UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

AN INVESTIGATION OF STRESS-RESPONSES IN PREGNANT WOMEN EXPOSED TO AMBIENT AIR POLLUTION IN DURBAN, SOUTH AFRICA

By

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DECLARATION

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PRESENTATIONS

The paper entitled: The effect of NOx pollution on oxidative stress in pregnant women living in Durban, South Africa by Anderson, S.M., Naidoo, R.N., Ramkaran, P., Phulukdaree, A., Muttoo, S., Asharam, K., Chuturgoon, A.A. was presented at the following local and international conferences (Poster):

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The paper entitled: OGG1 Ser326Cys polymorphism, HIV, obesity and air pollution exposure influences adverse birth outcome susceptibility, within South African Women by Anderson, S.M., Naidoo, R.N., Ramkaran, P., Asharam, K., Muttoo, S., Chuturgoon, A.A. was presented at the following local conferences (Poster):

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8-OHdG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AA	Amino acid
AAP	Ambient air pollution
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AP	Air pollution
ARE	Antioxidant response element
ART	Antiretroviral treatment
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BD	Becton Dickinson
BER	Base excision repair
BH4	Heme and tetrahydrobiopterin
BiP	Binding immunoglobulin protein
BMI	Body mass index
bp	Base pair
BP	Blood pressure
BTB	Broad complex/tramtrack/bric-a-brac
BW	Birthweight
bZip	Basic leucine-zipper
C	Carboxyl
Ca^{2+}	Calcium ion
CAT	Catalase
CD4	Cluster of differentiation 4
cDNA	complementary deoxyribonucleic acid
CHOP	CCAAT/enhancer-binding protein (C/EBP) homologous protein
CI	Confidence interval
CNC	Cap 'n' collar
cNOS	Constitutive nitric oxide synthases
CO	Carbon monoxide
Cu	Copper
Cul	Cullin
Cys	Cysteine

DGR	Double glycine receptor
dH_2O	deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ds	Double stranded
DSIB	Durban south industrial basin
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthases
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ETC	Electron transport chain
ETS	Environmental tobacco smoke
FAD	Flavin adenine dinucleotide
Fe	Iron
Fig.	Figure
FMN	Flavin mononucleotide
Fpg	Zinc-finger motif
GA	Gestational age
gp	Glycoprotein
GPx	Glutathione peroxidase
GRP78	78 kDa glucose-regulated protein
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S transferase
GSTM1	Glutathione S transferase Mu 1
GSTP1	Glutathione S transferase Pi 1
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
Hb	Haemoglobin
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HIV-	Human immunodeficiency virus negative

HIV+	Human immunodeficiency virus positive
IC	Intracellular
IFN	Interferon
IL	Interleukin
Ile	Isoleucine
Inc.	Incorporation
iNOS	Inducible nitric oxide synthases
IP3R	Inositol-1,4,5,triphosphate receptor
IRE	Inositol-requiring enzyme
IUGR	Intrauterine growth restriction
IVR	Intervening region or linker region
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein
LBW	Low birthweight
LP	Lipid peroxidation
m	Minutes
MACE	Mother and Child in the Environment
Maf	Musculoaponeurotic fibrosarcoma
MDA	Malondialdehyde
methHB	Methaemoglobin
$MgCl_2$	Magnesium chloride
min	Minutes
miR	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
N	Nitrogen
n	Sample size
NADPH	Nicotinamide adenine dinucleotide phosphate
NBW	Normal birthweight
ND	North Durban
NEDD	N-(1-Naphthyl)ethylenediamine
Neh	Nrf2-ECH homology
nNOS	Neural nitric oxide synthases
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthases

NOx	Oxides of nitrogen
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ns	Non-significant
O_2	Molecular oxygen
O_2^-	Superoxide
OGG1	8-oxoguanine glycolase 1
OR	Odds ratio
ORAC	Oxygen radical absorbance capacity (Total antioxidant activity)
OS	Oxidative stress
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PK	Protein kinases
PM	Particulate matter
$PM_{0.1}$	Ultrafine particulate matter
PM_{10}	Coarse particulate matter
$PM_{2.5}$	Fine particulate matter
POM	Polycyclic organic matter
PTB	Pre-term birth
PTP	Permeability transition pore
PUFA	Polyunsaturated fatty acid side chains
QH·	Ubisemiquinone radical intermediate
RFLP	Restriction fragment length polymorphism
RISC	Ribonucleic acid -induced silencing complex
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Relative risk
RT	Room temperature
RT-PCR	Real-Time polymerase chain reaction
s	Seconds
S1P	Serine protease 1
S2P	Metalloprotease site-2 protease

SA	South Africa
SD	South Durban
sec	Seconds
Ser	Serine
SNP	Single nucleotide polymorphism
SO_2	Sulphur dioxide
SOD	Superoxide dismutase
Sp	Specific protein
ss	Single stranded
SST	Serum-separating tubes
SULF	Sulfanilic acid
T1	Trimester one
T3	Trimester three
TB	Term birth
TBA/BHT	Butylated hydroxytoluene solution
TBARS	Thiobarbituric acid reactive substances
TNF	Tumour necrosis factor
TSP	Total suspended particles
UP	Unfolded proteins
UPR	Unfolded protein response
USA	United States of America
UTR	Untranslated region
Val	Valine
VCl_3	Vanadium trichloride
wt	Wild-type
Zn	Zinc

ABSTRACT

Living or working within an unhealthy environment is attributed to 12.6 million deaths worldwide and 2.2 million deaths in Africa. Ambient air pollution (AAP) exposure is amongst the major contributors of environmental and air quality decay. Durban South Africa (SA) is a rapidly developing city that requires increased infrastructure, transportation, and energy production to support the growing urban population. This leads to air quality degradation, in addition to the heavy burden of human immunodeficiency virus (HIV) and obesity SA faces increase the susceptibility of pathological conditions including respiratory diseases and adverse birth outcomes. Infants *in utero* are particularly vulnerable to adverse AAP effects, attributed to oxidative stress (OS), inflammation and genetic susceptibility, due to their biological vulnerability, sensitivity to their environment and rapid differentiation and growth.

South Durban (SD) comprises a complex mix of dense residential settlements and heavily industrialised areas with high levels of air pollution (AP). This makes SD an ideal location to investigate the effects of AAP, in particular, traffic-related AP (atmospheric oxides of nitrogen (NOx)), on OS and endoplasmic reticulum (ER) stress responses within third trimester pregnant women. A comparison sample of pregnant women, located within north Durban (ND) of similar socio-economic status were used for this study. The susceptibility of OS markers, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) DNA adducts, lipid peroxidation (LP) and nitric oxide (NO) levels, on adverse birth outcomes, including low birthweight (LBW) and pre-term birth (PTB), were also determined. Additional risk factors such as HIV, obesity and single nucleotide polymorphisms (SNP) within genes of the antioxidant response pathway were investigated for OS and adverse birth outcome susceptibility. Atmospheric NOx pollution data were obtained from land use regression modelling that was previously reported.

Atmospheric NOx and maternal serum 8-OHdG adducts were significantly elevated within SD living pregnant women. This induction of DNA damage was found to be the direct consequence of NOx exposure. Pregnant women carrying the variant and wild-type (wt) genotypes of glutathione S transferase (GST) P1 and M1 SNPs, respectively, increased the susceptibility of NOx induced OS. Exposure to increased NOx levels significantly reduced the gestational age (GA) of these pregnant women, with increased susceptibility for mothers carrying male neonates.

The wt 8-oxoguanine glycosylase 1 (OGG1) Ser326Cys genotype was found to be associated with both HIV and obesity. Therefore pregnant women infected with HIV (HIV+) and carrying the wt genotype significantly increased the risk for HIV associated LBW and PTB. In addition, living within SD and being exposed to higher levels of AAP significantly increased the susceptibility for PTB. Comorbid HIV and obesity were identified as additional risk factors for birthweight (BW) reduction.

Increased maternal serum NO levels were observed within HIV+ women, with reciprocal activity on malondialdehyde (MDA) levels. Increased levels of NO directly reduced BW, especially for HIV+ and SD living women. This suggests NO may play a key role in LBW aetiology as a consequence of HIV infection and traffic-related AP. HIV was shown to differentially modulate MDA's effect on neonatal BW. Exposure to increased levels of NOx and HIV infection induced the expression of microRNA (miR)-144, which was shown to negatively regulate nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This transcription factor, Nrf2, was shown to significantly increase antioxidant gene expressions. Therefore the induction of miR-144 was implicated as a mechanism for increased OS due to HIV and NOx exposure. In addition, elevated ER stress genes were observed within HIV negative SD living patients. Hence, exposure to higher levels of AAP within SD led to increased ER stress, which may act reciprocally on the induction of ROS leading to increased OS.

These findings indicate that exposure to atmospheric NOx, elevated AAP levels within SD and exposure to HIV infection resulted in increased OS with increased susceptibility towards adverse birth outcomes within pregnant women. Further studies into the mechanisms proposed within a larger population including multiple pollutants and gene interactions may give additional insight into the aetiology of adverse birth outcomes as a consequence of AAP exposure.

INTRODUCTION

The atmosphere is an essential shared source that protects and supports life. However, the atmosphere is threatened by anthropogenic activities where large amounts of pollutants are released into the ambient air daily. This threatens the air quality, where pollution load has become greater than pollutant dispersion, leading to ambient air pollution (AAP).

Ambient air pollution has been implicated in a wide range of pathological conditions that have contributed to 5.4% of deaths worldwide [1]. These conditions include cardiovascular disease, asthma, acute respiratory infections and lung cancer [2, 3]. Adverse effects of AAP on human health have gained global interest. The most vulnerable population are infants *in utero*. Exposure to AAP has been linked to adverse birth outcomes such as low birth weight (LBW) and pre-term birth (PTB) and is associated with infant morbidity, mortality and risk of development of respiratory and neurocognitive disorders later in life [4]. The exact mechanism by which AAP exposure leads to adverse birth outcomes has yet to be determined, however oxidative stress (OS) and inflammation have been implicated including genetic susceptibility.

South Africa (SA) is a developing country with a growing urban population which requires increased infrastructure, energy and natural resources for human consumption [5]. This has led to the deterioration of the environment and air quality. A major consequence of urbanisation is increased road traffic due to the lack of functional public transport, increased vehicle fleets with increased emissions from traffic congestion, poor vehicle maintenance, and aged vehicles. Additionally, due to poor land use planning, dense human settlements are in close proximity to heavily industrialised areas [5]. This is most noticeably observed within the Durban south industrial basin (DSIB) or south Durban (SD) in SA, where a large portion of the population resides in low-income households or informal settlements. They form dense communities in close proximity to busy roads, for easy access to transport for daily activities, and amidst industrialised areas. These communities also rely heavily on domestic fuels, such as coal, paraffin, and wood, for cooking and heating. Upon burning of these fuels, pollutants such as oxides of nitrogen (NOx) are released [5]. As a result, large communities are exposed daily to large amounts of AAP and this in combination with the heavy burden of human immunodeficiency virus (HIV) and obesity that SA is facing, drastically impacts human health.

Oxides of nitrogen comprise 60-70% of pollution associated with vehicle emissions [5]. These nitrogen (N) centred free radicals, upon inhalation, are absorbed into the lung tissue and produce

free radicals which enter the blood stream. Subsequently, they react with macromolecules (proteins, lipids and deoxyribonucleic acids (DNA)) present causing damage including lipid peroxidation (LP) and the production of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) mutagenic lesions [6–8]. Cytoprotection against these insults are provided by antioxidant enzymes including glutathione S-transferases (GSTs) that scavenge and eliminate reactive oxygen species (ROS) to prevent oxidant-related damage [9, 10]. Repair mechanisms including the DNA repair pathway and its key enzyme human 8-oxoguanine glycosylase 1 (OGG1), help mitigate stress through the repair of damaged macromolecules [11, 12].

The antioxidant response is regulated via the antioxidant response element (ARE) and its key transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [13–15]. Upon increased oxidative damage, Nrf2 dissociates from kelch-like ECH-associated protein 1 (Keap1) and translocates to the nucleus. It then binds to the ARE resulting in transcriptional activation of specific target genes including antioxidants e.g., superoxide dismutase 2 (SOD2) and catalase (CAT) [9, 16]. However, if the production of ROS exceeds the scavenging capacity of antioxidants, OS results leading to extensive macromolecular and cellular damage.

Excessive OS may lead to the disruption of cellular calcium homeostasis. The endoplasmic reticulum (ER) acts as a major reservoir of intracellular (IC) calcium and therefore plays a critical role in calcium homeostasis [17, 18]. This homeostasis is necessary for the proper function of the protein folding machinery; disruption results in the activation of the unfolded protein response (UPR) pathway that endeavours to restore balance within the ER [19].

Genetic susceptibility is an important determinate of an individual's response to toxic insult, viz. AAP exposure, HIV infection or obesity [20]. Single nucleotide polymorphisms (SNP) within antioxidant genes including OGG1, GST Mu (M) 1 and GST Pi (P) 1 affect their enzymatic activity and scavenging potential which could lead to increased OS and subsequent damage [10, 21, 22]. Epigenetics influence and regulate gene expression [23]. Non-coding or micro- ribonucleic acids (RNA) (miR) are important for Nrf2 regulation. MiR-144 inhibits the expression of Nrf2 messenger RNA (mRNA) [24] whilst miR-28 regulates Nrf2 post-transcriptionally by promoting Nrf2 degradation [25]. These genetic changes in human genes may lead to increased susceptibility to disease and neonatal conditions including LBW and PTB.

An unhealthy environment is linked to ~2.2 million deaths within Africa, of these, ~270,000 deaths are due to neonatal conditions [26]. In addition, ~57% of the total SA population living with HIV are women [27] with 68% being obese [28], suggesting that women and infants *in utero* are greatly at risk for negative impacts of AAP exposure. Pregnancy, a physiological state characterised by its high-energy and elevated basal oxygen demand, favours ROS production and increased susceptibility to OS [29]. In the presence of external or internal stressors viz. AAP and infection, the antioxidant stores would become saturated leading to increased OS [30, 31]. Exposure to tobacco smoke and traffic-related air pollution (AP) have been associated with increased OS markers within pregnant women [32–34] and reduced foetal growth, LBW and PTB [35, 36]. Although OS has been implicated in adverse birth outcome aetiology [37], no mechanism linking AAP induced OS to adverse birth outcomes have been identified. In addition, HIV and obesity influences need to be considered, due to the heavy burden of these conditions in SA.

RESEARCH RATIONALE, AIM, HYPOTHESIS, OBJECTIVES AND STUDY DESIGN

Research Rationale

In order to reduce the incidence of adverse birth outcomes due to AAP exposure, improvements in diagnosis, interventions, and treatments are mandatory. For this to occur it is essential to understand how exposure to AAP induces adverse birth outcomes. This will ensure that novel biomarkers and targeted interventions can be developed. Oxidative stress has been implicated and exploring associated mechanisms may give insight into the aetiology of adverse birth outcomes which could help in its proper management.

In SA, we have a unique population, heavily burdened with HIV and obesity. In addition to genetic susceptibility, these conditions may affect an individual's response to environmental exposures. Investigating these interactions is therefore paramount for biomarker and intervention research.

Aim

The study aimed to investigate the effect of ambient air pollution exposure on oxidative and endoplasmic stress profiles within third trimester (T3) pregnant women, their subsequent effect on neonatal adverse birth outcomes and whether HIV and obesity influenced these effects. The study was conducted by comparing pregnant women living in the heavily polluted south Durban, with pregnant women living in the less-industrialised north Durban (ND), SA.

Hypothesis

Exposure to high pollution levels and HIV infection would induce oxidative stress profiles within pregnant women and lead to increased incidence of adverse birth outcomes including reduced foetal growth.

Objectives

The study was designed to determine:

1. The effect NOx pollution had on oxidative stress markers within T3 and their subsequent effect on adverse birth outcomes
2. Whether polymorphisms within genes of the antioxidant response pathways: GSTP1 and GSTM1, and DNA repair pathway: OGG1, affected the susceptibility of adverse birth outcomes within pregnant woman exposed to AAP, HIV, and obesity.

3. Whether epigenetic regulation of the Nrf2-Keap1 pathway by miR-144 and miR-28 within T3, was influenced by exposure to higher or lower pollution levels and HIV infection.
4. The effect NOx pollution exposure in SD and ND had on the induction of the endoplasmic reticulum stress pathway within T3 and their subsequent effect on adverse birth outcomes.

Study Design

This study was conducted as a branch of the Mother and Child in the Environment (MACE) birth cohort study. Ethical clearance from the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BF263/12) (Appendix 1) and informed consent from study participants was obtained.

Briefly, women were recruited from public antenatal clinics present within three hospitals (Wentworth, Prince Mshiyeni and King Edward VIII Hospitals) in the heavily polluted SD. Whilst, a comparison sample of women with similar socio-economic status were recruited from public antennal clinics present within three hospitals (Addington, Mahatma Gandhi and King George V Hospitals) in the less-industrialised ND, in SA.

Upon enrolment, a questionnaire was completed which included the following relevant information: demographics, residential and antenatal history and potential confounding factors such as environmental and occupational exposures, maternal smoking, dietary history and pre-existing medical conditions. Follow up interviews were conducted at each trimester subsequent to enrolment and prior to delivery, to evaluate any changes such as exposures, dietary changes and pregnancy complications. In addition, clinical parameters of the mother were assessed at trimester one (T1) and T3 visits including height, weight and the levels of haemoglobin (Hb), iron (Fe) and blood pressure (BP) that were used in subsequent analyse discussed in the following chapters.

The basic clinical data of all neonates were obtained from birth records, including intra-labour history, birthweight (BW) and other anthropometric data. These were used in subsequent analyse discussed in the following chapters.

Environmental exposure data and other biochemical data collection methods are discussed in detail within the relevant chapters.

CHAPTER 1

LITERATURE REVIEW

1.1. Ambient air pollution

The deterioration of air quality and general environmental degradation are frequently observed within developing countries. Rapid industrialisation, economic expansion, increased urbanisation due to the natural growth of the urban population and rural migration are implicated [5, 38]. This deterioration of air quality is of major public health concern, as exposure to AAP has been associated with adverse health consequences ranging from pulmonary to neurological conditions, including cardiovascular diseases, cancer and most notably adverse birth outcomes [2–4].

Anthropogenic activities are the major cause of environmental AP; emitted from industrial facilities, motor vehicles, forest fires, tyre burning and burning of fossil fuels among others. Various pollutants have been identified; differing in their chemical composition and reactivity, whether they are transported via long or short ranges and their persistence within the environment. Their composition within ambient air ranges from gaseous pollutants, such as NOx and sulphur dioxide (SO_2), to particulate matter (PM), such as dust, pollen and ultrafine particles ($PM_{0.1}$). The main route of exposure is via inhalation, with ingestion and dermal contact minor routes of exposure. Upon inhalation, pollutants are absorbed and depending on their mode of action may enter general circulation and propagate their effects [3, 38]. The induction of OS within the lung, whether by inducing oxidants or impairing antioxidants; which initiates a cascade of responses, has been implicated in AAP mode of toxicity on human health. An influx and activation of inflammatory cells is one such response, where large quantities of free radicals are generated and released which may potentiate the pollutant effects [6].

1.1.1. Gaseous pollutants

1.1.1.1. Nitrogen oxides

Several forms of NOx exist within the ambient air, however, the two main forms are nitrogen dioxide (NO_2) and nitric oxide (NO) [39]. Nitrogen oxides are generated by reactions between oxygen and N at high temperatures from combustion processes. Greater quantities of NO (90-95%) compared to NO_2 (5-10%) are released into the air. In ambient air, NO rapidly oxidises with oxidants present (oxygen, ozone, and volatile organic compounds) to form NO_2 [6, 39, 40].

Major outdoor sources of NOx, are combustion processes from motor vehicles and stationary sources (i.e. power generation and heating). In urban areas, road traffic is the major contributor to atmospheric NOx. Principal indoor sources include tobacco smoke and burning of fossil fuels, wood, gas, oil, and kerosene for cooking or heating (Figure 1.1). Indoor levels are also determined by outdoor sources; poor ventilation and close proximity to heavily congested roads, would increase NOx levels indoors [39, 40].

Figure 1.1 Sources of Nitrogen oxides. The major outdoor sources are depicted in A) motor vehicle emissions, which produce 60-70% total NOx emissions in urban areas, and E) stationary combustion sources such as factories. The main indoor sources are depicted in image B) smoking and burning of C) wood and D) gas, common practices in the majority of households for cooking and heating (Images adapted from the internet [41]).

Nitrogen dioxide has limited solubility in aqueous solutions and is an N-centred free radical with strong oxidising potential [6]. Inhalation of atmospheric NO_2 and subsequent absorption and degradation in the lung fluids, forms metabolically activate and stable nitrites and nitrates. These metabolites are rapidly absorbed and translocated via the blood stream. Due to their strong oxidising potential, they are capable of interacting directly with lung-tissue components (protein and lipids) present resulting in a cascade of radical production, thereby exerting its

effect [3, 34]. Studies have shown, in animal models and under environmentally relevant levels of NO_2, atmospheric NO_2 elevates LP (dose and time-dependent), reduces antioxidant enzyme activities including SOD, glutathione reductase and glutathione peroxidase (GPx) [42] and decreases reduced glutathione (GSH) [43–45]. Exposure *in utero* has been associated with placental LP, impaired neonatal development and PTB [34, 46, 47].

1.1.1.2. Ozone

Ozone is highly reactive and formed through the interaction of sunlight with NOx and reactive hydrocarbons [48]. It is the main component within photochemical smog and the inhalation of slightly elevated concentrations (60–120 ppb) may cause several respiratory symptoms including decreased lung function, pulmonary inflammation, and increased airway hyper-reactivity. Ozone is a relatively insoluble gas and powerful oxidant; it directly oxidises the substrate present (cells present at the air-tissue interface, including protein and lipids) resulting in a cascade of secondary ozonation products [6] and LP [48]. Studies have reported oxidative damage due to the activation of heat shock proteins, Nrf2, pro-inflammatory cytokines and c-Jun onco genes [49, 50].

1.1.1.3. Carbon monoxide

Carbon monoxide (CO) is formed through the incomplete combustion of carbonaceous fuels including wood, kerosene, petrol, coal and natural gas. The main outdoor sources of exposure are from road transportation and parking areas, whilst indoor sources include combustion of domestic fuels for cooking and heating [39, 40]. It is a reproductive toxicant that interferes with oxygen delivery to the foetus. Carbon monoxide displaces oxygen from Hb which shifts the oxyhaemoglobin dissociation equilibrium. It has been demonstrated that CO crosses the placental barrier which leads to its accumulation. This leads to low levels of oxygen within foetal blood as a result of CO-poisoning and causes oxidative injury [46, 51].

1.1.1.4. Sulphur dioxide

Sulphur dioxide is generated through the combustion of sulphur-containing fossil fuels, such as coal and heavy oils. It is also produced through the smelting of sulphur containing ores [3]. The induction of OS, as a consequence of SO_2 reactivity properties within an aqueous environment, has been implicated in the role of SO_2 toxicity [52]. Studies in mice have reported reduced antioxidant activity (SOD and GPx) [53], elevated LP in mice testicles [54], brains, liver, lungs,

and heart [53]. Exposure *in utero* has been associated with functional and developmental toxicities [46, 55].

1.1.2. Particulate matter

Particulate matter consists of a broad spectrum of particle types, of which a large portion induces OS via radical formation. The major sources of PM include power plants, factories, refuse incinerator fires, motor vehicles and windblown dust [40]. Particles are classified by their relative diameter (µm); coarse (PM_{10}), fine ($PM_{2.5}$), $PM_{0.1}$ and nanoparticles. Inhaled PM_{10} particles are mainly deposited on the upper airways and removed via mucociliary clearance; whilst $PM_{2.5}$ and $PM_{0.1}$ (about 50% of PM_{10} composition) are able to reach the lung parenchyma. Thus suggesting, $PM_{2.5}$ and $PM_{0.1}$ contribute more significantly to PM toxicity [3, 38, 56].

The relatively small size of PM allows it to escape phagocytosis, be absorbed into the blood stream and disperse into various organs, upon entry into the lung. This PM entry into the body could result in oxidative injury and pro-inflammatory processes within the lungs and other target organs, including the placenta [46, 57]. Ultrafine particles have been shown to cross the placental barrier and interact with membrane proteins, especially growth factor receptors and impair their function. This could lead to decreased placental size and impaired nutrient and oxygen exchange; which has been associated with increased blood coagulation and viscosity which alters blood perfusion [57].

Major constituents of PM are transition metals and polycyclic aromatic hydrocarbons (PAH). They have been associated with OS, cytokine induction, and inflammation [58, 59].

1.1.2.1. Transition metals

Transition metals present on the particle surface have the ability to induce OS. These metals enter the environment through waste-water discharges, combustion, and manufacturing facilities [3]. Metals such as Fe, cobalt, and chromium undergo redox cycling, whilst lead, nickel, and mercury deplete GSH and protein-sulphydryl groups resulting in the production of ROS [56]. Metal catalysts are important in the Fenton oxidation reaction, where superoxide and hydrogen peroxide interact with transition metals, i.e. Fe and copper (Cu), via Fenton- Haber-Weiss reaction (Figure 1.2) to form hydroxyl radicals. Metal ions are also capable of direct interaction with cellular molecules and generate radicals such as thiol radicals. Thiol radicals may react with other thiol molecules to generate singlet oxygen which is converted to hydrogen peroxide

and induces additional ROS generation [60]. Metal ions also bind to proteins which disrupt antioxidant enzyme function and attack nucleic acids and proteins that result in the disruption of gene expression [3].

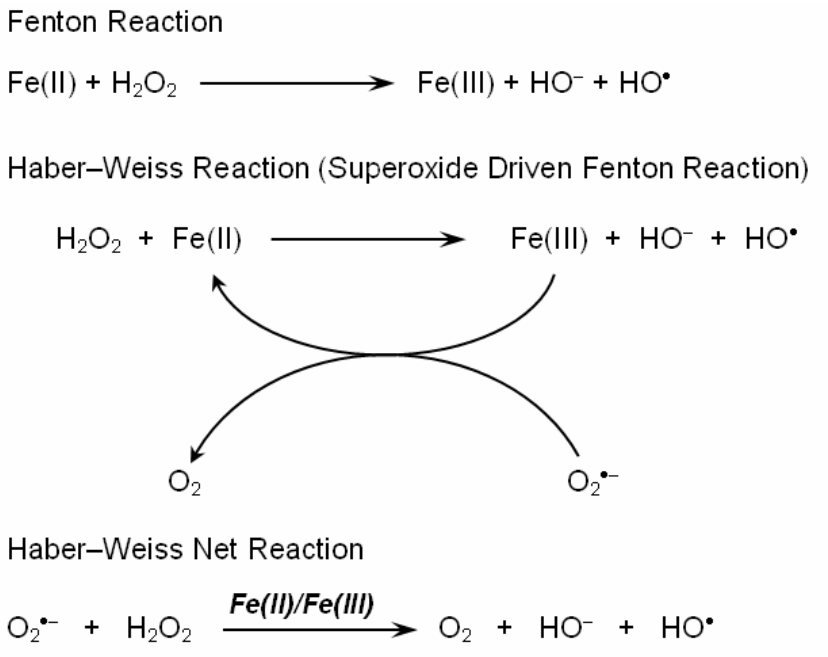

Figure 1.2 The Fenton- Haber-Weiss reaction. The Haber-Weiss net reaction produces hydroxyl radicals from hydrogen dioxide and superoxide, which is catalysed by Fe [61].

1.1.2.2. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are organic components carried on particulate surfaces. They indirectly induce OS, by cytochrome P450 biotransformation and generation of redox active quinones that act as free radical catalysts [6]. Polycyclic aromatic hydrocarbons have been associated with increased DNA-adduct formation [62–64], that is dose-dependent [65, 66]. Particulate matter has been implicated in the transfer of PAH from its surface across the placental barrier. The PAH within the placenta, interferes with placental development and the delivery of nutrients and oxygen to the foetus. This leads to reduced foetal nourishment *in utero*, as a consequence of increased blood viscosity and decreased placental blood flow [46, 58, 59].

Determining the exact mechanism by which pollutants exert their toxicity is challenging. This could be due to the differing concentrations of pollutants in the ambient air among locations, each possessing its own unique microenvironment. In addition, potential pollutant interactions, due to temperature differences and environmental pressures may affect their toxicity. Developing countries, such as SA, allow for the unique environment and ideal location for

investigating AAP effects on human health, especially those most vulnerable including woman, children, and infants *in utero*, due to the limited AAP monitoring sites and controls and a rapidly growing population in close proximity to pollution sources.

1.2. Durban South Industrial Basin

Durban, located within the province of KwaZulu-Natal, SA, is the second largest city by population in the country. It is home to the largest and busiest port including the primary route for exported petroleum and imported crude oil [67].

Apartheid-era policies that prioritised national strategic growth resulted in the heavy industrialisation of SD, with little regard for pollution load on the atmosphere and the effects of pollutant interactions. Thus it is considered one of the most heavily polluted and highly industrialised areas within SA. Today, SD comprises approximately 600 industries, including SA's two largest oil refineries , pulp and paper mills, petroleum industries, a sugar refinery, waste-water treatment works, major chemical and petrochemical storage facilities, motor industries, breweries and other small industries [67, 68].

Poor historic land use planning resulted in the juxtaposition of dense labour communities amongst industrial activity, with over 400,000 people living within this industrial hub today (Figure 1.3). In addition, to the continuous growth of vehicular traffic, aggravated by the dysfunctional rail service and public transport, shipping pollution and heavy truck traffic, creates a unique AP exposure setting [68, 69].

Figure 1.3 South Durban – Residential communities living within close proximity to industrial activity [70].

The south Durban or often termed "South Durban Industrial Basin" is approximately 24km long and 4km wide, ranging from the central Durban business district southward to Umbogintwini (Figure 1.4). The 'basin' like topography, due to the seaward ancient sand dunes at the coast (70-110m in height) and a ridge of hills landward (100-150m in height), in combination with the multiple pollutant sources in close proximity to dense residential areas in SD, create and possess adverse public health impacts. Dispersion of pollutants during the night and early morning is minimal, resulting in high levels of pollution; this deteriorates further in winter where surface temperature inversions prevent the upward migration of pollutants [67, 68].

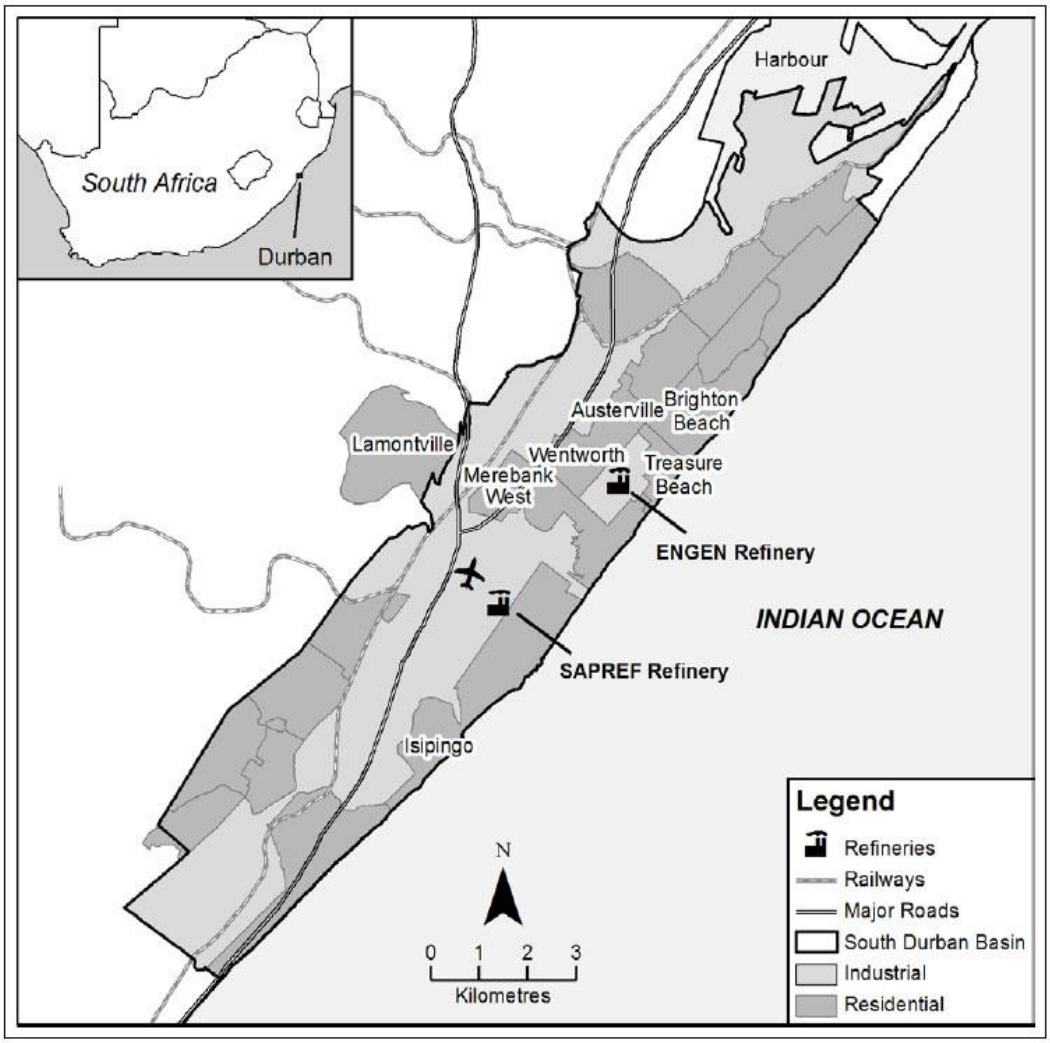

Figure 1.4 Location of south Durban in South Africa, depicting the entanglement of residential and industrial areas [71].

Major pollutants of interest within this area include: SO_2 emissions from stationary refineries and paper mills, NOx emitted from road traffic and industry and PM_{10} mainly emitted from traffic and industry with additional influences from biomass burning, dust, and salt. High levels

of these pollutants have been measured within SD [67, 72]. The communities living within SD are majority low-income households and informal settlements that rely heavily on domestic fuels such as coal, wood, and paraffin for cooking and heating. In addition to poor ventilation, the use of small-scale appliances that are poorly maintained, result in increased emissions of indoor AP including NOx, which puts them at risk for adverse health impacts of AAP [5].

Previous studies have shown that although the AAP levels fell below the national and international guidelines; SO_2 and PM_{10} were significantly associated with adverse health outcomes; including moderate to severe asthma documented within children of school going age, within SD [69, 73]. In addition, higher levels of OS markers were observed in pregnant women exposed to AAP in SD compared to the less industrialised ND [74]. However, this study did not link these increased markers of OS to a particular pollutant, nor determined what impact these markers had on neither neonatal birth outcomes nor health later in life, if any. The adverse health outcomes associated with SD AAP emissions resulted in the monitoring of these toxic emission levels from industries within SD [67].

South Durban located within the city's major transport routes including the national roads and highways, exposes residents to high levels of traffic-related AP, especially during morning and evening traffic (also times of low pollutant dispersion) [67]. Traffic-related AP, e.g. NOx, is rapidly worsening due to increased vehicle fleets, increased distance travelled, and high rates of emission from motor vehicles. High emission rates are due to road congestion which increases the rate of emission to km travelled, poor vehicle maintenance, and high age of vehicles [5]. The close proximity of these communities to heavily congested highways and roads, allows for the continuous exposure to traffic-related AP. Therefore, SD residents are highly vulnerable to traffic-related AP effects, especially those most susceptible to AAP adverse effects.

1.3. Pregnancy, Adverse birth outcomes and Ambient air pollution exposure

Infants *in utero* are among the most vulnerable to toxicant exposure, viz. AAP exposure. This susceptibility due to their biological vulnerability, sensitivity and rapid rates of replication and differentiation puts them at risk for impaired organ function and disease susceptibility later in life [38, 57].

1.3.1. Pregnancy

Pregnancy is a physiological state that is characterised by elevated basal oxygen demand and high-energy requirement for various organ functions including the foeto-placental unit [30, 31], which exhibits increased susceptibility to OS and OS-related injury. The human placenta is rich in mitochondria [29] and consumes about 1% of maternal basal energy production; it is highly vascular and exposed to high partial pressures of oxygen [31].

During the first few months of development, the placental barrier is >20µm thick and under hypoxic conditions; as no perfusion can occur. Nutrient exchange occurs only through passive diffusion. Increased capillary production, occurs as pregnancy continues, which decreases the placental barrier thickness [57]. This results in a fully developed foeto-placental unit that favours the exchange of gas, nutrients and metabolic products from the foetal to the maternal blood circulation.

Oxygen exchange across the placental barrier from maternal to foetal circulation is favoured by the low oxygen partial pressure, foetal Hb that has high affinity for oxygen and low pH of foetal blood [31]. The oxygen rich environment, increased energy consumption and abundant mitochondria mass within the placenta favours ROS production. The macrophage rich placenta also favours local production of free radicals, including NO metabolites and reactive nitrogen species (RNS) [31]. These increases in ROS and RNS can contribute to OS during *in utero* exposure to AAP.

Exposure to PM and PAH *in utero* have been shown to affect placental development [57, 59]. Studies have shown that OS in pregnancy has been associated with placental ischemia, repetitive hypoxia-reperfusion injury that results from improper spiral arteriole development [75, 76]. The alteration in utero-placental and umbilical cord flow, glucose and oxygen transport across the placental barrier negatively influences foetal growth [77].

1.3.2. Adverse birth outcomes and Ambient air exposure

Ambient air pollution exposure has been implicated in adverse birth outcomes including LBW, intrauterine growth retardation (IUGR), preeclampsia and PTB. These adverse birth outcomes have been significantly associated with increased infant morbidity; that ranges from neural to pulmonary conditions as well as infant mortality. They have also been shown to pre-dispose an individual to chronic diseases in adulthood [4].

Low birthweight affects 20 million people worldwide [78], whilst PTB has been implicated in 75% of neonatal morbidities and 70% of neonatal deaths [38]. These adverse birth outcomes occur as a result of restricted foetal growth and/or reduced length of gestation; with neonates weighing <2500 g at birth and premature delivery of a foetus before 37weeks of gestation, respectively. Environmental factors have been associated with reduced BW and gestational age (GA) [79].

Many studies have been done to determine whether environmental tobacco smoke (ETS) elevates the risk for adverse birth outcomes, including LBW (reviewed elsewhere [32, 33]). Among these Ahluwalia et al. found that ETS exposure increased the risk for adverse birth outcomes and was modified by maternal age [80]. However, exposure to ETS can potentially be avoided, unlike ambient air exposure.

The effects of AAP on foetal outcomes have become an important topic of study and are of great public health concern [51]. The first report of LBW babies were born to mothers living in highly polluted areas in Los Angeles (United States of America); in the early 1970s [81]. Following this initial report, publications sharply increased and extensive reviews were done which highlight all the current data about LBW [2, 4, 46, 57, 82] and PTB [4, 46, 83], and their association with AAP. Studies have shown both an association and no association between LBW, PTB and AAP; however, there is extensive evidence that supports the idea that exposure to AAP whilst pregnant, increases the risk for LBW and PTB (Table 1.1).

Table 1.1 Evidence of ambient air pollution and its association/non-association with low birthweight and pre-term birth

Author	AAP type	LBW- Association	Trimester of Exposure	Country (Year)
Wang et al. 1997	Total suspended particles (TSP), SO_2, complex pollution mix	Increased risk of LBW	3rd Trimester	Beijing (1988-1991)
Ritz & Yu 1999	CO	Significant increased risk for LBW	3rd Trimester	Los Angelas, CA (1989-1993)
Bobak & Leon 1998	SO_2, NO_2, TSP	LBW associated with SO_2 and TSP		Czech Republic (1986-1988)
Bobak 2000	SO_2, NO_2, TSP	LBW and PTB associated with SO_2 and TSP	1st Trimester	Czech Republic (1991)
Rogers et al. 2000	SO_2, TSP	VLBW associated with very high levels		George Health Care

		of SO_2, TSP		District 9, USA (1986-1988)
Maisonet et al. 2001	SO_2, CO, PM_{10}	SO_2 and CO increase risk of LBW PM_{10} no association to LBW Increased ambient levels associated increased risk of LBW	3rd Trimester	North-eastern USA (1994-1998)
Vassilev et al. 2001	Polycyclic organic matter (POM)	High POM concentrations associated with LBW and PTB		New Jersey (1990-1991)
Maroziene & Grazuleviciene 2002	NO_2, Ambient formaldehyde	Ambient formaldehyde associated with increased risk of LBW. 10µg/m^3 increase in NO_2 concentrations increased risk of PTB by 25%	1st Trimester	City of Kaunas (1998)
Liu et al. 2003	SO_2, CO, Ozone, PM_{10}, NO_2	Increased SO_2 associated with LBW PTB associated with SO_2 and CO exposure	1st Trimester (LBW) 3rd Trimester (PTB)	Vancouver, Canada (1985-1998)
Mishra et al. 2004	Biomass cooking fuels (wood, dung)	Preliminary data -high pollution cooking fuels associated with LBW		Zimbabwe (1994-1999)
Seo et al. 2007	PM_{10}, CO, SO_2, NO_2	PM_{10}, SO_2 and NO_2 increased risk of LBW CO increased personal risk of LBW	3rd Trimester	Seoul, South Korea (2002-2003)
Nascimento & Moreira 2009	SO_2, ozone, PM_{10}	SO_2 and ozone associated with LBW – also risk factors for LBW PM_{10} not associated with LBW		São Paulo State, Brazil (2001)
Ballester et al. 2010	NO_2	Exposure to NO_2 (>40µg/cm^3) associated with reduced birth length, BW and head circumference	1st Trimester	Valencia, Spain (2003-2005)
Kloog et al. 2012	$PM_{2.5}$	$PM_{2.5}$ associated with increased risk of LBW and PTB	3rd Trimester	Massachusetts (2000-2008)
Clemente et al. 2016	NO_2	10µg/m^3 increases in NO_2 associated with 48g decrease in BW	1st, 2nd and 3rd Trimester	Spain and Belgium (2004-2008)
Hao et al. 2016	NO_2, CO, $PM_{2.5}$	All pollutants investigated were associated with PTB	1st, 2nd and 3rd Trimester	Georgia, USA (2002-2006)

1.3.2. Adverse birth outcomes and Oxidative stress

The aetiology of adverse birth outcomes due to AAP exposure is not fully understood. However, factors that have been associated with AAP induced LBW and PTB pathology are OS, inflammation, endothelial dysfunction, reduced oxygen transport across the placenta and abnormalities of the placenta (Figure 1.5) [4].

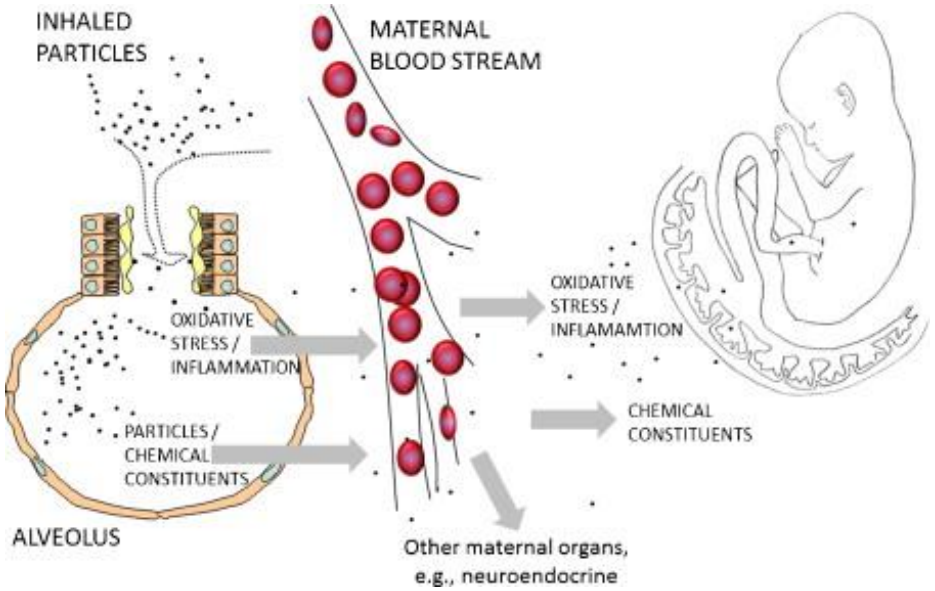

Figure 1.5 Ambient air pollution's potential mechanism for foetal toxicity leading to adverse birth outcomes. Inhaled AP enters the alveolus resulting in free radical production, which targets macromolecules present within the alveolus tissue. This results in OS and inflammation which is able to cross into the maternal blood stream and subsequently affect foetal growth through increased OS and inflammation within the placenta [98].

Pregnancy favours ROS production and presents with increased susceptibility to OS, due to the limited levels of foetal antioxidants [99], therefore several studies have investigated OS as a potential risk factor for LBW and PTB (Table 1.2). These studies demonstrate that increased OS markers as well as decreased antioxidant defence result in OS observed in LBW and PTB neonates.

Table 1.2 Evidence for Oxidative stress as a mechanism for LBW and PTB

Author	Oxidative stress markers	LBW- Association	Country (Year)
Matsubasa et al. 2002	Urinary 8-OHdG levels	Increased 8-OHdG levels associated with LBW and PTB	Japan
Gupta et al. 2004	Cord blood – SOD, CAT, GSH and serum malondialdehyde (MDA)	Increased MDA and decreased SOD, catalase, GSH (OS) associated with LBW	New Delhi, India
Kim et al. 2005	Maternal urinary 8-OHdG levels and MDA	Increased 8-OHdG and MDA levels associated with PTB and reduced BW in full-term neonates	Korea (2000-2001)
Kamath et al. 2006	Maternal and foetal MDA and proteolytic activity	Increased LP and protein oxidant damage associated with IUGR (SGA)	Manipal
Chadha et al. 2007	Maternal blood and urine NO metabolites levels	Increased levels of NO metabolites associated with PTB	India
Saker et al. 2008	Total antioxidant activity (ORAC), vitamin A, C and E, carbonyl proteins, hydro-peroxides, erythrocyte CAT, GPx, glutathione reductase, SOD, lipid and lipoprotein	Decreased ORAC, vitamin C and E values, high plasma hydro-peroxide and carbonyl protein, decreased SOD and CAT – OS associated with LBW	Algeria
Gveric-Ahmetasevic et al. 2009	MDA, serum peroxide and antioxidant capacity	Mothers and babies exposed to OS associated with LBW Placental LP may be associated with LBW pathophysiology	Croatia
Rossner et al. 2011	Placental- 8-OHdG levels	Increased 8-OHdG associated with LBW	Czech Republic (1994-1998)
Negi et al. 2012	Cord blood – MDA, carbonyl proteins, total antioxidant capacity, vitamin A, E and C	Increased LP and protein oxidation with decreased vitamin A, E, C and antioxidant capacity (OS) associated with preterm-LBW	India
Kumarathasan et al. 2016	Plasma 8-isoprostane (OS marker)	The 8-isoprostane was negatively associated with BW and GA	Canada

Although studies have reported AAP exposure and OS, individually increase the risk of adverse birth outcomes, and OS is implicated in AAP toxicity. Investigating the biological mechanisms through which OS induced by AAP affects neonatal birth outcomes, could allow for their targeted interventions and treatments. In addition, identifying other factors such as genetic predisposition and periods of infant vulnerability would allow for the better understanding of AAP induced toxicities and disease susceptibility.

