduling and blood processing were comparable regardless of whether subjects were exposed to FA or DE. Therefore, the fact that a diurnal pattern was seen for S100B, NSE and BDNF is not cause for concern. Neither the subject nor the researcher was informed of the exposure condition minimizing the risk of sampling bias. However, there were also weaknesses to the study design.

A considerable limitation was the use of a sample of convenience; the sample size goal (n = 30) was estimated powered to another endpoint (reaction time) from the pilot study and a power calculation performed from the results of the Bos *et al* study (145). Although no borderline significant trends were observed, there was a trend in the direction of the study hypothesis for BDNF. A power calculation indicated that an impractically large study size (n = 194) would be needed to provide 80% power to generate statistical significance at the 95% confidence level; already, a sample size of 27 subjects is quite significant for a controlled exposure study. Post-hoc calculations are controversial, and the calculation was performed in the interested only of highlighting how much smaller the EAPOC sample population was, relative to what may have been required to generate a positive result, assuming these trends were to continue. Given the lack of evident trends it seems unlikely that a larger sample size would change the outcomes of the study, however, the small sample size does remain a limitation of this work, as with most controlled exposure studies using a human model. An alternative post-hoc calculation suggested that a difference of 4999 pg/ml in delta (post – pre) BDNF levels would be required to yield positive findings given the sample size of 26 subjects at the post-exposure time point. Additionally, due to ethical and safety approval, the study was directed at healthy, middle aged individuals, despite the fact that the young and elderly appear to be more

susceptible to the CNS effects of air pollution (9,11,14,15,65). Similarly, evidence suggests that those with pre-existing cardiovascular and respiratory disease, metabolic syndromes (i.e. diabetes or obesity), and the elderly may be more susceptible to the cardiovascular, respiratory and inflammatory effects of TRAP and related exposures (23,59) and therefore, it is conceivable that these individuals may have increased susceptibility to the CNS effects of such exposures as well. For these reasons, controlled human exposure studies could instead consider exposures involving the elderly, those with known metabolic diseases or those already experiencing cognitive decline, presuming ethics approval was granted. For a similar reason, only a 2hr exposure was examined here, however, it is likely that the effects of TRAP on the CNS occur through a chronic, "multiple-hit" mechanism. As well, the fact that the exposure did not induce systemic inflammation suggests that oxidized DE may be more reflective of real world conditions, or might further highlight the lack of susceptibility of the population examined to the inflammatory effects of DE exposure. Finally, measurement of inflammatory markers and CNS specific proteins in the cerebrospinal fluid (CSF), opposed to serum, would have provided results which were more directly applicable to the CNS. However, CSF collection was not possible as ethics approval allowed only for the collection of blood.

Given these limitations, these findings do not take away from the important epidemiological research to find an association between residing in areas of higher TRAP and accelerated cognitive decline, or the animal research demonstrating that exposure to combustion-derived PM can induce CNS inflammation and alter brain morphology. There are many advantages of controlled human exposures over animal and epidemiological studies – notably the minimization of risk of confounding variables, and the elimination of the issue of interspecies differences. Further research that uses a human model and examines longer duration exposures, a different population, or more sensitive endpoints to detect CNS effects is clearly needed. While this work represents null findings, the results do not disprove a relationship between air pollution and adverse cognitive effects. In conclusion, the study suggests that the potential toxicological effects of TRAP on the CNS do not occur as a result of acute exposures.

5.5 Recommendations for Future Studies

It is clear from these findings that more sensitive outcomes to detect CNS effects are needed. As the BBB limits the flux of CNS proteins into systemic circulation, selecting an appropriate blood biomarker was a logistical challenge. The biomarkers chosen seemed the most appropriate as they are CNS specific – presuming no extra-cranial trauma (122) – and increase within 24 hours of CNS insults (133,176). However, they may not have been adequately sensitive as they are generally used as markers of relatively significant CNS trauma. Additionally, systemic inflammation could only be measured as a highly indirect indication of CNS inflammation. Although it would be challenging to receive ethical approval for such a study, collection and measurement of biomarkers and inflammatory cytokines in the CSF would provide results which are more brain-specific, and possibly more sensitive.

Other measures of cognitive function – including those examined within the EAPOC study, such as the CANTAB battery, balance assessment and a fMRI imaging – may also prove to be more sensitive markers of cognitive outcomes than S100B and NSE. Additionally, PET imaging is another example of a useful approach that could potentially be considered: a ligand for the peripheral benzodiazepine receptor (PK11195), which is upregulated in activated microglia, is being developed and is showing promise as a non-invasive approach to measure microglial activity in patients with neurodegenerative diseases such as Alzheimer's and Parkinson's disease (90). In future, a study which utilizes such a process, once the protocol has been adequately developed, could provide indication as to whether microglial cells are activated by DE exposure in an *in vivo* human model. Another method to detect changes in BBB permeability is the "CSF-serum albumin quotient" method, which is considered a "gold standard" to measure such an outcome. The principle is that albumin is a protein is synthesized in peripheral circulation and does not readily diffuse across the BBB, and therefore, the ratio of $[CSF_{albumin}]/[Serum_{albumin}]$ is typically low (approximately 0.0007). However, if the BBB is damaged then the ratio will increase as albumin gains access to the CSF through the disrupted BBB (166). If ethical approval were to

allow for CSF collection in volunteers, then the ratio of CSF to serum albumin could be measured following exposure to DE.