1.4. Oxidative Stress

Interest in prenatal exposure to AAP and its subsequent effects on adverse birth outcomes is growing rapidly. As the pollutants discussed previously, act either as free radicals or have the potential to generate free radicals, and pregnancy favours ROS production due to its highly oxygen-rich and energy requiring state, OS has therefore been suggested as a mediator of AAP toxicity leading to adverse birth outcomes.

Oxidative stress, within the human body, is the biochemical imbalance of oxidants and antioxidants that refers to a disturbance in the excessive production of oxidants over antioxidants [105]. Human redox biochemistry is predominantly made up of oxygen, as cells and cellular mechanisms require a reducing environment to function. Therefore, human cells are under constant threat by highly ROS as a natural consequence of aerobic respiration [106]. In analogy to OS, RNS function and are able to react with ROS to potentiate OS-damage [107]. Oxidative stress has been implicated in pathological conditions such as Parkinson's disease, Rheumatoid arthritis and adverse birth outcomes [105, 108].

1.4.1. Reactive oxygen species

Mitochondrial respiration is essential for energy production within human cells. A natural by-product of this process is ROS, which includes both radicals and nonradicals. A radical is a chemical species that possesses one or more unpaired electrons and the ability for its independent existence. Radicals are less stable than non-radicals [108]. Radicals of importance in humans: include hydroxyl ions, superoxide, NO, and singlet oxygen. Non-radicals contain only paired electrons, with most biological molecules being nonradicals. Other non-radical compounds produced in high concentrations include hydrogen peroxide, ozone, and molecular oxygen (O_2) [109]. This reactivity is necessary for normal molecular function through high-energy transfers within the mitochondria to produce adenosine triphosphate (ATP), an essential energy source for cellular processes [105].

Oxidative phosphorylation, a process that involves the transfer of electrons through the mitochondrial electron transport chain (ETC), creates a proton gradient across the inner mitochondrial membrane that drives ATP synthesis. This transfer of electrons results in the reduction of each protein complex until the electron is accepted by O_2 to form water. However, about 1-3% of electrons entering the ETC are not accepted by O_2 to form water but are instead catalysts for the reduction of oxygen into superoxide. These reduction reactions occur at

complexes I (nicotinamide adenine dinucleotide dehydrogenase) and III (ubiquinone-cytochrome c reductase), where complex III is the main site of superoxide production (Figure1.6) [110]. The ROS produced via the 'leaky' ETC are highly reactive and their close proximity to their targets which include DNA, protein and lipids have great potential to cause deleterious effects [106].

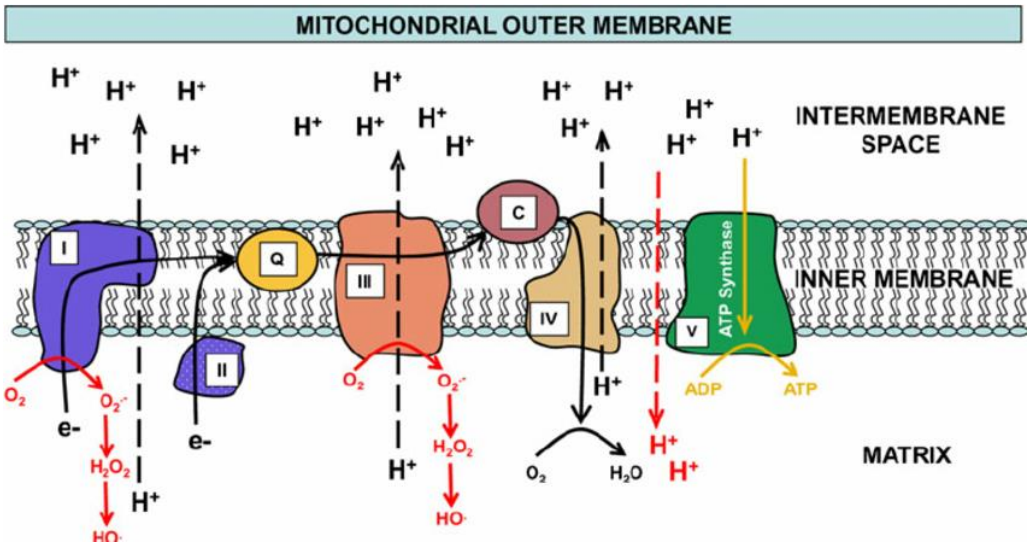

Figure 1.6 Mitochondrial reactive oxygen species production. During oxidative phosphorylation within the ETC, a leakage of electrons from complex I and III react with oxygen to form superoxide. This is a highly reactive molecule and undergoes the Fenton-Haber-Weiss reaction to produce hydroxyl radicals. The ROS are free to react and damage macromolecules within their proximity [111].

1.4.2. Antioxidants

The human body has developed an antioxidant defence system that scavengers free radicals, as a result of continuous reactive metabolic insult. This defence system maintains the delicate balance between oxidants and antioxidants to prevent cellular damage [112]. There are two types of antioxidants: enzymatic and non-enzymatic.

1.4.2.1. Enzymatic antioxidants

Enzymatic antioxidants possess a transition metal core which allows them to move between valences and scavenge free electrons from ROS, during the detoxification process.

1.4.2.1.1. Superoxide dismutase

The enzyme SOD exists as three isoforms: SOD1 which contains a metal co-factor comprising Cu-zinc (Zn) and is present within the cytosol, nucleus and plasma. Superoxide dismutase 2 has a manganese (Mn) core and is primarily located within the mitochondria. Lastly, the extracellular SOD3 comprises a Zn and Cu core similar to SOD1 [75, 109]. Superoxide dismutase catalyses the removal of superoxide and generates hydrogen peroxide as the reaction product (Figure 1.7).

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Figure 1.7 Dismutation of the superoxide radical into oxygen and hydrogen peroxide (Prepared by author).

1.4.2.1.2. Catalase

Catalase is an enzyme that removes hydrogen peroxide present in high concentrations, and is found in peroxisomes in most tissue. The enzyme consists of four protein subunits that contain ferric ions of the haem group. Two molecules of hydrogen peroxide are reduced to O_2 by CAT. The ferric ions of CAT are oxidised after interaction with the first molecule of hydrogen peroxide to produce Fe^{4+} (compound 1). The second molecule of hydrogen peroxide acts as an electron donor resulting in the production of O_2 (Figure 1.8) and the complete detoxification of two hydrogen peroxide molecules [109].

$$H_2O_2 \xrightarrow{CAT} \text{Compound 1}$$

$$H_2O_2 + \text{compound 1} \longrightarrow O_2 + 2H_2O$$

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O$$

Figure 1.8 Catalase detoxification of hydrogen peroxide (Prepared by author).

1.4.2.1.3. Glutathione peroxidase

Glutathione peroxidase exists in five isoforms and possesses high affinity for and removes hydrogen peroxide, generated by SOD in the cytosol and mitochondria. Its catalytic function is dependent on the presence of selenium within its active site and is important in protecting the lipid environment against oxidative damage [113].

Glutathione is an essential thiol antioxidant that is formed within all human cells of the body. The formation of GSH occurs within the cytosol; it comprises of cysteine (Cys), glycine and glutamate, and is regulated by its *de novo* synthesis that is catalysed by γ-glutamylcysteine synthase and glutathione synthase [75]. Glutathione participates in various detoxifying reactions forming glutathione disulphide (GSSG). Glutathione is restored by glutathione reductase which requires reduced nicotinamide adenine dinucleotide phosphate (NADPH); generated in the pentose phosphate pathway [105]. The removal of hydrogen peroxide by GPx utilises GSH, as electron donors, where it is oxidised to form GSSG. The oxidation of two GSH molecules by hydrogen peroxide results in the formation of oxidised GSSG and two molecules of water (Figure 1.9) [105, 109].

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

Figure 1.9 Glutathione peroxidase reduction of hydrogen peroxide to water (Prepared by author).

1.4.2.1.4. Glutathione S transferase

Glutathione S transferase inactivates secondary metabolites such as epoxides, unsaturated aldehydes and hydro-peroxides. There are three families of GSTs: cytosolic, mitochondrial and membrane-associated microsomal GSTs. The cytosolic GSTs are further divided into seven classes: Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta. During non-stressed cells, Mu and Pi classes of GST interact with kinases apoptosis signal-regulating kinase 1 and c-Jun N-terminal kinases (JNK). It has been shown that GSTP1 dissociates from JNK in response to OS [60]. However, GST function has been shown to be influenced by genotypic differences from SNPs, common polymorphisms have been found in GSTP1 and GSTM1 genes [114]. These genetic polymorphisms may influence an individual's susceptibility to OS and subsequent OS-related conditions, including adverse birth outcomes.

The GSTM1 gene, a member of the Mu family, is located on chromosome 1p13.3 [115]. A homozygous deletion of this gene results in the complete loss of enzyme activity; and is known as the null genotype [116, 117]. This deletion has been associated with increased asthma and wheezing among children who were exposed to ETS *in utero* [10, 118, 119], cardiovascular disease [120, 121] and increased levels of 8-OHdG adduct in individuals exposed to organic carbon and SO_2 [122]. This polymorphism was also suggested to increase the susceptibility of mothers to gestational diabetes mellitus [123].

The GSTP1 gene contains 7 exons and is located on chromosome 11q13 [115]. A functional polymorphism (Ile105Val; AA → AG/GG) in GSTP1 occurs due to the substitution of valine (Val) for isoleucine (Ile) at codon 105 within GSTP1. This affects the enzymes active site's substrate specificity [124, 125]. Studies have shown that the wt (A_{105}) has a threefold higher catalytic activity than the variant (G_{105}) for 1-chloro-2,4-dinitrobenzene; however, the reverse is observed for PAH diol epoxides with a seven-fold difference [126]. This polymorphism's variant genotype has been linked to an increased risk in cancer [127–129], increased susceptibility to NOx in individuals exposed to traffic-related AP [130] and increased levels of 8-OHdG in oesophageal cancer patients [131].

1.4.2.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants are mainly comprised of synthetic antioxidants and dietary supplements such as GSH, selenium, vitamin C and E, and Zn that act as ROS scavengers resulting in their neutralisation.

Ascorbic acid i.e., vitamin C is a water-soluble, redox catalyst that reduces and neutralises ROS. It is an essential cofactor for many enzymes, such as proline hydroxylase and dopamine-β-hydroxylase. It is an efficient scavenger that acts as an electron donor and neutralises ROS. The relative stability of ascorbyl makes it a powerful antioxidant [109].

Glutathione is a thiol-containing tripeptide; the reduced form of glutathione is glutamic acid-cysteine glycine. Glutathione is an important cofactor for enzyme peroxidase, therefore serves as an indirect antioxidant by donating electrons to detoxify hydrogen peroxide. It is important in many other biological functions including metabolism, Cu transport and preventing oxidation of sulphydryl protein groups. It also acts as a ROS scavenger where GSH interacts with hydroxyl, alkoxyl and peroxyl radicals as well as superoxide. Glutathione is also able to bind transition metals and prevent their participation in the Fenton- Haber-Weiss reaction, which requires transitional metals, to act as electron donors. In the absence of free transition metals the reaction does not occur which inhibits the production of hydroxyl radicals, as described in figure 1.2 [109].

1.4.3. Imbalance

Reactive oxygen species are of biological importance as they act as important signalling molecules and transcription factors in a diverse range of cell functions, this is comprehensively

reviewed elsewhere [105]. However, if ROS production exceeds the capacity of antioxidant scavenging, OS results leading to extensive damage of macromolecules such as lipids, proteins, and DNA.

Lipid peroxidation occurs as a result of the oxidation of polyunsaturated fatty acid side chains (PUFA) by hydroxyl radicals. Protein carbonyl groups are formed from the direct oxidation of protein side chains. Abstraction of hydrogen ions (H^+) within thiol groups of cysteine leads to disulfide bond generation which results in misfolded proteins. Hydroxyl radicals also attack DNA which generates DNA-adducts such as 8-OHdG.

Mitochondrial DNA is highly susceptible to oxidative attack. It is located close to the site of ROS production, has minimal repair mechanisms present, lacks protective histones and has a high mutation rate [105, 132, 133]. Mitochondrial DNA encodes proteins that are essential within the ETC, therefore mutations within the mitochondria due to ROS attack may lead to impaired ATP production which could further increase ROS production, potentiating the original OS (Figure 1.10) [105, 109].

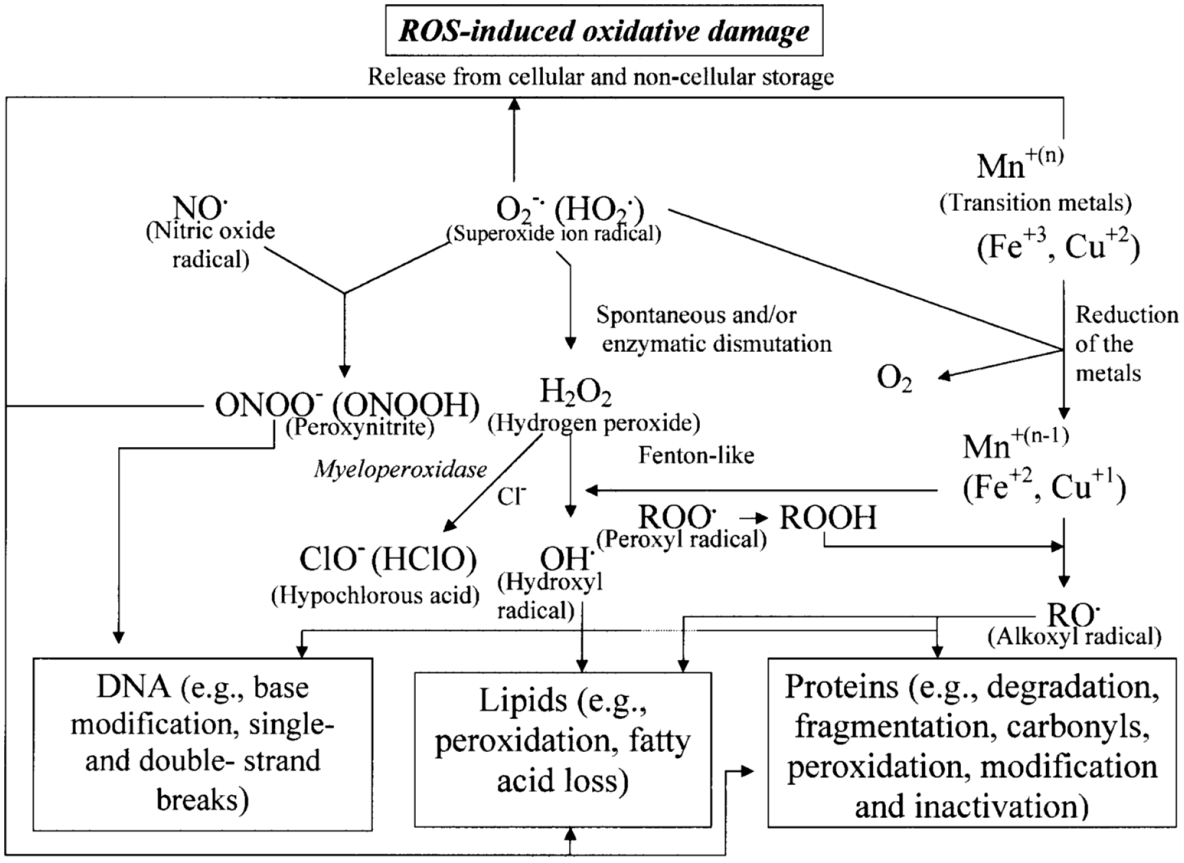

Figure 1.10 Oxidative damage induced by reactive oxygen species [109].

Pregnancy is a condition exhibiting increased susceptibility to OS, evidence from studies has associated increased OS markers [29] and raised lipid hydro-peroxides and MDA [134, 135] within normal pregnancies. It has been suggested that due to the high energy requirement necessary for placental and foetal development and increased inflammation, ROS production is favoured [136]. It should be noted that Fe supplementation during pregnancy, may also be a source for free radical production, as Fe is used within the Fenton- Haber-Weiss reaction to produce hydroxyl radicals [135]. Therefore any exogenous or endogenous toxicant is able to tip the balance between oxidant and antioxidant causing OS and OS-related damage. It has been reported that the DNA adduct, 8-OHdG, can be used as a biomarker of OS within clinical studies [7].

1.4.3.1. DNA damage- 7,8-dihydro-8-oxoguanine adduct

ROS generates a variety of mutagenic DNA lesions, including abasic or apurinic/apyrimidinic (AP) sites, DNA single (ss) or double strand (ds) breaks and base oxidation. Due to guanine's low redox potential, it is highly susceptible to oxidation. This results in the abundant production of 8-OHdG mutagenic lesions, with approximately 10^3 lesions generated per cell per day [137, 138].

The hydroxyl radical, when produced adjacent to nuclear and mitochondrial DNA, attacks DNA strands leading to the incorporation of radical adducts opposite 8-OHdG which induces the G:C → T:A transversion, where it aberrantly pairs with cytosine in an anti-conformation [139, 140]. This may lead to the accumulation of GC to TA mutations, if left unrepaired [22]. In addition, ROS are able to oxidise nucleotide pools [139].

Although an increase in modified DNA frequently occurs, the excessive accumulation of 8-OHdG in DNA has been linked to various inflammatory diseases and adverse birth outcomes [138]. The level of genomic 8-OHdG has been shown to correlate well with the dose and length of exposure to environmental agents, as well as inhaled environmental pollutant chemical composition and physical nature [138, 141, 142]. However, repair mechanisms are present within human cells in order to reduce or remove DNA modified bases.

1.5. Base excision repair pathway

Several repair DNA damage pathways exist, each targeting specific types of damage, to counteract the effects of ROS [139]. The base excision repair (BER) pathway preferentially

repairs oxidised bases within nuclear and mitochondrial DNA [131]. It comprises short- and long- patch pathways, which repair single and two or more nucleotides, respectively [106]. The BER preserves genome integrity by utilising glycosylases that excise the mutagenic adduct, which subsequently allows endonucleolytic cleavage and gap filling [138].

In eukaryotes, the 8-oxoguanine DNA glycosylase (OGG1) plays a pivotal role in the BER. It recognises 8-OHdG G:C base pairs, catalyses the expulsion of 8-OHdG and the cleavage of the DNA backbone which forms an AP site [11, 143]. The cleaved DNA is subsequently completed by AP endonuclease, DNA polymerase β and DNA ligase III to repair the oxidative damage (Figure 1.11) [144].

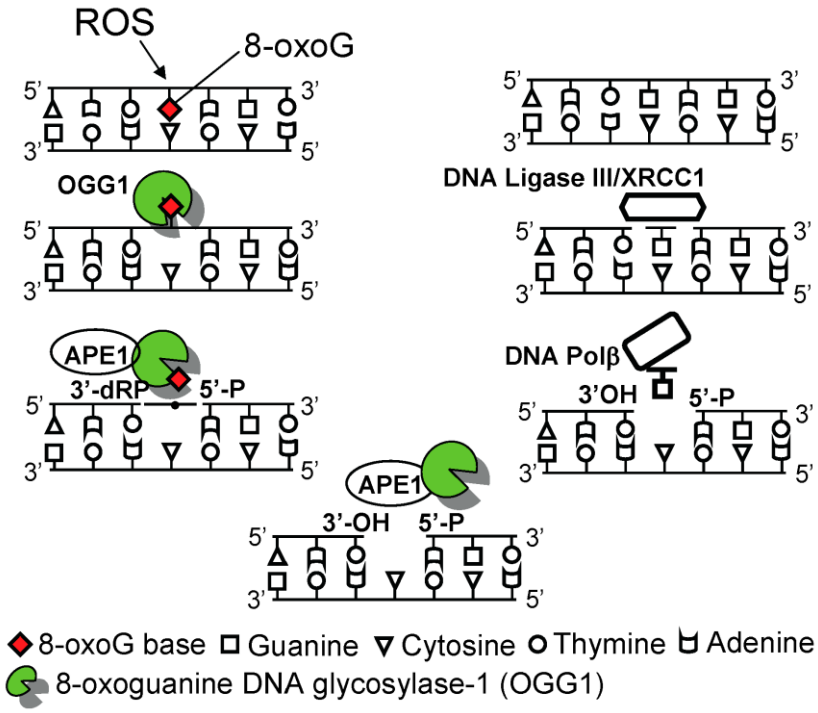

Figure 1.11 DNA damage repair initiated by 8-oxoguanine DNA glycosylase. The OGG1 recognises 8-OHdG and subsequently cleaves the DNA backbone which generates an AP site within the 3'-blocking end of the DNA. The cleaved DNA is subsequently cleaved by AP endonuclease 1 to remove the aldehyde residue to for a 3' hydroxyl end, followed by the addition of a guanine base by DNA polymerase β which is ligated by DNA ligase III to form a complete DNA strand [145].

1.5.1. Human 8-oxoguanine glycosylase 1

The OGG1 gene, mapped on chromosome 3p26.2 encodes the human OGG1 protein. This region consists of 7 exons and encodes 345 amino acids (AA) and a bifunctional glycosylase. The OGG1 carboxyl (C) –terminus undergoes alternative splicing and generates α-OGG1or β-

OGG1. The N-terminus, comprising a mitochondrial target sequence, is similar between α-OGG1 and β-OGG1 proteins, whilst the C-terminus is extensively different. Therefore, the production of either α- or β- OGG1 is dependent on the presence of a C-terminal nuclear localisation sequence. This sequence, present within α-OGG1 but absent from β-OGG1, suppresses the mitochondrial target sequence and is localised in the nucleus. The β-OGG1 which only consists of the mitochondrial target sequence is localised within the mitochondria [137, 139]. Although the α-isoform is located within the nucleus, it is thought to be responsible for nuclear and mitochondrial 8-OHdG repair [137].

The bifunctional glycosylase, OGG1, specifically recognises 8-OHdG paired with cytosine. This allows it to cleave oxidative base pairs and degrades adducts within the nucleoside pool [139]. The recognition of 8-OHdG by OGG1 occurs through the enzyme's ability to interact directly with a proton present on N7 instead of identifying its 8-oxo-carbonyl feature [106].

The human OGG1 protein comprising 8 cysteine residues, two of which (Cys253 and Cys255) are present within the active site, may be susceptible to regulation through oxidative modifications [11]. Cysteines are surrounded by a positive AA sequence which makes it susceptible to form reactive thiolate anions; which are particularly predisposed to oxidative modifications [22]. Reversible modifications of cysteine residues affect OGG1's enzyme activity [11, 22, 144].

The activity and expression of OGG1 has been found to be modulated post-transcriptional by OS [137] with the OGG1 promoter having binding sites for the specific protein 1 (Sp1) transcription factor and Nrf2 antioxidant response element. Its expression was also found to be inhibited by NO [11, 144, 146].

1.5.2. 8-oxoguanine glycosylase 1 serine-326-cysteine polymorphism

Several DNA repair genes have been shown to be highly polymorphic which alters protein structure and function. These changes could influence the genetic susceptibility individuals have towards certain diseases, such as carcinogenesis, cardiovascular and inflammatory diseases [147, 148].

Structural analysis of the OGG1 gene demonstrated the presence of several SNPs, the most common of these is the serine-326-cysteine (Ser326Cys) rs1052133 OGG1 SNP [143]. Within the OGG1 gene, a C→G transversion occurs at position 1245 in exon 7, which results in a

serine to cysteine AA substitution at codon 326 of the OGG1 protein. Ethnicity determines allelic frequency, where Caucasians have a 23-41% frequency for the G allele, the Asian population has a frequency of 40-60% [22, 139] and Sub-Saharan African populations were found to have 14.4% G allele frequencies [149]. However, limited data is available in African populations for the OGG1 Ser326Cys SNP and disease risk [149–152].

Functional studies have been broadly investigated, *in vitro*, with purified proteins and in cells/cells extracts, to determine the phenotypic impact the OGG1 Ser326Cys SNP has on enzyme repair activity [148]. Several studies revealed the Cys326 (G) allele was associated with reduced DNA adduct repair activity and posed an increased risk for 8-OHdG formation [22]. However, Janssen and colleagues reported no difference in repair activity amongst the wt and variant alleles [153].

Kohno and colleagues' study revealed the Ser326 wt allele had a repair capacity 7 fold greater than the Cys326 variant in *Escheria Coli* [154]. The greater repair rate of Ser326 compared to the C326 variant was also observed by Aka et al. [155]. A similar result was obtained in a lymphocyte study where the Cys326 variant protein had 40% reduced repair activity [156]. Bravard and colleagues' demonstrated the Cys326 variant cells presented with a greater genetic instability and decreased DNA repair rate [22]. They also showed Cys326 variant protein's repair capacity was sensitive to the cell redox status. Their analyses indicated the reduced enzymatic activity within the variant protein was due to the oxidation of its extra cysteine residue, which was also suggested by Lee et al. [157]. Bagryantseva and colleagues demonstrated a higher level of 8-OHdG in Cys326 variant patients compared to Ser326 wt patients exposed to traffic PAH [158]. A Japanese study reported reduced BW and birth length in neonates whose mother's carried the variant genotype, exposed to cigarette smoke; however, this association was removed when the study area was controlled [159].

Hill and Evans demonstrated, the Cys326 variant excised 8-OHdG from the dsDNA and cleaved AP sites at a rate 2-6 fold lower than Ser326 wt, depending on the base opposite to lesion [160]. This was further investigated by Simonelli and colleagues, who demonstrated that homozygous Cys326 variant carriers present with decreased cleavage activity, whilst the OGG1 mRNA expression remained unaffected [148]. The extra cysteine residue also promoted dimerization of Cys326 variant, and the formation of disulfide bond which suggested several factors occur in order to impair repair activity. The variant was shown to affect the efficiency of the BER, and

this affect in protein conformation and stability was amplified under mild oxidative conditions [148].

Although the variant protein is prone to oxidation and undergoes conformational modifications, which modulates its cell localisation and catalytic activity, the precise nature of the functional defect remains unclear. In addition, no studies have reported the effect of AAP such as NOx and PM, has on the susceptibility of adverse birth outcomes in pregnant women carrying the Ser326Cys OGG1 SNP.

1.6. Nuclear factor (erythroid-derived 2)-like 2 and Kelch-like ECH-associated protein 1 signalling

Cytoprotection against toxic insult within the human body is provided through the expression of antioxidant proteins and phase 2 detoxifying enzymes. Under normal conditions, these protective proteins are expressed minimally but are highly inducible by transcriptional activation, in the presence of low levels of ROS. This co-ordinated response is regulated through a *cis*-acting element known as the ARE which is located on the upstream regulatory sequence present on each gene in either single or multiple copies [9, 16].

The laboratories of Kan [13] and Yamamoto [14], simultaneously discovered that Nrf2 is the key transcription factor that regulates xenobiotic metabolism via ARE regulated genes. Jaiswal's group subsequently provided evidence for NADPH quinone oxidoreductase positive regulation by Nrf2 via its ARE [15]. Nrf2 belongs to a subset of basic leucine-zipper (bZip) genes that share a conserved structural domain known as a cap 'n' collar (CNC)-domain, therefore, the C-terminus of Nrf2 comprises a characteristic bZip CNC domain (Figure 1.12b) [13, 161]. DNA binding occurs at the basic region, whilst the leucine-zipper heterodimerises with small musculoaponeurotic fibrosarcoma (Maf) proteins. These Maf proteins act as essential heterodimeric partners for the binding of large CNC bZip proteins to the GC dinucleotide of ARE [16, 162, 163]. Transcriptional activation of Nrf2 is conferred within its N-terminus and at the end of its C-terminus. A Nrf2-ECH homology (Neh) 2 domain is present within the N-terminus, which has been shown to negatively regulate Nrf2 activation via Keap1 [164].

The suppressor of Nrf2, Keap1 was initially discovered by Yamamoto and colleagues, who demonstrated a negative regulatory control of Keap1 over Nrf2 by sequestering it within the cytoplasm [164]. Keap1 is a member of the Kelch family that contains two known protein-interaction domains: the broad complex/tramtrack/bric-a-brac (BTB) and the double glycine

receptor (DGR) domain. They are located within the N- and C-terminal region, respectively (Figure 1.12a) [9, 16, 165]. The BTB domain mediates homodimerisation and binding of Keap1 to Cullin 3 (Cul3), a Nrf2 ubiquitin ligase (E3) scaffolding protein. Whereas, binding of Keap1 to the Neh2 domain of Nrf2, is mediated by the DGR domain. Between the two domains, a region abundant in cysteine residues, known as the intervening region (IVR) or linker region exists [161, 166, 167]. These three components are important for the regulation of the protective response against environmental stresses.

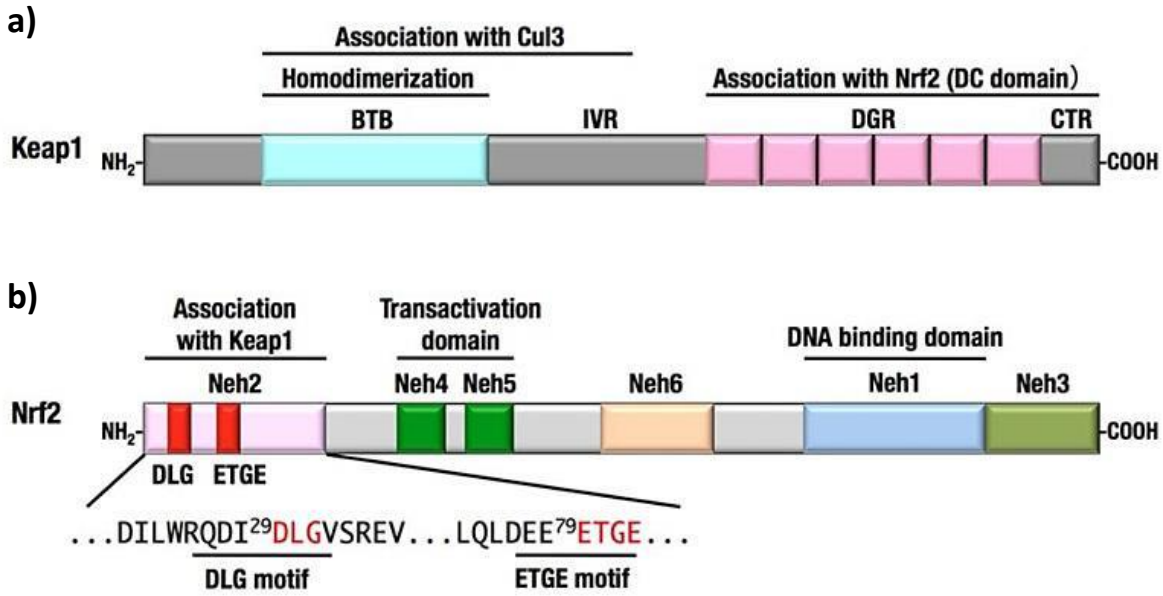

Figure 1.12 Domain structures present on a) Keap1 and b) Nrf2 genes. a) The DC domain present on the C-terminal of Keap1 binds Nrf2 whilst the N-terminal domain BTB homodimerises and binds Keap1 to Cul3. b) The Neh1 domain, present on the C-terminal region of Nrf2, mediates the heterodimerisation of small Maf proteins and the ARE on target genes, whilst the Neh2 domain present on the N-terminal binds to Keap1[168].

1.6.1. Under basal conditions, Nrf2 is suppressed by Keap1

Keap1 interacts directly with the actin cytoskeleton and is co-localised within the cytoplasm [169]. Under normal physiological conditions, Nrf2 is sequestered by Keap1 (Figure1.13a). The β-propeller within Keap1's DGR domain initially binds the conserved motif (ETGE) within the C-terminus of Nrf2's Neh2 domain, followed by the Nrf2's DLG motif. The stability of this complex is maintained through extensive inter- and intra-blade hydrogen bonds [9, 161, 167]. This Keap1-Nrf2 complex promotes the rapid degradation of Nrf2 by the ubiquitin-proteasome pathway, which accounts for the low levels of Nrf2 observed in many cell types due to its short

half-life of approximately 20 minutes. Keap1-Nrf2 interaction promotes the basal expression of cytoprotective genes and proteins within senescent cells [170, 171].

1.6.2. Activation of Nrf2 by ARE inducers

Upon oxidative insult, as a result of exogenous or endogenous stimuli viz. environmental pollution or infection, Nrf2 dissociates from Keap1 and translocates to the nucleus. Nrf2 and small Maf proteins form a heterodimer; which then binds to the ARE in the promoter region resulting in the transcriptional activation of specific target genes (Figure 1.13b) [9, 161]. Studies have shown that c-Jun and activating transcription factor (ATF) 4 can heterodimerise with Nrf2 and enhance the activation of ARE-driven reporter genes [172, 173].

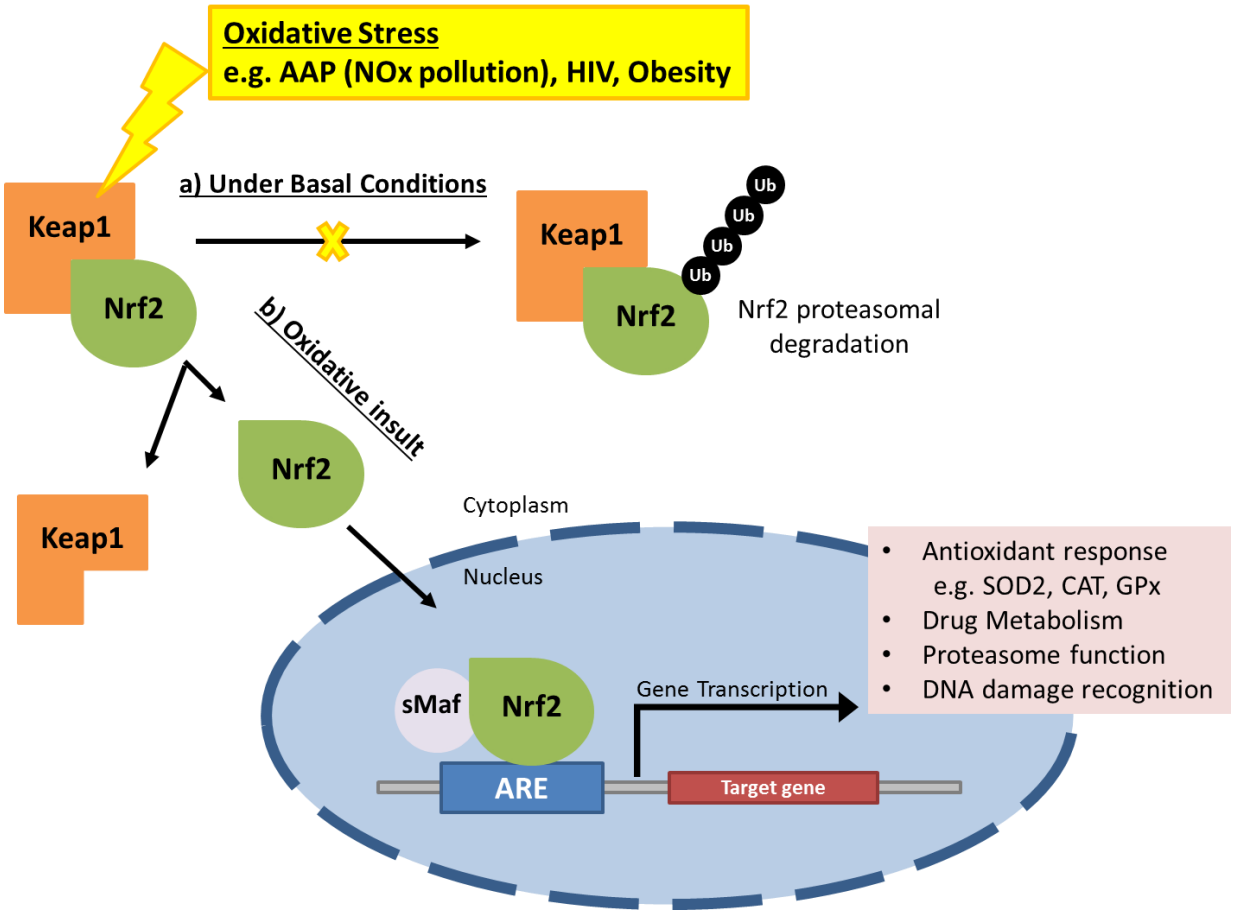

Figure 1.13 Transcriptional activation of Nrf2. a) Under Basal conditions, Nrf2 is bound to Keap1 within the cytoplasm and undergoes proteasomal degradation. b) Upon endogenous or exogenous toxic insult, Keap1's conformation is altered resulting in the release of Nrf2 into the cytoplasm where it translocates to the nucleus, heterodimers with small Maf proteins and binds to the ARE of its target gene resulting in its transcriptional activation (Prepared by author).

A variety of chemicals including environmental agents induce ARE genes through Nrf2 transcriptional activation [9, 161]. These inducers are all structurally diverse, however, share a common property; the ability to modify sulphydryl groups by alkylation, oxidation or reduction [174]. This ability of cells to recognise these inducer properties, suggest that they contain sensors rich in highly reactive cysteine residues. Therefore, as Keap1 contains 27 cysteine residues within its IVR, with Cys257, Cys273, Cys288 and Cys297 being the most reactive, it was proposed as a sensor for Nrf2 inducers [16, 174]. This was confirmed within mutational studies where Cys151, Cys273 and Cys288 were found to be essential for Keap1 regulation of Nrf2 [175, 176].

Several mechanisms result in the dissociation of Nrf2 from Keap1. Inducers are able to change the conformational structure of Nrf2-Keap1 complex, by modifying Keap1's cysteine thiols, resulting in the inhibition of Nrf2 ubiquitination. Whilst, other inducers bind directly to Keap1's IVR cysteines thereby preventing the formation of the stability complex, mentioned above, which prevents the ubiquitination of the Neh2 domain. Therefore, Nrf2 is able to bypass Keap1, enter the nucleus and facilitate transcriptional activation. However, other inducers such as toxic metals, are able to directly dissociate Nrf2 from Keap1, allowing Nrf2 stabilisation. In addition, Nrf2 transcriptional activation, nuclear translocation and degradation is regulated by covalent acetylation/deacetylation or phosphorylation/de-phosphorylation modifications within the Nrf2 gene [161].

Protein kinases (PK) have also be identified as possible sensors, where studies have demonstrated PKC and protein kinase RNA-like endoplasmic reticulum kinase (PERK) directly phosphorylate Nrf2 which results in the dissociation of Nrf2 from Keap1-Nrf2 complex [177–179]. Therefore suggesting, ER stress may play a role in Nrf2 activation and subsequent antioxidant transcription.

1.6.3. Target genes for Nrf2 regulation

Nrf2 regulates a substantial number of genes. The induction of the Nrf2 transcription factor, targets two main groups of regulation which include drug metabolism and disposition and antioxidant defence. In addition, Nrf2 regulates cell proliferation, proteasomal protein degradation, metabolic reprogramming and oxidative signalling [161, 168, 180, 181].

In response to environmental toxins, Nrf2's induction of drug metabolism enzymes and transporters such as cytochrome P450 [182] and GSTs [183, 184], control the metabolic fate of numerous pro-oxidants within the body.

An essential regulatory feature of Nrf2 transcriptional activation is the control of ROS homeostasis within the cell by its induction of antioxidant enzymes. This control occurs via several mechanisms: the induction of stress response proteins such as HO-1, regeneration of oxidised cofactors and proteins such as GSSG reduced to GSH, the synthesis of reducing factors such as NADPH and GSH and the induction of antioxidant enzymes such as SOD, CAT and GPx. Without Nrf2, the induction of cytoprotective proteins are insufficient resulting in increased susceptibility of tissues to toxic insult [161, 185, 186].

1.6.4. Epigenetic regulation of Nrf2

Human genetic susceptibility towards diseases has been an important feature in discovering how alterations in the genome predispose an individual to certain adverse conditions. The study of epigenetics has been highlighted most notably as a potential mechanism for the regulation of the Nrf2-Keap1 pathway. It is defined as the study of heritable changes in gene expression that does not alter the DNA sequence [187]. Several epigenetic mechanisms exist that regulate gene expression including non-coding RNAs, chromatin remodelling, histone modification and DNA methylation [188].

The non-coding RNAs or miRs are of particular interest in Nrf2 regulation. They are a class of small non-coding ssRNAs that are approximately 21-23 nucleotides in length. They exert their control over cellular functions including cell proliferation, differentiation and apoptosis through post-transcriptional regulation of gene expression. RNA polymerase II transcribes miRs from genomic loci, which is then processed by *Drosha* and transported to the nucleus as short hairpin precursors. Dicer then cleaves the miR precursors to generate mature miRs. The mature miRs are then loaded onto Argonaut proteins to form the RNA-induced silencing complex (RISC). This complex, together with base pairing of the miR onto the 3'- untranslated region (UTR) of target mRNAs, results in the inhibition of targeted gene expression. This occurs through either mRNA degradation or inhibition of protein translation (Figure 1.14) [24, 25, 189].

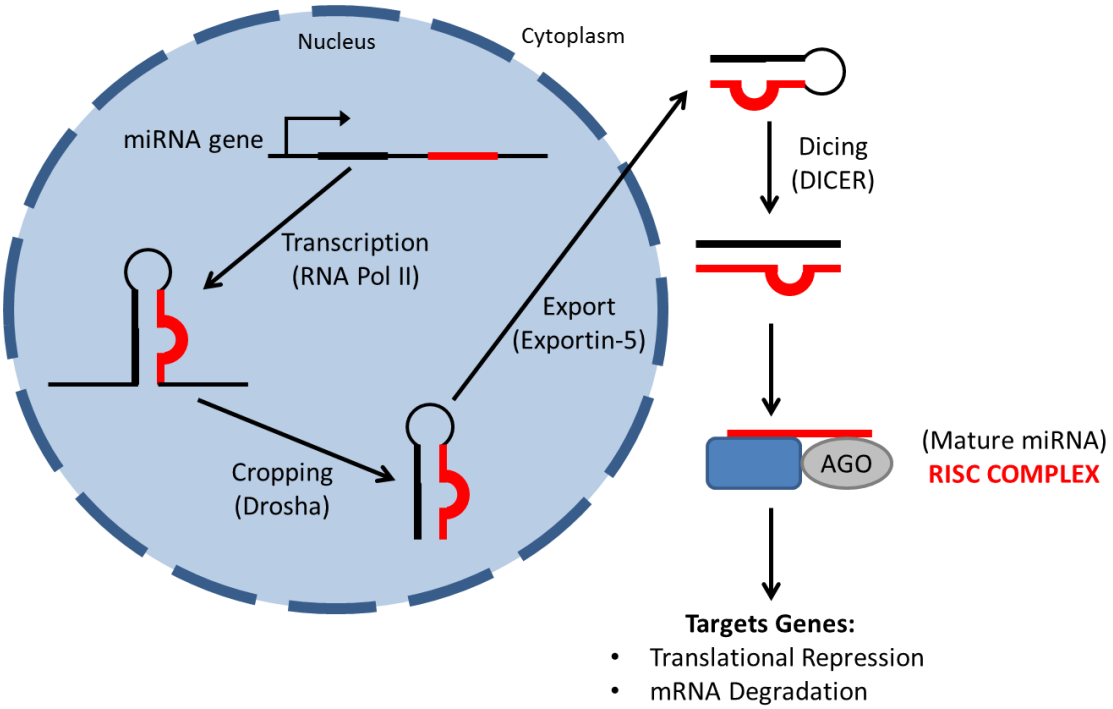

Figure 1.14 microRNA Biogenesis. The transcription of miRs from genomic DNA by RNA polymerase II, its subsequent cropping by *Drosha* and exportation out of the nucleus via Exportin-5, is followed by cleavage of the precursor miR by DICER. This results in the formation of mature miRs that binds to Argonaut proteins to form the RISC complex which leads to the transcriptional inhibition of target genes (Prepared by author).

Several studies have identified miRs that regulate Nrf2 and Keap1 signalling. Sangokoya and colleagues demonstrated that the miR-144 inhibited the expression of Nrf2 mRNA within a myelogenous leukaemia cell line [190]. This was later verified by Yamamoto et al., who showed a negative association of miR-144 with Nrf2 and downstream enzymes in response to diesel exhaust exposure [24]. The miR-28 was found to post-transcriptionally regulate Nrf2 expression by binding directly to Nrf2's mRNA 3'UTR which resulted in Nrf2 mRNA degradation. In addition, miR-28 also directly promoted Nrf2 protein degradation. This was not as a result of Keap1 protein expression or the Keap1-Nrf2 interaction but rather supressed mRNA expression through translational inhibition [25].

The controlled regulation and induction of Nrf2 is important to combat oxidative damage due to toxic insult from endogenous and exogenous sources to maintain homeostasis within the body. This prevents and helps fight against oxidative injury which may lead to inflammatory diseases, cardiovascular disorders, adverse birth outcomes and carcinogenesis. Investigating this pathway in response to AAP, and understanding how AAP affects this pathway may identify specific targets for potential therapeutic interventions for diseases associated with OS-damage such as adverse birth outcomes.

1.7. Reactive nitrogen species

Nitric oxide plays a major role in a variety of physiological processes including: the regulation of vasodilation, modification of neurotransmission, memory formation and has anti-microbial activity [191–193]. However, its elevated production has also been implicated in several inflammatory diseases, neurotoxicity, ischaemia and adverse birth outcomes [34, 107]. The term 'nitrosative stress' was coined by Stamler and colleagues [194], to describe the excessive production and dysregulated formation of NO and NO-metabolites.

1.7.1. Chemistry of NO

NO is a simple heterodimeric molecule that is highly reactive and unstable. It has a half-life of about 6-10 seconds [47] and degrades rapidly to form nitrites and nitrates. NO is produced endogenously and has exogenous sources. Within the body, NO is synthesised by a family of enzymes known as nitric oxide synthases (NOS). There are three isoforms that produce NO: neural (nNOS), endothelial (eNOS) and inducible (iNOS); each dependent on structural and functional properties including its tissue of origin [195, 196].

The constitutive NOS (cNOS) comprise eNOS and nNOS, which are found within vascular endothelial cells, neurons, smooth muscles and platelets. They have been shown to be positively modulated by IC levels of calcium and calmodulin binding [197]. NO produced via cNOS act as important signalling molecules within the cardiovascular and neural system.

The iNOS are found within immune cells such as macrophages, lymphocytes and neutrophils. It does not require activation by calcium and calmodulin, but is rather positively or negatively regulated by cell-cell contact in lymphocytes, inflammatory cytokines, bacterial and viral endotoxins. The production of NO via iNOS occurs over long periods of time and has been shown to be cytostatic or cytotoxic to tumour cells and microbial organisms [197]. Inflammatory cytokines that induce iNOS include interleukin (IL)- 1β, IL-6, IL-17, tumour necrosis factor (TNF)-α and interferon (IFN)- γ, whilst TNF-β, IL-4, 10, 11 and IL-13 suppress iNOS [198].

All NOS are haemoproteins that produce NO as a reaction by-product, during the catalytic conversion of L-arginine to L-citrulline. This reaction requires NADPH and O_2 with flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), heme and tetrahydrobiopterin (BH4) acting as cofactors (Figure 1.15) [195, 199].

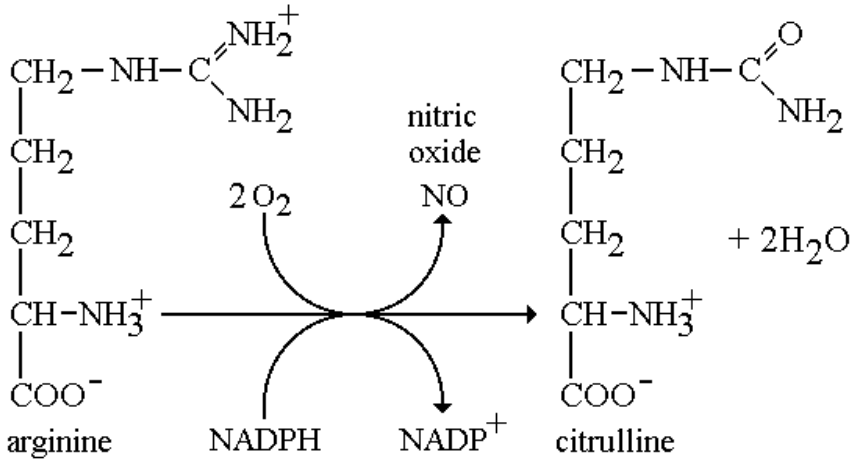

Figure 1.15 Synthesis of nitric oxide as a reaction by-product from the conversion of L-arginine to L-citrulline [200].

NO is able to diffuse across the cell membrane and has high affinity for Hb. At low levels of NO, it is rapidly inactivated by binding to Hb to form methaemoglobin (methHb) followed by degradation to inorganic nitrites and nitrates [201]. However, at higher levels of NO, it rapidly reacts with superoxide and O_2 to form peroxynitrite and dinitrogen trioxide, respectively. These NO intermediates and NO are highly reactive and result in macromolecular damage. The modification of peptides and proteins occurs as a result of S-nitrosylation, where NO directly modifies cysteine AA of target proteins to form S-nitrothiol adducts, as well as nitration of tyrosine in proteins. NO has been shown to directly oxidise to nitrite, which induces DNA damage, along with highly reactive peroxynitrite [107, 195, 196, 199].

Peroxynitrite is the most reactive intermediate of NO oxidation. It exists in two forms: nucleophilic or protonated peroxynitrite, it is highly reactive with a half-life <1second. Depending on its cellular environment and availability of reactive targets, it undergoes a variety of chemical reactions that not only cause nitration of tyrosine but also triggers LP, inactivate aconitases, inhibit the ETC and oxidise biological thiol-containing compounds. Peroxynitrite decays to form nitrates and undergoes homolytic decomposition to form highly reactive and toxic hydroxyl and nitrite radicals [196, 199].

1.7.2. Lipid peroxidation

Lipid peroxidation is the oxidative degradation of PUFA to products known as lipid peroxides or MDA. It consists of three stages: initiation, propagation and termination. Initiation strictly refers to the initial H^+ abstraction. Termination or LP decomposition is catalysed by transition metals and metal complexes [8].

The accumulation of NO within lipid bilayers and lipoproteins (hydrophobic cell regions) occurs due to its partition coefficient in octanol/water being 5.5 [199]. In addition, hydroperoxyl radicals, formed through the protonation of superoxide, a ROS produced via the ETC, are able to accumulate within this hydrophobic environment. This confers increased susceptibility of these areas to peroxynitrite attack and subsequent damage [199].

Peroxynitrite then undergoes decomposition to form hydroxyl and nitrite radicals that initiate LP chain reactions. These radicals abstract H^+ from the methylene group within the PUFA which leaves being an unpaired electron on the carbon. This C-centred radical undergoes molecular rearrangement to form conjugated dienes that react with O_2 to form peroxyl radicals, which is able to abstract H^+ itself, which subsequently sets up a chain reaction [199]. The peroxyl radical will continue to react with substrates present unless the chain reaction is terminated (Figure 1.16). Antioxidants such as vitamin E and other enzymes are able to end the reaction [8].

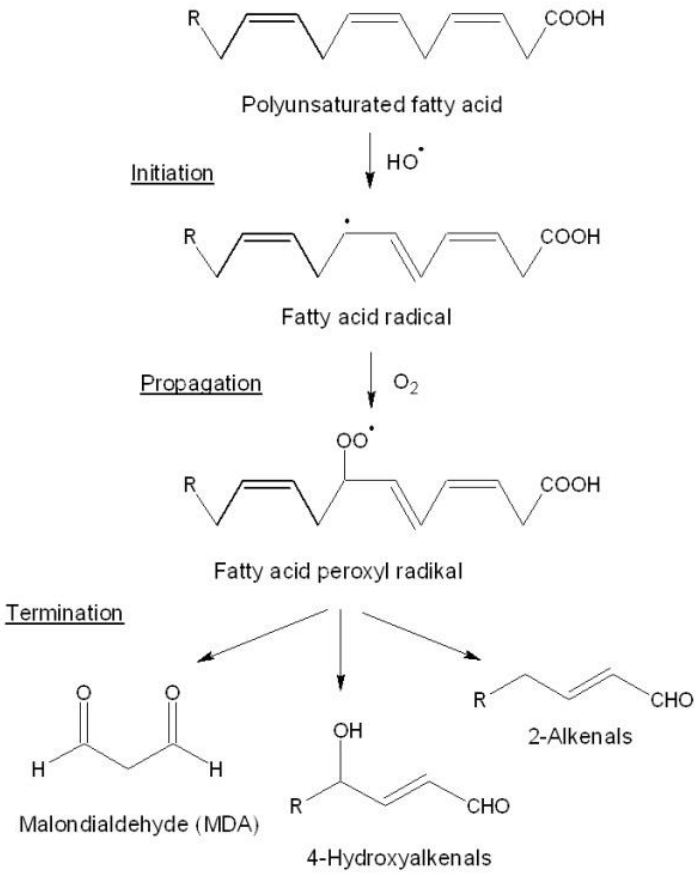

Figure 1.16 The lipid peroxidation chain reaction. Polyunsaturated fatty acids are oxidised to form fatty acid radicals that propagate the chain reaction until final termination into detectable products of LP including MDA, 4-hydroxyalkenals and 2-alkenals [202].

Lipid peroxidation has been associated with various inflammatory conditions [8, 203], cardiovascular disorders [204, 205] and adverse birth outcomes [103, 206, 207].

1.7.3. NO inhibition of OGG1 and other zinc-finger motifs

NO and its intermediates, not only directly target tissue components (lipids, DNA and proteins) but are able to inhibit or suppress antioxidant protective systems [208, 209]. The BER is an important pathway responsible for the repair of damaged DNA; with OGG1 function of utmost importance, as mentioned previously. OGG1, a Zn-finger protein, possess a cysteine rich-environment and critical thiol moieties that are necessary for catalytic activity [11]. NO and its intermediates, have a high affinity for cysteine-rich environments and are potent scavengers of cysteine, therefore suggests NO targets OGG1 directly [146]. A study by Jaiswal and colleagues, demonstrated that NO directly S-nitrosylates OGG1 which results in the loss of bound Zn and the irreversible loss of enzyme activity [12]. This was predated by Wink and Laval ‘s study on Zn-finger motif (Fpg) proteins; which showed that NO dramatically inhibited the Fpg proteins [146]. It was also found that the Cys326 variant of OGG1 SNP was more susceptible to oxidation by NO compared to the Ser326 wt [208]. Other enzymes containing Zn-finger motifs are possible targets of NO, with studies confirming this that showed cytochrome P450 [210] and SOD activity [211] were suppressed by NO action [146].

1.7.4. Human immunodeficiency virus’s induction of Nitric oxide

NO plays a pivotal role in the generation of the innate immune response against IC pathogens, such as HIV. NO passes readily into viral-infected cells and causes non-specific damage, which leads to various pathological events. NO acts as a host response modulator rather than an antiviral agent [198, 212].

HIV is a lentivirus that infects and kills vital immune cells within the body, which leads to acquired immune deficiency syndrome (AIDS) when the immune system is completely compromised and opportunistic infections are present [213]. HIV, is a ss-positive sense enveloped RNA virus, that consists of 9 genes within its genome. These genes encode 19 proteins essential for its function. The *pol* gene encodes reverse transcriptase, integrase and protease enzymes, the *env* gene generates glycoprotein (gp) 160 that is cleaved into gp120 and gp41 and the *gag* gene ensures the production of the capsid, matrix and nucleocapsid proteins. The 6 remaining genes regulate proteins that control the virus’s ability to infect cells and replicate new copies [214].

Upon entry into the target cell, HIV reverse transcribes its RNA genome to dsDNA, by reverse transcriptase, and is transported into the nucleus and integrated into the host genome by integrase. Once HIV is integrated into the host genome, it either becomes latent to avoid detection or it undergoes viral replication. During viral replication, the genome transcribes and translates new copies of the viral proteins that are packaged and released from the host cell as new viral particles that are able to infect other target cells [214, 215].

Several studies have found an increase in NO production within HIV infected individuals: adults with AIDS showed increased NO production [216], Zangerle and colleagues found HIV infected patients had greater nitrites and nitrates compared to blood controls, with even greater levels being observed in low CD4 count individuals [217]. The same effect was observed in HIV infected children, with an increase in circulating cytokines IL-1β, TNF-α and INF-γ, also observed [218]. Groeneveld's study showed serum nitrate was positively associated with cell-associated and plasma viral loads of HIV [219]. NO was reported to inhibit HIV replication and induce HIV reactivation within infected cells. It is speculated that the overproduction of NO is able to activate viral replication, especially within the primary infection and late stages of disease. However, the low production of NO may inhibit viral replication, most notably observed within the symptomless stage of disease or during treatment with antiretroviral drugs [198, 220–223]. HIV infected patients have been reported to be under chronic OS, other than the increased NO already noted, the *tat* gene has been shown to enhance Nrf2 expression, therefore, leading to the activation of the ARE and decreased GSH levels which induces RO. In addition, HIV infection has been reported to compromise SOD activity, increase LP and decrease the antioxidant response [212, 224–226].

HIV has been implicated in NO-mediated RNS, the degradation of the antioxidant system and increased risk for adverse birth outcomes such as LBW and PTB (reviewed by Xiao et al. [227]. This chronic OS environment could be detrimental to foetal growth and development within pregnant woman. However, no studies linking these effects of HIV and the effects of AAP on HIV toxicity have been reported. Understanding the combined effect of HIV and AAP exposure may identify potential biomarkers or targets of intervention; especially within SA.

1.8. Oxidative stress and Endoplasmic reticulum stress

The imbalance of ROS, as a result of endogenous or exogenous toxicants, leads to the disruption of cellular calcium homeostasis [105].

The ER acts as a major reservoir of IC calcium and therefore plays a critical role in calcium homeostasis [17, 18]. This homeostasis is necessary for the proper functioning of the protein folding machinery; disruption results in the activation of the UPR pathway that endeavours to restore balance within the ER (Figure 1.17). This is achieved through co-ordinated steps that reduce the misfolded/unfolded protein (UP) concentration through the suppression of protein synthesis, facilitation of protein degradation and increasing the protein folding capacity of the ER (reviewed elsewhere [18, 19, 228]). Failure to mitigate ER stress can lead to cellular death [105].

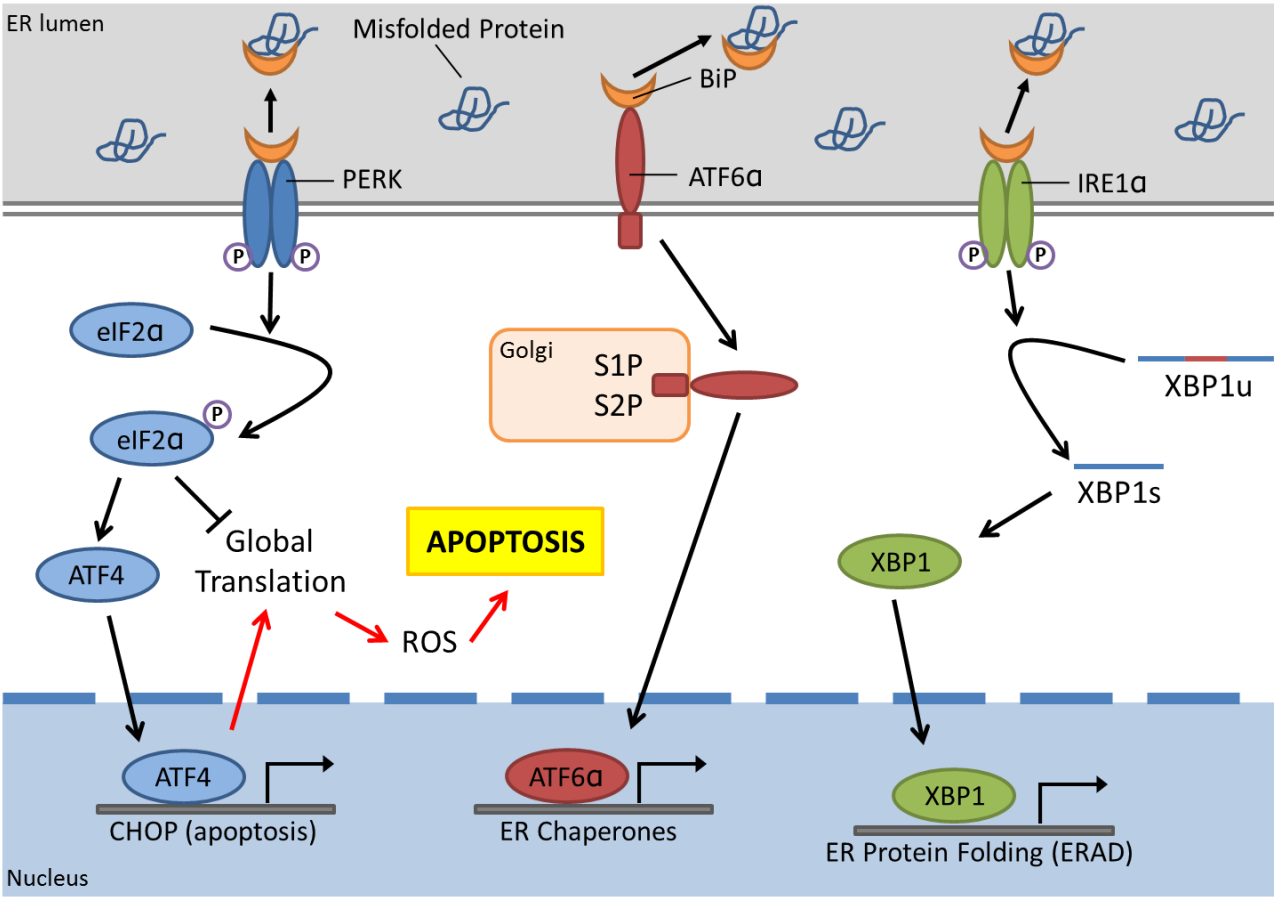

Figure 1.17 The unfolded protein response pathway. In stress free conditions, luminal protein chaperone binding immunoglobulin protein (BiP) also known as 78 kDa glucose-regulated protein (GRP78) binds to the intraluminal domain of the three UPR sensors: PERK, inositol-requiring enzyme 1α (IRE1α) and ATF6, rendering them inactive. Upon ER stress and the accumulation of UP and increased protein cargo within the ER, BiP dissociates from the UPR sensors and sequesters UP within the ER lumen, due to its higher affinity for UP. The dissociation of BiP from IRE1α and PERK causes the oligomerisation, auto-phosphorylation and activation of these sensors and downstream signalling pathways. The activation of the PERK arm leads to the phosphorylation of eukaryotic initiation factor 2 (eIF2) α which subsequently induces ATF4 mRNA translation and the inhibition of global protein translation. Pro-apoptotic genes

such as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) are induced by ATF4 resulting in cellular death. Global translational inhibition results in decreased translation of antioxidant proteins leading to increased ROS and apoptosis. Whilst the dissociation of BiP from ATF6, allows the translocation of ATF6 to the Golgi apparatus where it is processed by serine protease 1 (S1P) and metalloprotease site-2 protease (S2P) to yield an active transcription factor which enters the nucleus. The UP sequestered on BiP are translocated to the cytosol for proteasomal degradation by ER-associated degradation (ERAD) machinery. These three arms of UPR pathway endeavour to mitigate ER stress by facilitating protein degradation, increasing the ER's protein folding capacity by inducing protein chaperone production and by suppressing protein synthesis [18, 19, 228].

A reciprocal interplay between ROS production and increased ER stress due to calcium release has been suggested. Calcium release channels on the ER membrane, including the ryanodine receptor and the inositol-1,4,5-triphosphate receptor (IP3R), are activated by ROS. This induces calcium ions (Ca^{2+}) to migrate into the cytosol from the ER lumen. Increased IC Ca^{2+} results in the loss of ER chaperone proteins which impairs ER function. This leads to increased UP levels that can generate ROS in its attempt to repair UP. Increased IC Ca^{2+} also adversely affects mitochondrial activity which further increases ROS production [105]. This will be discussed in detail in sections: 1.8.1. and 1.8.2. below.

1.8.1. Mitochondrial ROS generation as a result of increased intracellular calcium occurs via the following mechanisms:

The mitochondria experiences increased calcium loading; which generates increased ROS production. Calcium loading inhibits the ETC at complex III through the opening of the permeability transition pore (PTP). This releases cytochrome c from the inner mitochondrial membrane which blocks complex III. Calcium inhibition of complex III results in the increase of ubisemiquinone radical intermediate (QH·) (the quantity of QH· reflects the amount of mitochondrial ROS produced) which increases ROS production. There is also an increase in QH· generation observed when the ETC turnover occurs more rapidly [229, 230].

Additional increases in ROS occur via the following mechanisms:

- Increased cytosolic Ca^{2+} stimulates the tricarboxylic acid cycle leading to increased O_2 consumption and ROS production.
- The Ca^{2+} induced opening of the PTP may result in GSH leakage into the cytosol from the mitochondrial matrix. This indirectly causes increased ROS generation as a result of reduced antioxidant capability.

The amplified levels of mitochondrial ROS generation; further activates Ca^{2+} release from the ER due to their close proximity. Increased Ca^{2+} close to the mitochondria causes increased ROS production, as a result of Ca^{2+} loading and the opening of the PTP. Generation of ROS then acts on Ca^{2+} release channels further increasing Ca^{2+}. A feedback loop is then established where increased IC Ca^{2+} induces ROS production which increases Ca^{2+} release into the cytosol, which loops back around. Therefore, suggesting a reciprocal interaction between ROS and ER stress that threatens cell survival [105, 229, 231].

1.8.2. Unfolded protein repair induces ROS generation by the following proposed mechanism:

The ER is a vast membranous organelle [18] that is the site of synthesis, folding, maturation and modification and trafficking of secreted and transmembrane proteins [19, 232]. The addition of a disulphide bonds are required for the stability, function and maturation of secretory proteins.

Misfolded proteins, formed as a result of incorrectly paired cysteine residues, may have an inappropriate disulfide bond that requires removal [229]. The formation of disulfide bonds generates about 25% of ROS in the cell, during ER oxidation protein folding [233]. Disulfide bond formation is driven by protein disulfide isomerase (PDI), where PDI becomes oxidized and subsequently reduced by ER oxidoreductin 1 that transfers electrons onto O_2. This leads to the generation of ROS. Reduced GSH may assist this process in UP which results in the generation of oxidized GSSG. The depletion of GSH induces ROS formation [229, 234].

Reactive oxygen species can be generated independent of disulfide bond formation. The accumulation of UP, within the ER lumen, causes Ca^{2+} to leak into the cytosol which generates ROS production. Alternatively, due to the high energy dependent processes of folding and refolding proteins within the ER, ATP depletion could result from UP accumulation. This would induce the mitochondrial oxidative phosphorylation pathway to increase ATP turnover thereby increasing ROS production [229].

1.8.3. Ambient air pollution, Adverse birth outcomes and Endoplasmic reticulum stress

There are a few studies that have investigated the effect of AAP on ER stress. Andersson et al. demonstrated that exposure to low levels of 1-nitropyrene (diesel exhaust) increased DNA damage which resulted in increased ROS and GRP78, a marker of ER stress [64]. Watterson et al. associated PM levels with the induction of ER stress [235]. Laing et al. demonstrated that

$PM_{2.5}$ induced PERK-dependent CHOP expression in mouse lung, liver and macrophage models [236]. This induction was dependent on ROS production for $PM_{2.5}$ induced apoptosis to occur. This gives evidence for AAP induced ER stress as a result of ROS production; demonstrating a reciprocal interaction between OS and ER stress.

The effect of ER stress on LBW has only been briefly investigated. Kawakami et al. has demonstrated that ER stress markers are increased in LBW neonates [237]. This area of research is important due to the reciprocal interaction shown between OS and ER stress, as well as the strong relationship observed between OS and LBW. It could be a potential mechanism linking OS and adverse birth outcome pathology to AAP toxicity.

1.9. Future considerations for ambient air pollution induction of adverse birth outcomes

In developing and developed countries, low birthweight and preterm birth are important indicators of infant morbidity [79]. Ambient air pollution has also increased within countries due to large industrial services such as power plants, coal-mines and petroleum factories. The vulnerable and highly susceptible state of foetuses *in utero*, due to their fast differentiation, high sensitivity to signals and rapid developmental processes, makes exposure to AAP during pregnancy highly risky and harmful.

As mentioned in this review, several studies have shown the negative effect of AAP and foetal development, with OS playing an important role in adverse birth outcomes. The mechanism by which OS causes these adverse birth outcomes needs further investigation, with ER stress and NO metabolism playing an important role in its pathology. The link between ER stress, RNS and OS is minimally understood and needs further analysis.

However, in the context of SA, two crucial factors need to be taken into account when investigating AAP exposure effects on adverse birth outcomes. These include obesity and the HIV status of the pregnant women.

Sub-Saharan Africa is heavily burdened with HIV where approximately 25.6 million people are living with HIV, of these about 27.4% are living within SA, with women accounting for more than half the total infected population [27]. In addition, SA has the highest obesity rate within sub-Saharan Africa, with more than 68% of women classified as overweight or obese [28]. The interaction between these conditions and AAP exposure may affect an individual's susceptibility to oxidative injury and disease, as both these conditions are associated with

chronic stress and inflammation. In addition, these conditions individually have been associated with adverse birth outcomes and infant morbidity; in combination, their effect may be potentiated [227, 238, 239].

The discovery of novel biomarkers and targeted interventions for the prevention of adverse birth outcomes, especially within the context of SA, requires the consideration of obesity and HIV status of the pregnant women. Identifying risk factors would allow for the better stratification of mother's disease susceptibility and risk, which could lead to better surveillance of AAP as well as guide potential targeted interventions.

1.10. References

1. WHO (2012) Mortality and burden of disease from ambient air pollution. In: WHO Glob. Heal. Obs. data. http://www.who.int/gho/phe/outdoor_air_pollution/burden/en/. Accessed 23 Oct 2017
2. Šrám RJ, Binková B, Dejmek J, Bobak M (2005) Ambient air pollution and pregnancy outcomes: A review of the literature. Environ Health Perspect 113:375–382. doi: 10.1289/ehp.6362
3. Kampa M, Castanas E (2008) Human health effects of air pollution. Environ Pollut 151:362–367. doi: 10.1016/j.envpol.2007.06.012
4. Fleischer NL, Merialdi M, Donkelaar A Van, et al (2014) Outdoor Air Pollution , Preterm Birth , and Low Birth Weight : Analysis of the World Health Organization Global Survey on Maternal and Perinatal Health. Environ Health Perspect 122:425–430. doi: 10.1289/ehp.1306837
5. DEA (2012) Chapter 5 : Air Quality Draft 2 version 2. http://soer.deat.gov.za/dm_documents/Chapter_5__Air_Quality_4kp8X.pdf. Accessed 30 Mar 2016
6. Kelly FJ (2003) Oxidative stress: its role in air pollution and adverse health effects. Occup Environ Med 60:612–616. doi: 10.1136/oem.60.8.612
7. Kim Y, Hong Y, Lee K, et al (2005) Oxidative stress in pregnant women and birth weight reduction. Reprod Toxicol 19:487–492. doi: 10.1016/j.reprotox.2004.10.007
8. Gutteridge JMC (1995) Lipid-peroxidation and antioxidants as biomarkers of tissue-damage. Clin Chem 41:1819–1828. doi: 7497639
9. Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47:89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

10. Romieu I, Ramirez-Aguilar M, Sienra-Monge JJ, et al (2006) GSTM1 and GSTP1 and respiratory health in asthmatic children exposed to ozone. Eur Respir J 28:953–9. doi: 10.1183/09031936.06.00114905
11. Bravard A, Vacher M, Gouget B, et al (2006) Redox regulation of human OGG1 activity in response to cellular oxidative stress. Mol Cell Biol 26:7430–6. doi: 10.1128/MCB.00624-06
12. Jaiswal M, LaRusso NF, Nishioka N, et al (2001) Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. Cancer Res 61:6388–6393.
13. Moi P, Chant K, Asunis I, et al (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the f-globin locus control region. Genetics 91:9926–9930. doi: 10.1073/pnas.91.21.9926
14. Itoh K, Igarashi K, Hayashi N, et al (1995) Cloning and Characterization of a Novel Erythroid Cell-Derived CNC Family Transcription Factor Heterodimerizing with the Small Maf Family Proteins. Mol Cell Biol 15:4184–4193. doi: 10.1128/MCB.15.8.4184
15. Venugopal R, Jaiswal AK (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase 1 gene. Proc Natl Acad Sci 93:14960–14965. doi: 10.1073/pnas.93.25.14960
16. Kobayashi M, Yamamoto M (2005) Molecular Mechanisms Activitating the Nrf2-Keap1 Pathway of Antioxidant Gene Regulation. Antioxid Redox Signal 7:385–394. doi: 10.1089/ars.2005.7.385
17. Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13:89–102.
18. Hotamisligil GS (2010) Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 140:900–917.
19. Saito A, Ochiai K, Kondo S, et al (2011) Endoplasmic reticulum stress response mediated by the PERK-eIF2 {alpha}-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. Sci Signal 286:4809–4818.
20. Romieu I, Moreno-Macias H, London SJ (2010) Gene by environment interaction and ambient air pollution. Proc Am Thorac Soc 7:116–22. doi: 10.1513/pats.200909-097RM
21. Mustafa MD, Pathak R, Ahmed T, et al (2010) Association of glutathione S-transferase M1 and T1 gene polymorphisms and oxidative stress markers in preterm labor. Clin Biochem 43:1124–1128. doi: 10.1016/j.clinbiochem.2010.06.018
22. Bravard A, Vacher M, Moritz E, et al (2009) Oxidation status of human ogg1-s326c

polymorphic variant determines cellular DNA repair capacity. Cancer Res 69:3642–3649. doi: 10.1158/0008-5472.CAN-08-3943

23. Cheng X, Ku CH, Siow RCM (2013) Regulation of the Nrf2 antioxidant pathway by microRNAs: New players in micromanaging redox homeostasis. Free Radic Biol Med 64:4–11. doi: 10.1016/j.freeradbiomed.2013.07.025
24. Yamamoto M, Singh A, Sava F, et al (2013) MicroRNA expression in response to controlled exposure to diesel exhaust: attenuation by the antioxidant N-acetylcysteine in a randomized crossover study. Environ Health Perspect 121:670–5. doi: 10.1289/ehp.1205963
25. Yang M, Yao Y, Eades G, et al (2011) MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. Breast Cancer Res Treat 129:983–91. doi: 10.1007/s10549-011-1604-1
26. WHO (2016) An estimated 12.6 million deaths each year are attributable to unhealthy environments. In: WHO News release. http://who.int/mediacentre/news/releases/2016/deaths-attributable-to-unhealthy-environments/en/. Accessed 20 Jan 2017
27. UNAIDS (2015) UNAIDS Fact sheet November 2015: Global HIV Statistics. http://www.unaids.org/en/regionscountries/countries/southafrica/. Accessed 16 Jan 2017
28. STATS SA (2016) South Africa Demographic and Health Survey 2016: Key Indicator Report. http://www.statssa.gov.za/publications/Report 03-00-09/Report 03-00-092016.pdf. Accessed 2 Aug 2017
29. Saker M, Soulimane Mokhtari N, Merzouk SA, et al (2008) Oxidant and antioxidant status in mothers and their newborns according to birthweight. Eur J Obstet Gynecol Reprod Biol 141:95–99. doi: 10.1016/j.ejogrb.2008.07.013
30. Mutlu B, Aksoy N, Cakir H, et al (2011) The effects of the mode of delivery on oxidative-antioxidative balance. J Matern Neonatal Med 24:1367–1370. doi: 10.3109/14767058.2010.548883
31. Casanueva E, Viteri FE (2003) Iron and oxidative stress in pregnancy. J Nutr 133:1700S–1708S.
32. Salmasi G, Grady R, Jones J, McDonald SD (2010) Environmental tobacco smoke exposure and perinatal outcomes: a systematic review and meta-analyses. Acta Obstet Gynecol Scand 89:423–441. doi: 10.3109/00016340903505748
33. Leonardi-Bee J, Smyth A, Britton J, Coleman T (2008) Environmental tobacco smoke and fetal health: systematic review and meta-analysis. Arch Dis Child Fetal Neonatal Ed 93:F351–F361. doi: 10.1136/adc.2007.133553

34. Tabacova S, Baird DD, Balabaeva L (1998) Exposure to oxidized nitrogen: lipid peroxidation and neonatal health risk. Arch Env Heal 53:214–221. doi: 10.1080/00039899809605698
35. Seo J, Ha E, Kim O, et al (2007) Environmental health surveillance of low birth weight in Seoul using air monitoring and birth data [in Korean]. J Prev Med Public Heal 40:363–370. doi: https://doi.org/10.3961/jpmph.2007.40.5.363
36. Darrow LA, Klein M, Strickland MJ, et al (2011) Ambient air pollution and birth weight in full-term infants in Atlanta, 1994-2004. Environ Health Perspect 119:731–737. doi: 10.1289/ehp.1002785
37. Rossner P, Tabashidze N, Dostal M, et al (2011) Genetic, biochemical, and environmental factors associated with pregnancy outcomes in newborns from the Czech Republic. Environ Health Perspect 119:265–271. doi: 10.1289/ehp.1002470
38. Backes C, Nelin T, Gorr M, Wold L (2013) Early life exposure to air pollution: how bad is it? Toxicol Lett 216:47–53. doi: http://dx.doi.org/10.1016/j.toxlet.2012.11.007
39. WHO (2010) Selected pollutants. WHO Guiledlines Indoor Air Qual. doi: 10.1186/2041-1480-2-S2-I1
40. Lodovici M, Bigagli E (2011) Oxidative stress and air pollution exposure. J Toxicol. doi: 10.1155/2011/487074
41. Internet (2017) Nitrogen Dioxide Sources. http://www.sabc.co.za/wps/wcm/connect/8ebee9004148d7609a729f3895839b19/factory-pollution.jpg?MOD=AJPERES&CACHEID=8ebee9004148d7609a729f3895839b19%0A. http://charlesandhudson.com/wp/photos/GasStove.jpg%0A. https://www.unitedfireplaceandstove.com/blog/wp-cont. Accessed 7 Nov 2017
42. Sagai M, Ichinose T (1991) Experimental study on lipid peroxide formation and tumor-promoting effects of nitrogen dioxide and ozone. In: Davies KJ. (ed) Oxidative Damage Repair Chem. Biol. Med. Asp. Pergamon Press, pp 511–516
43. Nakajima T, Kusumoto S (1968) Effects of nitrogen dioxide exposure on the contents of reduced glutathione in mouse lung. Proc Osaka Pref lnst Public Heal 6:17–21.
44. Nakajima T, Kusurnoto S, Chen C (1969) Studies on the contents of reduced glutathione and histopathological studies on the lungs of mice exposed to nitrogen dioxide gas. Proc Osaka Pref lnst Public Heafth 7:35–44.
45. Hatch C, Slade R, Selgrade M, Al E (1986) Nitrogen dioxide exposure and lung antioxidants in ascorbic acid-deficient guinea pigs. Toxicol Appl Pharrnacol 82:351–59.
46. Shah PS, Balkhair T (2011) Air pollution and birth outcomes: A systematic review. Environ Int 37:498–516. doi: 10.1016/j.envint.2010.10.009

47. Chadha S, Jain V, Gupta I, Khullar M (2007) Nitric oxide metabolite levels in preterm labor. J Obstet Gynaecol Res 33:710–7. doi: 10.1111/j.1447-0756.2007.00639.x
48. Kajekar R (2007) Environmental factors and developmental outcomes in the lung. Pharmacol Ther 114:129–145. doi: 10.1016/j.pharmthera.2007.01.011
49. Nichols BG, Woods JS, Luchtel DL, et al (2001) Effects of ozone exposure on nuclear factor-κB activation and tumor necrosis factro-α expression in human nasal epithelial cells. Toxicol Sci 60:356–362.
50. Bassett D, Elbon-Copp C, Otterbein S, et al (2001) Inflammatory cell availability affects ozone-induced lung damage. J Toxicol Environ Health 64:547–565.
51. Ha EH, Hong YC, Lee BE, et al (2001) Is air pollution a risk factor for low birth weight in Seoul? Epidemiology 12:643–648. doi: 10.1097/00001648-200111000-00011
52. Gumus S, Kipmen Korgun D, Bilmen S, et al (2000) Effects of Sulfur Dioxide Inhalation on Plasma Vitamin C and Ceruloplasmin in Ageing Rats. Ind Health 38:319–322.
53. Meng Z, Qin G, Etal (2003) Oxidative damage of sulfur dioxide inhalation on lungs and hearts of mice. Environ Res 93:285–292.
54. Meng Z, Bai W (2004) Oxidation damage of sulfur dioxide on testicles of mice. Environ Res 96:298–304.
55. Singh J (1989) Neonatal development altered by maternal sulfur dioxide exposure. Neurotoxicology 10:523–527.
56. Mazzoli-Rocha F, Fernandes S, Einicker-Lamas M, Zin WA (2010) Roles of oxidative stress in signaling and inflammation induced by particulate matter. Cell Biol Toxicol 26:481–498. doi: 10.1007/s10565-010-9158-2
57. Proietti E, Röösli M, Frey U, Latzin P (2013) Air Pollution During Pregnancy and Neonatal Outcome: A Review. J Aerosol Med Pulm Drug Deliv 26:9–23. doi: 10.1089/jamp.2011.0932
58. Ritz B, Wilhelm M, Hoggatt KJ, Ghosh JKC (2007) Ambient air pollution and preterm birth in the environment and pregnancy outcomes study at the University of California, Los Angeles. Am J Epidemiol 166:1045–1052. doi: 10.1093/aje/kwm181
59. Ritz B, Wilhelm M (2008) Ambient air pollution and adverse birth outcomes: Methodologic issues in an emerging field. Basic Clin Pharmacol Toxicol 102:182–190. doi: 10.1111/j.1742-7843.2007.00161.x
60. Birben E, Sahiner UM, Sackesen C, et al (2012) Oxidative Stress and Antioxidant Defense. World Allergy Organ J 5:9–19. doi: 10.1097/WOX.0b013e3182439613
61. Barbusinski K (2009) Fenton reaction - Controversy concerning the chemistry. Ecol Chem Eng S 16:347–358.

62. Lewtas J (2007) Air pollution combustion emissions: Characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat Res - Rev Mutat Res 636:95–133. doi: 10.1016/j.mrrev.2007.08.003
63. Mordukhovich I, Beyea J, Herring AH, et al (2016) Polymorphisms in DNA repair genes, traffic-related polycyclic aromatic hydrocarbon exposure and breast cancer incidence. Int J Cancer 139:310–321. doi: 10.1002/ijc.30079
64. Andersson H, Piras E, Demma J, et al (2009) Low levels of the air pollutant 1-nitropyrene induce DNA damage, increased levels of reactive oxygen species and endoplasmic reticulum stress in human endothelial cells. Toxicology 262:57–64. doi: 10.1016/j.tox.2009.05.008
65. Kuang D, Zhang W, Deng Q, et al (2013) Dose-response relationships of polycyclic aromatic hydrocarbons exposure and oxidative damage to DNA and lipid in coke oven workers. Envioronmental Sci Technol 47:7446–56. doi: 10.1021/es401639x
66. Li J, Lu S, Liu G, et al (2015) Co-exposure to polycyclic aromatic hydrocarbons, benzene and toluene and their dose-effects on oxidative stress damage in kindergarten-aged children in Guangzhou, China. Sci Total Environ 524–525:74–80. doi: 10.1016/j.scitotenv.2015.04.020
67. Guastella L, Knudsen S (2007) South Durban Basin Multi-Point Plan: Case Study Report. Output A2 DEAT AQA Implement Air Qual Manag Plan E. A. a. Tourism. Republic of South Africa, a. Govern.
68. Muttoo S, Ramsay L, Brunekreef B, et al (2017) Land Use Regression Modelling Estimating Nitrogen Oxides Exposure in Industrial South Durban, South Africa. Sci Total Environ 610–611:1439–1447. doi: 10.1016/j.scitotenv.2017.07.278
69. Kistnasamy EJ, Robins TG, Naidoo R, et al (2008) The relationship between asthma and ambient air pollutants among primary school students in Durban, South Africa. Int J Environ Heal 2:365. doi: 10.1504/IJENVH.2008.020929
70. UKZN (2011) UKZN SCIENTISTS TAKE ON SOUTH DURBAN BASIN. UKZN enewsletter Volume 5, Issue 24. http://enewsletter.ukzn.ac.za/Story.aspx?id=41&storyid=451. Accessed 7 Nov 2017
71. Brooks S, Sutherland C, Scott D, Guy H (2010) Integrating qualitative methodologies into risk assessment: Insights from South Durban. S Afr J Sci 106:1–10. doi: 10.4102/sajs.v106i9/10.324
72. Kistnasamy EJ (2005) The relationship between asthma and outdoor air pollution concetrations of sulphur dioxide (SO2), oxides of nitrogen (NOx), ozone (O3), total reduced sulphates (TRS), carbon monoxide (CO) and respirable particulate matter less

than 10 microns (PM10) in le.

73. Naidoo RN, Robins T, Batterman S, et al (2013) Ambient pollution and respiratory outcomes among schoolchildren in Durban, South Africa. South African J Child Heal 7:127–134. doi: 10.7196/sajch.598
74. Nagiah S, Phulukdaree A, Naidoo D, et al (2015) Oxidative stress and air pollution exposure during pregnancy: A molecular assessment. Hum Exp Toxicol 34:838–847. doi: 10.1177/0960327114559992
75. Agarwal A, Aponte-Mellado A, Premkumar BJ, et al (2012) The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 10:49. doi: 10.1186/1477-7827-10-49
76. Veerbeek JHW, Tissot Van Patot MC, Burton GJ, Yung HW (2015) Endoplasmic reticulum stress is induced in the human placenta during labour. Placenta 36:88–92. doi: 10.1016/j.placenta.2014.11.005
77. Slama R, Darrow L, Parker J, et al (2008) Meeting Report: Atmospheric Pollution and Human Reproduction. Env Heal Persect 116:791–798.
78. Negi R, Pande D, Kumar A, et al (2012) Evaluation of biomarkers of oxidative stress and antioxidant capacity in the cord blood of preterm low birth weight neonates. J Matern Neonatal Med 25:1338–1341. doi: 10.3109/14767058.2011.633672
79. Wang X, Ding H, Ryan L, Xu X (1997) Association between air pollution and low birth weight: a community-based study. Environ Health Perspect 105:514–520. doi: 10.1289/ehp.97105514
80. Ahluwalia IB, Grummer-Strawn L, Scanlon KS (1997) Exposure to environmental tobacco smoke and birth outcome: increased effects on pregnant women aged 30 years or older. Am J Epidemiol 146:42–47.
81. Williams L, Spence A, Tideman S (1977) Implication of the observed effect of air pollution on birth weight. Biodemography Soc Biol 24:1–9.
82. Glinianaia S V, Rankin J, Bell R, et al (2004) Particulate air pollution and fetal health: a systematic review of the epidemiologic evidence. Epidemiology 15:36–45. doi: 10.1097/01.ede.0000101023.41844.ac
83. Darrow L, Klein M, Flanders W, et al (2009) Ambient air pollution an dpreterm birth: a time series analysis. Epidemiology 20:689–698.
84. Ritz B, Yu F (1999) The effect of ambient carbon monoxide on low birth weight among children born in southern California between 1989 and 1993. Environ Health Perspect 107:17–25. doi: 10.1289/ehp.9910717
85. Bobak M, Leon D (1999) Pregnancy outcomes and outdoor levels of particulates,

sulphur dioxide and nitrogen oxides. An ecological study in districts of the Czech Republic 1986-88. Occup Environ Med 56:539–543.

86. Bobak M (2000) Outdoor air pollution, low birth weight, and prematurity. Environ Health Perspect 108:173–176. doi: 10.2307/3454517
87. Rogers JF, Thompson SJ, Addy CL, et al (2000) Association of very low birth weight with exposures to environmental sulfur dioxide and total suspended particulates. Am J Epidemiol 151:602–613.
88. Maisonet M, Bush TJ, Correa A, Jaakkola JJ (2001) Relation between ambient air pollution and low birth weight in the Northeastern United States. Environ Health Perspect 109:351–356.
89. Vassilev ZP, Robson MG, Klotz JB (2001) Outdoor exposure to airborne polycyclic organic matter and adverse reproductive outcomes: A pilot study. Am J Ind Med 40:255–262.
90. Maroziene L, Grazuleviciene R (2002) Maternal exposure to low-level air pollution and pregnancy outcomes: a population-based study. Environ Heal 1:6. doi: 10.1186/1476-069X-1-6
91. Liu S, Krewski D, Shi Y, et al (2003) Association between gaseous ambient air pollutants and adverse pregnancy outcomes in Vancouver, Canada. Environ Health Perspect 111:1773–1778. doi: 10.1289/ehp.6251
92. Mishra V, Dai X, Smith KR, Mika L (2004) Maternal Exposure to Biomass Smoke and Reduced Birth Weight in Zimbabwe. Ann Epidemiol 14:740–747.
93. Nascimento LF, Moreira D a (2009) Are environmental pollutants risk factors for low birth weight? Cad Saude Publica 25:1791–1796. doi: S0102-311X2009000800015 [pii]
94. Ballester F, Estarlich M, Iñiguez C, et al (2010) Air pollution exposure during pregnancy and reduced birth size : a prospective birth cohort study in Valencia , Spain. 14–16.
95. Kloog I, Melly SJ, Ridgway WL, et al (2012) Using new satellite based exposure methods to study the association between pregnancy pm2.5 exposure, premature birth and birth weight in Massachusetts. Environ Heal 11:40. doi: 10.1186/1476-069X-11-40
96. Clemente DBP, Casas M, Vilahur N, et al (2016) Prenatal ambient air pollution, placental mitochondrial DNA content, and birth weight in the INMA (Spain) and ENVIRONAGE (Belgium) birth Cohorts. Environ Health Perspect 124:659–665. doi: 10.1289/ehp.1408981
97. Hao H, Chang HH, Holmes HA, et al (2016) Air pollution and preterm birth in the U.S. State of Georgia (2002–2006): Associations with concentrations of 11 ambient air pollutants estimated by combining community multiscale air quality model (CMAQ)

simulations with stationary monitor measurements. Environ Health Perspect 124:875–880. doi: 10.1289/ehp.1409651

98. Chavatte-palmer P, Rousseau D (2015) A perspective on the developmental toxicity of inhaled nanoparticles. Reprod Toxicol. doi: 10.1016/j.reprotox.2015.05.015
99. Wells PG, Mccallum GP, Chen CS, et al (2009) Oxidative stress in developmental origins of disease: Teratogenesis, neurodevelopmental deficits, and cancer. Toxicol Sci 108:4–18. doi: 10.1093/toxsci/kfn263
100. Matsubasa T, Uchino T, Karashima S, et al (2002) Oxidative Stress in Very Low Birth Weight Infants As Measured by Urinary 8-OHdG. Free Radic Res 36:189–193. doi: 10.1080/10715760290006510
101. Gupta P, Narang M, Banerjee BD, Basu S (2004) Oxidative stress in term small for gestational age neonates born to undernourished mothers: a case control study. BMC Pediatr 4:14. doi: 10.1186/1471-2431-4-14
102. Kamath U, Rao G, Kamath SU, Rai L (2006) Maternal and fetal indicators of oxidative stress during intrauterine growth retardation (IUGR). Indian J Clin Biochem 21:111–115. doi: 10.1007/BF02913077
103. Gveric-Ahmetasevic S, Sunjic SB, Skala H, et al (2009) Oxidative stress in small-for-gestational age (SGA) term newborns and their mothers. Free Radic Res 43:376–384. doi: 10.1080/10715760902783285
104. Kumarathasan P, Vincent R, Bielecki A, et al (2016) Infant birth weight and third trimester maternal plasma markers of vascular integrity: the MIREC study. Biomarkers 21:257–266. doi: 10.3109/1354750X.2015.1134663
105. Burton GJ, Jauniaux E (2011) Oxidative stress. Best Pract Res Clin Obstet Gynaecol 25:287–299. doi: 10.1016/j.bpobgyn.2010.10.016
106. Storr SJ, Woolston CM, Zhang Y, Martin SG (2013) Redox environment, free radical, and oxidative DNA damage. Antioxid Redox Signal 18:2399–2408. doi: 10.1089/ars.2012.4920
107. Klatt P, Lamas S (2000) Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. 4944:4928–4944.
108. Aruoma OI (1998) Free radicals, oxidative stress, and antioxidants in human health and disease. J Am Oil Chem Soc 75:199–212. doi: 10.1007/s11746-998-0032-9
109. Kohen R, Nyska A (2002) Toxicologic Pathology. Toxicol Pathol 30:620–650. doi: 10.1080/0192623029016672
110. Al-Ghouleh I, Khoo N, Knaus U, et al (2011) Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. Free

Radic Biol Med 51:1271–1288.

111. McEwen ML, Sullivan PG, Rabchevsky AG, Springer JE (2011) Targeting Mitochondrial Function for the Treatment of Acute Spinal Cord Injury. Neurotherapeutics 8:168–179. doi: 10.1007/s13311-011-0031-7
112. Halliwell B, Gutteridge JM (1999) Free radicals in biology and medicine, 3rd Editio. Oxford Univeristy Press, Oxford
113. Halliwell B (1994) Free radicals, antioxidants, and human disease: Curiosity, cause, or consequence? Lancet 344:721–724. doi: 10.1016/S0140-6736(94)92211-X
114. Wong AW, McCallum GP, Jeng W, Wells PG (2008) Oxoguanine glycosylase 1 protects against methamphetamine-enhanced fetal brain oxidative DNA damage and neurodevelopmental deficits. J Neurosci 28:9047–54. doi: 10.1523/JNEUROSCI.2557-08.2008
115. Parl FF (2005) Glutathione S-transferase genotypes cancer risk. Cancer Lett 221:123–129. doi: 10.1016/j.canlet.2004.06.016
116. Seidegård J, Vorachek WR, Pero RW, Pearson WR (1988) Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. Proc Natl Acad Sci U S A 85:7293–7. doi: 10.1073/pnas.85.19.7293
117. Xu SJ, Wang YP, Roe B, Pearson WR (1998) Characterization of the human class Mu glutathione S-transferase gene cluster and the GSTM1 deletion. J Biol Chem 273:3517–3527. doi: 10.1074/jbc.273.6.3517
118. Gilliland FD, Li YF, Dubeau L, et al (2002) Effects of glutathione S-transferase M1, maternal smoking during pregnancy, and environmental tobacco smoke on asthma and wheezing in children. Am J Respir Crit Care Med 166:457–463. doi: 10.1164/rccm.2112064
119. Kabesch M, Hoefler C, Carr D, et al (2004) Glutathione S transferase deficiency and passive smoking increase childhood asthma. Thorax 59:569–73. doi: 10.1136/thx.2003.016667
120. de Waart F, Kok F, Smilde T, et al (2001) Effect of glutathione S-transferase M1 genotype on progression of atherosclerosis in lifelong male smokers. Atherosclerosis 158:227–231.
121. Tong H, Rappold A, Caughey M, et al (2014) Cardiovascular effects caused by increasing concentrations of diesel exhaust in middle-aged healthy GSTM1 null human volunteers. Inhal Toxicol 26:319–326. doi: 10.3109/08958378.2014.889257
122. Ren C, Vokonas PS, Suh H, et al (2010) Effect modification of air pollution on urinary 8-hydroxy-2'-deoxyguanosine by genotypes: an application of the multiple testing

procedure to identify significant SNP interactions. Environ Heal 9:78. doi: 10.1186/1476-069X-9-78

123. Qiu YH, Xu YL, Zhang WH (2015) Effect of GSTM1, GSTT1, and GSTP1 IIe105Val polymorphisms on susceptiblity to gestational diabetes mellitus. Genet Mol Res. doi: 10.4238/gmr.15027711

124. Johanssona A-S, Stenberga G, Widerstena M, Mannervik B (1998) Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. J Mol Biol 278:687–698. doi: http://dx.doi.org/10.1006/jmbi.1998.1708

125. Moyer AM, Salavaggione OE, Wu TY, et al (2008) Glutathione s-transferase p1: gene sequence variation and functional genomic studies. Cancer Res 68:4791–4801. doi: 10.1158/0008-5472.can-07-6724

126. Harris MJ, Coggan M, Langton L, et al (1998) Polymorphism of the pi class glutathione Stransferase in normal populations and cancer patients. Pharmacogenetics 8:27–31.