Despite the limitations in this study there was a, weak, trend that DE exposure was associated with a relative "increase" in NSE levels from baseline (or rather, less of a decrease) relative to FA exposure, at the 24hr post-exposure timepoint. It is critical to emphasize that this trend likely occurred by chance. Furthermore, it is most probable that, due to error in blood draws, several samples were slightly hemolysed on the morning of the FA exposure, causing NSE levels to be higher before the FA exposure than before DE. It is recommended that any future study that examines NSE in a controlled human exposure ensures that all venipuncturists are well experienced drawing blood prior to the study. Similarly, having the same technician perform the blood draw on each day will minimize the risk of random error.

The weak trend in the direction of the hypothesis for BDNF was also a noteworthy finding, although again, this trend was not statistically significant and therefore likely occurred as a result of random chance. To adequately test the findings from Bos *et al* (2011), future studies using controlled exposure chambers could examine BDNF levels following sufficient exercise to stimulate BDNF. Additionally, measurement of BDNF in plasma following acute exposure to DE would be another interesting avenue of research. It is thought that the concentration of BDNF in plasma is a more acute representation of fluctuations in BDNF levels in the brain (33,156) and, furthermore, there is evidence that current smokers have a decreased plasma concentration of BDNF (170). As a separate avenue, it may be possible to examine BDNF levels using a significantly larger sample size: over the past years, several controlled human studies in the APEL laboratory using a similar exposure protocol have collected plasma and serum samples, and many of these still remain in the -80^{o}C freezer. If resources were to allow, a future study in the laboratory could consider levels of BDNF in these samples before and after exposure to DE and FA.

Finally, given the biological mechanism that has been proposed pertaining to how PM exposure may impact the CNS, it seems likely that this relationship applies to more chronic exposures. Although an animal study did find that a 2hr exposure of DE induced an inflammatory response in the cortex and cerebellum of mice, the exposure involved was also substantially higher (1900 μg/m^3 DEPM) than what would ever be seen in a real world ambient scenario or allowed in a human exposure study (95). Research in human models examining the cognitive impacts of air pollution exposures should be designed to consider chronic exposures. Hypothetically, a controlled study could examine levels of inflammatory cytokines, S100b, NSE, and BDNF in workers frequently exposed to DE in their workplace. In such a study, samples could be drawn on a Sunday afternoon after a weekend away from work, and then again on Friday afternoon after a full work week. Controls could be an occupational group who perform a comparable job with regards to exercise, work hours, and stress levels, but not exposed to significant sources of TRAP. Similarly, environmental studies could examine measures of these biomarkers following an air pollution episode or compare levels of the biomarkers between individuals living in polluted and non-polluted cities. In these study designs care would be needed to ensure controls are adequately selected. For example, for BDNF, a significant source of bias could be introduced if research subjects exercise less during the air pollution episode.

In conclusion, these results show that a 2hr exposure to DE does not increase BBB permeability or induce CNS damage at a level that can be detectable using serum S100b or NSE as biomarkers. Similarly, a controlled exposure to DE did not impact BDNF levels in serum. Future studies in human models may provide further evidence as to the effects of air pollution on the CNS, particularly if the study design utilizes more sensitive biomarkers of CNS pathology or more proximal biomaterial, such as cerebrospinal fluid.

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Appendix I: Summary of the Cognitive Function Parameters Considered in EAPOC Study

**Focus of work by PhD student Jason Curran but provided here for context*

a) CANTAB Battery: A CANTAB battery was administered to each subject a total of 8 times (4 batteries for each exposure: prior to and immediately following exposure, 3-hours post-exposure, and 24-hours post-exposure). Specifically, each battery includes the following tests:

i. RVP: Rapid Visual Processing is a task of continuous performance and visual sustained attention. It is sensitive to changes in the parietal and frontal lobe areas of the brain. The task consists of a 2-minute practice stage and a 7-minute assessed stage during which single numerals appear at a rate of 100 digits per minute. Participants must identify a series of target sequences (2-4-6, 3-5-7, and 4-6-8) and touch a button immediately after to indicate they have detected the sequence. A' prime is a signal detection measure that reflects target sensitivity, regardless of the participant's tendency, or bias, to respond.