127. Miller DP, Neuberg D, de Vivo I, et al (2003) Smoking and the risk of lung cancer: susceptibility with GSTP1 polymorphisms. Epidemiology 14:545–51. doi: 10.1097/01.ede.0000073120.46981.24

128. Girdhar Y, Singh N, Behera D, Sharma S (2016) Combinations of the Variant Genotypes of CYP1A1, GSTM1 and GSTT1 are Associated with an Increased Lung Cancer Risk in North Indian Population: a Case-Control Study. Pathol Oncol Res 22:647–652. doi: 10.1007/s12253-016-0058-5

129. Olshan AF, Weissler MC, Watson M a, et al (2000) Tobacco Use , and the Risk of Head and Neck Cancer Use , and the Risk of Head and Neck Cancer 1. 9:185–191.

130. Melén E, Nyberg F, Lindgren CM, et al (2008) Interactions between glutathione S-Transferase P1, tumor necrosis factor, and traffic-related air pollution for development of childhood allergic disease. Environ Health Perspect 116:1077–1084. doi: 10.1289/ehp.11117

131. Lagadu S, Lechevrel M, Sichel F, et al (2010) 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker of oxidative damage in oesophageal cancer patients: lack of association with antioxidant vitamins and polymorphism of hOGG1 and GST. J Exp Clin Cancer Res 29:157. doi: 10.1186/1756-9966-29-157

132. Janssen B, Munters E, Pieters N, et al (2012) Placental Mitochondrial DNA Content and Particulate Air Pollution During in Utero Life. Env Heal Persect 120:1346–1352. doi: 10.1289/ehp.1104458

133. Linnane A, Marzuki S, Ozawa T, Tanaka M (1989) Mitochondrial DNA mutations as an

important contributor to ageing and degenerative diseases. Lancet 1:642–645.

134. Morris JM, Gopaul NK, Endresen MJ, et al (1998) Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. Br J Obstet Gynaecol 105:1195–1199.

135. Leal CAM, Schetinger MRC, Leal DBR, et al (2011) Oxidative stress and antioxidant defenses in pregnant women. Redox Rep 16:230–236. doi: 10.1179/230 1351000211Y.0000000013.

136. Fialová L, Malbohan I, Kalousová M, et al (2006) Oxidative stress and inflammation in pregnancy. Scand J Clin Lab Invest 66:121–127. doi: 10.1080/00365510500375230

137. Kershaw RM, Hodges NJ (2012) Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. Mutagenesis 27:501–510. doi: 10.1093/mutage/ges012

138. Ba X, Aguilera-Aguirre L, Sur S, Boldogh I (2015) 8-Oxoguanine DNA glycosylase-1-driven DNA base excision repair. Curr Opin Allergy Clin Immunol 15:89–97. doi: 10.1097/ACI.0000000000000135

139. Sameer a. S, Nissar S, Abdullah S, et al (2012) DNA Repair Gene 8-Oxoguanine DNA Glycosylase Ser326Cys Polymorphism and Colorectal Cancer Risk in a Kashmiri Population. DNA Cell Biol 31:541–546. doi: 10.1089/dna.2011.1349

140. Sidorenko VS, Zharkov DO (2008) [The role of glycosylases of the base excision DNA repair in pathogenesis of hereditary and infectious human diseases]. Mol Biol 42:891–903. doi: 10.1134/S0026893308050166

141. Loft S, Poulsen HE, Vistisen K, Knudsen LE (1999) Increased urinary excretion of 8-oxo-2'-deoxyguanosine, a biomarker of oxidative DNA damage, in urban bus drivers. Mutat Res 441:11–19.

142. Barbato DL, Tomei G, Tomei F, Sancini A (2010) Traffic air pollution and oxidatively generated DNA damage: can urinary 8-oxo-7,8-dihydro-2-deoxiguanosine be considered a good biomarker? A meta-analysis. Biomarkers 15:538–545. doi: 10.3109/1354750X.2010.493974

143. Ji G, Yan L, Liu W, et al (2013) OGG1 Ser326Cys polymorphism interacts with cigarette smoking to increase oxidative DNA damage in human sperm and the risk of male infertility. Toxicol Lett 218:144–149. doi: 10.1016/j.toxlet.2013.01.017

144. Kumar A, Pant MC, Singh HS, Khandelwal S (2011) Role of OGG1 Ser326Cys polymorphism and 8-oxoguanine DNA damage in risk assessment of squamous cell carcinoma of head and neck in north Indian population. Mutat Res - Genet Toxicol Environ Mutagen 726:227–233. doi: 10.1016/j.mrgentox.2011.09.015

145. Ba X, Aguilera-Aguirre L, Rashid QTAN, et al (2014) The role of 8-oxoguanine DNA glycosylase-1 in inflammation. Int J Mol Sci 15:16975–16997. doi: 10.3390/ijms150916975
146. Wink D, Laval J (1994) The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. Carcinogenesis 15:2125–2129.
147. Bosshard M, Markkanen E, van Loon B (2012) Base excision repair in physiology and pathology of the central nervous system. Int J Mol Sci. doi: 10.3390/ijms131216172
148. Simonelli V, Camerini S, Mazzei F, et al (2013) Genotype-phenotype analysis of S326C OGG1 polymorphism: a risk factor for oxidative pathologies. Free Radic Biol Med 63:401–9. doi: 10.1016/j.freeradbiomed.2013.05.031
149. Kang SW, Kim SK, Park HJ, et al (2015) Human 8-oxoguanine DNA glycosylase gene polymorphism (Ser326Cys) and cancer risk: updated meta-analysis. Oncotarget 8:44761–44775. doi: 10.18632/oncotarget.16226
150. Chang JS, Wrensch MR, Hansen HM, et al (2009) Base excision repair genes and risk of lung cancer among San Francisco Bay Area Latinos and African-Americans. Carcinogenesis 30:78–87. doi: 10.1093/carcin/bgn261
151. Laantri N, Jalbout M, Khyatti M, et al (2011) XRCC1 and hOGG1 genes and risk of nasopharyngeal carcinoma in North African countries. Mol Carcinog 50:732–737. doi: 10.1002/mc.20754
152. Duan WX, Hua RX, Yi W, et al (2012) The association between OGG1 Ser326Cys polymorphism and lung cancer susceptibility: A meta-analysis of 27 studies. PLoS One 7:1–7. doi: 10.1371/journal.pone.0035970
153. Janssen K, Schlink K, Götte W, et al (2001) DNA repair activity of 8-oxoguanine DNA glycosylase 1 (OGG1) in human lymphocytes is not dependent on genetic polymorphism Ser326/Cys326. Mutat Res 486:207–216.
154. Kohno T, Shinmura K, Tosaka M, et al (1998) Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. Oncogene 16:3219–3225. doi: 10.1038/sj.onc.1201872
155. Aka P, Mateuca R, Buchet JP, et al (2004) Are genetic polymorphisms in OGG1, XRCC1 and XRCC3 genes predictive for the DNA strand break repair phenotype and genotoxicity in workers exposed to low dose ionising radiations? Mutat Res - Fundam Mol Mech Mutagen 556:169–181. doi: 10.1016/j.mrfmmm.2004.08.002
156. Chen S-K, Hsieh WA, Tsai M-H, et al (2003) Age-associated decrease of oxidative repair enzymes, human 8-oxoguanine DNA glycosylases (hOgg1), in human aging. J Radiat Res 44:31–5. doi: 10.1269/jrr.44.31

157. Lee A, Hodges N, Chipman J (2005) Interindividual Variability in Response to Sodium Dichromate − Induced Oxidative DNA Damage : Role of the Ser 326 Cys Polymorphism in the DNA-Repair Protein of Interindividual Variability in Response to Sodium Dichromate – Induced Oxidative DNA Damage : R. Cancer Epidemiol biomarkers prev 14:497–505.
158. Bagryantseva Y, Novotna B, Rossner P, et al (2010) Oxidative damage to biological macromolecules in Prague bus drivers and garagemen: Impact of air pollution and genetic polymorphisms. Toxicol Lett 199:60–68. doi: 10.1016/j.toxlet.2010.08.007
159. Karahalil B, Emerce E, Kocabaş NA, Akkas E (2011) Associations between GSTM1 and OGG1 Ser326Cys polymorphisms and smoking on chromosomal damage and birth growth in mothers. Mol Biol Rep 38:2911–2918. doi: 10.1007/s11033-010-9953-0
160. Hill JW, Evans MK (2006) Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. Nucleic Acids Res 34:1620–1632. doi: 10.1093/nar/gkl060
161. Ma Q (2013) Role of Nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 53:401–426.
162. Kataoka K, Igarashi K, Itoh K, et al (1995) Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. Mol Cell Biol 15:2180–90. doi: 10.1128/MCB.15.4.2180
163. Kusunoki H, Motohashi H, Katsuoka F, et al (2002) Solution structure of the DNA-binding domain of MafG. Nat Struct Biol 9:252–256. doi: 10.1038/nsb771
164. Itoh K, Wakabayashi N, Katoh Y, et al (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76–86. doi: 10.1101/gad.13.1.76
165. Adams J, Kelso R, Cooley L (2000) The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol 10:17–24.
166. Li X, Zhang D, Hannink M, Beamer LJ (2004) Crystal structure of the Kelch domain of human Keap1. J Biol Chem 279:54750–54758. doi: 10.1074/jbc.M410073200
167. Padmanabhan B, Tong KI, Ohta T, et al (2006) Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. Mol Cell 21:689–700. doi: 10.1016/j.molcel.2006.01.013
168. Mitsuishi Y, Motohashi H, Yamamoto M (2012) The Keap1–Nrf2 system in cancers: stress response and anabolic metabolism. Front Oncol 2:1–13. doi: 10.3389/fonc.2012.00200
169. Kang M-I, Kobayashi A, Wakabayashi N, et al (2004) Scaffolding of Keap1 to the actin

cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. Proc Natl Acad Sci U S A 101:2046–2051. doi: 10.1073/pnas.0308347100

170. Zhang DD, Lo S, Cross J V, et al (2004) Keap1 Is a Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex Keap1 Is a Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex. Am Soc Microbiol 24:10941–10953. doi: 10.1128/MCB.24.24.10941
171. Kobayashi A, Kang M, Okawa H, et al (2004) Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2 Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2. Mol Cell Biol 24:7130–7139. doi: 10.1128/MCB.24.16.7130
172. He CH, Gong P, Hu B, et al (2001) Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. J Biol Chem 276:20858–20865. doi: 10.1074/jbc.M101198200
173. Venugopal R, Jaiswal a K (1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene 17:3145–3156. doi: 10.1038/sj.onc.1202237
174. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, et al (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci 99:11908–11913. doi: 10.1073/pnas.172398899
175. Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, et al (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers. Proc Natl Acad Sci 101:2040–2045. doi: 10.1073/pnas.0307301101
176. Zhang DD, Hannink M (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23:8137–51. doi: 10.1128/MCB.23.22.8137-8151.2003
177. Cullinan SB, Zhang D, Hannink M, et al (2003) Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 23:7198–7209.
178. Huang H-C, Nguyen T, Pickett CB (2000) Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. Proc Natl Acad Sci 97:12475–12480. doi: 10.1073/pnas.220418997

179. Huang HC, Nguyen T, Pickett CB (2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 277:42769–42774. doi: 10.1074/jbc.M206911200
180. Kwak M-K, Wakabayashi N, Greenlaw JL, et al (2003) Antioxidants Enhance Mammalian Proteasome Expression through the Keap1-Nrf2 Signaling Pathway. Mol Cell Biol 23:8786–8794. doi: 10.1128/MCB.23.23.8786-8794.2003
181. Malhotra D, Portales-Casamar E, Singh A, et al (2010) Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through chip-seq profiling and network analysis. Nucleic Acids Res 38:5718–5734. doi: 10.1093/nar/gkq212
182. Lämsä V, Levonen A, Leinonen H, et al (2010) Cytochrome P450 2A5 constitutive expression and induction by heavy metals is dependent on redox-sensitive transcription factor Nrf2 in liver. Chem Res Toxicol 23:977–985. doi: 10.1021/tx100084c
183. Jowsey IR, Jiang Q, Itoh K, et al (2003) Expression of the aflatoxin B1-8,9-epoxide-metabolizing murine glutathione S-transferase A3 subunit is regulated by the Nrf2 transcription factor through an antioxidant response element. Mol Pharmacol 64:1018–1028. doi: 10.1124/mol.64.5.1018
184. Rushmore TH, Pickett CB (1990) Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. J Biol Chem 265:14648–14653.
185. Reichard JF, Motz GT, Puga A (2007) Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. Nucleic Acids Res 35:7074–7086. doi: 10.1093/nar/gkm638
186. Harvey CJ, Thimmulappa RK, Singh A, et al (2009) Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. Free Radic Biol Med 46:443–453. doi: 10.1016/j.freeradbiomed.2008.10.040.
187. Wolffe AP, Matzke MA (1999) Epigenetics: regulation through repression. Science (80-) 286:481–486. doi: 10.1126/science.286.5439.481
188. Cheng D, Wu R, Guo Y, Kong ANT (2016) Regulation of Keap1–Nrf2 signaling: The role of epigenetics. Curr Opin Toxicol 1:134–138. doi: 10.1016/j.cotox.2016.10.008
189. Eades G, Yang M, Yao Y, et al (2011) miR-200a Regulates Nrf2 Activation by Targeting Keap1 mRNA in Breast Cancer Cells. J Biol Chem 286:40725–40733. doi: 10.1074/jbc.M111.275495
190. Sangokoya C, Telen MJ, Chi J, Dc W (2010) microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. Blood

116:4338–48. doi: 10.1182/blood-2009-04-214817

191. Thippeswamy T, McKay J, Quinn J, Morris R (2006) Nitric oxide, a biological double-faced janus--is this good or bad? Histol Histopathol 4:445–58. doi: 10.14670/HH-21.445
192. Eiserich J, Patel R, O'Donnell V (1998) Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. Mol Asp Med 19:221–357.
193. Murphy MP (1999) Nitric oxide and cell death. Biochim Biophys Acta - Bioenerg 1411:401–414. doi: 10.1016/S0005-2728(99)00029-8
194. Hausladen A, Stamler JS (1999) Nitrosative stress. Methods Enzymol 300:389–395. doi: https://doi.org/10.1016/S0076-6879(99)00143-3
195. Deng X, Deitrich RA (2007) Ethanol Metabolism and Effects: Nitric Oxide and its Interaction. Curr Clin Pharmacol 2:145–153.
196. Bakan E, Taysi S, Polat MF, et al (2002) Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. Jpn J Clin Oncol 32:162–166.
197. Singh AK, Pandita S, Chandra G, et al (2012) Role of Nitric Oxide in Immunity – a Review. Wayamba J Anim Sci 97–102.
198. Torre D, Pugliese A, Speranza F (2002) Reviews Role of nitric oxide in HIV-1 infection : friend or foe ? 2:273–280.
199. Calabrese V, Cornelius C, Rizzarelli E, et al (2009) Nitric Oxide in Cell Survival: A Janus Molecule. Antioxid Redox Signal 11:2717–2739. doi: 10.1089/ars.2009.2721
200. Chhabra N (2015) An amino acid that acts as a component of histones and a precursor for Nitric oxide (NO). In: Chem. Biomol. http://usmle.biochemistryformedics.com/an-amino-acid-that-acts-as-a-component-of-histones-and-a-precursor-for-nitric-oxide-no/. Accessed 10 Nov 2017
201. Sekar K (2006) Inhaled nitric oxide in term and preterm infants. J Perinatol 26 Suppl 1:S4-7–3. doi: 10.1038/sj.jp.7211497
202. Mimica-dukić N, Simin N, Svirčev E (2012) The Effect of Plant Secondary Metabolites on Lipid Peroxidation and Eicosanoid Pathway. Lipid Peroxidation. doi: 10.5772/48193
203. Simons K, Ehehalt R (2002) Cholesterol, lipid rafts, and disease. J Clin Invest 110:597–604.
204. Voutilainen S, Morrow JD, Roberts LJ, et al (1999) Enhanced in vivo lipid peroxidation at elevated plasma total homocysteine levels. ArteriosclerThrombVascBiol 19:1263–1266.
205. Aviram M (2000) Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. Free Radic Res 33:S85-97.

206. Paşaoğlu H, Bulduk G, Oğüş E, et al (2004) Nitric oxide, lipid peroxides, and uric acid levels in pre-eclampsia and eclampsia. Tohoku J Exp Med 202:87–92.
207. Negi R, Pande D, Kumar A, et al (2012) In vivo oxidative DNA damage and lipid peroxidation as a biomarker of oxidative stress in preterm low-birthweight infants. J Trop Pediatr 58:326–328. doi: 10.1093/tropej/fmr078
208. Moritz E, Pauly K, Bravard A, et al (2014) hOGG1-Cys326 variant cells are hypersensitive to DNA repair inhibition by nitric oxide. Carcinogenesis 35:1426–1433. doi: 10.1093/carcin/bgu066
209. Chien Y-H, Bau D-T, Jan K-Y (2004) Nitric oxide inhibits DNA-adduct excision in nucleotide excision repair. Free Radic Biol Med 36:1011–7. doi: 10.1016/j.freeradbiomed.2004.01.011
210. Wink DA, Osawa Y, Darbyshire JF, et al (1993) Inhibition of cytochrome P450 by nitric oxide and a nitric-oxide releasing agent. Arch Biochem Biophys 300:115–123.
211. Ischiropoulus H, Zhu L, Tsai MJ., et al (1993) Peroxynitrite formation from macrophage-derived nitric oxide. Arch Biochem Biophys 298:431–437.
212. Simão ANC, Victorino VJ, Morimoto HK, et al (2015) Redox-driven events in the human immunodeficiency virus type 1 (HIV-1) infection and their clinical implications. Curr HIV Res 13:143–50.
213. WHO (2017) HIV/AIDS. In: Media Cent. - Fact Sheet. http://www.who.int/mediacentre/factsheets/fs360/en/. Accessed 7 Nov 2017
214. Ivanov A, Values-Elliston VT, Ivanova ON, et al (2016) Oxidative Stress During HIV Infection: Mechanisms and Consequences. Oxid Med Cell Longev 2016:1–18. doi: 10.1155/2016/8910396
215. Freed E, Martin M (2007) HIVs and their replication. In: Knipe D., Howley P. (eds) Fields Virol. Lippimcott, Williams and Wilkins, Philadelphia, PA, USA, pp 2107–2185
216. Torre D, Ferrario G, Speranza F, et al (1996) Serum concentrations of nitrite in patients with HIV-1 infection. J Clin Pathol 49:574–576. doi: 10.1136/jcp.49.7.574
217. Zangerle R, Fuchus D, Reibnegger G, et al (1995) Serum Nitrite Plus Nitrate in Infection with Human Immunodeficiency Virus Type-1. Immunobiology 193:59–70. doi: https://doi.org/10.1016/S0171-2985(11)80155-5
218. Torre D, Ferrario G, Speranza F, et al (1996) Increased levels of nitrite in the sera of children infected with human immunodeficiency virus type 1. Clin Infect Dis 22:650–3.
219. Groeneveld PH, Kroon FP, Nibbering PH, et al (1996) Increased production of nitric oxide correlates with viral load and activation of mononuclear phagocytes in HIV-infected patients. Scand J Infect Dis 28:341–345. doi:

http://dx.doi.org/10.3109/00365549609037916

220. Jimenez J, Gonzalez-Nicolas J, Alvarez S, et al (2001) Regulation of human immunodeficiency virus type 1 replication in human T lymphocytes by nitric oxide. J Virol 75:4655–4663.

221. Albina J, Abate J, Henry W (1991) Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN-gamma in the induction of the nitric oxidesynthesizing pathway. J Immunol 147:144–148.

222. Takagi K, Nukaya I, Yasukawa K, Suketa Y (1994) Inhibitory mechanisms of antibody production by nitrogen oxides released from activated macrophages during the immune response: relationship to energy consumption. Immuno Cell Biol 72:241–248.

223. Torre G, Ferrario G (1996) Immunological aspects of nitric oxide in HIV-1 infection. Med Hypoth 47:405–407.

224. Zhang H, Li H, Zhou Y, et al (2009) Nrf2 is involved in inhibiting Tat-induced HIV-1 long terminal repeat transactivation. Free Radic Biol Med 47:261–268.

225. Suresh D, Annam V, Pratibha K, Et-al (2009) Total antioxidant capacity – a novel early biochemical marker of oxidative stress in HIV infected individuals. J Biom Sci 16:61–64.

226. Reshi M, Su Y, Hong J (2014) RNA Viruses: ROS-Mediated Cell Death. Int J Cell Biol 467452.

227. Xiao P-L, Zhou Y-B, Chen Y, et al (2015) Association between maternal HIV infection and low birth weight and prematurity: a meta-analysis of cohort studies. BMC Pregnancy Childbirth 15:1–11. doi: 10.1186/s12884-015-0684-z

228. Tam AB, Mercado EL, Hoffmann A, Niwa M (2012) ER Stress Activates NF-κB by Integrating Functions of Basal IKK Activity, IRE1 and PERK. PLoS One 7:e45078. doi: 10.1371/journal.pone.0045078

229. Malhotra JD, Kaufman RJ (2007) The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol 18:716–731.

230. Gorlach A, Klappa P, Kietzmann T (2006) The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid Redox Signal 8:1391–1418.

231. Jacobson J, Duchen M (2002) Mitochondrial oxidative stress and cell death in astrocytes: requirement for stored Ca2+ and sustained opening of the permeability transition pore. J Cell Sci 115:1175–1188.

232. Rutkowski DT, Kaufman RJ (2007) That which does not kill me makes me stronger: adapting to chronic ER stress. Trends Biochem Sci 32:469–476. doi:

10.1016/j.tibs.2007.09.003

233. Tu B, Weissman J (2004) Oxidative protein folding in eukaryotes: mechanisms and consequences. J Cell Biol 164:341–346.

234. van der Vlies D, Makkinje M, Jansens A, et al (2003) Oxidation of ER resident proteins upon oxidative stress: effects of altering cellular redox/antioxidant status and implications for protein maturation. Antioxid Redox Signal 5:381–387.

235. Watterson TL, Hamilton B, Martin R, Coulombe R a (2009) Urban particulate matter causes ER stress and the unfolded protein response in human lung cells. Toxicol Sci 112:111–122. doi: 10.1093/toxsci/kfp186

236. Laing S, Wang G, Briazova T, et al (2010) Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues. Am J Physiol Cell Physiol 299:C736–C749. doi: 10.1152/ajpcell.00529.2009

237. Kawakami T, Yoshimi M, Kadota Y, et al (2014) Prolonged endoplasmic reticulum stress alters placental morphology and causes low birth weight. Toxicol Appl Pharmacol 275:134–144. doi: 10.1016/j.taap.2013.12.008

238. Huda SS, Brodie LE, Sattar N (2010) Obesity in pregnancy: prevalence and metabolic consequences. Semin Fetal Neonatal Med 15:70–76. doi: 10.1016/j.siny.2009.09.006

239. Bukrinsky MI, Nottet HS, Schmidtmayerova H, et al (1995) Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease. J Exp Med 181:735–45.

CHAPTER 2

The effect of NOx pollution on oxidative stress in pregnant women living in Durban, South Africa

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Abstract

The purpose of the study was to evaluate the effect nitric oxide (NOx) pollution had on maternal serum 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) levels and neonatal outcomes in pregnant women living in Durban, South Africa (SA). Women, in their third trimester with singleton pregnancies, were recruited from the heavily industrialised south (n=225) and less industrialised north (n=152). Biomarker levels of serum 8-OHdG concentrations were analysed and the women were genotyped for glutathione-S-transferases pi 1 (GSTP1) and glutathione-S-transferases mu 1 (GSTM1) polymorphisms. The level of NOx pollution in the two regions was determined using land use regression modelling. The serum 8-OHdG was shown to significantly correlate with NOx levels; this relationship was strengthened in the south ($p<0.05$). This relationship was still observed after adjusting for maternal characteristics. GSTP1 was significantly associated with the south region, where the variant (AG+GG) genotype was associated with increased 8-OHdG levels as a result of NOx exposure ($p<0.05$). GSTM1 null genotype was associated with a positive correlation between NOx and 8-OHdG levels ($p<0.05$). NOx levels was found to marginally reduce gestational age ($p<0.05$), with mothers carrying male neonates, variant GSTP1 and living in the north being factors that contributed to gestational age reduction ($p<0.05$). Our study demonstrated that NOx exposure resulted in increased 8-OHdG levels in pregnant women living in Durban, SA which then led to gestational age reduction. The GSTP1 variant increased susceptibility of individuals to harmful effects of NOx.

Keywords

Nitrogen oxides, 8-OHdG, ambient air pollution, pre-term birth, pregnancy

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Ethical Approval

The Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BF263/12) approved the study, including the use of human subjects. Informed consent from all study participants was obtained.

1. Introduction

Durban, South Africa (SA), is a rapidly developing city with increased road traffic and industrial development in close proximity to residential areas. Durban is divided into a heavily industrial south region (also known as the South Durban Industrial Basin) and a less industrial north region; however both regions are undergoing increased urbanisation and development. This is of major health concern due to increased levels of ambient air pollution (AAP) in both these areas (Naidoo et al. 2013). Ambient air pollution and associated oxidative stress has been implicated in many pathological conditions, including cancer, asthma, acute respiratory infections and adverse birth outcomes (Šrám et al. 2005; Kampa and Castanas 2008; Wu et al. 2009; Fleischer et al. 2014; Chen et al. 2015; Moorthy et al. 2015). Pregnant females are highly susceptible to oxidative stress due to increased basal oxygen and changes in energy consumption during placental and foetal development. Infants *in utero* are highly susceptible to the harmful effects of AAP; exposure has been associated with low birth weight (LBW), inter-utero growth restrictions, preterm birth (PTB) and pre-eclampsia (Glinianaia et al. 2004; Negi et al. 2012a; Proietti et al. 2013). High levels of pollutants, including sulphur dioxide, carbon monoxide (CO), particulate matter (PM) and nitric oxides (NOx), have been reported (Kistnasamy et al. 2008). Oxides of nitrogen are of particular interest as they are by-products of vehicle combustion, smoking and cooking with gas, and have a nitrogen-centred free radical. They interact directly with macromolecules (DNA, lipids and proteins) and often result in a cascade of radical production and compromised cellular antioxidant function (Kelly 2003).

The increase in reactive oxygen species (ROS) and decrease in antioxidants lead to oxidative stress. The premutagenic deoxyguanosine DNA lesion is highly susceptible to oxidative stress, resulting in the hydroxylation of the guanosine residue at position C_8. This produces oxidative 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) and acts as a biomarker for oxidative DNA damage (Kim et al. 2005).

Glutathione-S-transferases (GST) are a family of phase II isoenzymes that protect against oxidative stress. This occurs through the conjugation of glutathione to electrophilic species that can react with and form protein or DNA adducts (Romieu et al. 2006). Two common and highly polymorphic antioxidants that have been implicated in health effects in response to chemical exposure are GST mu (M) and pi (P) 1(Wong et al. 2008). Common single-nucleotide polymorphisms (SNP) in the GSTM1 GSTP1 affect the enzymatic activity of GST, and have been associated with AAP associated health effects and increased oxidative stress (Romieu et al.

2006; Mustafa et al. 2010). A homozygous deletion in the GSTM1 gene results in the complete absence of enzyme activity (Seidegård et al. 1988; Xu et al. 1998), whilst a single nucleotide substitution of adenine (A) for guanine (G) results in the amino acid change of isoleucine for valine in GSTP1, at codon 105. This codon forms part of the active site of the enzyme, therefore this change results in the alteration of the substrate-specificity of the enzymes' binding site (Johanssona et al. 1998; Moyer et al. 2008). These polymorphisms have been associated with adverse birth outcomes due to AAP, such as increased risk of PTB (Sram et al. 2006; Mustafa et al. 2010; Slama et al. 2010).

To investigate a possible correlation between NOx and maternal oxidative stress, this study measured serum 8-OHdG in third trimester bloods of women, living in the south and north regions of Durban. To determine whether NOx and maternal oxidative stress impacted on neonatal birth weight (BW) and gestational age (GA), associations were investigated between NOx, 8-OHdG adduct concentration and neonatal BW and GA. Multivariate analyses were performed to assess whether the relationship held, when confounding factors were controlled. The study further investigated the prevalence of GST polymorphisms in South African women and their potential to affect the susceptibility of mothers, exposed to AAP, to oxidative stress.

2. Methodology

2.1 Study Population

The Mother and Child in the Environment (MACE) longitudinal cohort study recruited pregnant women from public sector anti-natal clinics in the heavily-polluted south Durban (n=225). A comparison sample of women with similar socio-economic statuses was recruited from the less-industrialised north Durban (n=152). The women were residents of the geographical area for the full duration of the pregnancy. Women with hypertension, multiple pregnancies, diabetes, placenta previa, genital tract infections and other complications which result in adverse growth effects were excluded from the study.

The study was approved by the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BF263/12) (Appendix 1) and informed consent from study participants was obtained.

2.2 Nitric Oxide levels

Land use regression modelling was used to determine the exposure levels of NOx for individual study participants. The method was developed following the ESCAPE approach (Beelen and Hoek 2010). Model development entailed measurement of NOx at selected locations and regressing these measurements against site-specific a priori defined (i.e. direction of effect) geographic predictors such as road length, land use types, topography and population and housing density in a multivariate regression model. NOx measurements were conducted over two, two-week periods during mid-winter and mid-summer using Ogawa samplers which were deployed at 40 randomly selected sites in the north & south Durban areas. The sampling periods selected are representative of the two distinct seasons to occur in Durban (Tyson and Preston-Whyte 2004; Tularam and Ramsay 2013), thus accounting for seasonal variation. The adjusted NOx measurements were then used in model development of which the regression coefficients were applied to each participant. This determined individual NOx exposure levels for each study participant (Muttoo et al. 2017).

2.3 Collection and Preparation of Samples

Third trimester blood was collected from pregnant women during the period between 2013 and 2015. The serum and whole blood was stored (-80°C) for analyses. Isolated serum was used for 8-OHdG adduct quantification and whole blood for genotyping of polymorphisms.

2.4 Polymorphisms of GSTM1 and GSTP1

DNA was isolated from whole blood using the Qiagen FlexiGene® DNA Kit (as per manufacturer's instructions). Isolated DNA was quantified using the Nanodrop 2000 spectrophotometer and standardised to 10ng/µl.

Differentiation polymerase chain reaction (PCR) was performed to assess the GSTM1 polymorphism (n=372), using β-globin as a reference gene. GSTM1 (215 base pair (bp)) and β-globin (268bp) PCR products were amplified using 40pmol of GSTM1 and β-globin primers (Inqaba Biotech, SA - Table 1) in a 30µl reaction (1x Green GoTaq Flexi buffer, 1.25mM $MgCl_2$, 0.5U GoTaq DNA polymerase (Promega), 200µM of each deoxyribonucleotide (dNTP), 10ng DNA template). Initial denaturation was applied (96°C, 5min), followed by 30 cycles of denaturation (96°C, 30sec), annealing (57°C, 30sec) and extension (72°C, 30sec), concluding

with final extension occurring at 72°C for 5min. Amplification products were electrophoresed on agarose gel (4%, 2µl GelRed) and visualised on the Bio-Rad ChemiDoc™ XRS+ System, using the Image Lab™ software. The presence of a single 268bp is indicative of homozygous null genotype and the presence of 218bp indicates either a homozygous positive or heterozygous (wild-type (wt)) genotype (Appendix 2:Fig.A2.1).

PCR- restriction fragment length polymorphism (RFLP) was used to investigate GSTP1 genotypes (n=377). A 176bp PCR product was amplified using 10pmol of GSTP1 primers (Inqaba Biotech, SA - Table 1) in a 25µl reaction (1x Green GoTaq Flexi buffer, 1.5mM $MgCl_2$, 0.5U GoTaq DNA polymerase (Promega), 200µM of each dNTP, 10ng DNA template). Initial denaturation was applied (96°C, 5min), followed by 30 cycles of denaturation (96°C, 30sec), annealing (55°C, 30sec) and extension (72°C, 30sec), concluding with final extension (72°C, 5min). Amplification products were electrophoresed on agarose gel (3%, 1µl GelRed) and visualised. The PCR amplicon underwent restriction endonuclease digestion to determine the presence of the polymorphic restriction site. An overnight digestion (37°C) was performed in 28µl reactions: 10µl PCR product and 18µl (18µl nuclease free water, 2µl 10x Buffer Tango, 1µl *Alw261 (BsmA1)* (Thermo Fisher Scientific)). Amplicons completely digested into 2 restriction fragments (91bp and 85bp) were homozygous for G_{105} allele (Appendix 2: Fig.A2.2). The restriction fragments were electrophoresed on an agarose gel (3%, 2µl GelRed) and visualised on the Bio-Rad ChemiDoc™ XRS+ System, using the Image Lab™ software.

Table 1: Primer sequences for PCR

Primer	Primer Sequence
GSTM1 forward	5'-GAACTCCCTGAAAAGCTAAAGC-3'
GSTM1 reverse	5'-GTTGGGCTCAAATATACGGTGG-3'
β-globin forward	5'-CAACTTCATCCACGTTCACC-3'
β-globin reverse	5'-GAAGAGCCAAGGACAGGTAC-3'
GSTP1 forward	5'-ACCCCAGGGCTCTATGGGAA-3'
GSTP1 reverse	5'-TGAGGGCACAAGAAGCCCCT-3'

2.5 Determination of 8-Hydroxydeoxyguanosine

The amount of serum 8-OHdG adduct was determined using a competitive OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc.), with a sensitivity range of 100pg/mL to 20ng/mL. Non-haemolysed serum samples (n=166) were chosen, at random by region, from the study population; diluted (1:5) in assay diluent and assayed as per manufacturer's instructions. A 1:5 dilution was recommended as per manufacture protocol, an initial

experiment was performed using this dilution and the levels of 8-OHdG fell within the range of the 8-OHdG standards and therefore this dilution was used for all subsequent analysis. A standard curve was set up using known concentrations of 8-OHdG standards (0-20ng/mL); the logarithmic equation for the best fit line was used to extrapolate the concentrations of the unknown samples (Appendix 3: Fig.A3.1). Each 96-well plate that was used had its own set of standards, to ensure human-error and variation between experiments was accounted for. The final concentration of 8-OHdG adduct was the anti-log multiplied by the dilution factor of 5.

2.6 Statistical Analysis

Statistical analyses were preformed using GraphPad Prism V5 Software Package (GraphPad Software Inc., San Diego, California, USA). Comparisons between north and south groups for maternal and neonatal characteristics, atmospheric NOx levels and maternal serum 8-OHdG adduct concentrations were determined using the Student t test. Data was log transformed to ensure normalcy, and allow graphical representation of the data. Correlations among atmospheric NOx, maternal serum 8-OHdG and neonatal BW, neonatal GA and genotypes of GSTM1 and GSTP1 were done using the non-parametric Spearman correlation. The Chi square and Fischer's exact tests were used to test the significant difference in the prevalence of GSTM1 and GSTP1 genotypes between north and south groups. A one way ANOVA was performed to determine the level of difference for maternal serum 8-OHdG adduct concentrations among the genotypes of GSTM1 and GSTP1. All statistical tests were two-sided. Multivariate linear regression was used to determine whether the relationship between atmospheric NOx and maternal serum 8-OHdG was affected by potential confounders, namely: maternal age, maternal body mass index (BMI), HIV status, area, maternal systolic and diastolic blood pressure (BP) and haemoglobin (Hb) levels. The relationship between neonatal GA and atmospheric NOx and maternal serum 8-OHdG was also determined using linear regression, and potential confounders were controlled. The linear regression analyses were done using STATA version 13.1.

3. Results

Maternal and neonatal characteristics of study participants are described in Table 2. The maternal age was slightly higher in the north compared to the south ($p=0.0844$). Mothers in the north were significantly shorter with higher BMI levels; compared to the south mothers who were taller and had lower BMI levels ($p<0.0001$). Maternal Hb levels were higher in the north compared to the south ($p=0.0002$). The systolic and diastolic BP measurements were

significantly lower in the north compared to the south (p<0.05). Mean GA and BW were lower in the north compared to the south (p=0.0540 and 0.0951 (Table 2), respectively) but did not reach significance.

Table 2: Maternal and neonate characteristics

	North		South		
	n	Mean (SD)	n	Mean (SD)	p-value
Maternal Age (years)	152	26.38 (5.90)	225	25.32 (5.67)	0.0844
Maternal height (cm)	152	142.3 (19.85)	224	159.0 (6.28)	***<0.0001
Maternal BMI	152	34.12 (12.41)	224	25.99 (6.64)	***<0.0001
Haemoglobin (g/dL)	152	10.96 (1.73)	224	7.79 (5.50)	***0.0002
BP systolic (mmHg)	152	109.1 (13.20)	224	111.8 (11.97)	*0.0143
BP diastolic (mmHg)	152	67.75 (9.187)	224	70.34 (8.58)	**0.002
Gestational age (weeks)	137	38.55 (1.81)	202	38.85 (1.76)	0.0540
Birthweight (g)	139	3003 (670.7)	202	3125 (610.1)	0.0951

n= sample size, SD= standard deviation. Statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.0001$

The level of atmospheric NOx in the south (37.04 ± 7.46) was significantly greater than in the north (33.26 ± 8.51, p<0.0001) (Fig.1A). This corresponds to a significant increase in maternal serum 8-OhdG concentration observed in the south (20.26 ± 40.88ng/mL) compared to the north (11.51 ± 18.51ng/mL, p=0.0197) (Fig.1B).

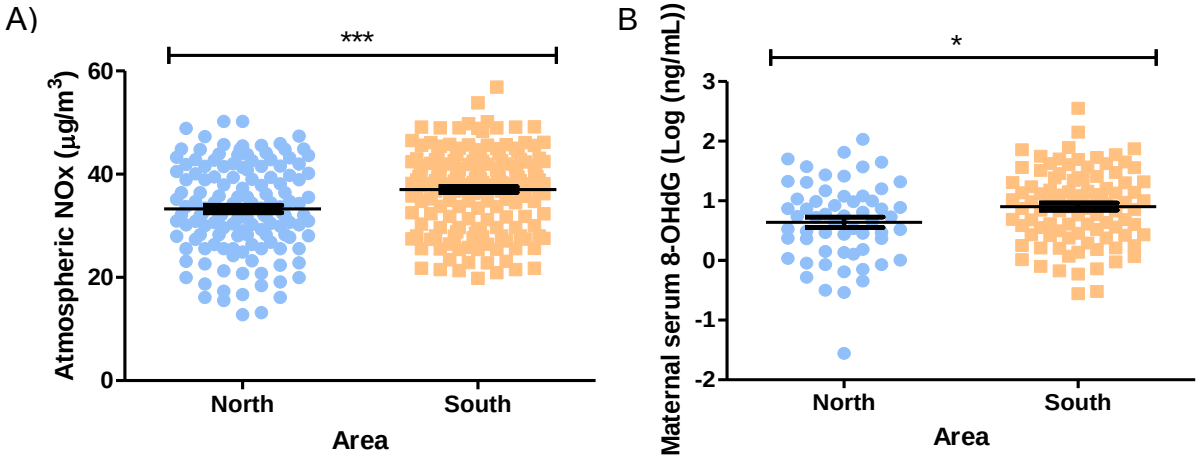

Figure 1: The concentrations of (A) atmospheric NOx (µg/cm3) and (B) maternal serum 8-OHdG (log(ng/mL)) for patients living in the North [(A) n=142; (B) n=59] and South [(A) n=185; (B) n=97]. Statistical significance: *** $p<0.0001$ and * $p<0.05$.

A significant positive correlation (Spearman r=0.2173; p=0.0158) was found between the levels of maternal serum 8-OHdG and atmospheric NOx (Fig.2A). When the specific area was taken into consideration, the south (Spearman r= 0.2337; p=0.0466) atmospheric NOx was correlated significantly with maternal serum 8-OHdG concentration (Fig.2C) whilst the north (Spearman r=0.1270; p=0.3795) showed a positive trend, although not significant (Fig.2B). No relationship was observed between NOx and neonate BW (Spearman r=0.09865; 95%CI -0.016-0.210; p=0.0814) and NOx and neonatal GA (Spearman r= -0.0716; 95%CI -0.185 – 0.043; p=0.0814). When area was considered a negative trend is suggested for NOx and BW in both north (Spearman r= -0.1135; 95%CI -0.282-0.062; p=0.19) and south (Spearman r=-0.060; 95%CI -0.211 – 0.093; p=0.43). A similar negative trend is observed between NOx and GA in both the north (Spearman r= -0.1135; 95%CI -0.282 – 0.062; p=0.19) and south (Spearman r= -0.060; 95%CI -0.211 – 0.093; p=0.43). Maternal serum 8-OHdG was not shown to correlate with BW (Spearman r=0.058; 95%CI -0.1521 – 0.2635; p=0.58) and GA (Spearman r= 0.044; 95%CI -0.121 – 0.207; p=0.59).

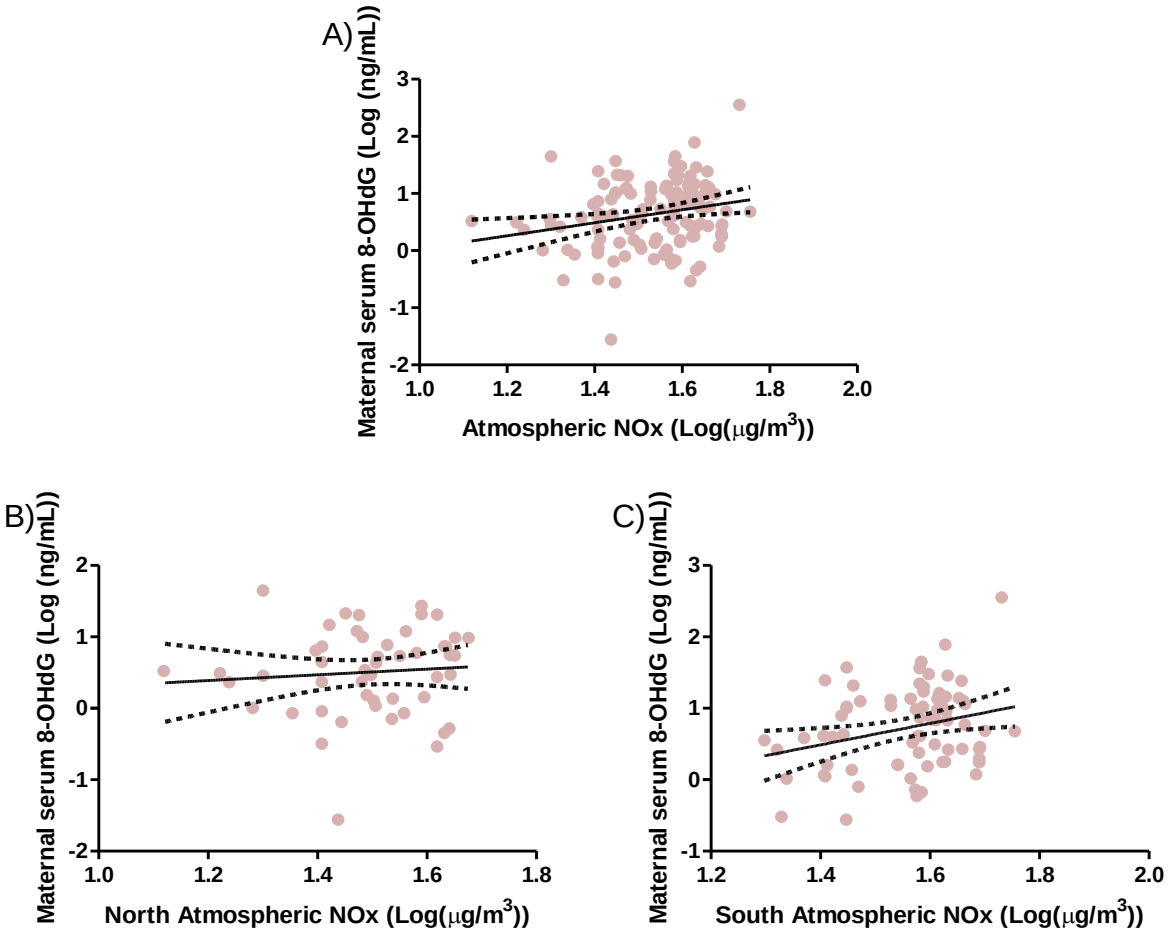

Figure 2: Relationship between the concentrations of atmospheric NOx (log(μg/cm^3)) and maternal serum 8-OHdG (log(ng/mL)) for all patients (A) [Spearman r=0.2173; 95%CI 0.03657 to 0.3843; *p=0.0158;

n=123], patients living in the North (B) [Spearman r=0.1270; 95%CI -0.1652 to 0.3987; p=0.3795; n=50] and South (C) [Spearman r= 0.2337; 95%CI -0.003195 to 0.4457; *p=0.0466; n=73]. Dotted lines represent 95%CI interval. Statistical significance: *$p<0.05$*

Maternal HIV status was thought to influence maternal serum 8-OHdG adduct concentration, however no difference between HIV positive (16.02±21.39ng/mL) and negative (18.48 ±40.23ng/mL) 8-OHdG concentration was observed (Fig.3).

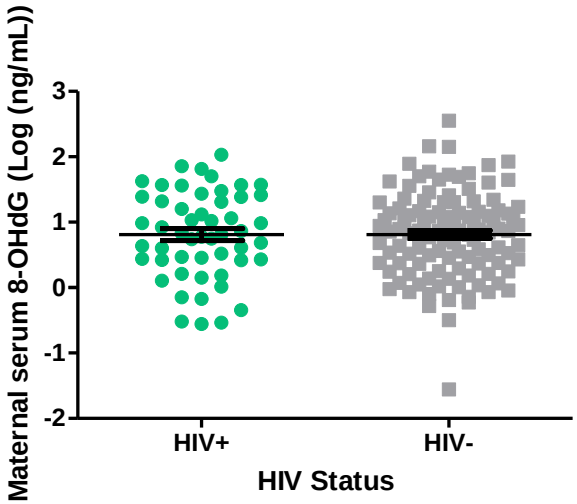

Figure 3: Maternal serum 8-OHdG adduct (log(ng/mL)) concentration between HIV positive (n=52) and HIV negative (n=113) patients in the total sample group.

The genotypic prevalence of GSTM1 and GSTP1 amongst study participants are shown in Table 3 and Table 4, respectively. For all subjects, the prevalence of the GSTM1 null (0/0) and wt (0/+ or +/+) type was 25.3% and 75.7%, respectively. The frequencies of the A_{105} and G_{105} allele of GSTP1 were 44.2% and 55.8%, respectively. A significantly greater fold increase (p=0.0144) was observed in the south for the GSTP1 A_{105} allelotype compared to G_{105} allelotype (1.8 and 1.2, respectively). The prevalence of GSTP1 AA (wt) and AG+GG (variant) (p=0.0281) was significantly different between the north and south; whilst the prevalence of GSTM1 (p=0.9038) did not differ significantly. The GSTP1 AG and GG genotypes were combined for analysis, as it has been shown that subjects with a single GSTP1 G_{105} allele have reduced enzyme activity compared to those with the GSTP1 A_{105} allele (Zimniak et al. 1994).

Table 3: Genotypic frequency of GSTM1 in patients living in the North and South (n=372).

	Total n (%)	North n (%)	South n (%)	p-value
Genotype frequency				
GSTM1 0/0	94 (25.3)	39 (41.5)	55 (59.5)	0.9038[#]
GSTM1 0/+ or +/+	278 (75.7)	113 (40.7)	165 (59.3)	

[#]RR (1.021, 95%CI: 0.7722 - 1.349); OR (1.035, 95%CI: 0.6438 – 1.665)

n= sample size, RR= relative risk, OR= Odds ratio, CI= confidence interval

Table 4: Genotype and allele frequencies of GSTP1 in patients living in the North and South (n=377).

	Total n (%)	North n (%)	South n (%)	p-value
Genotype frequency				
GSTP1 A_{105}/A_{105}	92 (24.4)	28 (30.4)	64 (69.6)	***0.0281**[#1]
GSTP1 A_{105}/G_{105} and G_{105}/G_{105}	285 (75.6)	124 (43.5)	161 (56.5)	
Allelotype frequency				
GSTP1 A_{105}	361 (44.2)	129 (35.7)	232 (64.3)	***0.0144**[#2]
GSTP1 G_{105}	456 (55.8)	175 (44.5)	218 (55.5)	

[#1]RR(0.6995; 95%CI: 0.4998 - 0.9790); OR(0.5680; 95%CI: 0.3438 - 0.9386)

[#2]RR(0.8025, 95%CI: 0.6723 - 0.9579); OR (0.6927, 95%CI: 0.5166 - 0.9286)

n= sample size, RR= relative risk, OR= Odds ratio, CI= confidence interval.
Statistical significance: *$p<0.05$*

No difference was observed in the levels of maternal serum 8-OHdG among the GSTM1 genotypes between the north and south (Fig.4A). Maternal serum 8-OHdG concentration was higher in AA genotyped mothers (29.31±10.10ng/mL, n=38) compared to AG+GG genotyped mothers (13.16±1.772, n=127, p=0.1589), although not significant. When area was considered, the level of maternal serum 8-OHdG was higher in the north GSTP1 AA (21.11±8.9ng/mL) mothers compared to the GSTP1 AG+GG (8.70±2.0ng/mL) mothers but did not reach

significance (p=0.1421). The south GSTP1 AG+GG (13.50±2.0ng/mL) mothers had significantly greater levels of maternal serum 8-OHdG compared to the north (8.70±2.0ng/mL, p=0.0188) (Fig .4B).

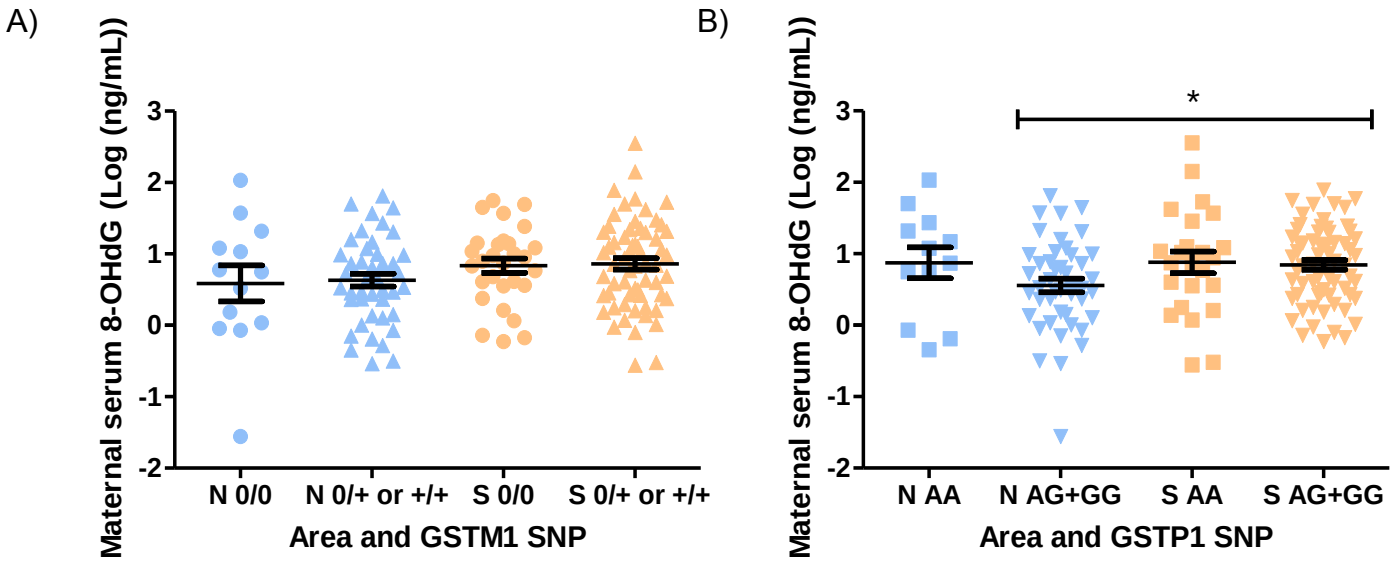

Figure 4: Maternal serum 8-OHdG adduct (log(ng/mL)) concentration between the GSTM1 (A) and GSTP1 (B) genotypes for patients living in the North and South. Statistical significance: *$p<0.05$.

The relationship between the atmospheric NOx and maternal serum 8-OHdG concentration was investigated among the different genotypes in Table 5. A significant correlation was observed between the atmospheric NOx and the level of maternal serum 8-OHdG within the GSTM1 null genotype (Spearman r=0.4227, p=0.0199) and the GSTP1 AA+GG genotype (Spearman r=0.2105, p=0.0395). No relationship was observed when north and south was taken into consideration.

Table 5: Relationship between the concentrations of atmospheric NOx (log(µg/cm^3)) and maternal serum 8-OHdG (log(ng/mL)) among the different SNP genotypes (GSTM1 and GSTP1) for the total patient sample and those living in the North and South.

		GSTM1 SNP Genotypes	
		0/0	0/+ or +/+
Total	Spearman r (95%CI)	**0.4227 (0.06250 to 0.6855)**	0.1566 (-0.05478 to 0.3546)
	p-value	***0.0199**	0.1338
North	Spearman r (95%CI)	0.5758 (0.0816)	0.03247 (-0.2907 to 0.3490)
	p-value	ns	0.8424
South	Spearman r (95%CI)	0.3046 (-0.1732 to 0.6663)	0.2218 (-0.05980 to 0.4707)
	p-value	0.1916	0.1104
		GSTP1 SNP Genotypes	
		A_{105}/A_{105}	A_{105}/G_{105} and G_{105}/G_{105}
Total	Spearman r (95%CI)	0.2525 (-0.1527 to 0.5851)	**0.2105 (0.004413 to 0.3995)**
	p-value	0.2038	***0.0395**
North	Spearman r (95%CI)	-0.1459 (0.6876)	0.07900 (-0.2474 to 0.3893)
	p-value	ns	0.6280
South	Spearman r (95%CI)	0.3887 (-0.1284 to 0.7397)	0.1864 (-0.08845 to 0.4348)
	p-value	0.1231	0.1690

CI= confidence interval, ns= not significant. Statistical significance: *$p<0.05$*

The relationship between atmospheric NOx and maternal serum 8-OHdG concentration, controlled for potential confounders, is described in Table 6. The β-coefficient and regression model for the total samples was strengthened when maternal characteristics were controlled (unadjusted =1.14, adjusted^2=1.21, $p<0.05$). For every 1% increase in NOx it would lead to a 1.21% increase in maternal serum 8-OHdG. This almost doubled when area was taken into consideration, where a 1% increase in atmospheric NOx would result in a 1.99% increase in maternal 8-OHdG, in the south (p=0.004). When polymorphisms were considered, a 1% increase in atmospheric NOx would lead to a 1.14% increase in maternal 8-OHdG for the variant GSTP1 genotype (p=0.026) whilst an almost equal change in percent was observed between NOx and 8-OHdG for the GSTM1 wt genotype (p=0.076). The neonate gender was shown to influence the concentration of maternal serum 8-OHdG, where a 1% increase in NOx would result in a 2.48% increase in maternal serum 8-OHdG for women carrying female neonates (p=0.002).

Table 6: Impact of atmospheric NOx (log) concentration on maternal serum 8-OHdG adduct (log) concentration – linear regression analysis for total mothers and subdivided into area, GSTM1 and GSTP1 SNP with adjustments for maternal characteristics.

		Maternal serum 8-OHdG (log) concentration			
		β-coefficient	(95%CI)	p-value	R-squared (p-value)
Atmospheric NOx (log)					
Total					
	Unadjusted (n=123)	**1.14**	**(0.251 – 2.02)**	***0.012**	**0.0507 (*0.012)**
	Adjusted[1] (n=123)	**1.14**	**(0.256 – 2.03)**	***0.012**	**0.0517 (*0.041)**
	Adjusted[2] (n=123)	**1.21**	**(0.244 – 2.18)**	***0.015**	**0.1423 (*0.022)**
Area					
	North (n=50)	0.317	(-1.41 – 2.04)	0.713	0.1061 (0.662)
	South (n=73)	**1.99**	**(0.679 – 3.30)**	****0.004**	**0.1818 (0.060)**
GSTP1					
	AA (n=27)	1.29	(-1.70 – 4.28)	0.378	0.3063 (0.350)
	AG+GG (n=96)	**1.14**	**(0.137 – 2.14)**	***0.026**	**0.1195 (0.118)**
GSTM1					
	0/+ or +/+ (n=93)	1.01	(-0.108 – 2.13)	0.076	0.1171 (0.144)
	0/0 (n=30)	0.824	(-1.36 – 3.01)	0.442	0.3835 (0.101)
Neonate Gender					
	Male (n=70)	0.75	(-0.588 – 2.08)	0.268	0.1445 (0.185)
	Female (n=48)	**2.48**	**(1.02 – 3.95)**	****0.001**	**0.2932 (*0.040)**

Adjusted[1]: HIV status; Adjusted[2]: HIV status, area, maternal age, body mass index, haemoglobin, blood pressure systolic and diastolic.
Area, GSTP1 and GSTM1 results were all adjusted for HIV status, maternal age, body mass index, haemoglobin, blood pressure systolic and diastolic.
CI= confidence interval, n= sample size. Statistical significance: *$p<0.05$, **$p<0.01$

The relationship between atmospheric NOx and GA, controlled for potential confounders, is described in Table 7. Controlling for potential maternal and neonate confounders, strengthened the β-coefficient and regression model for the total samples (unadjusted =-0.464, adjusted^2=-1.64, $p<0.05$). Therefore for every 1% increase in NOx there would be a 0.0164% reduction in GA. The maternal serum 8-OhdG did not significantly impact GA; however when it was not included in the regression model, NOx was also not found to influence GA. Controlling for 8-OHdG, therefore was an important factor for NOx influencing GA. When area was considered, a 1% increase in NOx would result in a 0.0196% decrease in GA ($p=0.023$). The GSTP1 variant caused a 0.013% reduction in GA, whilst the GSTM1 wt genotype caused a 0.0126% decrease in GA if a 1% increase in NOx is observed ($p<0.05$, Table 7). Mothers carrying a male foetus, were significantly associated with a 0.0156% decrease in GA if NOx were to increase by 1% ($p=0.027$). This decrease was almost doubled (0.0203% change) with mothers carrying a female foetus, although not significant ($p=0.097$).

Table 7: Impact of atmospheric NOx (log) concentration on gestational age – linear regression analysis for total mothers and subdivided into area, GSTM1 and GSTP1 SNP with adjustments for maternal characteristics.

		Gestational Age (weeks)			
		β-coefficient	(95%CI)	p-value	R-squared (p-value)
Atmospheric NOx (log)					
Total					
	Unadjusted (n=311)	-0.464	(-1.12 – 0.194)	0.166	0.2075 (***<0.0001)
	Adjusted[1] (n=118)	**-1.49**	**(-2.60 – -0.377)**	****0.009**	**0.3384 (***0.003)**
	Adjusted[2] (n=64)	**-1.64**	**(-3.21 – -0.066)**	***0.042**	**0.5597 (***0.0008)**
Area					
	North (n=49)	**-1.96**	**(-3.63 – -0.287)**	***0.023**	**0.5254 (**0.0093)**
	South (n=69)	-0.688	(-2.59 – 1.21)	0.471	0.3030 (0.0884)
GSTP1					
	AA (n=27)	-1.35	(-4.15 – 1.46)	0.316	0.7943 (*0.0222)
	AG+GG (n=91)	**-1.30**	**(-2.52 – -0.072)**	***0.038**	**0.2553 (*0.0445)**
GSTM1					
	0/+ or +/+ (n=88)	**-1.26**	**(-2.66 – -0.109)**	***0.034**	**0.3115 (**0.0093)**
	0/0 (n=30)	-1.38	(-4.39 – 1.88)	0.407	0.6875 (0.0554)
Neonate Gender					
	Male (n=70)	**-1.56**	**(-2.95 – -0.184)**	***0.027**	**0.3541 (*0.0135)**
	Female (n=48)	-2.03	(-4.44 – 0.385)	0.097	0.3550 (0.1922)

Unadjusted: birthweight; Adjusted[1]: neonatal characteristics: birthweight , child gender, Apgar scores: 1m and 5m, body: brain ratio, ponderal index and maternal characteristics: HIV status, area, maternal age, body mass index, haemoglobin, blood pressure systolic and diastolic, GSTP1 and GSTM1; Adjusted[2]: same as Adjusted[1] with parity included

Area, GSTP1, GSTM1 and neonate gender results were all adjusted for neonatal characteristics: birthweight, child gender, Apgar scores: 1m and 5m, body: brain ratio, ponderal index and maternal characteristics: HIV status, maternal age, body mass index, haemoglobin, blood pressure systolic and diastolic.

CI= confidence interval, n= sample size. Statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.0001$

4. Discussion

Exposure to NOx was shown to directly influence maternal serum 8-OHdG concentrations in pregnant women living in Durban, SA. Gestational age of these women was also shown to significantly decrease as a result of increased NOx exposure. This is the first study in Durban, SA to link the increase in oxidative stress in pregnant women to increased NOx pollution.

Oxides of nitrogen, a by-product of combustion, have been linked to several adverse health conditions, including respiratory diseases, cardiovascular diseases, low birth weight and preterm birth (Seo et al. 2007; Wu et al. 2009; César et al. 2015). This nitrogen centred free radical, upon inhalation, is absorbed in lung fluids producing free radical products that enter the blood stream. These free radicals are then able to react directly with macromolecules (protein, lipids and DNA) present resulting in ROS production (Tabacova et al. 1998). Guanine, having the lowest redox potential among the nucleic bases, is highly susceptible to oxidation by ROS; which results in the production of 8-OHdG mutagenic lesions (Kershaw and Hodges 2012; Ba et al. 2015). Therefore this accounts for the significant increase in maternal serum 8-OHdG adduct concentration as a result of NOx exposure (Fig. 1 and 2).

The Durban south region has been shown previously to have higher levels of air pollution (Naidoo et al. 2013) and in the present study with significantly increased NOx concentration (Fig.1A) compared to the north. Previously, pregnant women in the south region have been shown to exhibit increased markers of oxidative stress compared to the north (Nagiah et al. 2015). This finding was corroborated in our study, where pregnant women in the south had increased levels of serum 8-OHdG compared to the north (Fig.1B). Our study went further and investigated the relationship between NOx and 8-OHdG, the influence of GST polymorphisms as well as linking NOx to GA.

Several studies have found an association of pollution (polycyclic aromatic hydrocarbon (PAH), diesel-exhaust smoke and smoking) to increased 8-OHdG and oxidative stress (Risom et al. 2005; Lewtas 2007; Leonardi-Bee et al. 2008; Ren et al. 2010). Studies have also observed that a dose-effect relationship occurs between PAH exposure and levels of urinary 8-OHdG (Kuang et al. 2013; Li et al. 2015). Our study found a significantly positive correlation between atmospheric NOx and maternal serum 8-OHdG (Fig.2A, $p=0.158$). As mentioned above, the south region is considered to have a higher pollution level than the north. When area was taken into consideration, the relationship between NOx and 8-OHdG was strengthened in the heavily

industrialised south (Fig.2C, p=0.0466) whilst the relationship was lost in the less industrialised north (Fig.2B). This relationship was further investigated by controlling for potential confounding factors, to determine whether this effect was indeed a response to NOx exposure. The results confirmed that NOx exposure caused a significant increase in 8-OHdG concentration when controlled for maternal characteristics (Table 7). It was found that a 1% increase in NOx results in a 1.21% (p=0.015) increase in 8-OHdG, with an even higher increase (1.99%, p=0.004) observed for south living mothers. The results provide evidence that exposure to atmospheric NOx increases serum 8-OHdG levels in pregnant women.

An important controller of oxidative stress, are antioxidants that help reduce and eliminate oxidants to prevent oxidative stress-related damage. However, genetic susceptibility plays an important role in determining the effect and responses an individual has to oxidative damage. Therefore the risk for cancer, adverse reproductive outcomes and cardiovascular diseases are a consequence of air pollution exposure and genetic susceptibility (Lewtas 2007; Lagadu et al. 2010). GSTs are antioxidant enzymes that protect against oxidative stress by conjugating electrophilic species and thereby neutralising their effect. These GSTs have two common polymorphisms that influence an individual's genetic susceptibility to oxidants. A homozygous deletion in GSTM1 results in the enzyme inactivation (Mustafa et al. 2010), while the substitution of isoleucine for valine at codon 105 in GSTP1 reduces substrate specificity (Wong et al. 2008).

Our results indicate that GSTP1 is associated with the heavily industrialised south (Table 4, p=0.0281), with increased serum 8-OHdG observed in the GSTP1 AG+GG genotypes compared to the AA genotype. This suggests that wt GSTP1 is able to scavenge oxidants more effectively than the variant which leads to reduced serum 8-OHdG concentrations observed. When subdivided into areas, no difference in 8-OHdG was observed in north and south wt GSTP1 whilst the north variant mothers had significantly lower 8-OHdG levels compared to the south variant mothers (Fig.4B, $p<0.05$). This suggests that at low pollution levels (north) the variant GSTP1 enzymes, with its reduced specificity is still able to conjugate electrophiles and reduce their effect whilst at higher pollution levels (south) the variant genotype is overwhelmed and becomes inefficient at scavenging which leads to increased oxidants present that attack DNA leading to increased 8-OHdG levels.

It has been shown that the variant GSTP1 allele has a 7 fold greater efficacy against PAH diol epoxides than wt allele, whilst 3 fold less effective against 1-chloro-2,4-diinitrobenzene

(Strange et al. 2000). However, this study showed that the variant genotype is less efficient compared to the wt, at detoxifying oxidant products. A significant positive correlation was observed between NOx and 8-OHdG levels in AG+GG GSTP1 genotyped mothers (Table 5, p=0.0395); further confirming that the variant GSTP1 mothers when exposed to NOx are unable to effectively scavenge oxidants leading to increased DNA damage. No association was observed between GSTM1 and the heavily industrialised south (Table 3), with no difference in serum 8-OHdG levels observed between the null and wt GSTM1 genotypes. A significant positive correlation was observed between NOx and serum 8-OHdG for null GSTM1 mothers (Table 5, p=0.0199), thus suggesting that the inactive enzyme GSTM1 was unable to neutralise oxidants leading to increased DNA damage. These results were further analysed in multivariate analyses to determine whether the results observed in bivariate correlations remained when controlling for maternal characteristics as potential confounders.

The GSTP1 variant mothers where shown to have increased serum 8-OHdG (1.14%) with increasing NOx (1%, p=0.026, Table 6), whilst in GSTM1 null mothers this relationship was lost. This could be a result of small sample size, once all confounding factors were taken into account, only 30 GSTM1 null mothers remained, which could account for the lack of association between NOx and 8-OHdG. Other studies have shown no association between GSTM1 null individuals, DNA damage and pollution (PAH (Marczynski et al. 2002; Garte et al. 2007) and particulate matter (Sørensen et al. 2003)). The multivariate analysis however, suggests a parallel increase in 8-OHdG (1.01%) as a result of NOx (1%) exposure for GSTM1 wt mothers (Table 6, p=0.076). The results provide evidence that GSTP1 variant genotype increases the susceptibility of mothers to NOx exposure leading to increased oxidative stress.

Pregnancy, a physiological state characterised by increased basal oxygen demand and high energy requirement, favours ROS production and has been shown to exhibit increased susceptibility to oxidative stress in normal pregnancies (Saker et al. 2008). This already highly susceptible condition in the presence of high AAP would exasperate antioxidant stores and lead to increased oxidative stress. Exposure to traffic-related air pollution (i.e. NOx, CO and primary exhaust particles) have been implicated in deceased foetal growth, LBW and PTB (Seo et al. 2007; Darrow et al. 2011). Several studies have also reported oxidative stress as a potential mechanism for LBW and PTB, with reports showing increased 8-OHdG in LBW and PTB (Kim et al. 2005; Mustafa et al. 2010; Rossner et al. 2011).

Our study first set out to find an association between NOx and serum 8-OHdG with neonate BW and GA. Using simple correlations, no association was observed between serum 8-OHdG and BW, with a negative trend suggested between NOx and BW in north and south.. This negative trend was not significant; however this could be due to our small sample size as previous studies have shown a link between AAP and BW reduction (Lacasana et al. 2005; Darrow et al. 2011; Wilhelm et al. 2012). Next our study used bivariate linear regression to determine whether NOx was associated with reductions in GA. The results from the unadjusted bivariate analysis again suggested a negative trend but remained non- significant; also observed using simple correlation. We then controlled for maternal and neonatal characteristics, which revealed a small significant reduction in gestational age as a result of NOx exposure (Table 7, $p=0.042$). This relationship was only found to be significant when maternal serum 8-OHdG was controlled for, suggesting 8-OHdG may affect GA.

Previous studies have found increased 8-OHdG in mothers who give birth prematurely (Matsubasa et al. 2002; Nassi et al. 2009; Darrow et al. 2011; Negi et al. 2012b). When area was considered, the north showed a significantly higher reduction in GA compared to the total mothers, with south mothers having no reduction observed. This study only measured the levels of NOx; however other pollutants or environmental factors may have been present in high concentrations within the north area. These pollutants or environmental factors may be potent enough to cause the reduction in GA observed within the north. This would be an ideal follow up study. The GTSP1 variant and GSTM1 wt mothers were susceptible to GA reduction as a consequence of NOx exposure ($p=0.034$ and $p=0.038$ (Table 7), respectively). Neonatal gender was also found to be associated with reduced gestational age due to NOx exposure, where mothers carrying male infants exposed to NOx had a significant reduction in GA. These reductions in GA observed, were small (<0.1 weeks per percent change in NOx), therefore would not impact on clinical significance for individual neonate. However, a negative shift in GA on the population level could result in increased PTB nationwide.

The findings of our study must be interpreted in light of the following limitations. Firstly, upon subdivision, by area and genotype, our sample size becomes relatively small. Secondly, due to our relatively small sample size, ethnicity was not taken into account; this could be addressed in a future study with increased population numbers. Although significant results are obtained, increasing population numbers in future studies could give further insight into the conclusions observed. Thirdly, measuring other pollutants in study areas could give further insight into their

effects on 8-OHdG levels and GA. A combined effect of NOx and other pollutions such as particulate matter would also be interesting to investigate.

In conclusion, this study demonstrated increased maternal serum 8-OHdG in pregnant women exposed to higher levels of NOx pollution in the south. This increase in DNA damage was found to be a direct consequence of increased NOx exposure, with increased susceptibility found in GSTP1 variant carriers and GSTM1 wt carriers. Gestational age was also found to be reduced as a consequence of NOx exposure, with male neonates making mothers more susceptible to GA reduction. This study highlights the need for better systems in place to reduce traffic-related air pollution close to residential areas, so that vulnerable individuals are better protected against oxidative stress related injury.

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6. References

Ba X, Aguilera-Aguirre L, Sur S, Boldogh I (2015) 8-Oxoguanine DNA glycosylase-1-driven DNA base excision repair. Curr Opin Allergy Clin Immunol 15:89–97. doi: 10.1097/ACI.0000000000000135

Beelen R, Hoek G (2010) ESCAPE Exposure assessment manual.

César ACG, Carvalho JA, Nascimento LFC (2015) Association between NOx exposure and deaths caused by respiratory diseases in a medium-sized Brazilian city. Brazilian J Med Biol Res 48:1130–1135. doi: 10.1590/1414-431X20154396

Chen Z, Salam MT, Eckel SP, Breton C V, Gilliland FD (2015) Chronic effects of air pollution on respiratory health in Southern California children: findings from the Southern California Children's Health Study. J Thorac Dis 7:46–58. doi: 10.3978/j.issn.2072-1439.2014.12.20

Darrow LA, Klein M, Strickland MJ, Mulholland JA, Tolbert PE (2011) Ambient air pollution and birth weight in full-term infants in Atlanta, 1994-2004. Environ Health Perspect 119:731–737. doi: 10.1289/ehp.1002785

Fleischer NL, Merialdi M, Donkelaar A Van, Vadillo-Ortega F, Martin R V., van Donkelaar A, Vadillo-Ortega F, Martin R V., Betran AP, Souza JP, O'Neill MS, Donkelaar A Van, Vadillo-Ortega F, Martin R V., van Donkelaar A, Vadillo-Ortega F, Martin R V., Betran AP, Souza JP, O'Neill MS (2014) Outdoor Air Pollution , Preterm Birth , and Low Birth Weight : Analysis of the World Health Organization Global Survey on Maternal and Perinatal Health. Environ Health Perspect 122:425–430. doi: 10.1289/ehp.1306837

Garte S, Taioli E, Raimondi S, Paracchini V, Binkova B, Sram RJ, Kalina I, Popov TA, Singh R, Farmer PB (2007) Effects of metabolic genotypes on intermediary biomarkers in subjects exposed to PAHS: Results from the EXPAH study. Mutat Res - Fundam Mol Mech Mutagen 620:7–15. doi: 10.1016/j.mrfmmm.2007.02.017

Glinianaia S V, Rankin J, Bell R, Pless-Mulloli T, Howel D (2004) Particulate air pollution and fetal health: a systematic review of the epidemiologic evidence. Epidemiology 15:36–45. doi: 10.1097/01.ede.0000101023.41844.ac

Johanssona A-S, Stenberga G, Widerstena M, Mannervik B (1998) Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. J Mol Biol 278:687–698. doi: http://dx.doi.org/10.1006/jmbi.1998.1708

Kampa M, Castanas E (2008) Human health effects of air pollution. Environ Pollut 151:362–367. doi: 10.1016/j.envpol.2007.06.012

Kelly FJ (2003) Oxidative stress: its role in air pollution and adverse health effects. Occup Environ Med 60:612–616. doi: 10.1136/oem.60.8.612

Kershaw RM, Hodges NJ (2012) Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. Mutagenesis 27:501–510. doi: 10.1093/mutage/ges012

Kim Y, Hong Y, Lee K, Park H, Park E, Moon H, Ha E (2005) Oxidative stress in pregnant women and birth weight reduction. Reprod Toxicol 19:487–492. doi: 10.1016/j.reprotox.2004.10.007

Kistnasamy EJ, Robins TG, Naidoo R, Batterman S, Mentz GB, Jack C, Irusen E (2008) The relationship between asthma and ambient air pollutants among primary school students in Durban, South Africa. Int J Environ Heal 2:365. doi: 10.1504/IJENVH.2008.020929

Kuang D, Zhang W, Deng Q, Zhang X, Huang K, Guan L, Hu D, Wu T, Guo H (2013) Dose-response relationships of polycyclic aromatic hydrocarbons exposure and oxidative damage to

DNA and lipid in coke oven workers. Envioronmental Sci Technol 47:7446–56. doi: 10.1021/es401639x

Lacasana M, Esplugues A, Ballester F (2005) Exposure to ambient air pollution and prenatal and early childhood health effects. Eur J Epidemiol 20:183–199. doi: http://dx.doi.org/10.1007/s10654-004-3005-9

Lagadu S, Lechevrel M, Sichel F, Breton J, Pottier D, Couderc R, Moussa F, Prevost V (2010) 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker of oxidative damage in oesophageal cancer patients: lack of association with antioxidant vitamins and polymorphism of hOGG1 and GST. J Exp Clin Cancer Res 29:157. doi: 10.1186/1756-9966-29-157

Leonardi-Bee J, Smyth A, Britton J, Coleman T (2008) Environmental tobacco smoke and fetal health: systematic review and meta-analysis. Arch Dis Child Fetal Neonatal Ed 93:F351–F361. doi: 10.1136/adc.2007.133553

Lewtas J (2007) Air pollution combustion emissions: Characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat Res - Rev Mutat Res 636:95–133. doi: 10.1016/j.mrrev.2007.08.003

Li J, Lu S, Liu G, Zhou Y, Lv Y, She J, Fan R (2015) Co-exposure to polycyclic aromatic hydrocarbons, benzene and toluene and their dose-effects on oxidative stress damage in kindergarten-aged children in Guangzhou, China. Sci Total Environ 524–525:74–80. doi: 10.1016/j.scitotenv.2015.04.020

Marczynski B, Rihs H-P, Rossbach B, Hölzer J, Angerer J, Scherenberg M, Hoffmann G, Brüning T, Wilhelm M (2002) Analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine and DNA strand breaks in white blood cells of occupationally exposed workers: comparison with ambient monitoring, urinary metabolites and enzyme polymorphisms. Carcinogenesis 23:273–81. doi: https://doi.org/10.1093/carcin/23.2.273

Matsubasa T, Uchino T, Karashima S, Tanimura M, Endo F (2002) Oxidative Stress in Very Low Birth Weight Infants As Measured by Urinary 8-OHdG. Free Radic Res 36:189–193. doi: 10.1080/10715760290006510

Moorthy B, Chu C, Carlin DJ (2015) Polycyclic Aromatic Hydrocarbons: From Metabolism to Lung Cancer. Toxicol Sci 145:5–15. doi: 10.1093/toxsci/kfv040

Moyer AM, Salavaggione OE, Wu TY, Moon I, Eckloff BW, Hildebrandt MA, Schaid DJ, Wieben ED, Weinshilboum RM (2008) Glutathione s-transferase p1: gene sequence variation

and functional genomic studies. Cancer Res 68:4791–4801. doi: 10.1158/0008-5472.can-07-6724

Mustafa MD, Pathak R, Ahmed T, Ahmed RS, Tripathi AK, Guleria K, Banerjee BD (2010) Association of glutathione S-transferase M1 and T1 gene polymorphisms and oxidative stress markers in preterm labor. Clin Biochem 43:1124–1128. doi: 10.1016/j.clinbiochem.2010.06.018

Muttoo S, Ramsay L, Brunekreef B, Beelen R, Meliefste K, Naidoo RN (2017) Land Use Regression Modelling Estimating Nitrogen Oxides Exposure in Industrial South Durban, South Africa. Sci Total Environ 610–611:1439–1447. doi: 10.1016/j.scitotenv.2017.07.278

Nagiah S, Phulukdaree A, Naidoo D, Ramcharan K, Naidoo R, Moodley D, Chuturgoon A (2015) Oxidative stress and air pollution exposure during pregnancy: A molecular assessment. Hum Exp Toxicol 34:838–847. doi: 10.1177/0960327114559992

Naidoo RN, Robins T, Batterman S, Mentz G, Jack C (2013) Ambient pollution and respiratory outcomes among schoolchildren in Durban, South Africa. South African J Child Heal 7:127–134. doi: 10.7196/sajch.598

Nassi N, Ponziani V, Becatti M, Galvan P, Donzelli G (2009) Anti-oxidant enzymes and related elements in term and preterm newborns. Pediatr Int 51:183–187. doi: 10.1111/j.1442-200X.2008.02662.x

Negi R, Pande D, Kumar A, Khanna RS, Khanna HD (2012a) Evaluation of biomarkers of oxidative stress and antioxidant capacity in the cord blood of preterm low birth weight neonates. J Matern Neonatal Med 25:1338–1341. doi: 10.3109/14767058.2011.633672

Negi R, Pande D, Kumar A, Khanna RS, Khanna HD (2012b) In vivo oxidative DNA damage and lipid peroxidation as a biomarker of oxidative stress in preterm low-birthweight infants. J Trop Pediatr 58:326–328. doi: 10.1093/tropej/fmr078

Proietti E, Röösli M, Frey U, Latzin P (2013) Air Pollution During Pregnancy and Neonatal Outcome: A Review. J Aerosol Med Pulm Drug Deliv 26:9–23. doi: 10.1089/jamp.2011.0932

Ren C, Vokonas PS, Suh H, Fang S, Christiani DC, Schwartz J (2010) Effect modification of air pollution on urinary 8-hydroxy-2'-deoxyguanosine by genotypes: an application of the multiple testing procedure to identify significant SNP interactions. Environ Heal 9:78. doi: 10.1186/1476-069X-9-78

Risom L, Møller P, Loft S (2005) Oxidative stress-induced DNA damage by particulate air pollution. 592:119–137. doi: 10.1016/j.mrfmmm.2005.06.012

Romieu I, Ramirez-Aguilar M, Sienra-Monge JJ, Moreno-Macías H, del Rio-Navarro BE, David G, Marzec J, Hernández-Avila M, London S (2006) GSTM1 and GSTP1 and respiratory health in asthmatic children exposed to ozone. Eur Respir J 28:953–9. doi: 10.1183/09031936.06.00114905

Rossner P, Tabashidze N, Dostal M, Novakova Z, Chvatalova I, Spatova M, Sram RJ (2011) Genetic, biochemical, and environmental factors associated with pregnancy outcomes in newborns from the Czech Republic. Environ Health Perspect 119:265–271. doi: 10.1289/ehp.1002470

Saker M, Soulimane Mokhtari N, Merzouk SA, Merzouk H, Belarbi B, Narce M (2008) Oxidant and antioxidant status in mothers and their newborns according to birthweight. Eur J Obstet Gynecol Reprod Biol 141:95–99. doi: 10.1016/j.ejogrb.2008.07.013

Seidegård J, Vorachek WR, Pero RW, Pearson WR (1988) Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. Proc Natl Acad Sci U S A 85:7293–7. doi: 10.1073/pnas.85.19.7293

Seo J, Ha E, Kim O, Kim B, Park H, Leem J, Hong Y, Kim Y (2007) Environmental health surveillance of low birth weight in Seoul using air monitoring and birth data [in Korean]. J Prev Med Public Heal 40:363–370. doi: https://doi.org/10.3961/jpmph.2007.40.5.363

Slama R, Gräbsch C, Lepeule J, Siroux V, Cyrys J, Sausenthaler S, Herbarth O, Bauer M, Borte M, Wichmann HE, Heinrich J (2010) Maternal fine particulate matter exposure, polymorphism in xenobiotic-metabolizing genes and offspring birth weight. Reprod Toxicol 30:600–612. doi: 10.1016/j.reprotox.2010.07.001

Sørensen M, Autrup H, Hertel O, Wallin H, Knudsen LE, Loft S (2003) Personal exposure to PM2. 5 and biomarkers of DNA damage. Cancer Epidemiol Biomarkers Prev 12:191–196.

Šrám RJ, Binková B, Dejmek J, Bobak M (2005) Ambient air pollution and pregnancy outcomes: A review of the literature. Environ Health Perspect 113:375–382. doi: 10.1289/ehp.6362

Sram RJ, Binkova B, Dejmek J, Chvatalova I, Solansky I, Topinka J (2006) Association of DNA adducts and genotypes with birth weight. Mutat Res - Genet Toxicol Environ Mutagen 608:121–128. doi: 10.1016/j.mrgentox.2006.04.022

Strange RC, Jones PW, Fryer AA (2000) Glutathione S-transferase: Genetics and role in toxicology. Toxicol Lett 112–113:357–363. doi: 10.1016/S0378-4274(99)00230-1

Tabacova S, Baird DD, Balabaeva L (1998) Exposure to oxidized nitrogen: lipid peroxidation and neonatal health risk. Arch Env Heal 53:214–221. doi: 10.1080/00039899809605698

Tularam H, Ramsay L (2013) Synoptic influences on air pollution events in the Durban South Basin, 2006 to 2010. University of Kwa-Zulu Natal

Tyson P, Preston-Whyte R (2004) The weather and climate of southern Africa, 2nd edn. Oxford University Press, Oxford; New York

Wilhelm M, Ghosh JK, Su J, Cockburn M, Jerrett M, Ritz B (2012) Traffic-Related Air Toxics and Term Low Birth Weight in Los Angeles County, California. Environ Health Perspect 120:132–138. doi: 10.1289/ehp.1103408

Wong RH, Hu CW, Yeh CY, Chao MR, Chen CC, Huang JH, Chang SH, Lee SI, Lee HS (2008) Sulfotransferase 1A1 and glutathione S-transferase P1 genetic polymorphisms modulate the levels of urinary 8-hydroxy-2′-deoxyguanosine in betel quid chewers. Arch Toxicol 82:313–321. doi: 10.1007/s00204-007-0248-8

Wu J, Ren C, Delfino RJ, Chung J, Wilhelm M, Ritz B (2009) Association between Local Traffic-Generated Air Pollution and Preeclampsia and Preterm Delivery in the South Coast Air Basin of California. Environ Health Perspect 117:1773–1779. doi: 10.1289/ ehp.0800334

Xu SJ, Wang YP, Roe B, Pearson WR (1998) Characterization of the human class Mu glutathione S-transferase gene cluster and the GSTM1 deletion. J Biol Chem 273:3517–3527. doi: 10.1074/jbc.273.6.3517

Zimniak P, Nanduri B, Pikula S, Bandorowicz-Pikula J, Singhal SS, Srivastava SK, Awasthi S, Awasthi YC (1994) Naturally occurring human glutathione S-transferase GSTP1-1 isoforms with isoleucine and valine in position 104 differ in enzymic properties. Eur J Biochem 224:893–899. doi: 10.1111/j.1432-1033.1994.00893.x

CHAPTER 3

OGG1 Ser326Cys polymorphism, HIV, obesity and air pollution exposure influences adverse birth outcome susceptibility, within South African Women.

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Abstract:

The global HIV and obesity epidemics are major public health concerns; particularly as both are associated with increased risk of adverse birth outcomes. Despite extensive research, their combined effect, in terms of birth outcomes, has not been investigated. A single-nucleotide polymorphism (SNP) within 8-oxoguanine glycosylase 1 (OGG1) (Ser326Cys) has been suggested to affect body mass indices and therefore could predispose South African (SA) women to adverse effects of obesity. This study investigated the associations of OGG1 Ser326Cys SNP in relation to HIV and obesity on the susceptibility of low-birthweight (LBW) and pre-term birth (PTB) in SA women exposed to ambient air-pollution living in Durban. In our study population, the OGG1 SNP was associated with HIV and obesity. Wild-type (CC)-carrying patients had increased susceptibility for HIV-associated LBW and PTB. Co-morbid HIV and obese patients delivered neonates with decreased birthweights. Living within the heavily-polluted south-Durban and carrying the CC-genotype increased the risk for PTB within our study population.

Keywords:

Low birthweight, HIV, obesity, OGG1 Ser326Cys polymorphism, pre-term birth, air pollution

Highlights

- OGG1 Ser326Cys polymorphism associated with HIV
- Comorbid HIV and obesity associated with decreased neonatal BW
- Wild-type OGG1 Ser326Cys genotype increased risk of PTB and HIV associated LBW
- Living in heavily polluted south Durban increased susceptibility of PTB

Introduction

The global human immunodeficiency virus (HIV) epidemic affects approximately 36.5 million people worldwide, with majority of these infected people living in low- and middle-income countries. Sub-Saharan Africa represents the highest number of people living with HIV (25.6 million people) with approximately 27.4% living within South Africa (SA). Women account for more than half the infected population [1]. In addition to the HIV epidemic, SA has the highest obesity rate in sub-Sahara Africa, with more than 68% of women classified as overweight or obese [2]. Women being the most vulnerable to these diseases are cause for concern because not only does it affect the health of women but the health of future generations. With obesity and HIV both individually associated with increased susceptibility to adverse birth outcomes,

neonate morbidity and mortality, together they could potentially pose a significant threat to public health [3–6].

Various adverse birth outcomes linked to obesity include gestational diabetes and hypertension, neonatal macrosomia, intrauterine growth restriction and pre-term birth (PTB) [5,7,8]. HIV infection has been shown to increase the risk for low birthweight (LBW) and PTB [3,4,9]. As HIV infection was most commonly associated with reduced weight, which has been shown to increase the risk of LBW and PTB, a shift to increased obesity in HIV infected patients has been observed in developed countries [10]. Studies in developing countries however have shown decreased BMI in HIV positive (HIV+) patients, even with the increased use of antiretroviral therapy [11,12]. Therefore the combined effect of obesity and HIV could further potentiate risk for adverse birth outcomes. Studies have shown that both HIV and obesity affect the metabolic state of the infected person, with oxidative stress and inflammation implicated in LBW and PTB aetiology [13,14].

It has been reported that ambient air pollution (AAP) exposure is linked to increased risk of LBW and PTB [13,15,16]. In developing countries, e.g. SA, increased industrialisation and urbanisation leads to increased AAP which negatively impacts on the health of the population. This is most notable observed within the Durban South Industrial basin (DSIB) where high levels of AAP have been reported. Studies have associated these AAP to adverse health effects in children including moderate to severe asthma, wheezing and airway hyperactivity [17,18].

The combined effect of both external and internal exposures, either through infection, obesity or pollution exposure, on birth outcomes is important to fully understand the complex aetiology of these adverse outcomes. However, an important determinate of an individual's response to toxic insult, either by HIV infection, obesity or AAP exposure, is their genetic susceptibility. Genes of the DNA repair pathway have been shown to be highly polymorphic which affects structure and function of its proteins. The human 8-oxoguanine glycosylase 1 (OGG1) gene, a key component of the DNA repair pathway, consists of eight cysteine residues within its active site and is susceptible to oxidative modification [19–21]. A functional single nucleotide polymorphism (SNP) exists within the OGG1 gene, as a result of an amino acid substitution of serine with cysteine at positon 326 (Ser326Cys) within exon 7 [22]. This polymorphism has been suggested to reduce the repair activity of OGG1, with carriers of the homozygous C326 (G) allele (rs1052133 GG variant) and heterozygous (CG) genotypes having reduced activity

compared with the homozygous S326 (CC) wild-type (wt) genotype, and may affect an individual's response to external insults [22,23].

Limited data is available within the indigenous African population for the OGG1 Ser326Cys SNP, and its effect on adverse birth outcomes. The high incidence of obesity and HIV within this population group together with their known metabolic abnormalities and well-established link between HIV and adverse birth outcomes, necessitates an investigation of OGG1 (an enzyme involved in metabolic activities) Ser326Cys SNP that may give further insight to the susceptibility of adverse birth outcomes within this population. This longitudinal birth cohort study was therefore conducted to investigate the effects of the OGG1 Ser326Cys SNP, HIV and obesity on the susceptibility of LBW and PTB in woman exposed to AAP living in Durban, SA. Additionally, the effect of the exposed woman's clinical parameters on LBW and PTB susceptibility was also explored.

Methods

Study Population

The Mother and Child in the Environment (MACE) longitudinal cohort study recruited HIV negative (HIV-) (n=282) and HIV+ (n=155) pregnant women from public sector anti-natal clinics in the heavily-polluted DSIB and less-industrialised Durban north region. Ethical approval from the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BF263/12) (Appendix 1) and informed consent from study participants was obtained. The female participants were residents of the geographical area for the full duration of the pregnancy. Women with hypertension, multiple pregnancies, diabetes, placenta previa, genital tract infections and other complications which result in adverse growth effects were excluded from the study.

Clinical Parameters of Study Population

Upon enrolment, at trimester one (T1), study participants underwent clinical tests to determine the following parameters: weight, height, body mass index (BMI), iron and haemoglobin (Hb) levels, systolic and diastolic blood pressure (BP) and age. The study population was followed up at trimester three (T3), where the above parameters were measured once again.

Collection and Preparation of Samples

Third trimester blood was collected in BD Vacutainer® EDTA, aliquoted and stored at -80°C for genotyping of polymorphisms.

OGG1 rs1052133 Ser326Cys Polymorphism

DNA was extracted from whole blood using the Qiagen FlexiGene® DNA Kit (Qiagen, ID:51206), as per manufacturer's instructions. Extracted DNA was quantified using the Nanodrop 2000 spectrophotometer and standardised to $10ng\mu l^{-1}$.

Polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) was used to investigate OGG1 SNP genotypes (n=309). A 234bp PCR product was amplified using 6pmol of primers (Inqaba Biotech, SA; OGG1 forward: 5'-CCCAACCCCAGTGGATTCTCATTGC-3', OGG1 reverse: 5'-GTGCCCCATCTAGCCTTGCGGCCCTT-3'). The 30µl reaction consisted of 1x Green GoTaq Flexi buffer, 1.25mM $MgCl_2$, 0.5U GoTaq DNA polymerase (Promega), 200µM of each dNTP, and 10ng DNA template. Initial denaturation was applied (94°C, 4min), followed by 35 cycles of denaturation (94°C, 30sec), annealing (60°C, 1min) and extension (72°C, 30sec), concluding with final extension (72°C, 5min). Amplification products were electrophoresed on agarose gel (1.8%, 1µl Gel Red (catalogue no.41003, Biotium Inc., Hayward, CA)) and visualised on the Bio-Rad ChemiDocTM XRS+ System, using the Image Lab™ software. The PCR amplicon underwent restriction endonuclease digestion to determine the presence of the polymorphic restriction site. An overnight digestion (37°C) was performed in 28µl reactions; each reaction consisting of 10µl PCR product, nuclease-free water, 2µl 10x Buffer G, and 1µl *Sat*1 (*Fnu*4HI) (Thermo Fisher Scientific). A 21bp fragment was present in all samples due to a *Sat*1 invariant site induced by the mismatch. Amplicons digested completely with 164bp, 49bp and 21bp restriction fragments were homozygous for the G-allele (Appendix 2: Fig.A2.3). The restriction fragments were electrophoresed on an agarose gel (1.8%, 2µl GelRed) and visualised as mentioned above.