- *Main outcome for analysis: A' prime; mean latency*
- Subsidiary outcome: total false alarms

ii. DMS: Delayed Matching to Sample is a task which assesses forced choice recognition memory for non-verbalisable patterns. DMS tests both simultaneous and delayed (short-term) visual recognition memory. It is sensitive to changes in the medial temporal lobe area, with some input from the frontal lobes. Participants were shown a complex visual pattern and then, after a delay of 0s, 4s or 12s, will be shown four choice patterns. Participants must identify the pattern which exactly matches the target image.

- *Main outcome for analysis: mean correct latency (all delays)*
- Subsidiary outcome measures: % correct (all delays, 12s, 4s, 0s), median correct latency (12s, 4s, 0s), probability of error

iii. RTI: Reaction Time is a task of simple and choice reaction time, movement and vigilance. The participant must hold down a button until a yellow spot appears on the screen, then release the button and touch where the yellow spot appeared. The spot appears in a single location in the simple reaction time task and in one of five locations in the choice reaction time task.

- *Main outcome for analysis: mean five-choice reaction time*
- Subsidiary outcome measures: median of reaction time (simple and five-choice test), median movement time, (simple and five-choice tests), error score (simple and five-choice tests).

iv. AST: Attention Switching Task is a sensitive measure of frontal lobe and 'executive' function. The test measures the ability of the participant to switch attention between the direction or location of an arrow on the screen. In the task, an arrow appears on the screen and can be

located on the left or right side and pointing either to the left or to the right. Each trial is preceded by a cue, 'which side?' or 'which direction?', indicating how the participant should respond. The task assesses two aspects of cognitive function: it allows for detection of a Stroop-like effect – by comparing latencies and errors from trials in which arrow direction and location are congruent – and a task-switching effect – by comparing response latencies and errors from trials in which participants have to follow the same rule versus a switch rule.

- *Main outcome analysis: median response latency (switched, non-switched), median response latency (congruent, incongruent) → these are used to calculate 'switch cost' (switched – non-switched) and 'congruency cost' (incongruent – congruent)*
- Subsidiary outcome measures: total correct trials, median reaction latency, switch errors, non-switch errors, congruent errors, incongruent errors

v. SWM: Spatial Working Memory is a test sensitive to measure of the frontal lobe and 'executive' function which tests a subject's ability to retain spatial information and to manipulate remembered items in working memory. The test begins with a number of coloured squares being shown on the screen. The aim of the test is that, by process of elimination, the subject will find one blue 'tolken' in each of a number of boxes until all are found.

b) VAS, Mood state assessment: The mood state assessment questionnaire will be administered using the CANTAB touchscreen tablet following each CANTA battery.

c) Static Balance Assessment: Balance assessment will be conducted before and immediately after each exposure condition using the Balance Error Scoring System (BESS). In brief, the test is an easily administered challenge which incorporates three different stances (double, single leg and tandem). This protocol is commonly used in sports medicine.

d) fMRI: Functional MR and spectroscopy wase performed at the Child and family Research Imaging Facility (Women's and Children's Hospital) Each subject underwent a functional MRI of the brain before and after exposure to DE and FA. Functional neuroimaging (fMRI) is a magnetic resonance imaging technique which assesses the organization of the brain and its activity through detection of the change in magnetiation between oxygen-rich and oxygen-poor blood to assess changes in blood flow related to neural activity in the brain. The scanning protocol was be as follows: resting stat fMRI scan, following by fMRI administration of the Sternberg test of working memory.

The major goal of the MRI portion of the project iwa to compare changes in the active network from baseline (pre-exposure) to post-exposure in both FA and DE conditions and demonstrate changes in the network of active brain regions following DE exposure. Specifically, the Sternberg test of working memory is employed as the aim of this study, in particular, is to assess changes in the active brain regions associated with the task of working memory.

Appendix II: Typical Plate Design of Samples

All ELISA analysis was performed on a 96-well (12*8) plate. In order to prevent error from "batch effects" care was taken to ensure all samples from each subject were ran on the same plate with its own internal control (standard curve). However, within each plate the samples were not randomized, which may have potentially created errors due to "edge effect" on the plate. This was not initially concerned a concern as subject exposures were supposedly counter balanced; this would mean that half of the DE exposures would be ran in the upper 4 wells of the ELISA plate, and the other half would be run on the lower 4 wells, and similar for FA. However, after the ELISA kits had been run it was discovered that the subjects were not properly counter balanced as more subjects were exposed to FA first (n=17) than DE first (n=10). This means that more of the FA exposures were ran on the top 4 wells of the plate than the bottom. Reasons why this was not concerning are as follows: (1) a 4-tip micropipette tip was used, meaning that reagents were added to the top 4 wells using the same respective pipette tip as the bottom 4 wells and (2) with the exception of TNF-α, exposure day (which was directly correlated with location on the ELISA plate) was not associated with levels of the other biomarkers in the linear mixed effect models.

The following schematic shows the typical design of the plates for subjects who had a complete set of blood draws; note that the subject numbers are interchangeable and that all samples were run in duplicates with the exception of TNF-α.