Statistical Analysis

Statistical Analyses were performed using GraphPad Prism V5 Software Package (GraphPad Software Inc., San Diego, California, USA) and STATA version 13.1. Measurements taken during the mothers' T1 and T3 were used for BMI, iron, Hb and systolic and diastolic BP data. The Student t test with Welch's correction was used to determine statistical significance between the HIV-/+ and obesity groups for maternal and neonatal characteristics. The Chi square and Fischer's exact tests were used to test the significant difference in the prevalence of OGG1 genotypes between HIV-/+ and obesity groups, including PTB and LBW and maternal characteristics: BMI, Hb, iron and diastolic and systolic BP. All statistical tests were two-sided. The relationship between PTB, HIV and location of birth was determined using logistic regression, and potential confounders were controlled.

Results

Maternal Clinical Characteristics

The study participants were divided into two categories: normal birthweight (NBW)/LBW and term birth (TB)/PTB, these were then further subdivided by HIV status. The mean gestational age (GA) for women giving birth to NBW and LBW infants were 39.1 weeks and 36.9 weeks, respectively ($p<0.0001$). The mean birthweight (BW) for term and pre-term infants were 3135.95g and 2393.02g, respectively ($p<0.0001$). The distribution of age, height and BMI was significantly different among all groups in LBW/NBW and TB/PTB categories ($p<0.0001$) (Table.1).

Table 1 Maternal clinical characteristics

	NBW HIV- (n=260)	NBW HIV+ (n=125)	LBW HIV- (n=34)	LBW HIV+ (n=27)	[a]p-value	TB HIV- (n=270)	TB HIV+ (n=132)	PTB HIV- (n=23)	PTB HIV+ (n=18)	[a]p-value
Age (years)	24.35 ± 0.319	28.37 ± 0.476	23 ± 0.931	29.07 ± 1.41	***<**0.0001**	24.17 ± 0.312	28.82 ± 0.479	24.65 ± 1.25	26.67 ± 1.62	***<**0.0001**
Age Range (years)	15 – 43	17 – 42	15 – 37	16 – 44		15-43	19-44	15-37	16 - 38	
Height (m)	1.55 ± 0.009	1.47 ± 0.017	1.54 ± 0.021	1.45 ± 0.040	**0.0053	1.55 ± 0.008	1.47 ± 0.017	1.54 ± 0.029	1.48 ± 0.052	**0.0076
Height Range (m)	1 – 1.76	1 – 1.82	1.06 – 1.68	1.02 – 1.66		1 – 1.76	1 – 1.82	1.06 – 1.68	1 – 1.67	
BMI T1 (kg/m^2)	28.77 ± 0.641	34.21 ± 1.20	24.67 ± 1.69	35.64 ± 2.71	***<**0.0001**	28.28 ± 0.624	34.93 ± 1.19	27 ± 2.25	29.78 ± 2.78	***<**0.0001**
BMI T3 (kg/m^2)	31.92 ±0.694	37.95 ± 1.31	28.19 ± 2.09	37.35 ± 2.53	***<**0.0001**	31.50 ± 0.687	38.23 ± 1.28	30.35 ± 2.44	33.14 ± 2.32	***<**0.0001**

Results described: mean ± standard error
NBW = normal birth weight, LBW = low birth weight, TB = term birth, PTB = pre-term birth, n=sample size, T1= trimester one, T3= trimester three, BMI= body mass index
[a]Comparison between all four groups
Statistical significance: ** **p<0.0001*, ** p<0.01*

Association between OGG1 polymorphism, HIV and obesity

A significant association between HIV and obesity for both T1 and T3 are shown in Table 2. The frequency of obese patients in T1 increased 1.4 fold in HIV+ patients compared to the 0.4 fold decrease observed in non-obese HIV+ patients ($p<0.0001$). In T3, a 0.9 decrease in frequency was observed in HIV+ obese compared to a 0.4 fold decrease in HIV+ non-obese patients ($p<0.0001$).

Table 2 Association of HIV and Obesity for trimester one (n=496) and three (n=472)

	Total n (%)	HIV- n (%)	HIV+ n (%)	p-value
Obesity				
Trimester 1				
Non-obese	306 (66.9)	222 (72.6)	84 (27.4)	***<0.0001[a]
Obese	190 (33.1)	104 (54.7)	86 (45.3)	
Trimester 3				
Non-obese	240 (50.8)	186 (77.5)	54 (22.5)	***<0.0001[b]
Obese	232 (49.2)	126 (54.3)	106 (45.7)	

n=sample size, RR= risk ratio, OR= odds ratio, CI= confidence interval, BMI= body mass index, non-obese (BMI<30), obese (BMI>30)
Statistical significance: ****p<0.0001; **p<0.01*
[a]RR(1.325; 95%CI 1.145, 1.535); OR(2.185; 95%CI 1.494, 3.197)
[b]RR(1.427, 95%CI 1.245, 1.635); OR (2.898, 95%CI 1.946, 4.315)

The genotypic and allelotypic prevalence of OGG1 amongst HIV and obese study participants are shown in Table 3. For all subjects, the prevalence of the OGG1 CC and CG+GG genotypes was 66.9% and 33.1%, respectively. The C and G allele frequencies were 81.1% and 18.9%, respectively. The frequency and prevalence of the allelotypes and genotypes were significantly different among HIV+ and HIV- individuals. The frequency of the G-allelotype decreased 0.7 fold whilst the C-allelotype increased 1.1 fold (Table.3: $p=0.0089$) in HIV+ patients.

The prevalence of OGG1 allelotypes and genotypes were significantly different between obese and non-obese individuals in T1 (Table.3). In T1, both CC and CG+GG genotype frequencies were decreased in obese patients by 0.7 and 0.4, respectively (Table.3: $p=0.0324$). However, the C-allelotype increased by 1.1 fold versus the 0.6 decrease in G-allele frequency observed in obese patients (Table.3: $p=0.0410$). In T3, the CC-genotype frequency increased 1.1 fold whilst the CG+GG-genotype decreased 0.7 fold in obese patients (Table.3: $p=0.08$). Both allele

frequencies decreased in obese patients by 0.99 fold and 0.7 fold, respectively (Table.3: p=0.0863). The genotypic frequency distributions were consistent with the Hardy-Weinberg equilibrium for this study (Chi^2 test: p>0.05).

Table 3 Genotypic and allelotypic frequencies of OGG1 patients that are HIV+ or HIV- (n=302) and non-obese (BMI<30) or obese (BMI>30) in trimester one (n=301) and three (n=288)

	HIV			
	Total n (%)	HIV- n (%)	HIV+ n (%)	p-value
Genotype frequency				
OGG1 CC	202 (66.9)	82 (40.6)	120 (59.4)	****0.0048**[a]
OGG1 CG and GG	100 (33.1)	24 (24)	76 (76)	
Allelotype frequency				
OGG1 C	490 (81.1)	306 (78.1)	184 (86.8)	****0.0089**[b]
OGG1 G	114 (18.9)	86 (21.9)	28 (13.2)	
	Obesity			
	Total n (%)	Non-obese n (%)	Obese n (%)	p-value
Genotype frequency				
Trimester 1				
OGG1 CC	201 (66.8)	117 (58.2)	84 (41.8)	***0.0324**[c]
OGG1 CG and GG	100 (33.1)	71 (71)	29 (29)	
Trimester 3				
OGG1 CC	194 (67.4)	94 (48.5)	100 (51.6)	0.08[d]
OGG1 CG and GG	94 (32.6)	56 (59.6)	38 (40.4)	
Allelotype frequency				
Trimester 1				
OGG1 C	488 (81.1)	295 (78.1)	193 (86.8)	***0.0410**[e]
OGG1 G	114 (18.9)	81 (21.9)	33 (13.2)	
Trimester 3				
OGG1 C	469 (81.4)	236 (50.3)	233 (49.7)	0.0863[f]
OGG1 G	107 (18.6)	64 (59.8)	43 (40.2)	

n=sample size, RR= risk ratio, OR= odds ratio, CI= confidence interval, BMI= body mass index
Statistical significance: **p<0.05*
[a]RR(0.7817; 95%CI 0.6671, 0.9160); OR(0.4621; 95%CI 0.2698, 0.7916)
[b]RR(0.8278, 95%CI 0.7303, 0.9383); OR (0.5415, 95%CI 0.3404, 0.8612)
[c]RR(0.8198; 95%CI 0.6906, 0.9733); OR(0.5689; 95%CI 0.3399, 0.9522)
[d]RR(0.8133, 95%CI 0.6521, 1.014); OR (0.6379, 95%CI 0.3872, 1.051)
[e]RR(0.8508, 95%CI 0.7415, 0.9761); OR (0.6227, 95%CI 0.3996, 0.9705)
[f]RR(0.8413, 95%CI 0.7030, 1.007); OR (0.6805, 95%CI 0.4441, 1.043)

Effect of OGG1 polymorphism and HIV on the risk of LBW and PTB

Obesity and the genotypic influence of OGG1 Ser326Cys genotypes on the association between HIV, obesity and LBW or PTB are described in Table 4. HIV infection was suggested to be associated with LBW in the total population (p=0.071), this was found to be significant for CC-carrying patients (p=0.034). Where being HIV+ increased the susceptibility of having LBW by a 1.45 fold change and PTB by a 2.26 fold increase (Table.4, p=0.034 and p=0.036, respectively).

Being obese in both T1 and T3 increased the susceptibility of delivering a LBW infant for HIV+ patients by 5.4 fold and 2.4 fold, respectively (Table.4: p=0.006 and p=0.017, respectively). Additionally, being obese in T3 and carrying CC-genotype increased the susceptibility of delivering a LBW infant by 3.4 fold in HIV+ patients (Appendix 4: Table A4.1: p=0.029) and being obese in T1 and living in the south increased the risk of HIV associated LBW by 8.01 fold (Appendix 4: Table A4.1: p=0.022). Whilst, carrying the CC-genotype and being non-obese in T3 increased the susceptibility of PTB in HIV+ patients by 3 fold (Appendix 4: Table A4.1: p=0.024).

Table 4 OGG1 Genotypic and obesity frequency distributions among patients delivering LBW or NBW neonates, at term (TB) or pre-term (PTB) that are HIV+ and HIV- (n=302)

	Birth Weight (g)			Gestational Age (weeks)		
	NBW n(%)	LBW n(%)	p-value	TB n(%)	PTB n(%)	p-value
Total						
HIV-	260 (88.44)	34 (11.56)	0.071	270 (92.15)	23 (7.85)	0.154
HIV+	125 (82.24)	27 (17.76)		132 (88)	18 (12)	
CC						
HIV-	98 (89.91)	11 (10.09)	***0.034**	100 (91.74)	9 (8.26)	***0.036**
HIV+	59 (78.67)	16 (14.67)		61 (81.33)	14 (18.67)	
CG+GG						
HIV-	62 (86.11)	10 (13.89)	0.621	66 (91.67)	6 (8.33)	0.864
HIV+	18 (81.82)	4 (18.18)		19 (90.48)	2 (9.52)	
Non-obese						
T1						
HIV-	172 (85.57)	29 (14.43)	0.680	183 (91.04)	18 (8.96)	0.146
HIV+	61 (83.56)	12 (16.44)		62 (84.93)	11 (15.07)	
T3						
HIV-	146 (85.38)	25 (14.62)	0.851	155 (90.64)	16 (9.36)	0.100
HIV+	43 (84.31)	8 (15.69)		42 (82.35)	9 (17.65)	
Obese						
T1						
HIV-	88 (94.62)	5 (5.38)	****0.006**	87 (94.57)	5 (5.43)	0.357
HIV+	64 (81.01)	15 (28.99)		70 (90.91)	7 (9.09)	
T3						
HIV-	111 (92.5)	9 (7.5)	***0.017**	112 (94.12)	7 (5.88)	0.496
HIV+	81 (81.82)	18 (18.18)		89 (91.75)	8 (8.25)	

n=sample size, NBW= normal birth weight, LBW= low birth weight, TB=term birth, PTB= pre-term birth, T1= trimester one, T3= trimester three, BMI= body mass index
NBW (>2500g), LBW (<2500g), TB (>37 weeks), PTB (<37 weeks), non-obese (BMI<30), obese (BMI>30)
Statistical significance: ***p<0.01, *p<0.05*

The distribution of BW for obesity and HIV are shown in Figure 1. In T1, obese patients gave birth to higher BW infants compared to non-obese (Fig.1A, p=0.0331) whilst HIV+ patients gave birth to smaller BW infants compared to HIV- (Fig.1A, p=0.0547). In HIV- patients this trend was also observed where obese patients gave birth to higher BW infants than non-obese,

however HIV+ obese patients gave birth to significantly smaller BW infants than HIV- obese patients (Fig.1A p=0.0039). A similar trend was also observed in T3 (Fig.1B, p=0.0177), where HIV+ obese patients gave birth to smaller BW infants compared to HIV- obese patients. The OGG1 SNP genotypes showed no significant change in BW among HIV-/+ and obesity, however CC-carrying HIV+ patients (2952.23±84.05) had lower BW than CC HIV- patients (3115.85±46.51, p=0.0911). This same trend was observed in obese HIV+ CC patients (T1: 2990.15±116.77; T3: 2967.08±112.11) compared to obese HIV- CC patients (T1: 3167±67.07, p=0.1939; T3: 3143.60±58.09, p=0.1663). The effect of OGG1 SNP, obesity and HIV on GA was also determined. No change was observed between obese patients, however in non-obese patients those that were HIV- CC-carrying (T1: 38.96±0.207; T3: 38.90±0.225) gave birth later than compared to HIV+ CC-carrying patients (T1:37.92±0.329, p=0.0095; T3: 37.75±0.357, p=0.0091).

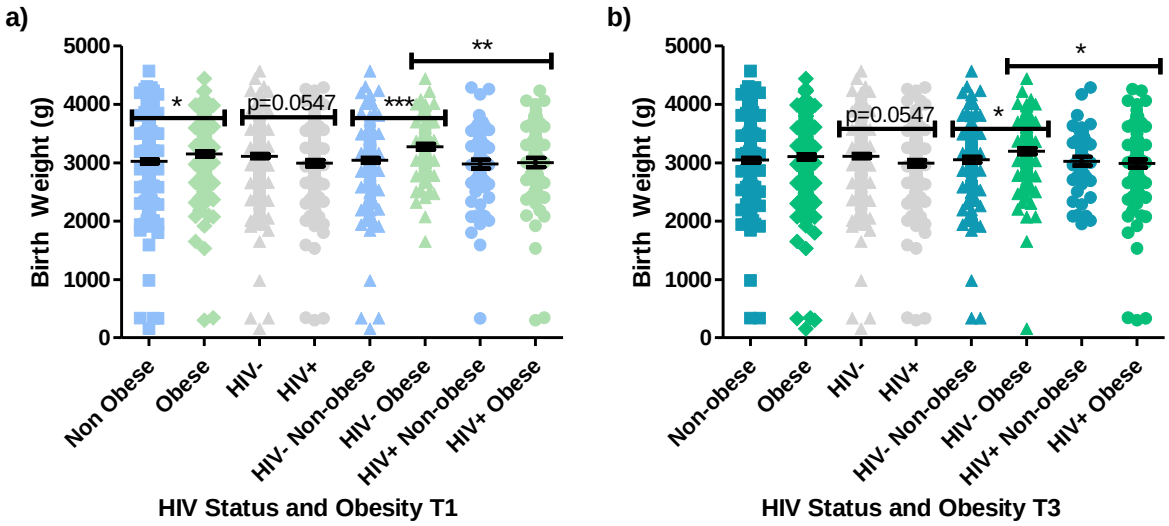

Figure 1 Birthweight disruption within obese (BMI>30) and non-obese (BMI<30) patients that are HIV+ and HIV- for trimester one (a) and three (b) Statistical significance: ***p<0.01, *p<0.05*

OGG1 polymorphism association with maternal clinical parameters

The association between the OGG1 CC and CG+GG genotypes and LBW/PTB among the maternal clinical characteristics are described in Table 5. No significant difference between the genotypes for clinical characteristics was observed in PTB. However, a significant increase in BMI for both T1 and T3 was observed in LBW for CC-carrying patients compared to CG+GG (T1:p=0.0104 and T3:p=0.0076, Table.5). This was also observed for iron, where the CC-carrying patients had significantly higher iron levels compared to CG+GG for both trimesters

for LBW (T1:p=0.0314, T3:p=0.0454, Table.5). A similar trend was observed for PTB, where iron in T3 was higher in CC-carrying patients compared to CG+GG (p=0.0992).

Table 5 Association of OGG1 Ser326Cys genotypes with maternal clinical parameters in LBW and PTB neonates

	(a) LBW (n=41)		(b) PTB (n=31)	
Variable	CC vs CG+GG	p-value	CC vs CG+GG	p-value
BMI T1	33.55 ± 2.71 vs 23.06 ± 1.33	***0.0104**	30.04 ± 2.10 vs 26.56 ± 2.25	0.3701
BMI T3	36.50 ± 2.66 vs 25.19 ± 2.34	****0.0076**	32.00 ± 1.82 vs 29.17 ± 2.57	0.4139
Hb T1	11.20 ± 0.246 vs 9.29 ± 1.41	0.0776	9.23 ± 0.992 vs 9.53 ± 1.47	0.8759
Hb T3	10.48 ± 0.469 vs 9.46 ± 1.14	0.3318	9.70 ± 0.796 vs 8.19 ± 1.88	0.3898
Iron T1	10.16 ± 0.599 vs 6.88 ± 1.70	***0.0314**	7.89 ± 1.13 vs 4.11 ± 2.08	0.1073
Iron T3	10.01 ± 0.536 vs 7.24 ± 1.54	***0.0454**	9.07 ± 0.918 vs 5.65 ± 2.21	0.0992
BP systolic T1	110.07 ± 2.44 vs 106 ± 3.40	0.3356	111.17 ± 2.51 vs 115.5 ± 3.91	0.3795
BP systolic T3	110.12 ± 2.13 vs 108 ± 2.99	0.5643	105.05 ± 2.37 vs 103 ± 5.68	0.6958
BP diastolic T1	66.63 ± 1.44 vs 67 ± 2.11	0.8836	67.57 ± 1.95 vs 70.38 ± 2.63	0.4490
BP diastolic T3	65.46 ± 1.86 vs 66.64 ± 1.68	0.6789	64.27 ± 1.69 vs 63.88 ± 2.73	0.9034

Results described: mean ± standard error of mean (n=sample size)
n=sample size, LBW= low birth weight, PTB= pre-term birth, BMI= body mass index, Hb= haemoglobin, BP= blood pressure, T1= trimester one, T3= trimester three
Statistical significance: *$p<0.01$, *$p<0.05$

A sub-analysis of BMI and iron for T1 and T3 are represented in Table 6. The data showed that the BMI for T1 and T3 was significantly greater in CC-carrying patients compared to CG+GG for all LBW and NBW (T1: p=0.0104 and p=0.0269, T3: p=0.0076 and p=0.0453, respectively). HIV infection was associated with a higher BMI (T1) in LBW compared to NBW (p=0.621: 35.64±2.71 and 34.21±1.20, respectively) whilst HIV- was significantly associated with lower BMI (T1) in LBW compared to NBW (p=0.030: 24.67±1.67 and 28.77±0.641, respectively) for all patients. This pattern was also observed between BMI T1/T3 and LBW for CC- and CG+GG-carrying patients, although not significant.

Iron is significantly greater in CC-carrying patients compared to CG+GG patients giving birth to LBW infants (Table.6: T1:p=0.0314 and T3:p=0.0454). HIV infection greatly influences this relationship where HIV+ CC-carrying patients have greater iron levels compared to HIV- CC patients whilst the opposite is observed within CG+GG-carrying patients. No association between iron for T1/T3 was observed for NBW and OGG1 genotypes, however it was observed that HIV infection increased iron levels significantly for both CC and CG+GG-carrying patients (p=0.0022 and p=0.0424, respectively).

Table 6 Sub-analysis of body mass index, iron and OGG1 Ser326Cys for all groups

Groups	CC vs CG+GG	p-value
BMI T1		
All LBW	33.55 ± 2.71 (27) vs 23.06 ± 1.33 (14)	***0.0104**
LBW HIV-	24.96 ± 2.18 (11) vs 22.30 ± 1.54 (10)	0.3401
LBW HIV+	39.46 ± 3.68 (16) vs 24.94 ± 2.70 (4)	**0.0728**
All NBW	30.67 ± 0.875 (157) vs 27.62 ± 0.855 (80)	***0.0269**
NBW HIV-	29.58 ± 0.917 (98) vs 26.27 ± 0.818 (62)	0.0824
NBW HIV+	34.13 ± 1.68 (59) vs 32.27 ± 2.27 (18)	0.5732
BMI T3		
All LBW	36.50 ± 2.66 (26) vs 25.19 ± 2.34 (14)	****0.0076**
LBW HIV-	30.07 ± 2.95 (11) vs 24.51 ± 3.14 (10)	0.2121
LBW HIV+	41.22 ± 3.67 (15) vs 26.89 ± 2.78 (4)	**0.0691**
All NBW	34.09 ± 0.972 (157) vs 31.01 ± 0.926 (78)	***0.0453**
NBW HIV-	31.82 ± 1.04 (98) vs 29.79 ± 0.929 (60)	0.1822
NBW HIV+	37.85 ± 1.83 (59) vs 35.10 ± 2.36 (18)	0.4443
Iron T1		
All LBW	10.16 ± 0.599 (27) vs 6.88 ± 1.70 (14)	***0.0314**
LBW HIV-	9.3 ± 1.24 (11) vs 8.13 ± 1.82 (10)	0.5957
LBW HIV+	10.74 ± 0.539 (16) vs 3.75 ± 3.75 (4)	****0.0029**
All NBW	7.61 ± 0.411 (157) vs 7.56 ± 0.600 (80)	0.9498
NBW HIV-	6.75 ± 0.540 (98) vs 6.71 ± 0.729 (62)	0.9621
NBW HIV+	9.04 ± 0.584 (59) vs 10.53 ± 0.445 (18)	0.1774
Iron T3		
All LBW	10.02 ± 0.536 (26) vs 7.24 ± 1.54 (14)	***0.0454**
LBW HIV-	9.4 ± 1.08 (11) vs 8.74 ± 1.52 (10)	0.7243
LBW HIV+	10.47 ± 0.489 (15) vs 3.5 ± 3.5 (4)	****0.0021**
All NBW	8.50 ± 0.378 (157) vs 8.33 ± 0.564 (78)	0.7963
NBW HIV-	7.62 ± 0.520 (98) vs 7.71 ± 0.700 (60)	0.9172
NBW HIV+	9.97 ± 0.458 (59) vs 10.41 ± 0.496 (18)	0.6195

Results described: mean ± standard error of mean (n=sample size)
NBW= normal birth weight, LBW= low birth weight, T=trimester
Statistical significance: **p<0.01, *p<0.05*

Multiple-variables logistic regression analysis

We further analysed the impact of maternal characteristics on PTB as shown in Table 7. The logistic regression was used to control for any potential confounding factors that may have influenced the relationships observed thus far. The results show that HIV increased the odds of

PTB although not significantly (p=0.244). Patients living in the heavily polluted DSIB or carrying the CC-genotype have significantly greater odds (13.06 and 15.66, respectively) for PTB. Maternal clinical characteristics had no impact on the risk of PTB. The impact of maternal characteristics on LBW showed no significant results (Appendix 4: Table A4.2).

Table 7 Impact of maternal clinical characteristics on the risk of pre-term birth – a multi-variate logistic regression analysis (n=128)

Variable	OR[a] (95%CI)	p-value
	Pre-term Birth	
HIV	2.73 (0.505, 14.74)	0.244
Maternal age	0.922 (0.771, 1.10)	0.372
BMI	1.00 (0.998, 1.00)	0.482
Hb	0.984 (0.953, 1.02)	0.324
Iron	1.00 (0.973, 1.03)	0.886
BP systolic	1.00 (0.999, 1.00)	0.170
BP diastolic	1.00 (0.998, 1.00)	0.491
OGG1 (CC genotype)	**15.66** (1.10, 222.32)	***0.042**
Area (South)	**13.06** (1.10, 155.17)	***0.042**
NOx levels	0.912 (0.809, 1.03)	0.132
	R^2 = 0.4248 (**p=0.0040)	

Results described: OR, (95%CI), p-value with regression model R^2-value and p-value
n= sample size, OR= odds ratio, CI= confidence interval, HIV= HIV positive, BMI= body mass index, Hb= haemoglobin, BP= blood pressure, OGG1 represents CC genotype, Area represents heavily polluted south
[a]Adjusted for maternal characteristics: HIV status, BMI, area, NOx levels, maternal age, haemoglobin, iron, blood pressure systolic and diastolic, OGG1 CC SNP and neonatal characteristics: low birth weight, neonate gender, neonate length, head circumference, parity, body: brain index, ponderal index, Apgar scores 1min and 5min
Statistical significance: ***p<0.01, *p<0.05*

Discussion

To the best of our knowledge, this is the first study to show the association between Ser326Cys OGG1 SNP with HIV infection and its influence on LBW and PTB, in a South African population. It was observed that co-morbid HIV infection and obesity resulted in decreased neonatal BW; with the wild-type OGG1 genotype increasing the risk for PTB and HIV associated LBW. Patients exposed to higher levels of AAP within the DSIB were also shown to have an increased risk for PTB.

In developed countries, a shift from HIV associated wasting to obesity has been observed. This shift is in response to HIV being a chronic condition with infected people living longer and

gaining weight, similar to the general population [10,14]. This study is in agreement to those done in developed countries, where being HIV+ significantly increases the odds for being obese (Table.2, T1:OR 2.185, T3: OR:2.898) [14,24,25]. Other studies done in SA have shown the opposite where HIV+ patients have been reported to have reduced BMI, even with ART treatment [11,12]. The discrepancy between this study and those previously done in SA, could be due to the increased use of ART therapy within HIV+ patients from 31.2% in 2012 to 47% in 2014 [26]. In addition to the socio-behavioural attitudes, where ethnic black obese women are perceived as attractive and is associated with affluence, wealth, respect and dignity. Excessive loss of weight is also associated with HIV; therefore being overtly overweight masks the disease from relatives and acquaintances [12,14].

Individual genetic susceptibility is highly important when considering interactions between the environment and health outcomes. These genetic differences influence the response individuals have to external and internal stresses. Both obesity and HIV infection have been associated with metabolic abnormalities and increased oxidative stress [14]. Increased BMI has been associated with the OGG1 Ser326Cys SNP within a Japanese population; however this association was removed when the population area was controlled [27]. Our study investigated and showed that obesity in T1 and HIV infection was associated with OGG1 Ser326Cys SNP (Table.3: p=0.0048 and p=0.0324, respectively). To our best knowledge, this is the first study to show an association between HIV infection and OGG1 Ser326Cys SNP.

Our study then investigated the effect HIV and obesity had on adverse birth outcomes. It was observed that HIV was suggested to increase the frequency of LBW (Table.4, p=0.071). In CC-carrying patients, this association strengthened and became significant. Carrying the CC-genotype therefore increased the susceptibility of HIV+ associated PTB and LBW (Table.4). It is well known that male neonates generally have larger BW for GA than female neonates [28] therefore it was interesting to note that carrying a female neonate and the CC genotype increased the susceptibility of HIV associated LBW (Appendix 4: Table A4.3, p=0.024); whilst carrying a male neonate increased the susceptibility of HIV associated PTB (Appendix 4: Table A4.3, p=0.024).

There was increased BMI observed in CC-carrying versus variant mothers, for both LBW and NBW neonates. However an increased BMI was observed for LBW delivering HIV+ CC-carrying mothers compared to NBW mothers, whilst the opposite is true for HIV- CC-carrying mothers. Therefore, HIV infection has been shown to increase the BMI of LBW delivering

mothers (Table 6, p=0.621) whilst being HIV- significantly reduced BMI in LBW delivering mothers (Table.6, p=0.03). This may suggest a differential effect of HIV and the OGG1 SNP on BMI levels within mother's delivering LBW neonates. This correlates well with the observed increased risk of HIV associated LBW, in patients that are obese (Table.4, T1:p=0.006 and T3:p=0.017). Although obesity has been shown previously to be associated with macrosomia (large BW) and reduced BMI associated with LBW [5,8], HIV infection has never been taken into consideration. It was observed that neonates born to obese versus non-obese mothers had significantly greater BW; this is in line with what is observed within literature [5,29]. However, obese mothers with HIV co-infection gave birth to neonates with significantly smaller BW than HIV- obese mothers (Fig.1, p=0.0039). It has been suggested that restriction of foetal growth is related to increased oxidative stress, coagulation and alterations in endothelial and vascular functions which compromises blood flow and oxygen transfer between the foetus and mother [4,13]. HIV and obesity both result in increased inflammation and oxidative stress, with obesity linked to endothelial dysfunction and atherosclerosis which could result in reduced blood flow to the foetus resulting in foetal growth restriction [5,30].

Although the OGG1 SNP had no direct impact on neonatal BW, it was observed that carrying the CC-genotype increased the susceptibility of PTB by 15.66 (Table.7, p=0.042). Previously, the variant was shown to have reduced BW compared to the wt, although not significant and the study sample size was small [31]. Studies have also reported conflicting reports that the variant has reduced repair capacity [22,32], whilst others show no difference in repair capacity [22,33,34]. The combined effect of HIV, obesity and pollution on the OGG1 SNP has not be considered, therefore in light of these interactions this study suggests that that co-morbid infection with HIV and OGG1 CC-genotype increases the risk for LBW and PTB.

The DSIB has been shown previously to have high levels of AAP that affect children's health [17,18,35]. This study shows that those living with the DSIB have a 13.06 fold increased risk of PTB (Table.7, p=0.042). The DSIB has been shown to have increased levels of nitrogen oxides (NOx), compared to the less polluted north Durban [36]. Nitrogen oxides are common by-products of vehicle combustion, cooking with gas and smoking [37]. These pollutants are free-radicals and as such upon inhalation result in increased oxidative stress, with high levels of NO being reported in PTB [38]. Several studies have reported AAP increases the risk of PTB [39–41]. Therefore patients exposed to higher levels of AAP within the DSIB, compared to the north Durban are at greater risk for delivering prematurely, which puts them at a greater risk for PTB-

associated morbidities, such as asthma, respiratory complications and neurodevelopmental impairments [42].

Limitations of our study include the relatively small sample size; a larger study population would increase the power of our results. Additionally, the effect antiretroviral drug use and ethnicity was not controlled for; this could be further investigated in future larger studies.

In conclusion this study identifies patient's carrying the OGG1 Ser326Cys CC genotype are at greater risk for HIV associated LBW, and delivering prematurely. Living within the DSIB and being exposed to higher levels of AAP increased the susceptibility of PTB. Co-morbid HIV infection and obesity are additional risk factors for BW reduction within this South African population. To better improve the prevalence of adverse birth outcomes, education on the effect of HIV and obesity on pregnancy should be improved to reduce pre-pregnancy HIV and obesity rates. This is of particular importance in SA, due to the high incidence of obesity and HIV. Education and identifying risk factors are important preventive measures which are essential for reducing adverse birth outcomes in all populations.

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References

[1] UNAIDS, UNAIDS Fact sheet November 2015: Global HIV Statistics, 2015. http://www.unaids.org/en/regionscountries/countries/southafrica/ (accessed January 16, 2017).

[2] STATS SA, South Africa Demographic and Health Survey 2016: Key Indicator Report, 2016. http://www.statssa.gov.za/publications/Report 03-00-09/Report 03-00-092016.pdf (accessed August 2, 2017).

[3] E.S. Machado, C.B. Hofer, T.T. Costa, S.A. Nogueira, R.H. Oliveira, T.F. Abreu, L.A. Evangelista, I.F.A. Farias, R.T.C. Mercadante, M. de F.L. Garcia, R.C. Neves, V.M. Costa, J.S. Lambert, Pregnancy Outcome in HIV-1-infected Women Receiving

Combination Antiretroviral Therapy Prior versus After Conception., Sex Transm Infect. 85 (2009) 82–87. doi:10.1136/sti.2008.032300.

[4] P.-L. Xiao, Y.-B. Zhou, Y. Chen, M.-X. Yang, X.-X. Song, Y. Shi, Q.-W. Jiang, Association between maternal HIV infection and low birth weight and prematurity: a meta-analysis of cohort studies, BMC Pregnancy Childbirth. 15 (2015) 1–11. doi:10.1186/s12884-015-0684-z.

[5] S.S. Huda, L.E. Brodie, N. Sattar, Obesity in pregnancy: prevalence and metabolic consequences, Semin. Fetal Neonatal Med. 15 (2010) 70–76. doi:10.1016/j.siny.2009.09.006.

[6] H. Kruger, Pregnancy outcomes of overweight and normal weight women in a South African outpatient clinic, J. Hum. Ecol. (2005) 67–75. http://www.krepublishers.com/06-Special Volume-Journal/JHE-00-Special Volumes/JHE-13-Hum-Obesity-Web/JHE-SI-13-09-065-075-Kruger-H-S/JHE-SI-13-09-065-075-Kruger-H-S-Text.pdf.

[7] G.C.S. Smith, I. Shah, J.P. Pell, J.A. Crossley, R. Dobbie, Maternal obesity in early pregnancy and risk of spontaneous and elective preterm deliveries: A retrospective cohort study, Am. J. Public Health. 97 (2007) 157–162. doi:10.2105/AJPH.2005.074294.

[8] H.S. Kruger, Maternal anthropometry and pregnancy outcomes: a proposal for the monitoring of pregnancy weight gain in outpatient clinics in South Africa., Curationis. 28 (2005) 40–49. doi:10.4102/curationis.v28i4.1012.

[9] P.J. Goldstein, R. Smit, M. Stevens, J.L. Sever, Association between HIV in pregnancy and antiretroviral therapy, including protease inhibitors and low birth weight infants., Infect. Dis. Obstet. Gynecol. 8 (2000) 94–98. doi:10.1155/S1064744900000089.

[10] N. Crum-Cianflone, R. Tejidor, S. Medina, A. Ganesan, Obesity among HIV Patients: The Latest Epidemic, AIDS Patient Care STDS. 22 (2009) 925–930. doi:10.1089/apc.2008.0082.Obesity.

[11] T. Bärnighausen, T. Welz, V. Hosegood, J. Bätzing-Feigenbaum, F. Tanser, K. Herbst, C. Hill, M.-L. Newell, Hiding in the shadows of the HIV epidemic: obesity and hypertension in a rural population with very high HIV prevalence in South Africa, J. Hum. Hypertens. 22 (2007) 236–239. doi:10.1038/sj.jhh.1002308.

[12] A. Malaza, J. Mossong, T. Bärnighausen, M.-L. Newell, Hypertension and obesity in adults living in a high HIV prevalence rural area in South Africa., PLoS One. 7 (2012) e47761. doi:10.1371/journal.pone.0047761.

[13] N.L. Fleischer, M. Merialdi, A. Van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A. van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A.P. Betran, J.P. Souza, M.S. O'Neill, A. Van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A. van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A.P. Betran, J.P. Souza, M.S. O'Neill, Outdoor Air Pollution , Preterm Birth , and Low Birth Weight : Analysis of the World Health Organization Global Survey on Maternal and Perinatal Health, Environ. Health Perspect. 122 (2014) 425–430. doi:10.1289/ehp.1306837.

[14] V. Amorosa, M. Synnestvedt, R. Gross, H. Friedman, R.R. MacGregor, D. Gudonis, I. Frank, P. Tebas, A tale of 2 epidemics: the intersection between obesity and HIV infection in Philadelphia, J Acquir Immune Defic Syndr. 39 (2005) 557–561. http://www.ncbi.nlm.nih.gov/pubmed/16044007.

[15] E.H. Ha, Y.C. Hong, B.E. Lee, B.H. Woo, J. Schwartz, D.C. Christiani, Is air pollution a risk factor for low birth weight in Seoul?, Epidemiology. 12 (2001) 643–648. doi:10.1097/00001648-200111000-00011.

[16] I. Kloog, S.J. Melly, W.L. Ridgway, B. a Coull, J. Schwartz, Using new satellite based exposure methods to study the association between pregnancy pm2.5 exposure, premature birth and birth weight in Massachusetts, Environ. Heal. 11 (2012) 40. doi:10.1186/1476-069X-11-40.

[17] R.N. Naidoo, T. Robins, S. Batterman, G. Mentz, C. Jack, Ambient pollution and respiratory outcomes among schoolchildren in Durban, South Africa, South African J. Child Heal. 7 (2013) 127–134. doi:10.7196/sajch.598.

[18] J. Nriagu, T. Robins, L. Gary, G. Liggans, R. Davila, K. Supuwood, C. Harvey, C.C. Jinabhai, R. Naidoo, Prevalence of asthma and respiratory symptoms in south-central Durban, South Africa., Eur. J. Epidemiol. 15 (1999) 747–755. doi:10.1023/A:1007653709188.

[19] A. Bravard, M. Vacher, B. Gouget, A. Coutant, F.H. de Boisferon, S. Marsin, S. Chevillard, J.P. Radicella, Redox regulation of human OGG1 activity in response to cellular oxidative stress., Mol. Cell. Biol. 26 (2006) 7430–6. doi:10.1128/MCB.00624-06.

[20] A. Kumar, M.C. Pant, H.S. Singh, S. Khandelwal, Role of OGG1 Ser326Cys polymorphism and 8-oxoguanine DNA damage in risk assessment of squamous cell carcinoma of head and neck in north Indian population, Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 726 (2011) 227–233. doi:10.1016/j.mrgentox.2011.09.015.

[21] M. Jaiswal, N.F. LaRusso, N. Nishioka, Y. Nakabeppu, G.J. Gores, Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide, Cancer Res. 61 (2001) 6388–6393.

[22] A. Bravard, M. Vacher, E. Moritz, L. Vaslin, J. Hall, B. Epe, J.P. Radicella, Oxidation status of human ogg1-s326c polymorphic variant determines cellular DNA repair capacity, Cancer Res. 69 (2009) 3642–3649. doi:10.1158/0008-5472.CAN-08-3943.

[23] E. Moritz, K. Pauly, A. Bravard, J. Hall, J. Pablo Radicella, B. Epe, hOGG1-Cys326 variant cells are hypersensitive to DNA repair inhibition by nitric oxide, Carcinogenesis. 35 (2014) 1426–1433. doi:10.1093/carcin/bgu066.

[24] B.S. Taylor, Y. Liang, D. Ph, L.S. Garduño, E.A. Walter, M. Gerardi, D. Ph, G.M. Anstead, D. Ph, D. Bullock, B.J. Turner, M.S. Ed, High Risk of Obesity and Weight Gain for HIV-Infected Uninsured Minorities, J Acquir Immune Defic Syndr. 65 (2014) e33–e40. doi:10.1097/QAI.0000000000000010.High.

[25] T. Tate, A.L. Willig, J.H. Willig, J.L. Raper, L. Moneyham, M.C. Kempf, M.S. Saag, M.J. Mugavero, HIV infection and obesity: Where did all the wasting go?, Antivir. Ther. 17 (2012) 1281–1289. doi:10.3851/IMP2348.

[26] S.A.N.A.C. SANAC, South Africa: Global AIDS Response Progress Report, 2015. http://sanac.org.za/wp-content/uploads/2016/06/GARPR_report-high-res-for-print-June-15-2016.pdf (accessed August 7, 2017).

[27] M. Hara, K. Nakamura, H. Nanri, Y. Nishida, A. Hishida, S. Kawai, N. Hamajima, Y. Kita, S. Suzuki, E.M. Mantjoro, K. Ohnaka, H. Uemura, D. Matsui, I. Oze, H. Mikami, M. Kubo, H. Tanaka, Japan Multi-Institutional Collaborative Cohort (J-MICC) Study Group, Associations between hOGG1 Ser326Cys polymorphism and increased body mass index and fasting glucose level in the Japanese general population., J. Epidemiol. 24 (2014) 379–84. doi:http://dx.doi.org/10.2188/jea.JE20140002.

[28] M.S. Kramer, R.W. Platt, S.W. Wen, K.S. Joseph, A. Allen, A New and Improved Population-Based Birth Weight for Gestational Age, Pediatrics. 108 (2001) e35. doi:10.1542/peds.108.2.e35.

[29] Z. Yu, S. Han, J. Zhu, X. Sun, C. Ji, X. Guo, Pre-Pregnancy Body Mass Index in Relation to Infant Birth Weight and Offspring Overweight/Obesity: A Systematic Review and Meta-Analysis, PLoS One. 8 (2013) e61627. doi:10.1371/journal.pone.0061627.

[30] M.I. Bukrinsky, H.S. Nottet, H. Schmidtmayerova, L. Dubrovsky, C.R. Flanagan, M.E. Mullins, S.A. Lipton, H.E. Gendelman, Regulation of nitric oxide synthase activity in

human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease., J. Exp. Med. 181 (1995) 735–45. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2191885&tool=pmcentrez&rendertype=abstract.

[31] B. Karahalil, E. Emerce, N.A. Kocabaş, E. Akkas, Associations between GSTM1 and OGG1 Ser326Cys polymorphisms and smoking on chromosomal damage and birth growth in mothers, Mol. Biol. Rep. 38 (2011) 2911–2918. doi:10.1007/s11033-010-9953-0.

[32] P. Aka, R. Mateuca, J.P. Buchet, H. Thierens, M. Kirsch-Volders, Are genetic polymorphisms in OGG1, XRCC1 and XRCC3 genes predictive for the DNA strand break repair phenotype and genotoxicity in workers exposed to low dose ionising radiations?, Mutat. Res. - Fundam. Mol. Mech. Mutagen. 556 (2004) 169–181. doi:10.1016/j.mrfmmm.2004.08.002.

[33] V. Simonelli, S. Camerini, F. Mazzei, B. Van Loon, A. Allione, M. D'Errico, F. Barone, A. Minoprio, F. Ricceri, S. Guarrera, A. Russo, B. Dalhus, M. Crescenzi, U. Hübscher, M. Bjørås, G. Matullo, E. Dogliotti, Genotype-phenotype analysis of S326C OGG1 polymorphism: a risk factor for oxidative pathologies., Free Radic. Biol. Med. 63 (2013) 401–9. doi:10.1016/j.freeradbiomed.2013.05.031.

[34] S. Lagadu, M. Lechevrel, F. Sichel, J. Breton, D. Pottier, R. Couderc, F. Moussa, V. Prevost, 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker of oxidative damage in oesophageal cancer patients: lack of association with antioxidant vitamins and polymorphism of hOGG1 and GST., J. Exp. Clin. Cancer Res. 29 (2010) 157. doi:10.1186/1756-9966-29-157.

[35] E.J. Kistnasamy, T.G. Robins, R. Naidoo, S. Batterman, G.B. Mentz, C. Jack, E. Irusen, The relationship between asthma and ambient air pollutants among primary school students in Durban, South Africa, Int. J. Environ. Heal. 2 (2008) 365. doi:10.1504/IJENVH.2008.020929.

[36] S.M. Anderson, R.N. Naidoo, P. Ramkaran, A. Phulukdaree, S. Muttoo, K. Asharam, A.A. Chuturgoon, The Effect of Nitric Oxide Pollution on Oxidative Stress in Pregnant Women Living in Durban, South Africa, Arch. Environ. Contam. Toxicol. (2017). doi:10.1007/s00244-017-0465-y.

[37] F.J. Kelly, Oxidative stress: its role in air pollution and adverse health effects., Occup. Environ. Med. 60 (2003) 612–616. doi:10.1136/oem.60.8.612.

[38] S. Chadha, V. Jain, I. Gupta, M. Khullar, Nitric oxide metabolite levels in preterm labor., J. Obstet. Gynaecol. Res. 33 (2007) 710–7. doi:10.1111/j.1447-0756.2007.00639.x.

[39] C. Backes, T. Nelin, M. Gorr, L. Wold, Early life exposure to air pollution: how bad is it?, Toxicol. Lett. 216 (2013) 47–53. doi:http://dx.doi.org/10.1016/j.toxlet.2012.11.007.

[40] P.S. Shah, T. Balkhair, Air pollution and birth outcomes: A systematic review, Environ. Int. 37 (2011) 498–516. doi:10.1016/j.envint.2010.10.009.

[41] M. Bobak, Outdoor air pollution, low birth weight, and prematurity., Environ. Health Perspect. 108 (2000) 173–176. doi:10.2307/3454517.

[42] R.L. Goldenberg, J.F. Culhane, J.D. Iams, R. Romero, Epidemiology and causes of preterm birth, Lancet. 371 (2008) 75–84.

CHAPTER 4

HIV induces nitric oxide and lipid peroxidation, which influences neonatal birthweight in a South African population

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Abstract

HIV has been implicated in adverse birth outcomes, due to increased oxidative stress and inflammation. In addition, HIV has been reported to increase nitric oxide levels. Therefore the combined exposures to HIV and traffic-related air pollution, within south Durban, South Africa (SA), may lead to adverse birth outcomes. However, the exact mechanism of action is still unknown, therefore this study aimed to identify a potential mechanism. HIV's influence on oxidative and nitrosative stress markers within pregnant women was first assessed. Second, the effect of these stress makers and oxides of nitrogen (NOx) exposure have on neonatal birthweight (BW) was evaluated. Finally, the effect HIV and traffic-related pollution exposure has on the oxidative and endoplasmic profile and epigenetic regulation of Nrf2-Keap1 pathway by miR-144 and miR-28 in pregnant women was determined. Women, in their third trimester with singleton pregnancies, who were HIV+ and HIV-, were recruited from Durban, SA. Biomarker levels of serum nitrites/nitrates (NO) and malondialdehyde (MDA) were analysed and mRNA expression levels of oxidative and endoplasmic stress response genes were assessed. Land regression modelling was performed to determine NOx exposure levels. HIV exposure during pregnancy was associated with increased NO levels. NO was shown to reduce neonatal BW. NO and MDA was found to reciprocally increase each other, with HIV differentially influencing MDA effect on BW. HIV downregulated miR-144 which was negatively associated with *Nrf2*, suggesting a potential mechanism for HIV associated chronic oxidative stress. This study proposes that NO plays a key role in neonatal BW reduction in response to HIV and traffic-related air pollution.

Keywords

HIV, oxidative stress, NO, MDA, adverse birth outcomes, traffic-pollution, Nrf2-Keap1 pathway, ER stress, epigenetics

Highlights

- HIV exposure increased serum NO, with reciprocal action on MDA levels
- HIV induced miR-144 suggested as a mechanism for increased oxidative stress
- Exposure to heavily polluted south Durban activated the ER stress
- Maternal serum NO reduced neonatal birthweight, in heavily polluted Durban south

Introduction

An estimated 36.7 million people in the world are living with the human immunodeficiency virus (HIV). Of these, 7 million are living in South Africa (SA) and women account for more than half of the total HIV infected population in SA [1]. This is of major concern, not only due to the associated morbidity and mortality, but due to increased risk of adverse birth outcomes associated with HIV infection [2].

HIV infection in pregnant women has been reported to increase the susceptibility of neonates to shunting, wasting and reduced weight with increased risk of low birth weight (LBW) and preterm births (PTB) [2]. Factors that affect LBW and PTB pathology include oxidative stress, inflammation, endothelial dysfunction, reduced oxygen transport across the placenta and abnormalities of the placenta [3]. HIV is a lentivirus that infects and kills vital cells of the immune system [4]. Upon HIV infection, it triggers the innate immune response activating macrophages to produce nitric oxide (NO), which is a major mediator of inflammation and apoptosis [5]. Studies have shown increased levels of NO and inflammatory markers in HIV infected patients [5,6]. HIV has also been associated with increased oxidative stress, where decreased antioxidant capacity and increased oxidative damage have been reported [4,7]. Therefore HIV could illicit LBW and PTB as a result of increased inflammation and oxidative stress, with NO potentially playing a key role.