Figure AII.1: typical layout of ELISA plate

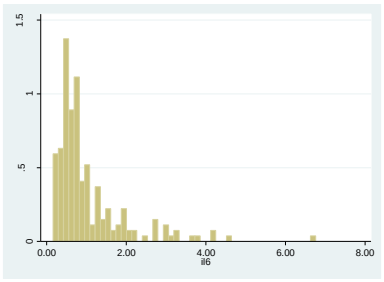
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	STD Curve [highest]	STD Curve [highest]	Sub# 101 Day*: 1 Time#: 1	#101 Day: 1 Time: 1	#102 Day: 1 Time: 1	#102 Day: 1 Time: 1	#103 Day: 1 Time: 1	#103 Day: 1 Time: 1	#104 Day: 1 Time: 1	#104 Day: 1 Time: 1	#105 Day: 1 Time: 1	#105 Day: 1 Time: 1
B	STD Curve dilution 1	STD Curve dilution 1	#101 Day: 1 Time: 2	#101 Day: 1 Time: 2	#102 Day: 1 Time: 2	#102 Day: 1 Time: 2	#103 Day: 1 Time: 2	#103 Day: 1 Time: 2	#104 Day: 1 Time: 2	#104 Day: 1 Time: 2	#105 Day: 1 Time: 2	#105 Day: 1 Time: 2
C	STD Curve dilution 2	STD Curve dilution 2	#101 Day: 1 Time: 3	#101 Day: 1 Time: 3	#102 Day: 1 Time: 3	#102 Day: 1 Time: 3	#103 Day: 1 Time: 3	#103 Day: 1 Time: 3	#104 Day: 1 Time: 3	#104 Day: 1 Time: 3	#105 Day: 1 Time: 3	#105 Day: 1 Time: 3
D	STD Curve dilution 3	STD Curve dilution 3	#101 Day: 1 Time: 4	#101 Day: 1 Time: 4	#102 Day: 1 Time: 4	#102 Day: 1 Time: 4	#103 Day: 1 Time: 4	#103 Day: 1 Time: 4	#104 Day: 1 Time: 4	#104 Day: 1 Time: 4	#105 Day: 1 Time: 4	#105 Day: 1 Time: 4
E	STD Curve dilution 4	STD Curve dilution 4	#101 Day: 2 Time: 1	#101 Day: 2 Time: 1	#102 Day: 2 Time: 1	#102 Day: 2 Time: 1	#103 Day: 2 Time: 1	#103 Day: 2 Time: 1	#104 Day: 2 Time: 1	#104 Day: 2 Time: 1	#105 Day: 2 Time: 1	#105 Day: 2 Time: 1
F	STD Curve dilution 5	STD Curve dilution 5	#101 Day: 2 Time: 2	#101 Day: 2 Time: 2	#102 Day: 2 Time: 2	#102 Day: 2 Time: 2	#103 Day: 2 Time: 2	#103 Day: 2 Time: 2	#104 Day: 2 Time: 2	#104 Day: 2 Time: 2	#105 Day: 2 Time: 2	#105 Day: 2 Time: 2
G	STD Curve dilution 6	STD Curve dilution 6	#101 Day: 2 Time: 3	#101 Day: 2 Time: 3	#102 Day: 2 Time: 3	#102 Day: 2 Time: 3	#103 Day: 2 Time: 3	#103 Day: 2 Time: 3	#104 Day: 2 Time: 3	#104 Day: 2 Time: 3	#105 Day: 2 Time: 3	#105 Day: 2 Time: 3
H	BLANK 1	BLANK 2	#101 Day: 2 Time: 4	#101 Day: 2 Time: 4	#102 Day: 2 Time: 4	#102 Day: 2 Time: 4	#103 Day: 2 Time: 4	#103 Day: 2 Time: 4	#104 Day: 2 Time: 4	#104 Day: 2 Time: 4	#105 Day: 2 Time: 4	#105 Day: 2 Time: 4

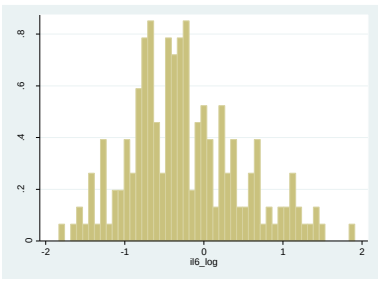
*Day represents exposure day

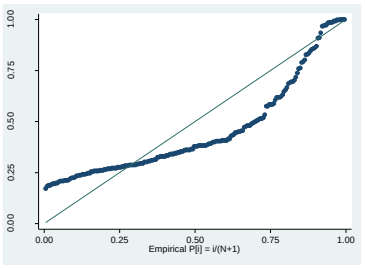
#time represents exposure time point.

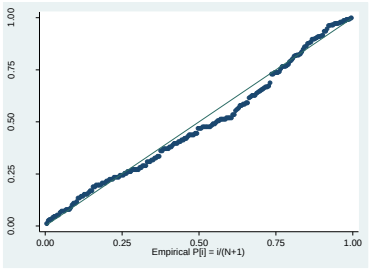
Appendix III: Histograms and Information to Determine Skewedness of Data

Marker: IL-6







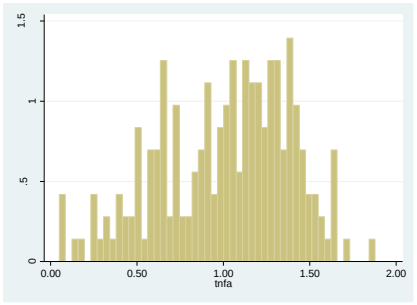


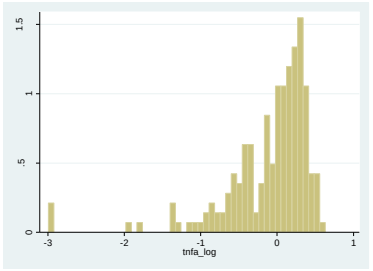
Marker	Parameter	Value	
		Untransformed data	Log-transformed data
IL-6	Arithmetic mean	1.025	-0.246
	Median	0.740	-0.301
	Standard deviation (SD)	0.916	0.701
	Geometric SD (S_g = e^SD) (GSD)	2.02	

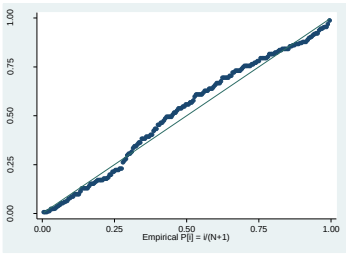
Marker	Test	p-value	
		Untransformed data	Log-transformed data
IL-6	Skewness	0.0000	0.0048
	Kurtosis	0.0000	0.7784
	Shapiro-wilk test	0.00000	0.00473

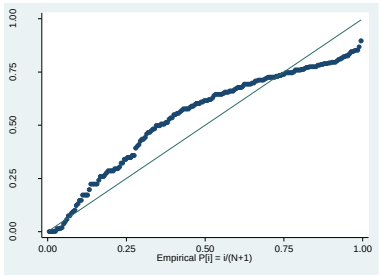
Conclusion: neither the untransformed nor the log-transformed IL-6 data matched a perfect distribution, however, the decision to log-transform the data was made based on the following rationale: the geometric standard deviation was larger than 1.5 and the arithmetic mean was also larger than the median (1.025 vs. 0.740), suggesting skewed data. Although the statistical testing for skewness and the Shapiro-Wilk test suggested that neither the transformed or untransformed data sets followed a normal distribution (p-value > 0.05), however, the un-transformed data had yielded considerably lower p-values and therefore failed the tests “more strongly”. Finally, through visualization of the histograms and quantile plots it appeared that the data was more closely normally distributed once log-transformed.

Marker: TNF-α







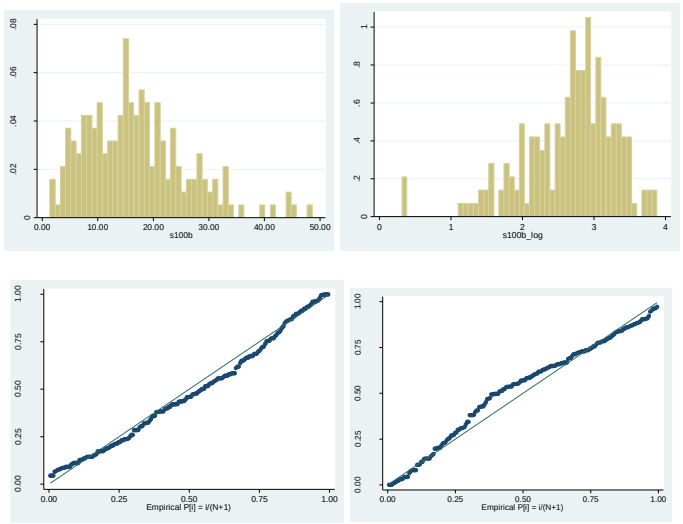


Marker	Parameter	Value	
		Untransformed data	Log-transformed data
TNF-α	Arithmetic mean	1.015	-0.102
	Median	1.070	0.677
	Standard deviation (SD)	0.384	0.582
	Geometric SD (S_g = e^SD) (GSD)	1.790	

Marker	Test	p-value	
		Untransformed data	Log-transformed data
TNF-α	Skewness	0.0166	0.0000
	Kurtosis	0.1104	0.0000
	Shapiro-wilk test	0.00191	0.0000

Conclusion: neither the untransformed nor the log-transformed TNF-α data matched a perfect distribution, however, the decision to NOT log-transform the data was made based on the following rationale: although the geometric standard deviation was larger than 1.5 (1.790) suggesting the data was slightly skewed, the arithmetic mean was comparable to the median (1.015 vs. 1.070). Although the statistical testing for skewness and the Shapiro-Wilk test suggested that neither the transformed or untransformed data sets followed a normal distribution (p-value > 0.05), the transformed data yielded considerably lower p-values and therefore failed the tests “more strongly”. Finally, through visualization of the histograms and quantile plots it appeared that the data was more closely normally distributed prior to being log-transformed.