Nitric oxide is an inorganic free radical that is produced endogenously by a family of isoenzymes as a reaction by-product, during the catalytic conversion of L-arginine to L-citrulline [8]. Three isoenzymes of NO synthases (NOS) have been identified; neural (nNOS), endothelial (eNOS) and inducible (iNOS). NO readily diffuses across cell membranes; at low concentrations it binds to haemoglobin (Hb) and becomes inactive, whilst in excess NO reacts with superoxide and oxygen to form peroxynitrite and dinitrogen trioxide, respectively. NO and its intermediates are highly reactive and result in macromolecular damage; including modifying cysteine amino acids of target proteins, triggering lipid peroxidation (LP), inhibiting the electron transport chain and oxidising biological thiol-containing compounds. Both NO and peroxynitrite are highly unstable and degrade to nitrates and nitrites, which act as markers of NO concentration [9,10]. Increased levels of NO have been associated with PTB [11].

Lipid peroxidation is the oxidative conversion of polyunsaturated fatty acids (PUFA) to products known as malondialdehyde (MDA) or lipid peroxides, which act as biomarkers. The decomposition of NO and peroxynitrite to form hydroxyl radicals and nitrite radicals initiate LP

chain reactions, where hydroxyl radicals and nitrite abstract hydrogen ions from the methylene group of PUFA resulting in an unpaired electron on carbon that reacts with oxygen present to form peroxyl radicals. This sets up the chain reaction of LP, which can continue to cause macromolecular damage or is terminated by antioxidants [12]. Several studies have associated increased LP with adverse birth outcomes, especially PTB [13,14].

Alternatively, exogenous sources of NO include vehicle exhaust fumes and cigarette smoke [15,16]. Durban, in SA, is a rapidly developing city with increased urbanisation and road traffic, all factors associated with an increase in oxides of nitrogen (NOx) pollution within the atmosphere. This in combination with residential areas in close proximity to busy highways and roads makes individuals highly susceptible to adverse effects of NOx exposure, with infants' *in utero* being highly vulnerable to the negative effects. It has been reported previously that exposure to NOx and other air pollution (AP) have been associated with increased risk of LBW and PTB [13,17–20]. Durban, consisting of a highly industrialised south region (SD) and a less industrialised north region (ND), is an ideal location to investigate the effects of traffic-related AP on birth outcomes.

Endogenous and exogenous toxic insult, viz. exposure to HIV or traffic-related AP, results in macromolecular and cellular damage leading to increased stress responses. Cytoprotection against these insults is provided by antioxidant enzymes such as catalase (CAT), superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx) [21] and repair enzymes including the human 8-oxoguanine glycosylase 1 (OGG1) gene which is a key component within the DNA repair pathway. OGG1 is highly susceptible to oxidative modification and has been shown to be inhibited by NO [22,23]. These responses are regulated via the antioxidant response element (ARE) where nuclear factor (erythroid-derived 2)-like 2 (Nrf2) acts as the key transcription factor. Upon increased oxidative damage and subsequent dissociation from kelch-like ECH-associated protein 1 (Keap1), Nrf2 translocates to the nucleus where it binds to the ARE resulting in transcriptional activation of specific target genes, including antioxidants [24]. Epigenetic regulation of Nrf2-Keap1 pathway by microRNA (miR)-144 and miR-28 has been found to be an important determinate of an individual's response to certain adverse conditions [25,26]. The endoplasmic reticulum (ER) stress response is also activated when there is increased oxidative insult resulting in increased unfolded proteins (UP). Increased UP and ER stress is sensed by ER chaperones, including BiP, which activates the UP response pathway (UPR) to mitigate ER stress [27].

A pilot study conducted by Nagiah and colleagues reported increased markers of oxidative stress within pregnant women living in the heavily industrialised south [28]. However this study did not investigate the effects HIV may have on these stress profiles, due to their small sample size, nor the subsequent effect on adverse birth outcomes. As a follow on study we aimed to determine whether HIV infection influenced oxidative and nitrosative stress markers within pregnant women, with consideration taken for the NOx exposure and living area of each women. The effect of the stress markers and NOx exposure on neonatal birth weight (BW) was then investigated. To further understand the oxidative profile of HIV and locational effect on these mothers, gene expression for oxidative and ER stress markers, including epigenetic regulation of the Nrf2-Keap1 pathway by miR-144 and miR-28 were also investigated.

Methodology

Study population

The Mother and Child in the Environment (MACE) longitudinal cohort study recruited HIV negative (HIV-) (n=230) and positive (HIV+) (n=126) pregnant women from public sector anti-natal clinics in the heavily-polluted Durban south region and less-industrialised Durban north region KwaZulu-Natal, SA). Ethical approval from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF263/12) (Appendix 1) and informed consent from study participants was obtained. The women participants were residents of the geographical area for the full duration of the pregnancy. Women with hypertension, multiple pregnancies, diabetes, placenta previa, genital tract infections and other complications which result in adverse growth effects were excluded from the study.

NOx pollution

The exposure levels of atmospheric NOx for individual study participants was determined using land use regression modelling, developed following the ESCAPE approach [29]. NOx measurements were collected using Ogawa samplers over two-week periods during mid-summer and mid-winter. Measurements were taken at 40 randomly selected sites in the north and south Durban areas. The regression-derived parameter estimates were used to determine pollutant levels at un-monitored locations such as participant homes [30].

Sample collection and preparation

Third trimester blood was collected in BD Vacutainer® EDTA and SST tubes. The SST tubes were centrifuged at 1000xg for 10min to isolate serum and stored at -80°C until NO and MDA

quantification. Equal parts whole blood and RNALater® (ThermoFischer Scientific; AM7021) was stored at -80°C for the assessment of various gene expressions.

Nitrates and Nitrites Assay

NO has a short half-life, approximately 6-10s, and thus degrades rapidly to nitrites and nitrates [11]. The detection of these NO metabolites was used to determine the NO concentration within each individual. This was performed using vanadium (III) chloride as the reducing agent and an acidic Griess reaction for detection. Non-haemolysed serum samples (n=301) were chosen, at random, from the study population recruited above and diluted (1:4) in deionised water (dH_2O). Sodium nitrate (200µM) was serial diluted to make up standards (0-200µM). 50µl of samples and standards (in duplicate) were pipetted into a 96-well microtitre plate. Into each well, 50µl VCl_3 was added. Rapidly 25µl SULF followed by 50µl NEDD was added into each well. The plate was incubated for 45min at 37°C. The absorbance was measured at 540nm using the BioTek® µQuant™ Microplate Spectrophotometer (BioTek® Instruments, Inc.). A standard curve was constructed and the linear equation used to extrapolate the concentration of the unknown samples (Appendix 5: Fig.A5.1). The final concentration (µM) of NO was multiplied by the dilution factor (4).

Lipid peroxidation

The amount of serum lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay, which quantified MDA as described by Halliwell and Gutteridge [31]. Non-haemolysed serum samples (n=299) were diluted (1:2) in phosphate-buffered saline (PBS). Then, 100µl 2% H_3PO_4, 100µl 7% H_3PO_4 and 200µl thiobarbituric acid (1%,w/v)/0.1 mM butylated hydroxytoluene solution (TBA/BHT) was added to the 1.5ml tube containing 100µl diluted serum. A positive and negative control were prepared, 0.5µl 1% MDA (200µl TBA/BHT) and 200µl 3mM HCl, respectively. Samples were vortexed, 100µl 1M HCl was added to ensure optimal pH. The samples were boiled for 45min (100°C) and allowed to cool to room temperature (RT). Butanol (750µl) was added to each tube, vortexed and allowed to separate for 20min at RT into two phases. The butanol phase was transferred to a 96-well plate (100µl), following aspiration and centrifugation at 18,506xg for 6min (RT). The absorbance was measured at wavelength 532nm, with a reference wavelength of 600nm, using the BioTek® µQuant™ Microplate Spectrophotometer (BioTek® Instruments, Inc.). The MDA concentration was calculated by dividing the mean absorbance of the samples by the absorption coefficient $156mM^{-1}$.

RNA isolation

Complementary DNA (cDNA) was synthesised from total RNA and used in Real-Time (RT) PCR for analysis of gene expression. Total RNA was isolated from whole blood stored in equal parts RNALater® using an in house protocol. Briefly, 500µl of whole blood was mixed with red blood cell lysis buffer (2,500µl), incubated on ice for 15min and centrifuged (400xg, 10min, 4°C). The supernatant was discarded, 1000µl lysis buffer was added, vortexed and centrifuged (400xg, 10min, 4°C). Thereafter, the supernatant was removed and discarded. QIAzol lysis reagent (Qiagen #79306) (500µl) was added and incubated overnight at -80°C. Following incubation, chloroform (100µl) was added, and centrifuged (12,000xg, 15min, 4°C). The aqueous phase was transferred to a fresh 1.5ml tube, cold isopropanol (250µl) added and stored overnight at -80°C. Following incubation, samples were centrifuged (12,000xg, 20min, 4°C) and the supernatant was removed. The pellet was washed with cold 75% ethanol and finally centrifuged (7,400xg, 15min, 4°C). The ethanol was aspirated and the pellet was allowed to air-dry. The pellet was resuspended in 15µl nuclease free water and quantified using the Nanodrop 2000 spectrophotometer. The RNA were subsequently standardised to a concentration of 300ng/µl.

cDNA synthesis and mRNA expression

Isolated RNA was pooled into groups (25 participants per group) according to their geographical area (ND and SD) and HIV status. The pooled RNA was converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad #170-8891). A total reaction volume of 20µl (4µl 5x iScript reaction mix, 1µl iScript Reverse transcriptase, 1000ng of RNA template and nuclease free water) was incubated under the following conditions: 25°C for 5min, 42°C for 30min, 85°C for 5min and a final hold at 4°C.

Antioxidant gene expression, ER and oxidative stress markers were investigated using RT-PCR. The mRNA expression levels of the relevant genes (Table 1) were measured using the commercially available iQ™ SYBR® Green Supermix kit (Bio-Rad #170-8882). Briefly, a 10µl reaction volume consisting of 25µM of each sense and antisense primer (Inqaba Biotech, SA - Table 1), 1µl cDNA template, 5µl IQ™ SYBR® Green supermix and nuclease free water) was used. Following initial denaturation (95°C, 8min), amplification was carried out over 37cycles of denaturation (95°C, 15sec), annealing (Table 1, 40sec) and extension (72°C, 30sec). A housekeeping gene (ribosomal 18S, Table 1) was amplified under the same conditions. The RT-PCR was carried out using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

The fold change ($2^{-\Delta\Delta Ct}$) was calculated using the method described by Livak and Schmittgen [32].

Table 1 RT-PCR Primer sequences and annealing temperatures

Gene		Sequence	Annealing Temperature (°C)
Antioxidant Genes			
SOD2	Sense	5' GAGATGTTACACGCCCAGATAGC 3'	57
	Antisense	5' AATCCCCAGCAGTGGAATAAGG 3'	
CAT	Sense	5' TAAGACTGACCAGGGCATC 3'	58
	Antisense	5' CAACCTTGGTGAGATCGAA 3'	
GPx	Sense	5' GACTACACCCAGATGAACGAGC 3'	58
	Antisense	5' CCCACCAGGAACTTCTCAAAG 3'	
Oxidative stress markers			
Nrf2	Sense	5' AGTGGATCTGCCAACTACTC 3'	58
	Antisense	5' CATCTACAAACGGGAATGTCTG 3'	
Keap1	Sense	5' CCTTCAGCTACACCCTGGAG 3'	57
	Antisense	5' AACATGGCCTTGAAGACAGG 3'	
OGG1	Sense	5' GCATCGTACTCTAGCCTCCAC 3'	60
	Antisense	5' AGGACTTTGCTCCCTCCAC 3'	
ER Stress Markers			
BiP	Sense	5' CGGGCAAAGATGTCAGGAAAG 3'	55
	Antisense	5' TTCTGGACGGGCTTCATAGTAGAC 3'	
eIF2	Sense	5' CCTCACCATTTGCCTAAGGA 3'	57
	Antisense	5' GGGGGACTTTCCTTCTTCTG 3'	
ATF4	Sense	5' GTTCTCCAGCGACAAGGCTA 3'	65
	Antisense	5' ATCCTCCTTGCTGTTGTTGG 3'	
CHOP	Sense	5' ACCAAGGGAGAACCAGGAAACG 3'	55
	Antisense	5' TCACCATTCGGTCAATCAGAGC 3'	
Housekeeping			
18S	Sense	5' CAAATCGCTCCACCAACTAA 3'	
	Antisense	5' ACACGGACAGGATTGACAGA 3'	

miScript cDNA synthesis and microRNA expression

RNA grouped according to their geographical area (ND and SD) and HIV status, was converted to miScript cDNA using the miScript II RT kit as per manufacturer instructions (Qiagen #218160). Briefly, a 20µl total reaction volume (4µl 5x miScript HiFlex, 2µl 10x miScript nucleics mix, 2µl miScript reverse transcriptase mix, 1000ng of RNA template and nuclease free water) was incubated for 60min at 37°C followed by 5min at 95°C. The miScript cDNA was then diluted (1:3).

The expression of miR-144 and miR-28 were investigated using RT-PCR. The miRNA expression of miR-144 (Qiagen #218300, MS00020328) and miR-28 (Qiagen #218300, MS00003255) were determined using commercially available primer assays and the miScript SYBR Green PCR Kit (Qiagen #218073). The RT-PCR control RNU6-2 (Qiagen #218300, MS00033740) was used alongside the miRNA of interest to ensure normalcy. The reaction was carried out according to the manufacturer's instructions. Briefly a total reaction volume of 12.5µl (6.25µl 2x Quantitect SYBR green master mix, 1.25µl Universal primer, 10x primer assay (miR-144/miR-28/RNU6-2), 1µl cDNA and nuclease free water) underwent initial denaturation at 95°C for 15min, followed by 40 cycles of denaturation (94°C, 15s), annealing (55°C, 30s) and extension (70°C, 30s). The RT-PCR was carried out using the CFX96 Touch™ Real-Time PCR Detection System (BioRad). The fold change ($2^{-\Delta\Delta Ct}$) was calculated using the method described by Livak and Schmittgen [32].

Statistical Analysis

Statistical Analyses were preformed using GraphPad Prism V5 Software Package (GraphPad Software Inc., San Diego, California, USA). NO, NOx and MDA data was log transformed to ensure normalcy, and allow graphical representation of the data. Measurements taken during the mothers' third trimester was used for body mass index (BMI), iron, Hb, systolic and diastolic blood pressure (BP) data. The Student t test was used to determine statistical significance between the HIV- and HIV+ groups for maternal and neonatal characteristics, atmospheric NOx levels and maternal NO and MDA concentrations. Correlations among atmospheric NOx, maternal serum NO and MDA and neonatal BW were done using the non-parametric Spearman correlation. A one way ANOVA was performed to determine the level of difference for the oxidative and ER stress markers and miRNA analysed in RT-PCR. Multivariate linear regression was used to determine whether the relationship between maternal serum NO and MDA and atmospheric NOx was affected by potential confounders, namely: maternal age, maternal BMI, HIV status, area, maternal systolic and diastolic blood pressure and Hb and iron levels. The relationship between neonatal BW and atmospheric NOx and maternal serum NO and MDA was also determined using linear regression, and potential confounders were controlled. The linear regression analyses were performed using STATA version 13.1. Statistical significance was set at $p < 0.05$.

Results

Maternal and Neonatal Characteristics

Maternal and neonatal characteristics of study participants are described in Table 2. The maternal age is greater in HIV+ compared to HIV- (*p<0.0001*) patients. HIV+ mothers are shorter (*p<0.0001*) and have larger BMIs (*p<0.0001*) compared to HIV- mothers (Table 2). The level of Hb and iron in HIV+ are greater than HIV- mothers (p=0.0186 and p<0.0001, respectively). BP measurements showed no difference (Table 2). The mean gestational age (GA) is shorter (*p=0.0344*), and BBR greater (0.0175) for HIV+ mothers compared to HIV- mothers. No difference was observed for PI, 1min and 5min Apgar scores (Table 2).

Table 2 Maternal and neonatal characteristics

	HIV-		**HIV+**		
	n	**Mean (SD)**	**n**	**Mean (SD)**	***p*-value**
Maternal Characteristics					
Maternal Age (years)	230	24.48 (5.39)	126	28.21 (5.67)	***<0.0001**
Maternal height (cm)	230	156.0 (11.65)	125	148.5 (18.56)	***<0.0001**
Maternal BMI	219	30.53 (9.58)	120	37.23 (13.94)	***<0.0001**
Haemoglobin (g/dL)	219	9.40 (4.49)	120	10.36 (3.01)	***0.0186**
Iron	219	7.51 (5.25)	120	9.69 (3.82)	***<0.0001**
BP systolic (mmHg)	219	108.8 (12.42)	120	109.0 (13.68)	0.9002
BP diastolic (mmHg)	219	67.34 (9.29)	120	67.28 (7.83)	0.9543
Neonatal Characteristics					
Gestational age (weeks)	207	38.86 (1.84)	113	38.44 (2.03)	***0.0448**
Birthweight (g)	208	3107 (625.9)	114	2974 (668.7)	0.0874
Body: brain ratio (BBR)	208	12.49 (10.94)	114	13.41 (12.43)	***0.0175**
Ponderal Index (PI)	208	2.59 (0.845)	114	2.54 (0.999)	0.0965
Apgar score (1min)	208	8.26 (0.901)	114	8.184 (1.28)	0.6703
Apgar score (5min)	208	9.29 (0.685)	114	9.254 (1.189)	0.3466

n= sample size, SD= standard deviation, g= grams, BMI= body mass index
Statistical significance: ****p<0.0001*, **p<0.05*.

HIV and location effects on maternal serum NO and MDA levels

The level of atmospheric NOx is significantly greater in the south (37.35±0.66µM) compared to the north (33.54 ± 0.80µM, *p=0.0007*) (Fig.1A). Maternal serum NO concentration is significantly higher in the north (67.75 ± 4.45µM) compared to the south (56.18 ± 2.69µM, *p=0.0111*) (Fig.1B). However, no correlation was observed between total atmospheric NOx and

maternal serum NO levels (Spearman r=-0.06334, 95%CI -0.2076 to 0.08358, *p=0.3840*, including correlations between patients living in the north (Spearman r=0.02235, 95%CI -0.1979 to 0.2404, *p=0.8391*) and south (Spearman r=-0.08259, 95%CI -0.2744 to 0.1156, *p=0.4000*). The maternal serum MDA concentrations did not differ between the north and south. A significant positive correlation was observed between maternal serum NO and MDA concentration as well as for patients living in the north and south (Total: Spearman r=0.2339, 95%CI 0.1202 to 0.3415, *p<0.0001*; North: Spearman r=0.2502, 95%CI 0.06309 to 0.4203, *p=0.0075*; South: Spearman r=0.2113, 95%CI 0.063136 to 0.3503, *p=0.0043*).

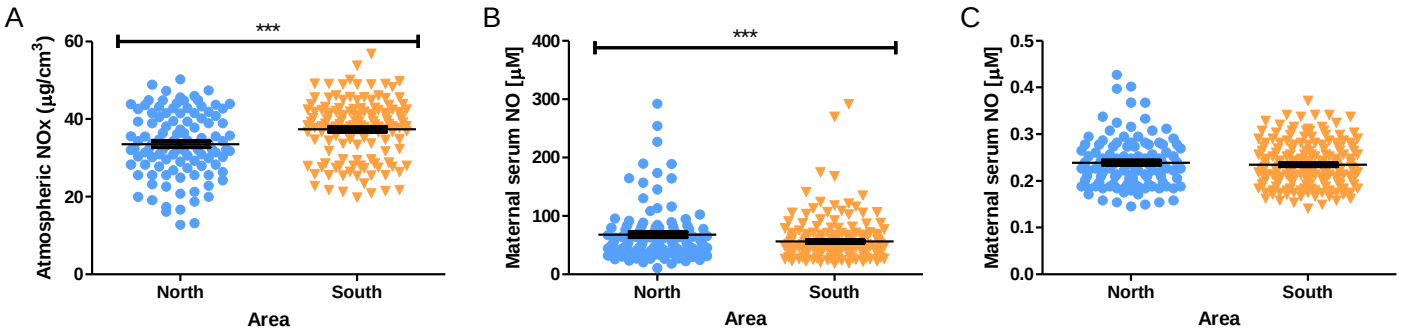

Figure 1 The concentrations of atmospheric NOx (µg/cm^3) (A), maternal serum NO (µM) (B) and maternal serum MDA (µM) (C) for patients living in the North ([A] n=109, [B] n=114, [C] n=113) and South ([A] n=135, [B] n=183, [C] n=181). Statistical significance: ****p<0.0001*, **p<0.05*.

Atmospheric NOx showed no difference between HIV+ (36.64 ± 0.80µg/cm^3) and HIV- (35.07 ± 0.69µg/cm^3, *p=0.1911*) patients (Fig.2A), whilst maternal serum NO levels were significantly greater in HIV+ (75.29 ± 4.31µM) compared to HIV- (52.84 ± 2.72µM, *p<0.0001*) patients (Fig.2B). The atmospheric NOx levels in HIV+ patients living in the north (36.47 ± 1.09µg/cm^3) were significantly higher compared to HIV- patients (31.31 ± 1.07µg/cm^3, *p=0.0021*), with north HIV- patients having significantly lower atmospheric NOx levels compared to south HIV- patients (37.60 ± 0.79µg/cm^3, *p<0.0001*) (Fig.2D). The maternal serum NO concentration was significantly higher in HIV+ patients in the north (76.35 ± 5.89µM, *p=0.0022*) and south (74.03 ± 6.38µM, *p<0.0001*) compared to their respective HIV- patients (North= 59.45 ± 6.50µM, South= 50.02 ± 2.69µM) (Fig.2E). No relationship between atmospheric NOx and maternal serum NO was observed among HIV+ and HIV- patients living in the north and south. Maternal serum MDA concentrations showed no difference among patients irrespective of HIV status and living location (Fig.2C and 2F). A significant negative correlation was observed in HIV- patients between atmospheric NOx and maternal serum MDA (Spearman r=-0.1926, 95%CI -0.3635 to -0.009263, *p=0.0343*), this correlation was strengthened in south living HIV- patients (Spearman r=-0.2368, 95%CI -0.4470 to -0.001832,

p=0.0422). However, no correlation is observed in north living patients (Spearman r=-0.09837, 95%CI -0.3825 to 0.2027, *p=0.5106*). A significant positive correlation was observed between NO and MDA across all subdivisions of HIV status and living location, except HIV+ north patients (Table 3).

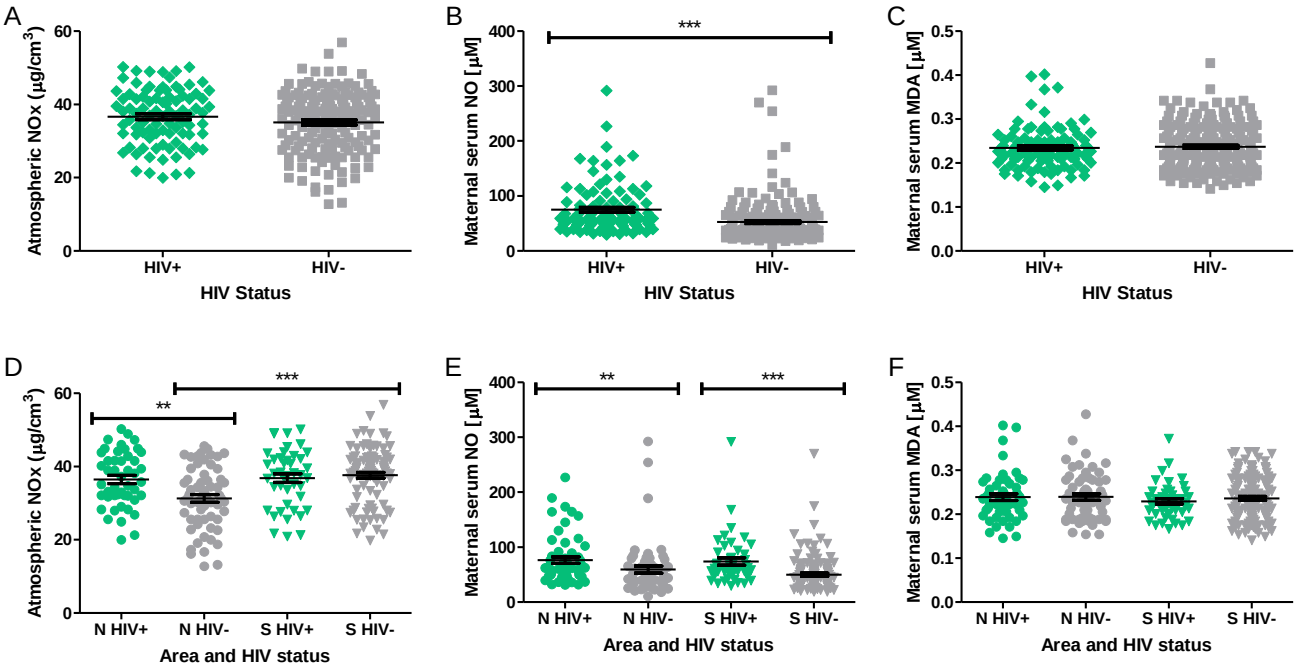

Figure 2 Atmospheric NOx (A and D), maternal serum NO (B and E) and maternal serum MDA (C and F) concentrations in HIV+ and HIV- patients, including patients subdivided by their living location (D, E and F). Statistical significance: ****p<0.0001*, ***p<0.001*.

Table 3 Relationship between the maternal serum NO (log(µM)) concentration and maternal serum MDA (log(µM)) for all patients living in the North and South, as well as HIV status

		Total	**HIV+**	**HIV-**
Total	Spearman r	**0.2339**	**0.2301**	**0.2525**
	(95%CI)	**(0.1202 to 0.3415)**	**(0.03045 to 0.4122)**	**(0.1112 to 0.3838)**
	p-value	****<0.0001**	***0.0206**	****0.0004**
North	Spearman r	**0.2502**	0.09260	**0.3378**
	(95%CI)	**(0.06309 to 0.4203)**	(-0.1849 to 0.3564)	**(0.07932 to 0.5538)**
	p-value	***0.0075**	0.5013	***0.0095**
South	Spearman r	**0.2113**	**0.4450**	**0.2099**
	(95%CI)	**(0.06316 to 0.3503)**	**(0.1690 to 0.6563)**	**(0.03738 to 0.3703)**
	p-value	***0.0043**	***0.0019**	***0.0145**

Statistical significance: ****p<0.0001, **p<0.01 *p<0.05*

Multivariate analysis for inducers of NO and MDA

The relationship among maternal serum NO and MDA, atmospheric NOx and HIV status, controlled for maternal characteristics, is described in Table 4. An increase in maternal serum MDA was associated with a significant increase in maternal serum NO, when controlled for potential confounders (Table 4). For every 1% increase in MDA there would be a 0.6% increase in NO for the total population (*p<0.0001*), 0.76% increase for HIV- mothers (*p<0.0001*), 0.71% for HIV+ mothers (*p=0.037*), 0.56% for the north (*p=0.026*) and 0.84% for the south (*p<0.0001*). A reciprocal increase in MDA resulted from an increase in NO was observed in Table 4. Where every 1% increase in NO would lead to a 0.12% increase in MDA for the total population (*p<0.0001*), 0.14% for HIV- mothers (*p<0.0001*), 0.1% for HIV+ mothers (*p=0.037*), 0.11% for the north (*p=0.026*) and 0.14% for the south (*p<0.0001*). In HIV+ south living mothers a 1% increase in serum NO would result in a 1.56% increase in MDA (β-coefficient: 1.562, 95%CI 0.571 to 2.55, *p=0.0003*; R^2=0.4301, *p=0.0699*). Mothers living in the north was associated with a 0.5% reduction in maternal serum NO if atmospheric NOx increased by 1% (*p=0.051*). The negative correlation between atmospheric NOx and serum MDA observed in HIV- patients previously, was further investigated controlling for potential confounders. This result was confirmed, were every 1% increase in atmospheric NOx would cause a 0.16% reduction in MDA (*p=0.045*) in HIV- mothers. In HIV- south living mothers a reduction of 0.24% in MDA (β-coefficient: -2.375, 95%CI -0.457 to -0.018, *p=0.034*; R^2=0.1890, *p=0.0814*) would result from a 1% increase in atmospheric NOx. Being HIV+ significantly increased maternal serum NO for the total population, those living in the north and south (Table 4).

Table 4 Impact of atmospheric NOx (log) and HIV on maternal serum NO and MDA (log) concentrations – linear regression analysis for total mothers and subdivided into HIV status and area with adjustments for maternal characteristics

	Maternal serum NO (log) concentration				
	Total	HIV Status		Area	
	(n=189)	HIV- (n=120)	HIV+ (n=69)	North (n=84)	South (n=105)
Serum MDA (log)	0.672 (0.342 - 1.00) ***<0.0001	0.759 (0.344 - 1.17) ***<0.0001	0.707 (0.043 - 1.37) *0.037	0.564 (0.068 - 1.06) *0.026	0.837 (0.379 - 1.30) ***<0.0001
Atmospheric NOx (log)	-0.153 (-0.441 - 0.134) 0.282	-0.037 (-0.411 - 0.337) 0.845	-0.325 (-0.854 - 0.203) 0.369	-0.444 (-0.89 - 0.002) 0.051	0.113 (-0.294 - 0.521) 0.582
HIV+	0.373 (0.222-0.524) ***<0.0001			0.493 (0.236 - 0.750) ***<0.0001	0.332 (0.132 - 0.531) **0.001
	R^2 = 0.2576 (***p<0.0001)	R^2 = 0.1667 (*p=0.0142)	R^2 = 0.1585 (p=0.2920)	R^2 = 0.2720 (**p=0.0035)	R^2 = 0.3032 (***p<0.0001)
	Maternal serum MDA (log) concentration				
	Total	HIV Status		Area	
	(n=189)	HIV- (n=120)	HIV+ (n=69)	North (n=84)	South (n=105)
Serum NO (log)	0.124 (0.063 - 0.184) ***<0.0001	0.141 (0.064 - 0.218) ***<0.0001	0.101 (0.006 - 0.196) *0.037	0.115 (0.014 - 0.216) *0.026	0.145 (0.066 - 0.225) ***<0.0001
Atmospheric NOx (log)	-0.081 (-0.205 - 0.042) 0.193	-0.161 (-0.320 - -0.003) *0.045	0.0001 (-0.202 - 0.203) 0.999	-0.031 (-0.237 - 0.176) 0.768	-0.136 (-0.304 - 0.031) 0.110
HIV+	-0.047 (-0.116 - 0.021) 0.174			-0.034 (-0.160 - 0.093) 0.599	-0.056 (-0.143 - 0.031) 0.204
	R^2 = 0.1213 (**p=0.009)	R^2 = 0.1800 (**p=0.0074)	R^2 = 0.32752 (*p=0.0175)	R^2 = 0.0999 (p=0.5191)	R^2 = 0.1713 (*p=0.0299)

Results described: β-coefficient, (95%CI), p-value with regression model R^2-value and p-value.
Adjusted for maternal characteristics: HIV status, BMI, area, maternal age, Hb, iron and blood pressure systolic and diastolic
Statistical significance: ****p<0.0001, **p<0.01, *p<0.05*

The effects of HIV, location and maternal serum NO and MDA levels on neonatal BW

Maternal serum NO was significantly greater in patients who delivered babies with a LBW (70.00 ± 6.761µM) compared to normal BW (NBW) (57.59 ± 2.340µM, *p=0.0292*) (Fig.3A). No difference was observed for maternal serum MDA (Fig.3B). No difference was observed between NO and MDA levels when the patients for LBW and NBW were divided into HIV+ and HIV- (Appendix 6: Fig.A6.1).

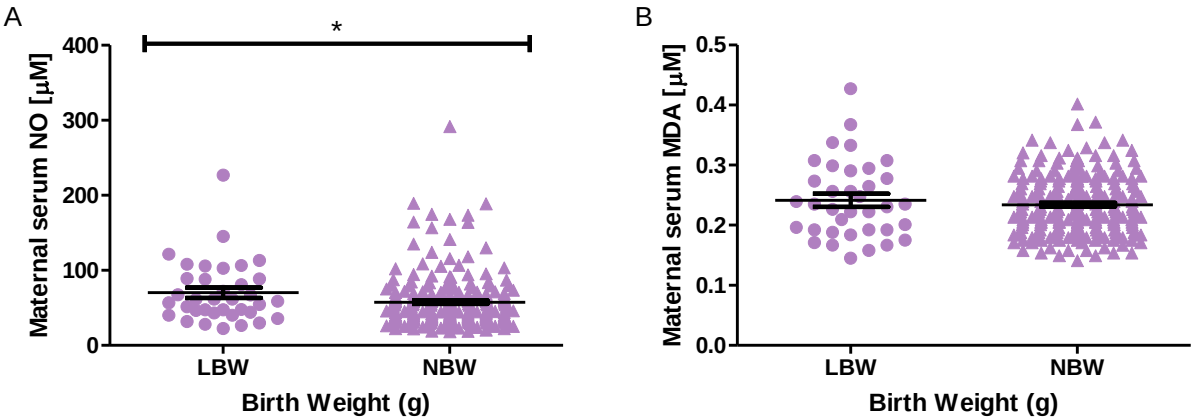

Figure 3 Maternal serum NO (µM) (A) and MDA (µM) (B) concentrations in patients who delivered infants with low BW (LBW) (<2500g) ([A] n=36 and [B] n=36) and normal BW (NBW) (>2500g) ([A] n=230 and [B] n=228). Statistical significance: *$p<0.05$.

A negative trend is suggested between neonatal BW and maternal serum NO (Table 5, $p=0.0843$) concentration. When the HIV status of the patient is taken into consideration the BW is significantly correlated with NO, with an increase in NO resulting in a decrease in BW (Table 5, $p=0.0249$). This is also observed in patients living in the south (Table 5, $p=0.0224$). This relationship between BW and NO strengthens and is most strongly correlated in HIV+ patients living in the south (Spearman r= -0.5167, $p=0.0005$; Table 5).

Table 5 Relationship between the maternal serum NO (log(µM)) concentration and neonatal birthweight (log(g)) for all patients living in the North and South, as well as HIV status

		Total	**HIV+**	**HIV-**
Total	Spearman r	-0.1060	**-0.2325**	0.009826
	(95%CI)	(-0.2269 to 0.01802)	**(-0.4215 to -0.02405)**	(-0.1440 to 0.1632)
	p-value	0.0843	***0.0249**	0.8979
North	Spearman r	0.04468	-0.04905	0.2173
	(95%CI)	(-0.1578 to 0.2436)	(-0.3279 to 0.2376)	(-0.07349 to 0.4740)
	p-value	0.6573	0.7325	0.1296
South	Spearman r	**-0.1777**	**-0.5167**	-0.05843
	(95%CI)	**(-0.3259 to -0.02107)**	**(-0.7139 to -0.2437)**	(-0.2381 to 0.1251)
	p-value	***0.0224**	****0.0005**	0.5209

Statistical significance: ***$p<0.0001$, *$p<0.05$

In HIV+ patients, a significantly negative correlation was observed between maternal serum MDA and neonatal BW (Table 6, $p=0.02$). This correlation was strengthened in south living

patients (Table 6, *p=0.0129*), but lost in north living patients (Table 6, *p=0.3213*). No correlation was observed in HIV- patients (Table 6).

Table 6 Relationship between the maternal serum MDA (log(µM)) and neonatal birthweight (log(g)) for all patients living in the North and South, as well as HIV status

		Total	**HIV+**	**HIV-**
Total	Spearman r	-0.01026	**-0.2423**	0.09462
	(95%CI)	(-0.1344 to 0.1142)	**(-0.4310 to -0.03326)**	(-0.06028 to 0.2451)
	p-value	0.8682	***0.0200**	0.2169
North	Spearman r	-0.01905	-0.1431	0.1039
	(95%CI)	(-0.2203 to 0.1838)	(-0.4124 to 0.1492)	(-0.1879 to 0.3789)
	p-value	0.8508	0.3213	0.4726
South	Spearman r	-0.007495	**-0.3807**	0.08815
	(95%CI)	(-0.1650 to 0.1504)	**(-0.6195 to -0.07756)**	(-0.09635 to 0.2668)
	p-value	0.9241	***0.0129**	0.3343

Statistical significance:*$p<0.05$

Multivariate analysis determined factors associated with BW reduction

Factors affecting neonatal BW, controlled for maternal and neonatal characteristics, are described in Table 7. Gestational age increased BW significantly across all divisions (Table 7). For every 1 unit increase in parity there would be a 9.271g increase in BW for the total population (*p=0.049*) and a 18.1g increase in BW for HIV- mothers (*p=0.017*). Maternal serum NO was negatively correlated with BW in Table 5, this was further investigated and controlled for potential confounders in Table 7, which was shown to cause a significant decrease in BW. Every 1% increase in maternal serum NO would cause a 18.93g decrease in BW for the total population (*p=0.022*). This strengthened in the HIV- population, where a 1% increase in NO resulted in a 23.85g decrease in BW (*p=0.02*). For south living mothers, a 36.5g decrease in BW (*p=0.01*) resulted from every 1% increase in NO. Mothers carrying male neonates were associated with a 37.5g decrease in BW for every 1% increase in NO (*p=0.006*). Maternal serum MDA showed both an increase and decrease in neonatal BW (Table 7). Every 1% increase in MDA for HIV- mothers would cause a 61.9g increase in BW (*p=0.011*) while for HIV+ mothers a 107.6g decrease (*p=0.052*) in BW was observed. Mothers carrying female neonates were associated with a 66.8g increase in BW when a 1% increase is observed in atmospheric NOx. Controlling for HIV+ patients showed no significant change in BW (Table 7).

Table 7 Impact of atmospheric NOx (log), maternal serum NO and MDA (log) concentrations on neonate birthweight (g) – linear regression analysis for total mothers and subdivided into HIV status, area and neonate gender with adjustments for maternal and neonate characteristics

	Neonate Birthweight (g)						
	Total	HIV Status		Area		Neonatal Gender	
	(n=103)	HIV- (n=56)	HIV+ (n=47)	North (n=50)	South (n=53)	Male (n=60)	Female (n=43)
Gestational Age	**101.13** (50.23 - 152.02) ***<0.0001	**100.62** (30.68 - 170.62) **0.006	**167.41** (48.43 - 286.39) **0.007	**74.5** (-13.15 - 162.14) *0.093	**109.7** (33.45 - 185.95) **0.006	**123.74** (33.62 - 213.86) **0.008	**68.14** (0.610 - 135.68) *0.048
Parity	**92.71** (0.244 - 185.17) *0.049	**180.91** (34.20 - 327.61) *0.017	22.52 (-118.49 - 163.52) 0.746	65.25 (-59.60 - 190.1) 0.295	129.85 (-47.59 - 307.29) 0.146	96.85 (-57.75 - 251.45) 0.213	124.28 (-3.64 - 252.20) 0.056
Serum NO (log)	**-189.29** (-350.57 - -28.01) *0.022	**-238.54** (-437.09 - -39.99) *0.02	-55.82 (-358.88 - 247.23) 0.709	-48.57 (-289.09 - 191.95) 0.683	**-364.99** (-636.81 - -93.17) *0.010	**-375.17** (-636.66 - -113.69) **0.006	-19.38 (-312.78 - 274.02) 0.893
Serum MDA (log)	**372.55** (-30.87 - 775.967) 0.070	**619.03** (150.50 - 1087.55) *0.011	**-1075.65** (-2160.52 - 9.23) 0.052	309.54 (-314.97 - 934.04) 0.320	414.07 (-239.56 - 1067.71) 0.207	581.63 (-19.49 - 1182.76) 0.058	47.26 (-635.80 - 730.32) 0.888
HIV+	-33.01 (-202.53 - 136.50) 0.699			-155.57 (-476.21 - 165.08) 0.330	34.4 (-233.45 - 302.25) 0.796	-0.645 (-240.02 - 238.73) 0.996	-96.60 (-411.67 - 218.46) 0.533
Atmospheric NOx (log)	146.32 (-191.32 - 483.97) 0.391	221.93 (-211.34 - 655.20) 0.306	491.71 (-226.36 - 1209.79) 0.172	275.72 (-287.48 - 838.91) 0.326	121.05 (-424.8 - 666.91) 0.655	296.43 (-270.84 - 863.71) 0.297	**668.07** (9.12 - 1327.02) *0.047
	R^2 = 0.6600 (***p<0.0001)	R^2 = 0.7778 (***p<0.0001)	R^2 = 0.7057 (**p=0.0009)	R^2 = 0.7562 (***p<0.0001)	R^2 = 0.6881 (**p=0.0002)	R^2 = 0.6990 (***p<0.0001)	R^2 = 0.7918 (**p=0.0002)

Results described: β-coefficient, (95%CI), *p*-value with regression model R^2-value and *p*-value

Adjusted for maternal characteristics: HIV status, BMI, area, maternal age, Hb, iron, blood pressure systolic and diastolic, OGG1 Ser326Cys SNP (this polymorphism was taken into consideration due to it previously (unpublished data) being associated with HIV and influences birthweight) and neonatal characteristics: gestational age, parity, body: brain index, ponderal index, neonate gender, Apgar scores 1min and 5min.

Statistical significance: ****p<0.0001, **p<0.01, *p<0.05*

Effects of HIV and location on oxidative and ER stress markers

Oxidative stress markers' gene expressions are shown in Figure 4. HIV- mothers living in the south were shown to have decreased expression levels of *Keap1*, *OGG1* and *CAT* with increased *Nrf2*, *SOD2* and *GPx* expression levels compared to the north. In HIV+ south living mothers the gene expression of *Keap1*, *OGG1* and *GPx* decreased, whilst *Nrf2*, *SOD2* and *CAT* increased compared to the north. The *OGG1* gene expression (Fig.4C) was increased in HIV+ compared to HIV- mothers, however when area was considered, HIV+ south living patients had significantly reduced *OGG1* gene expression compared to the total HIV+ mothers. *Nrf2* was positively correlated with *CAT* and *GPx*, where an increase in *Nrf2* gene expression would lead to an increase in *CAT* and *GPx* gene expression (Table 9, $p<0.05$).

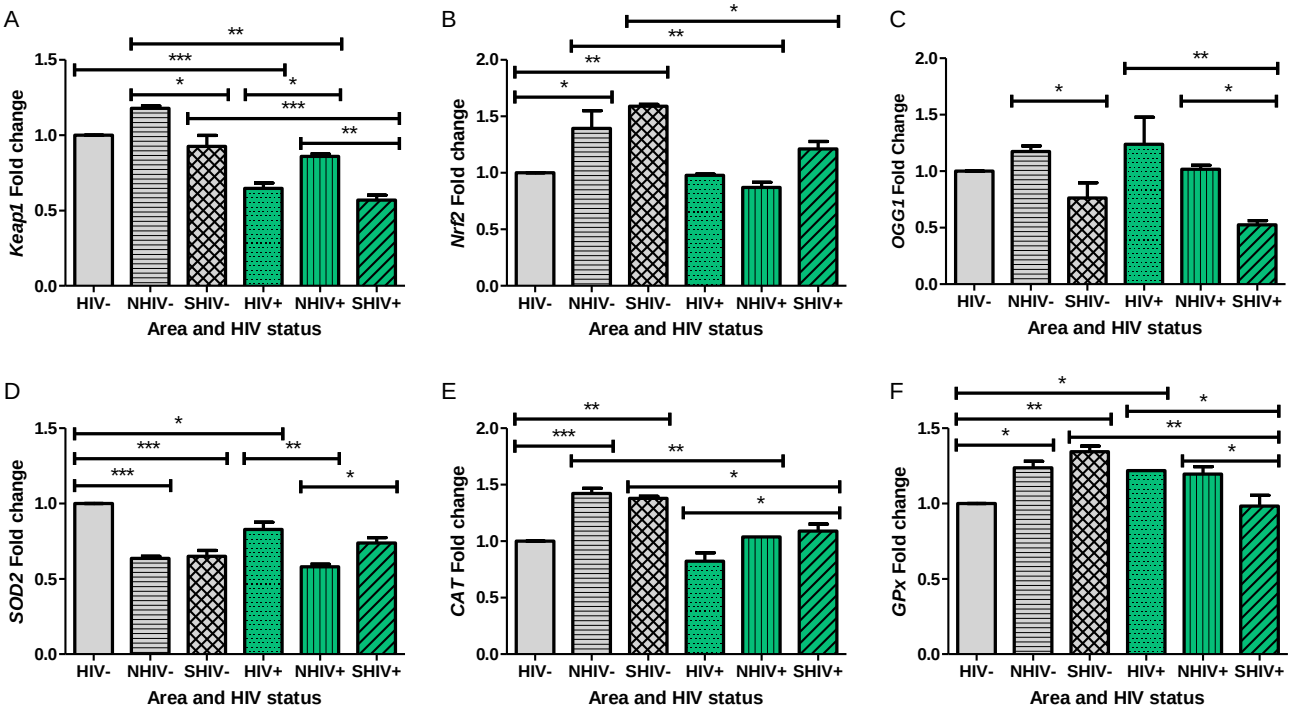

Figure 4 The mRNA fold change of oxidative stress markers: A) *Keap1*, B) *Nrf2*, C) *OGG1*, D) *SOD2*, E) *CAT*, F) *GPx* found within the third trimester for HIV- and HIV+ patients living in the north and south. Statistical significance: ***$p<0.0001$, **$p<0.01$, *$p<0.05$.

Table 9 Relationship among miR-28, miR-144 and *Nrf2* RT-PCR fold changes and the antioxidant and oxidative stress markers for all patients (n=76)

	Nrf2	*Keap1*	*OGG1*	*SOD2*	*CAT*	*GPx*
miR-144	-0.6290*	-0.0033	0.4995*	-0.0453	-0.1719	-0.0015
miR-28	-0.5965*	0.7977*	0.8158*	0.3228*	0.2440	0.1889
Nrf2	1.00	-0.0002	-0.1640	-0.7455	0.6167*	0.6020*

Results described: Spearman r.
Statistical significance:*$p<0.05$

The expression of miR-144 and miR-28 were significantly reduced in both HIV- and HIV+ south living patients compared to the north living patients (Fig.5, *p<0.0001*). The expression of miR-144 and miR-28 both were negatively correlated with *Nrf2* (Table 9, *p<0.05*) and miR-28 was positively associated with *Keap1*, *OGG1* and *SOD2* (Table 9, *p<0.05*). Living in the heavily polluted south reduced the expression of both miR-144 and miR-28 by -95.73 and -11.73 fold respectively (Table 10, *p<0.0001*). Being HIV positive decreased miR-28 expression by -4.5 fold whilst it increased the expression of miR-144 by 51.07 fold (Table 10, *p<0.0001*).