Marker: S100B

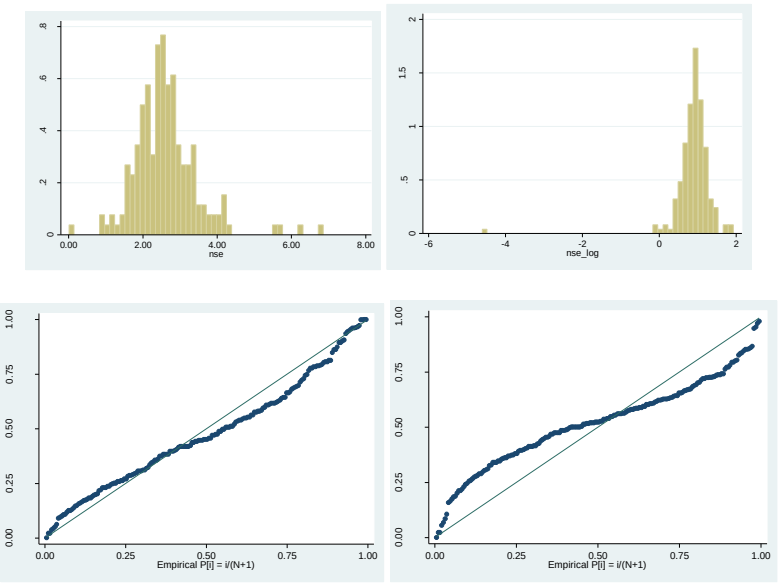


Marker	Parameter	Value	
		Untransformed data	Log-transformed data
S100B	Arithmetic mean	16.960	2.660
	Median	16.015	2.774
	Standard deviation (SD)	9.155	0.642
	Geometric SD (S_g = e^SD) (GSD)	1.90	

Marker	Test	p-value	
		Untransformed data	Log-transformed data
S100B	Skewness	0.000	0.0000
	Kurtosis	0.0493	0.0033
	Shapiro-wilk test	0.000001	0.0000

Conclusion: neither the untransformed nor the log-transformed S100B data matched a perfect normal distribution, however, the decision to NOT log-transform the data was made based on the following rationale: although the geometric standard deviation was larger than 1.5 (1.90), suggesting the data was slightly skewed, the arithmetic mean was comparable to the median (16.960 vs. 16.015). The statistical testing for skewness and the Shapiro-Wilk test suggested that neither the transformed or untransformed data sets followed a normal distribution (p-value > 0.05) and there was essentially no difference between the p-values yielded. Given this inconclusive evidence, more visualization of the histograms was critical. The histograms and quantile plots it appeared that the data was more closely normally distributed prior to being log-transformed.

Marker: NSE

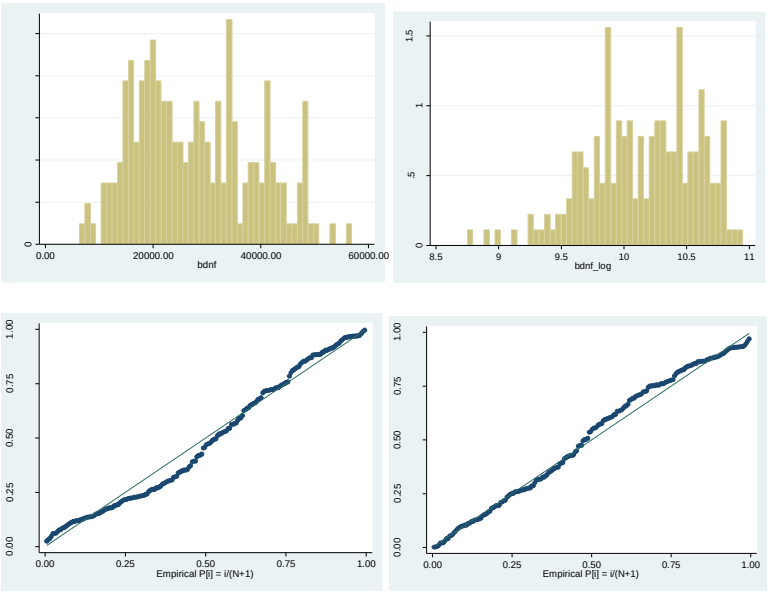


Marker	Parameter	Value	
		Untransformed data	Log-transformed data
NSE	Arithmetic mean	2.61	0.89
	Median	2.51	0.92
	Standard deviation (SD)	0.859	0.504
	Geometric SD (S_g = e^SD) (GSD)	1.66	

Marker	Test	p-value	
		Untransformed data	Log-transformed data
NSE	Skewness	0.0000	0.0000
	Kurtosis	0.0000	0.0000
	Shapiro-wilk test	0.00000	0.0000

Conclusion: neither the untransformed nor the log-transformed NSE data matched a perfect normal distribution, however, the decision to NOT log-transform the data was made based on the following rationale: the geometric standard deviation was slightly larger than 1.5 (1.66), suggesting the data may be skewed, the arithmetic mean was comparable to the median (2.61 vs. 2.51). The statistical testing for skewness and the Shapiro-Wilk test suggested that neither the transformed or untransformed data sets followed a normal distribution (p-value > 0.05) and there was essentially no difference between the p-values yielded. Given this inconclusive evidence, more visualization of the histograms was critical. The histograms and quantile plots it appeared that the data was more closely normally distributed prior to being log-transformed.

Marker: BDNF



Marker	Parameter	Value	
		Untransformed data	Log-transformed data
BDNF	Arithmetic mean	27779	10.147
	Median	26723	10.190
	Standard deviation (SD)	11026	0.429
	Geometric SD (S_g = e^SD) (GSD)	1.536	

Marker	Test	p-value	
		Untransformed data	Log-transformed data
BDNF	Skewness	0.0368	0.0097
	Kurtosis	0.0004	0.6594
	Shapiro-wilk test	0.00018	0.00074

Conclusion: neither the untransformed nor the log-transformed BDNF data matched a perfect normal distribution, and it was challenging to determine whether log-transformation should occur, however, the decision to log-transform the data was made based on the following rationale: the geometric standard deviation was only slightly above 1.5 (1.53), suggesting the data may be skewed. The arithmetic mean was significantly larger that the median in the untransformed data set (27779 vs. 26723), however, the values were comparable when the data was log-transformed (10.147 vs. 10.190), suggesting the un-transformed data was skewed, whereas the log-transformed data was not. The statistical testing for skewness and the Shapiro-Wilk tests suggested that neither the transformed or untransformed data sets followed a normal distribution (p-value > 0.05), although the skewness tests did suggest that the untransformed data set was less skewed. The histogram and quantile plots were comparable with both the

untransformed and transformed data, however, overall it appeared that the data was more closely normally distributed after being log-transformed.

Appendix IV: R Data and Analysis Codes for Mixed Effects Model

Data Sets used for statistical analysis

Classes ‘tbl_df’, ‘tbl’ and 'data.frame': 224 obs. of 41 variables:

```
$ SubjectID          :Factor w/ 27 levels "101","102","103",..: 1 1 1 1 1 1 1 1 2 2 ...
$ Gender             :Factor w/ 2 levels "M","F": 2 2 2 2 2 2 2 2 1 1 ...
$ screeningage       :num  22.5 22.5 22.5 22.5 22.5 ...
$ exposure1age       : num  22.6 22.6 22.6 22.6 22.6 ...
$ exposure2age       : num  22.7 22.7 22.7 22.7 22.7 ...
$ daysbetweenexp     : num  27.4 27.4 27.4 27.4 27.4 ...
$ exposuretype       : Factor w/ 2 levels "FA","DE": 1 1 1 1 2 2 2 2 2 2 ...
$ exposureday        : Factor w/ 2 levels "D1","D2": 1 1 1 1 2 2 2 2 1 1 ...
$ ordertype          : Factor w/ 2 levels "1st","2nd": 1 1 1 1 1 1 1 1 2 2 ...
$ timepoint          : Factor w/ 4 levels "Pre","Post","3hr",..: 1 2 3 4 1 2 3 4 1 2 ...
$ hemolysis          : num  NA NA NA NA NA NA NA NA NA NA ...
$ il6                : num  0.53 0.8 0.76 0.47 0.37 0.56 0.57 0.87 1.77 1.96 ...
$ tnfa                       : num  0.62 0.39 0.47 0.65 1.12 1.12 1.21 0.58 0.92 0.66 ...
$ tnfaorigional      : num  0.62 0.39 0.47 0.65 1.12 1.12 1.21 0.58 0.92 0.66 ...
$ s100b              : num  14.21 16.42 9.36 15.48 10.42 ...
$ nse                        : num  6.86 6.3 0.01 2.75 2.5 3.3 1.94 2.75 2.79 2.4
$ nseorigional       : num  6.86 6.3 0.01 2.75 2.5 3.3 1.94 2.75 2.79 2.41 ...
$ bdnf               : num  39593 31695 29510 41594 35405 ...
$ il6basedif         : num  NA 0.27 0.23 -0.06 NA ...
$ tnfabasedif        : num  NA -0.23 0.03 -0.15 NA ...
$ s100bbasedif       : num  NA 2.21 -4.85 1.27 NA ...
$ nsebasedif         : num  NA -0.56 -6.85 -4.11 NA 0.8 -0.56 0.25 NA -0.38 ...
$ bdnfbasedif        : num  NA -7898 -10083 2001 NA ...
$ sleepqual          : Factor w/ 5 levels "1","2","3","4",..: 3 3 3 4 2 2 2 NA 4 4 ...
 $ caffeine          : num  0 0 0 0 0 0 0 NA 1 1 ...
```

Mixed Effects Model

1. Testing for order interaction

```
>il6ordexp <- lmer(log(il6)~exposuretype*exposureday+(1|SubjectID),data=eapoc)
>anova(il6ordexp)

Analysis of Variance Table of type III  with  Satterthwaite
approximation for degrees of freedom
                           Sum Sq Mean Sq NumDF  DenDF F.value  Pr(>F)
exposuretype              0.11658 0.11658     1 174.77 0.75447 0.38626
exposureday               0.45190 0.45190     1 174.77 2.92445 0.08902 .
exposuretype:exposureday 0.10687 0.10687     1  24.83 0.69161 0.41354
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2. Testing for interaction between gender and age

```
>il6expgend <- lmer(log(il6)~exposuretype*Gender+(1|SubjectID),data=eapoc)
>anova(il6expgend)
```

```
Analysis of Variance Table of type III  with  Satterthwaite
approximation for degrees of freedom
                     Sum Sq  Mean Sq NumDF   DenDF F.value Pr(>F)
exposuretype        0.311756 0.311756     1 174.857 1.98504 0.1606
Gender              0.245260 0.245260     1  24.864 1.56164 0.2231
exposuretype:Gender 0.002631 0.002631     1 174.857 0.01675 0.8972
```

3. Determining individual fixed effect variables independently (exposure type, time-point, gender, age and exposure day)

>il6fix <-
lmer(log(il6)~exposuretype+Gender+timepoint+exposure1age+exposureday+(1|SubjectID),data=eapoc)
>anova(il6fix)

```
Analysis of Variance Table of type III  with  Satterthwaite
approximation for degrees of freedom
              Sum Sq Mean Sq NumDF   DenDF F.value  Pr(>F)
exposuretype 0.11658 0.11658     1 171.535  0.7616 0.38405
Gender       0.27359 0.27359     1  23.539  1.7872 0.19405
timepoint    0.70636 0.23545     3 172.125  1.5381 0.20642
exposure1age 0.63398 0.63398     1  23.482  4.1414 0.05329 .
exposureday  0.45190 0.45190     1 171.535  2.9520 0.08758 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

4. Determining effect of exposure and timepoint alone and as interaction

>il6mixed1 <- lmer(log(il6)~exposuretype*timepoint+(1|SubjectID),data=eapoc)
>anova(il6mixed1)

```
Analysis of Variance Table of type III  with  Satterthwaite
approximation for degrees of freedom
                        Sum Sq Mean Sq NumDF  DenDF F.value Pr(>F)
exposuretype           0.32827 0.32827     1 169.72 2.09170 0.1499
timepoint              0.70782 0.23594     3 170.27 1.50339 0.2155
exposuretype:timepoint 0.08871 0.02957     3 169.72 0.18842 0.9042
```

5. Determining effect of exposure and timepoint alone
>il6mixedf <- lmer(log(il6)~exposuretype+timepoint+(1|SubjectID),data=eapoc)
>anova(il6mixedf)

```
Analysis of Variance Table of type III  with  Satterthwaite
approximation for degrees of freedom
              Sum Sq Mean Sq NumDF  DenDF F.value Pr(>F)
exposuretype 0.31822 0.31822     1 172.73  2.0567 0.1533
timepoint    0.70811 0.23604     3 173.27  1.5255 0.2096
```

6. Post hoc-testing

```
>pw1 <- summary(lsmeans(il6mixedf,revpairwise~timepoint))
>pw1$contrasts

contrast       estimate         SE     df t.ratio p.value
 Post - Pre   0.13112421 0.07675723 173.30   1.708  0.3225
 3hr - Pre    0.02523862 0.07777141 173.43   0.325  0.9882
 3hr - Post  -0.10588559 0.07887151 173.77  -1.343  0.5372
 24hr - Pre  -0.02347522 0.07895164 173.71  -0.297  0.9908
 24hr - Post -0.15459942 0.07933781 173.45  -1.949  0.2118
 24hr - 3hr  -0.04871384 0.08038247 173.63  -0.606  0.9301

Results are averaged over the levels of: exposuretype
Results are given on the log scale.
P value adjustment: tukey method for comparing a family of 4 estimates

>pw2 <- summary(lsmeans(il6mixedf,revpairwise~exposuretype))
>pw2$contrasts

contrast    estimate         SE     df t.ratio p.value
 DE - FA  -0.04749634 0.03381105 173.01  -1.405  0.1619

Results are averaged over the levels of: timepoint
```