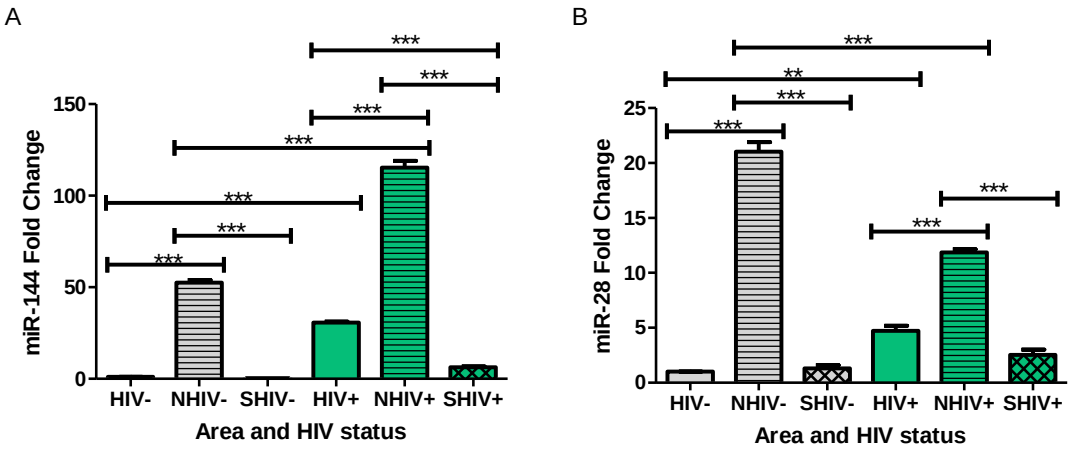

Figure 5 The expression of (A) miR-144 and (B) miR-28 within third trimester for HIV- and HIV+ patients living in the North and South. Statistical significance: ***p<0.0001*, ****p<0.01.*

Table 10 Impact of atmospheric NOx, HIV status and living in the south on the expressions of miR-144 and miR-28 (n=68)

	miR-144	**miR-28**
Atmospheric NOx (log)	17.08 (-7.8 to 41.97) 0.174	-2.29 (-5.62 to 1.04) 0.174
HIV+	**51.07** (37.58 to 64.55) ***<0.0001	**-4.50** (-6.29 to -2.68) ***<0.0001
Area (South)	**-95.73** (-108.26 to -83.20) ***<0.0001	**-11.73** (-13.41 to -10.05) ***<0.0001
	R^2=0.8908 (***<0.0001)	R^2= 0.8538 (***<0.0001)

Results described: β-coefficient, (95%CI), *p*-value with regression model R^2-value and *p*-value.
Adjusted for maternal characteristics: BMI, maternal age, Hb, iron and blood pressure systolic and diastolic, NO and MDA
Statistical significance: ****p<0.0001*

It was interesting to note that *SOD2* gene expression was shown to be influenced by maternal serum NO concentrations (Table 8). For the total population a positive correlation was observed between serum NO and *SOD2* fold change. When area is taken into account, the south living mothers had a stronger positive correlation (Table 8, $p<0.05$) whilst north living mothers had a stronger negative correlation (Table 8, $p<0.05$) between serum NO and *SOD2* gene expression.

The gene expression for ER stress markers are shown in Figure 6. In HIV- mothers the gene expression for *eIF2*, *ATF4* and *CHOP* were significantly increased within the south living (*eIF2*: 1.019±0.046; *ATF4*: 1.161±0.042; *CHOP*: 1.655±0.075) mothers compared to the north (*eIF2*: 0.763±0.021, $p<0.05$; *ATF4*:0.765±0.079, $p<0.01$; *CHOP*: 1.215±0.105, $p<0.01$). The expression of *BiP* increased (SHIV-:1.087±0.015; NHIV-: 1.057±0.029, $p=0.536$), although not significant. The gene expression of all ER stress markers were greater in HIV- south living patients compared to HIV+ south living patients (*eIF2*: 0.8593±0.080; *ATF4*: 1.141±0.061; *CHOP*: 0.5436±0.015; *BiP*: 0.7380±0.036), with *CHOP* ($p<0.0001$) and *BiP* ($p<0.0001$) fold changes significantly different. In HIV+ mothers the *eIF2* and *ATF4* gene expressions are greater in the south compared to north (*eIF2*: 0.8264±0.069, $p>0.05$; *ATF4*: 0.7967±0.019, $p<0.05$) living mothers, whilst both *CHOP* and *BiP* are significantly greater in the north (*CHOP*: 0.946±0.013, $p<0.05$; *BiP*: 0.9549±0.010, $p<0.0001$) compared to south living mothers. The gene expression of ER stress markers' *eIF2* and *ATF4* were found to have a negative correlation with maternal serum NO for the total population (Table 8). This negative correlation was strengthened in south living mothers (Table 8, $p<0.05$) whilst it was reversed to a positive correlation in north living mothers (Table 8, $p<0.05$).

Table 8 Relationship between maternal serum NO (log(uM)) and the fold change of antioxidant and ER stress markers for all patients (n=76), and those living in the North (n=39) and South (n=37)

	RT-PCR Fold Change ($2^{-\Delta\Delta Ct}$)									
	OGG1	*SOD2*	*CAT*	*GPx*	*Keap1*	*Nrf2*	*BiP*	*eIF2*	*ATF4*	*CHOP*
Maternal serum NO										
Total	-0.0653	0.2040	-0.3917*	-0.3917*	-0.3386*	-0.3290*	-0.3386*	-0.1412	-0.1412	-0.3917*
North	-0.4540*	**-0.4540***	-0.4540*	-0.4540*	-0.4540*	-0.4540*	-0.4540*	**0.4540***	**0.4540***	-0.4540*
South	-0.4346*	**0.4346***	-0.4346*	-0.4346*	-0.4346*	-0.4346*	-0.4346*	**-0.4346***	**-0.4346***	-0.4346*

Results described: Spearman r.
Statistical significance: *$p<0.05$.

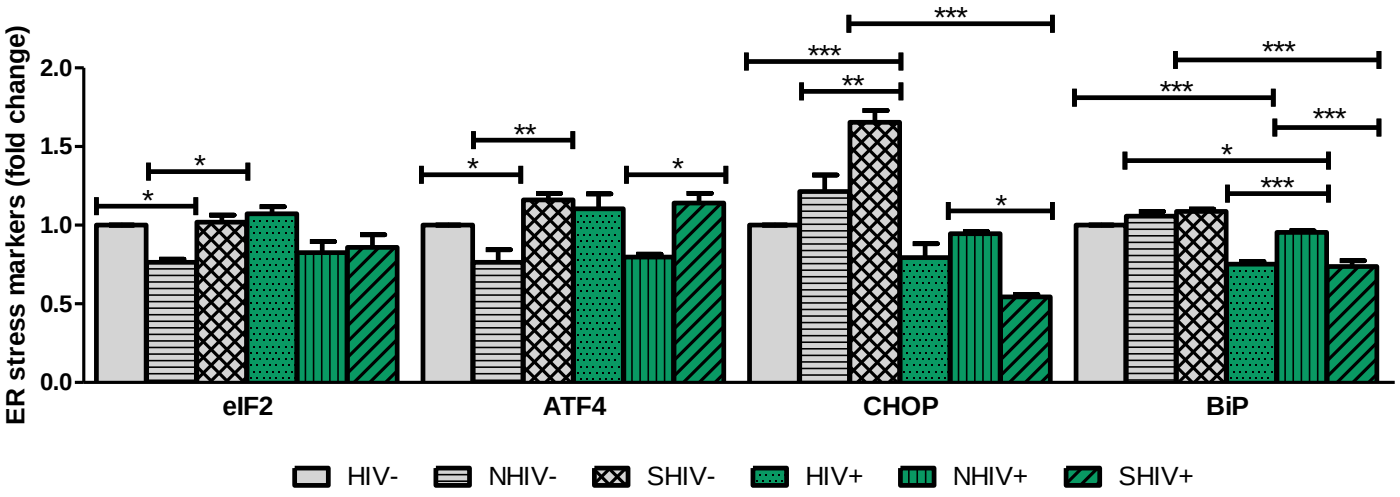

Figure 6 The mRNA fold change of ER stress markers found within third trimester for HIV- and HIV+ patients living in the North and South. Statistical significance: ****p<0.0001, **p<0.01, *p<0.05.*

Discussion

Macrophages, play a pivotal role in the innate immune response, are among the first cells in contact with HIV and have been implicated as a major reservoir of HIV during its subclinical infection [33]. HIV has shown to stimulate macrophages to produce NO, with HIV *env* gp120 [34] and regulatory *Tat* protein considered inducers of NO through the activation of iNOS and eNOS genes [5,33]. Several studies have also shown increased sera NO production in asymptomatic HIV-infected patients [35], children and adults with acquired immune deficiency syndrome (AIDS) and especially those with opportunistic diseases [36–38]. Pro-inflammatory cytokines, such as TNFα and INFγ, increase NO production from HIV-infected macrophages [36,39]. Increased levels of NO have been reported in blood plasma of women during gestation [40]. Our study showed increased levels of serum NO concentrations in HIV+ mothers compared to HIV- mothers (Fig.2B and 2E), with being HIV+ shown to induce NO production (Table 4).

NO's highly reactive intermediate peroxynitrite, inhibits the electron transport chain, causes oxidation of biological thiol compounds and induces LP [10]. Therefore an increase in NO, within a highly aerobic environment would lead to increased peroxynitrite formation and subsequent LP. Our study supports this observation where increased serum NO was shown to increase serum MDA concentrations (Table 3 and 4). Interestingly, a reciprocal activity was observed between NO and MDA concentrations, where one increased the other and vice versa

(Table 3 and 4). Although an increase in MDA concentrations in HIV patients has been reported previously [41,42], HIV was shown to have no direct effect on MDA concentrations within our study population (Table 4), suggesting MDA increased as a result of the indirect activity of HIV through increased NO production.

Additionally, exposure to pollutants in the heavily industrialised SD and less industrialised ND, especially traffic-related NOx AP, was hypothesised to have an effect on NO and MDA levels [28]. Atmospheric NOx was shown to have no effect on serum NO levels, however it was suggested to cause a decrease in NO levels in ND (Table 4, *p=0.051*). The north was shown to have a lower concentration of atmospheric NOx compared to the south (Fig.1A), thus proposing that low exogenous sources of NOx had a minimal effect on the endogenous production of NO. Inhalation of NOx from the environment enters the blood stream and is taken up by Hb, through this low levels of NOx become inactive [43]. Nagiah et al. reported increased MDA levels within SD [28], however this was not significant and increasing the sample size within this study showed no change was observed between ND and SD (Fig.1C). Taking into consideration HIV and a specific pollutant altered the initial finding. Atmospheric NOx was shown to decrease levels of MDA in HIV- patients (Table 4), with only a slight decrease in NOx levels observed in these patients (Fig.2A) and lower levels of NO (Fig.2B). This inhibition of LP as a result of NOx could be due to NO's ability to terminate LP propagation, as the unpaired NO radical reacts with an unpaired peroxyl radical, thereby neutralising the reaction [44].

Our study further investigated the effects of maternal serum NO and MDA, HIV-infection and atmospheric NOx had on neonatal BW. Previous studies have shown that NOx pollution was associated with LBW [17], increased levels of NO and MDA in PTB [11,13] and HIV associated with risk of LBW and PTB [2].

Our study shows NO may play a role in the aetiology of LBW, with HIV having no direct influence on the reduction of BW. The levels of NO increased within mothers delivering LBW neonates (Fig.3A), with the HIV status of the mother having no affect (Appendix 6: Fig.A6.1). In addition BW was reduced by increasing NO (Table 5 and 7). It has been proposed that the inhibition or restriction of foetal growth could be a consequence of pulmonary and placental inflammation, increased blood viscosity and coagulation. These alterations in endothelial and vascular functions thereby compromise placental blood flow and transfer of oxygen (O_2) and nutrients between mother and foetus [45]. As mentioned previously, NO is readily absorbed into the blood stream and binds to Hb which forms methemoglobin (MethHb) [16]. MethHb is

known to have reduced O_2 carrying capacity, which could in turn lead to reduced O_2 transfer at the utero-placental interface thereby negatively impacting on foetal growth [13]. Although SD NOx concentrations did not directly increase NO (Table 4), other pollutants in the area (not measured) [46] could be responsible for the increasing NO which may lead to BW reduction. It was interesting to note that carrying a male neonate significantly impacted on NO concentration leading to reduced BW, while carrying a female neonate and being exposed to high levels of NOx increased BW (Table 7). Since, it is well known that male infants compared to female infants have a larger BW for GA [47].

Our results indicate that HIV may play a differential role in MDA induced effect on BW (Table 7). It was observed that HIV- mothers were associated with an increase in BW ($p=0.011$) and HIV+ mothers were associated with a reduction in BW ($p=0.052$), as a consequence of increasing MDA (Table 6 and 7). Pregnancy is characterised by a high energy requirement and increased basal O_2 demand that favours reactive oxygen species (ROS) production [43], and as such increased levels of ROS would be expected in HIV- mothers leading to increased MDA levels. However, this may not impact negatively on the foetus as scavengers of oxidants are present to help reduce ROS. This is observed in Figure 4, where *SOD2* (Fig.4, $p<0.05$) and *CAT* (Fig.4, $p>0.05$) gene expression levels were greater in HIV- mothers compared to HIV+ mothers. The chronic oxidative stress observed in HIV+ patients [4] and increased NO production would lead to increased MDA concentrations, and potentially further increase NO levels (reciprocal interaction observed, Table 3 and 4), above antioxidants capacity. This could lead to vascular tissue damage, increased inflammation and alterations in placental O_2 transfer which could impact negatively on foetal growth [45].

The Nrf2-Keap1 pathway is responsible for the regulation of antioxidants in response to oxidative damage to maintain homeostasis. Upon oxidative damage, Nrf2 dissociates from the Keap1 complex, allowing it to translocate to the nucleus where it binds to the ARE and induces the transcriptional activation of specific target genes, including *SOD2*, *CAT* and *GPx* [48]. This study showed that *Nrf2* was positively associated with *CAT* and *GPx* (Table 9, $p<0.05$), with a significant increase observed in *Nrf2*, *CAT* and *GPx* expressions in HIV- south living patients compared to the total HIV- patients (Fig.4). A reduced *Nrf2* expression was observed by Nagiah et al. [28], however HIV infection was not considered in their study and may explain the difference observed as HIV+ SD living patients had significantly reduced *Nrf2* mRNA expression levels as compared to HIV- SD patients in this study (Fig.4B). It was interesting to note that the mRNA expression of *SOD2* was significantly reduced in HIV+ patients compared

to HIV- patients; whilst HIV+ patients living in SD had greater *SOD2* expressions than ND (Fig.4). The reduction of *SOD2* in HIV+ patients could be due to the inhibition of *SOD2* by the HIV *Tat* protein. It has been shown to inhibit the binding of Sp1 and Sp3 transcriptional factors to *SOD2* gene promoter and binds to its mRNA [4]. The increase in *SOD2* in south living HIV+ patients, also reported within Nagiah et al. SD patients [28], could be a result of increased maternal serum NO as it was observed within this study that serum NO was positively associated with *SOD2* gene expression for SD patients whilst ND patients had a negative correlation between serum NO and *SOD2* gene expression (Table 8). Reduced expression of *OGG1* was observed for both HIV- and HIV+ SD patients compared to ND, which was also reported by Nagiah et al. [28]. However, this study identified a negative association that exists between maternal serum NO and OGG1 gene expression for both ND and SD patients (Table 8). This is in agreement with literature where studies have reported that NO inhibits *OGG1* gene expression [23,49].

Genetic susceptibility is highly important when considering interactions between the environment and health outcomes [50]. The study of epigenetics, defined as the study of heritable differences in gene expression that does not alter the DNA sequence, has been most notably highlighted as an important potential mechanism for regulation of the Nrf2-Keap1 pathway [51]. Studies have shown that miR-144 and miR-28 are negatively associated with Nrf2 [25,26]. This study observed a similar effect, where an increase in miR-144 and miR-28 expression levels led to a decrease in *Nrf2* expression (Table 9), whilst miR-28 was positively associated with *Keap1* (Table 9). The expression of miR-144 was reduced as a consequence of living in the industrialised SD but increased in HIV+ patients and exposure to atmospheric NOx (Table 10). MiR-144 has been shown previously to be upregulated in high viral load HIV+ patients [52]. The expression of miR-28 was decreased in HIV+ patients living in the industrialised SD (Table 10). MiR-28 has been reported to target the 3'-UTR of the HIV genome, thereby inhibiting HIV replication and contributing to viral latency [53]. These results suggest that HIV and atmospheric NOx induced miR-144 expression leading to reduced *Nrf2* expression levels and thus reducing antioxidant capacity with increased oxidative stress.

The increase in oxidative stress, as a result of high pollution levels within the south could lead to the disruption of cellular calcium homeostasis. This homeostasis is necessary for the proper function of the ER's protein folding machinery and disruption would lead to the activation of the UPR pathway [54,55]. The UPR endeavours to restore balance to the ER, failure to mitigate ER stress can lead to cellular death [56]. Upon increase in ER stress, the ER chaperone BiP

dissociates from UP sensors and activates the UPR. The UPR comprises of three separate arms initiated by PERK, IRE1α or ATF6. The PERK arm phosphorylates eIF2α which in turn induces translation of *ATF4* mRNA. The PERK-eIF2α-ATF4 regulatory arm induces antioxidant stress-response genes and pro-apoptotic genes including *CHOP* [57]. HIV- south living patients had increased *eIF2*, *ATF4* and *CHOP* (Fig.6) gene expression levels compared to ND patients. Therefore the high levels of pollution found within the south, including atmospheric NOx would lead to increased ER stress via the PERK-eIF2-ATF4 pathway, thus increasing expression levels of *CHOP* which could lead to increased apoptosis.

In conclusion, this study demonstrated increased maternal serum NO as a consequence of HIV infection, with reciprocal action on MDA levels. The induction of the miR-144 was implicated as a mechanism for increased oxidative stress due to HIV exposure. The PERK-eIF2α-ATF4 pathway is proposed as a mechanism for increased ER stress due to exposure to the heavily polluted south Durban. Increased NO levels was observed to reduce BW, especially mothers exposed to higher levels of pollution. HIV was shown to differentially influence MDA effect on neonatal BW. The results of this study indicate that NO may play a key role in neonatal BW reduction as a consequence of traffic-related AP and HIV infection. This is of particular importance for SA, due to the high number of individuals with HIV infection and increased urbanisation. By identifying risks for LBW and PTB, it allows for the proper management and prevention of these adverse birth outcomes in developing countries.

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References

[1] UNAIDS, UNAIDS Fact sheet November 2015: Global HIV Statistics, 2015. http://www.unaids.org/en/regionscountries/countries/southafrica/ (accessed January 16, 2017).

[2] P.-L. Xiao, Y.-B. Zhou, Y. Chen, M.-X. Yang, X.-X. Song, Y. Shi, Q.-W. Jiang, Association between maternal HIV infection and low birth weight and prematurity: a meta-analysis of cohort studies, BMC Pregnancy Childbirth. 15 (2015) 1–11. doi:10.1186/s12884-015-0684-z.

[3] N.L. Fleischer, M. Merialdi, A. Van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A. van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A.P. Betran, J.P. Souza, M.S. O'Neill, A. Van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A. van Donkelaar, F. Vadillo-Ortega, R. V. Martin, A.P. Betran, J.P. Souza, M.S. O'Neill, Outdoor Air Pollution , Preterm Birth , and Low Birth Weight : Analysis of the World Health Organization Global Survey on Maternal and Perinatal Health, Environ. Health Perspect. 122 (2014) 425–430. doi:10.1289/ehp.1306837.

[4] A. Ivanov, V.T. Values-Elliston, O.N. Ivanova, S.N. Kochetkov, E. Starodubova, B. Bartosch, M. Isaguliants, Oxidative Stress During HIV Infection: Mechanisms and Consequences, Oxid. Med. Cell. Longev. 2016 (2016) 1–18. doi:10.1155/2016/8910396.

[5] D. Torre, A. Pugliese, F. Speranza, Reviews Role of nitric oxide in HIV-1 infection : friend or foe ?, 2 (2002) 273–280.

[6] G. Giovannoni, R.F. Miller, S.J.R. Heales, J.M. Land, M.J.G. Harrison, E.J. Thompson, Elevated cerebrospinal fluid and serum nitrate and nitrite levels in patients with central nervous system complications of HIV-1 infection: A correlation with blood-brain-barrier dysfunction, J. Neurol. Sci. 156 (1998) 53–58. doi:10.1016/S0022-510X(98)00021-5.

[7] A.N.C. Simão, V.J. Victorino, H.K. Morimoto, E.M.V. Reiche, C. Panis, Redox-driven events in the human immunodeficiency virus type 1 (HIV-1) infection and their clinical implications., Curr. HIV Res. 13 (2015) 143–50. http://www.ncbi.nlm.nih.gov/pubmed/25771095.

[8] X. Deng, R.A. Deitrich, Ethanol Metabolism and Effects: Nitric Oxide and its Interaction, Curr. Clin. Pharmacol. 2 (2007) 145–153.

[9] V. Calabrese, C. Cornelius, E. Rizzarelli, J.B. Owen, A.T. Dinkova-Kostova, D.A. Butterfield, Nitric Oxide in Cell Survival: A Janus Molecule, Antioxid. Redox Signal. 11 (2009) 2717–2739. doi:10.1089/ars.2009.2721.

[10] E. Bakan, S. Taysi, M.F. Polat, S. Dalga, Z. Umudum, N. Bakan, M. Gumus, Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer, Jpn J Clin Oncol. 32 (2002) 162–166. http://www.ncbi.nlm.nih.gov/pubmed/12110642.

[11] S. Chadha, V. Jain, I. Gupta, M. Khullar, Nitric oxide metabolite levels in preterm labor., J. Obstet. Gynaecol. Res. 33 (2007) 710–7. doi:10.1111/j.1447-0756.2007.00639.x.

[12] J.M.C. Gutteridge, Lipid-peroxidation and antioxidants as biomarkers of tissue-damage, Clin. Chem. 41 (1995) 1819–1828. doi:7497639.

[13] S. Tabacova, D.D. Baird, L. Balabaeva, Exposure to oxidized nitrogen: lipid peroxidation and neonatal health risk, Arch Env. Heal. 53 (1998) 214–221. doi:10.1080/00039899809605698.

[14] S.W. Walsh, Y. Wang, Secretion of lipid peroxides by the human placenta, Am. J. Obstet. Gynecol. 169 (1993) 1462–1466. doi:https://doi.org/10.1016/0002-9378(93)90419-J.

[15] F.J. Kelly, Oxidative stress: its role in air pollution and adverse health effects., Occup. Environ. Med. 60 (2003) 612–616. doi:10.1136/oem.60.8.612.

[16] N. Castiglione, S. Rinaldo, G. Giardina, V. Stelitano, F. Cutruzzolà, Nitrite and Nitrite Reductases: From Molecular Mechanisms to Significance in Human Health and Disease, Antioxid. Redox Signal. 17 (2012) 684–716. doi:10.1089/ars.2011.4196.

[17] J. Seo, E. Ha, O. Kim, B. Kim, H. Park, J. Leem, Y. Hong, Y. Kim, Environmental health surveillance of low birth weight in Seoul using air monitoring and birth data [in Korean]., J. Prev Med Public Heal. 40 (2007) 363–370. doi:https://doi.org/10.3961/jpmph.2007.40.5.363.

[18] I. Kloog, S.J. Melly, W.L. Ridgway, B. a Coull, J. Schwartz, Using new satellite based exposure methods to study the association between pregnancy pm2.5 exposure, premature birth and birth weight in Massachusetts, Environ. Heal. 11 (2012) 40. doi:10.1186/1476-069X-11-40.

[19] Z.P. Vassilev, M.G. Robson, J.B. Klotz, Outdoor exposure to airborne polycyclic organic matter and adverse reproductive outcomes: A pilot study, Am. J. Ind. Med. 40 (2001) 255–262.

[20] X. Wang, H. Ding, L. Ryan, X. Xu, Association between air pollution and low birth weight: a community-based study., Environ. Health Perspect. 105 (1997) 514–520. doi:10.1289/ehp.97105514.

[21] T.W. Kensler, N. Wakabayashi, S. Biswal, Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway., Annu. Rev. Pharmacol. Toxicol. 47 (2007) 89–116. doi:10.1146/annurev.pharmtox.46.120604.141046.

[22] M. Jaiswal, N.F. LaRusso, K. Nishioka, Y. Nakabeppu, G.J. Gores, Human Oggl, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide (vol 61, pg 6388, 2001), Cancer Res. 61 (2001) 7705.

[23] A. Bravard, M. Vacher, B. Gouget, A. Coutant, F.H. de Boisferon, S. Marsin, S. Chevillard, J.P. Radicella, Redox regulation of human OGG1 activity in response to cellular oxidative stress., Mol. Cell. Biol. 26 (2006) 7430–6. doi:10.1128/MCB.00624-06.

[24] M. Kobayashi, M. Yamamoto, Molecular Mechanisms Activitating the Nrf2-Keap1 Pathway of Antioxidant Gene Regulation, Antioxid. Redox Signal. 7 (2005) 385–394. doi:10.1089/ars.2005.7.385.

[25] M. Yamamoto, A. Singh, F. Sava, M. Pui, S.J. Tebbutt, C. Carlsten, MicroRNA expression in response to controlled exposure to diesel exhaust: attenuation by the antioxidant N-acetylcysteine in a randomized crossover study., Environ. Health Perspect. 121 (2013) 670–5. doi:10.1289/ehp.1205963.

[26] M. Yang, Y. Yao, G. Eades, Y. Zhang, Q. Zhou, MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism., Breast Cancer Res. Treat. 129 (2011) 983–91. doi:10.1007/s10549-011-1604-1.

[27] J.D. Malhotra, R.J. Kaufman, The Endoplasmic Reticulum and the Unfolded Protein Response, Semin. Cell Dev. Biol. 18 (2007) 716–731. doi:10.1016/j.semcdb.2007.09.003.The.

[28] S. Nagiah, A. Phulukdaree, D. Naidoo, K. Ramcharan, R. Naidoo, D. Moodley, A. Chuturgoon, Oxidative stress and air pollution exposure during pregnancy: A molecular assessment., Hum. Exp. Toxicol. 34 (2015) 838–847. doi:10.1177/0960327114559992.

[29] R. Beelen, G. Hoek, ESCAPE Exposure assessment manual, 2010. http://www.escapeproject.eu/manuals/ESCAPE_Exposure-manualv9.pdf.

[30] S. Muttoo, L. Ramsay, B. Brunekreef, R. Beelen, K. Meliefste, R.N. Naidoo, Land Use Regression Modelling Estimating Nitrogen Oxides Exposure in Industrial South Durban, South Africa, Sci. Total Environ. 610–611 (2017) 1439–1447. doi:10.1016/j.scitotenv.2017.07.278.

[31] B. Halliwell, J.M. Gutteridge, Free radicals in biology and medicine, 3rd Editio, Oxford Univeristy Press, Oxford, 1999.

[32] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCt Method, Methods. 25 (2001) 402–408. doi:10.1006/meth.2001.1262.

[33] F. Chen, Y. Lu, V. Castranova, Y. Rojanasakul, K. Miyahara, Y. Shizuta, V. Vallyathan, X. Shi, L.M. Demers, Nitric Oxide Inhibits HIV Tat-Induced NF- □ B Activation, Nitric Oxide. 155 (1999) 275–284. doi:10.1016/S0002-9440(10)65121-8.

[34] D. Pietraforte, E. Tritarelli, U. Tesra, M. Minetti, gp120 HIV envelope glycoprotein increases the production of nitric oxide in human monocyte-derived macrophages., J. Leukoc. Biol. 55 (1994) 175–182.

[35] P.H. Groeneveld, F.P. Kroon, P.H. Nibbering, S.M. Bruisten, P. Van Swieten, R. Van Furth, Increased production of nitric oxide correlates with viral load and activation of mononuclear phagocytes in HIV-infected patients, Scand. J. Infect. Dis. 28 (1996) 341–345. doi:http://dx.doi.org/10.3109/00365549609037916.

[36] D. Torre, G. Ferrario, F. Speranza, R. Martegani, C. Zeroli, Increased levels of nitrite in the sera of children infected with human immunodeficiency virus type 1., Clin. Infect. Dis. 22 (1996) 650–3. http://www.ncbi.nlm.nih.gov/pubmed/8729204.

[37] D. Torre, G. Ferrario, G. Bonetta, F. Speranza, C. Zeroli, Production of nitric oxide from peripheral blood mononuclear cells and polymorphonuclear leukocytes of patients with HIV-1, AIDS. 9 (1995) 979–980.

[38] R. Zangerlf, D. Fuchs, G. Reibnegger, G. Werner-Felmayer, H. Gallati, H. Wachter, E.R. Werner, Serum Nitrite Plus Nitrate in Infection with Human Immunodeficiency Virus Type-l, Immunobiol. 193 (1995) 59–70. doi:10.1016/S0171-2985(11)80155-5.

[39] M.I. Bukrinsky, H.S. Nottet, H. Schmidtmayerova, L. Dubrovsky, C.R. Flanagan, M.E. Mullins, S.A. Lipton, H.E. Gendelman, Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease., J. Exp. Med. 181 (1995) 735–45.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2191885&tool=pmcentrez&rendertype=abstract.

[40] R. Huozha, S. Pandita, M. Ashutosh, Production of nitric oxide by Murrah buffalo lymphocytes during gestation, Rev. Vet. 21 (2010) 895–899.

[41] P.T. Ronaldson, R. Bendayan, HIV-1 viral envelope glycoprotein gp120 produces oxidative stress and regulates the functional expression of multidrug resistance protein-1 (Mrp1) in glial cells, J. Neurochem. 106 (2008) 1298–1313. doi:10.1111/j.1471-4159.2008.05479.x.

[42] A. Shah, S. Kumar, S.D. Simon, D.P. Singh, A. Kumar, HIV gp120- and methamphetamine-mediated oxidative stress induces astrocyte apoptosis via cytochrome P450 2E1., Cell Death Dis. 4 (2013) e850. doi:10.1038/cddis.2013.374.

[43] M. Saker, N. Soulimane Mokhtari, S.A. Merzouk, H. Merzouk, B. Belarbi, M. Narce, Oxidant and antioxidant status in mothers and their newborns according to birthweight, Eur. J. Obstet. Gynecol. Reprod. Biol. 141 (2008) 95–99. doi:10.1016/j.ejogrb.2008.07.013.

[44] M.B. Grisham, D. Jourd'Heuil, D. a Wink, Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation., Am. J. Physiol. 276 (1999) G315–G321.

[45] L.A. Darrow, M. Klein, M.J. Strickland, J.A. Mulholland, P.E. Tolbert, Ambient air pollution and birth weight in full-term infants in Atlanta, 1994-2004, Environ. Health Perspect. 119 (2011) 731–737. doi:10.1289/ehp.1002785.

[46] R.N. Naidoo, T. Robins, S. Batterman, G. Mentz, C. Jack, Ambient pollution and respiratory outcomes among schoolchildren in Durban, South Africa, South African J. Child Heal. 7 (2013) 127–134. doi:10.7196/sajch.598.

[47] M.S. Kramer, R.W. Platt, S.W. Wen, K.S. Joseph, A. Allen, A New and Improved Population-Based Birth Weight for Gestational Age, Pediatrics. 108 (2001) e35. doi:10.1542/peds.108.2.e35.

[48] Q. Ma, Role of Nrf2 in oxidative stress and toxicity, Annu. Rev. Pharmacol. Toxicol. 53 (2013) 401–426.

[49] D. Wink, J. Laval, The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo., Carcinogenesis. 15 (1994) 2125–2129. http://www.ncbi.nlm.nih.gov/pubmed/7955043.

[50] I. Romieu, H. Moreno-Macias, S.J. London, Gene by environment interaction and ambient air pollution., Proc. Am. Thorac. Soc. 7 (2010) 116–22. doi:10.1513/pats.200909-097RM.

[51] X. Cheng, C.H. Ku, R.C.M. Siow, Regulation of the Nrf2 antioxidant pathway by microRNAs: New players in micromanaging redox homeostasis, Free Radic. Biol. Med. 64 (2013) 4–11. doi:10.1016/j.freeradbiomed.2013.07.025.

[52] K. Duskova, P. Nagilla, H.-S. Le, P. Iyer, A. Thalamuthu, J. Martinson, Z. Bar-Joseph, W. Buchanan, C. Rinaldo, V. Ayyavoo, MicroRNA regulation and its effects on cellular transcriptome in human immunodeficiency virus-1 (HIV-1) infected individuals with distinct viral load and CD4 cell counts., BMC Infect. Dis. 13 (2013) 250. doi:10.1186/1471-2334-13-250.

[53] J. Huang, F. Wang, E. Argyris, K. Chen, Z. Liang, H. Tian, W. Huang, K. Squires, G. Verlinghieri, H. Zhang, Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes, Nat. Med. 13 (2007) 1241–1247. doi:10.1038/nm1639.

[54] C. Hetz, The unfolded protein response: controlling cell fate decisions under ER stress and beyond, Nat. Rev. Mol. Cell Biol. 13 (2012) 89–102.

[55] G.S. Hotamisligil, Endoplasmic reticulum stress and the inflammatory basis of metabolic disease, Cell. 140 (2010) 900–917.

[56] G.J. Burton, E. Jauniaux, Oxidative stress., Best Pract. Res. Clin. Obstet. Gynaecol. 25 (2011) 287–299. doi:10.1016/j.bpobgyn.2010.10.016.

[57] J.D. Malhotra, R.J. Kaufman, Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?, Antioxid. Redox Signal. 9 (2007) 2277–2293. doi:10.1089/ars.2007.1782.

CHAPTER 5

Discussion, Conclusion and Recommendations

In the African region, approximately 2.2 million deaths per annum are linked to living or working within an unhealthy environment. Of these 270,000 deaths are associated with neonatal conditions including PTB and LBW [1]. South Africa, a rapidly developing country that is heavily burdened by HIV/AIDS [2] and obesity [3], faces rapid deterioration of the environment and air quality as a consequence of a growing urban population. This is most notably observed within SD, where residents that largely comprise low-income households or informal settlements are living within close proximity to heavily industrialised areas. In addition to these dense communities living near busy roads, they rely mostly on domestic fuels for cooking and heating [4]. Therefore, these dense human settlements are continuously exposed to large amounts of AAP and are greatly at risk for adverse AAP effects including respiratory conditions and adverse birth outcomes [5].

In SA, individuals living within low-income households or informal settlements rely largely on healthcare within government hospitals. These hospitals are often overcrowded and have limited provisions to care for premature babies or those born with adverse birth outcomes [6, 7]. Therefore, it is essential to identify novel biomarkers or interventional therapies to help reduce the burden of adverse birth outcomes. To do this understanding the aetiology of adverse birth outcomes is crucial and to determine risk factors associated with individual susceptibility to these conditions. Ambient air pollution and OS have been implicated in adverse birth outcome aetiology, including genetic susceptibility. Pregnant women living within the SD have been reported to have increased OS markers [8], in addition to high pollution levels detected within SD [9, 10] makes for an ideal follow up study to determine their effects on adverse birth outcome susceptibility within this population. Therefore this study was conducted to investigate oxidative and endoplasmic stress responses within pregnant women exposed to AAP living within Durban (SA) and determined their effect on PTB and LBW susceptibility. In addition, HIV infection, obesity and gene-environment interactions were determined; this is paramount within SA context due to the heavy burden of these conditions.

First, it was shown that an increase in atmospheric NOx exposure induced OS via the formation of 8-OHdG adducts within pregnant women, this was most noticeably observed within SD and for women carrying female neonates. This direct association was identified after the initial

finding that SD had higher levels of atmospheric NOx and pregnant women living in SD had greater concentrations of serum 8-OHdG. Inhalation of atmospheric NOx and subsequent absorption into the lung leads to the formation of free radicals that are able to directly interact with macromolecules present including DNA. This induces the production of mutagenic 8-OHdG lesions leading to increased OS [11, 12]. Antioxidants such as GST are important controllers of OS; however, polymorphisms exist within GSTM1 and GSTP1 which have been shown to influence an individual's response to toxic insult viz. exposure to AAP [13–15]. Pregnant women carrying the variant GSTP1 genotypes had increased susceptibility to OS following NOx exposure. Although an increase in 8-OHdG did not directly influence the susceptibility to adverse birth outcomes, it may play a role in the reduction of GA. This reduction of GA as a consequence of increased atmospheric NOx levels was found to be significant only after maternal serum 8-OHdG levels were controlled within the multi-regression analysis. In addition, carrying the variant GSTP1 genotypes and a male neonate increased the risk of reduced GA. It should be noted that although the individual reduction of GA was minimal, on a global scale this negative shift may lead to increased incidence of nationwide PTB.

A polymorphism exists within OOG1; the key component within the DNA repair pathway that removes 8-OHdG adducts and reduces OS. This polymorphism (OGG1 Ser326Cys) has been suggested to affect BMI and therefore may predispose SA women to adverse effects of obesity [16]. In addition to obesity, SA is also heavily burdened with HIV infections where more than half the infected population are women [2, 3], which makes them susceptible to oxidative-related injury including adverse birth outcomes [17–19]. The OGG1 Ser326Cys polymorphism was associated with HIV and obesity. Pregnant women carrying the wt OGG1 Ser326Cys genotype were at greater risk for HIV associated LBW and PTB, this risk was found to increase in comorbid obese and HIV infected CC-carrying patients with those living in SD even more susceptible to HIV associated LBW. Women who were HIV+ and obese delivered larger BW neonates compared to HIV- obese women, therefore suggesting that comorbid HIV and obesity increases the risk for reduced BW and delivering LBW neonates. Women living within the heavily polluted SD and carrying the wt OGG1 Ser326Cys genotype had increased risk of delivering PTB neonates. Further investigation into the possible mechanism by which HIV, obesity and carrying the wt Ser326Cys OGG1 genotype increases the susceptibility of LBW and PTB may identify potential intervention strategies to help reduce the incidence of PTB with SA.

It has been reported that NO inhibits zinc-finger proteins, such as OGG1 [20, 21]. In addition, HIV patients present with increased levels of NO [22–24]. The levels of maternal serum NO were next investigated, as it may identify a potential pathway for adverse birth outcome susceptibility as a result of HIV or NOx exposure. As NO metabolites are known to initiate LP [25], maternal serum MDA levels were investigated in conjunction with NO levels. HIV infection within pregnant women induced maternal serum NO production with reciprocal action on MDA levels. Atmospheric NOx was not shown to directly affect the concentration of NO, however living within SD strengthened the reciprocal activity of NO and MDA.

This increase in NO, as a result of HIV induction, was shown to play a role in LBW aetiology through its ability to reduce neonatal BW and increased levels of NO present within mothers giving birth to LBW neonates. The reduction or inhibition of foetal growth may be due to placental and pulmonary inflammation, with changes in endothelial and vascular functions leading to reduced placental blood flow and transfer of oxygen and nutrients between mother and foetus. Upon entry into the blood stream, NO binds to Hb and forms methHb that has reduced oxygen carrying capacity. This may prevent sufficient oxygenation of the foetus leading to reduced foetal growth [11, 26]. It was interesting to note that women carrying male neonates where highly susceptible to reduced BW as a consequence of increased NO concentrations, similarly observed above with reduced GA due to increased NOx exposure.

In addition, HIV was shown to differentially affect MDAs effect on BW. In the presence of HIV infection, increased levels of MDA reduced neonatal BW. Whilst in the absence of HIV, increased levels of MDA increased neonatal BW. In the absence of HIV, antioxidants are able to scavenge free radicals which reduce the harmful effect of ROS and therefore would not impact negatively on foetal growth. However in the presence of HIV, the body is under chronic OS which induces high levels of NO and MDA above antioxidant capacity (decreased expression of *SOD2* and *CAT* were observed). Furthermore, NO inhibited *OGG1* gene expression, evidence by a negative association between serum NO and *OGG1* expression, which may compromise the efficiency of the DNA repair pathway leading to increased DNA adducts and OS. This may cause vascular tissue damage, increased inflammation and alterations of placental oxygen exchange which may reduce foetal growth [27, 28].

The key regulator of the antioxidant response, that endeavours to control oxidant homeostasis, is the transcription factor *Nrf2* [29]. The expression of *Nrf2* is under epigenetic control, where non-coding miRs including miR-144 and miR-28 have been described to suppress its expression

thereby preventing it from preforming its function [30–32]. This study provides further evidence for the inhibition of *Nrf2* expression as a result of miR-144 and miR-28 induction, as observed by the negative association between miR-144/miR-28 and *Nrf2*. The exposure to HIV infection and increased levels of atmospheric NOx induced miR-144 expression within pregnant women. This has been proposed as a possible mechanism for OS induction and subsequent reduction in GA and BW as a consequence of HIV/NOx exposure. Targeting this pathway, through the suppression of Nrf2 inhibitors or the induction of Nrf2, may be a potential therapeutic invention strategy for the reduction of adverse birth outcome incidences. Where upon the activation of Nrf2, stimulates antioxidant gene transcription leading to reduced OS which may mitigate oxidative related injuries. However, further investigation into this pathway using a larger study size, is required to confirm these findings.

Excessive ROS production, as observed within OS in response to high levels of pollution within SD, may lead to cellular calcium homeostasis. This disruption impairs the efficacy of the ER protein folding machinery resulting in the accumulation of UP and subsequent activation of the UPR [33]. It was observed that HIV- south living pregnant women were at greatest risk for ER stress, via the activation of the PERK-eIF2-ATF4 pathway, evidence by increased expression of *eIF2*, *ATF4* and *CHOP*. This may further increase ROS production, due to the reciprocal activity of ER stress on OS and vice versa, potentiating oxidant-related injury [34]. This may predispose pregnant women living within SD to adverse birth outcomes. Investigating the other two arms of UPR, i.e. IRE1α and ATF6, in future studies would give further insight into the mechanism of ER stress induced OS and possible negative impacts on foetal growth.

In conclusion, pregnant women living within SD and exposed to increased levels of atmospheric NOx and HIV infection have elevated OS which predisposes them to adverse birth outcomes. Implicated in the elevation of OS: induction of miR-144 leading to reduced Nrf2 control and antioxidant response, activation of the PERK-eIF2-ATF4 UPR pathway and the induction of NO, which plays a key role in HIV associated LBW/PTB. Pregnant women carrying the variant GSTP1 and wt OGG1 Ser326Cys genotypes were found to be susceptible to reduced GA and PTB, respectively. However, as disease aetiologies involve complex interactions between different genes and the environment, future studies focusing on multiple gene and environment interactions may give further insight into adverse birth outcome susceptibility as a result of AAP exposure.

The limitations for this study include a relatively small sample size; the power of the results would increase with a larger study population. In addition, this study focused on only one specific pollutant (NOx). As individuals are exposed to several pollutants within the air at once (e.g. PM. SO_2, CO and NOx), studying pollutant interactions, gene-pollutant and multiple gene-pollutant interactions may give further insight into the mechanistic link between elevated OS and adverse birth outcomes. Furthermore, the ethnicity of study participants should be considered (even though the majority of our study participants were black South Africans), as polymorphic susceptibilities vary among different racial groups.

References

1. WHO (2016) An estimated 12.6 million deaths each year are attributable to unhealthy environments. In: WHO News release. http://who.int/mediacentre/news/releases/2016/deaths-attributable-to-unhealthy-environments/en/. Accessed 20 Jan 2017
2. UNAIDS (2015) UNAIDS Fact sheet November 2015: Global HIV Statistics. http://www.unaids.org/en/regionscountries/countries/southafrica/. Accessed 16 Jan 2017
3. STATS SA (2016) South Africa Demographic and Health Survey 2016: Key Indicator Report. http://www.statssa.gov.za/publications/Report 03-00-09/Report 03-00-092016.pdf. Accessed 2 Aug 2017
4. DEA (2012) Chapter 5 : Air Quality Draft 2 version 2. http://soer.deat.gov.za/dm_documents/Chapter_5__Air_Quality_4kp8X.pdf. Accessed 30 Mar 2016
5. Fleischer NL, Merialdi M, Donkelaar A Van, et al (2014) Outdoor Air Pollution , Preterm Birth , and Low Birth Weight : Analysis of the World Health Organization Global Survey on Maternal and Perinatal Health. Environ Health Perspect 122:425–430. doi: 10.1289/ehp.1306837
6. GET_IT (2016) Premature births, a cause for concern – World Prematurity Day. In: GET IT Durban Online. http://durban.getitonline.co.za/2016/10/31/premature-births-a-cause-for-concern-world-prematurity-day/#.WhfiuVWWbIV. Accessed 22 Nov 2017
7. Soodyal N (2017) More medical support needed for premature babies: expert. In: East Coast Radio News Watch. https://www.ecr.co.za/news/news/more-medical-support-needed-premature-babies-expert/. Accessed 22 Nov 2017
8. Nagiah S, Phulukdaree A, Naidoo D, et al (2015) Oxidative stress and air pollution exposure during pregnancy: A molecular assessment. Hum Exp Toxicol 34:838–847.

doi: 10.1177/0960327114559992

9. Naidoo RN, Robins T, Batterman S, et al (2013) Ambient pollution and respiratory outcomes among schoolchildren in Durban, South Africa. South African J Child Heal 7:127–134. doi: 10.7196/sajch.598

10. Kistnasamy EJ, Robins TG, Naidoo R, et al (2008) The relationship between asthma and ambient air pollutants among primary school students in Durban, South Africa. Int J Environ Heal 2:365. doi: 10.1504/IJENVH.2008.020929

11. Tabacova S, Baird DD, Balabaeva L (1998) Exposure to oxidized nitrogen: lipid peroxidation and neonatal health risk. Arch Env Heal 53:214–221. doi: 10.1080/00039899809605698

12. Kershaw RM, Hodges NJ (2012) Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. Mutagenesis 27:501–510. doi: 10.1093/mutage/ges012

13. Mustafa MD, Pathak R, Ahmed T, et al (2010) Association of glutathione S-transferase M1 and T1 gene polymorphisms and oxidative stress markers in preterm labor. Clin Biochem 43:1124–1128. doi: 10.1016/j.clinbiochem.2010.06.018

14. Slama R, Gräbsch C, Lepeule J, et al (2010) Maternal fine particulate matter exposure, polymorphism in xenobiotic-metabolizing genes and offspring birth weight. Reprod Toxicol 30:600–612. doi: 10.1016/j.reprotox.2010.07.001

15. Romieu I, Ramirez-Aguilar M, Sienra-Monge JJ, et al (2006) GSTM1 and GSTP1 and respiratory health in asthmatic children exposed to ozone. Eur Respir J 28:953–959. doi: 10.1183/09031936.06.00114905

16. Hara M, Nakamura K, Nanri H, et al (2014) Associations between hOGG1 Ser326Cys polymorphism and increased body mass index and fasting glucose level in the Japanese general population. J Epidemiol 24:379–84. doi: http://dx.doi.org/10.2188/jea.JE20140002

17. Amorosa V, Synnestvedt M, Gross R, et al (2005) A tale of 2 epidemics: the intersection between obesity and HIV infection in Philadelphia. J Acquir Immune Defic Syndr 39:557–561.

18. Smith GCS, Shah I, Pell JP, et al (2007) Maternal obesity in early pregnancy and risk of spontaneous and elective preterm deliveries: A retrospective cohort study. Am J Public Health 97:157–162. doi: 10.2105/AJPH.2005.074294

19. Xiao P-L, Zhou Y-B, Chen Y, et al (2015) Association between maternal HIV infection and low birth weight and prematurity: a meta-analysis of cohort studies. BMC Pregnancy

Childbirth 15:1–11. doi: 10.1186/s12884-015-0684-z

20. Wink D, Laval J (1994) The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. Carcinogenesis 15:2125–2129. doi: 10.1093/carcin/15.10.2125

21. Jaiswal M, LaRusso NF, Nishioka N, et al (2001) Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. Cancer Res 61:6388–6393.

22. Torre D, Pugliese A, Speranza F (2002) Reviews Role of nitric oxide in HIV-1 infection : friend or foe ? 2:273–280.

23. Giovannoni G, Miller RF, Heales SJR, et al (1998) Elevated cerebrospinal fluid and serum nitrate and nitrite levels in patients with central nervous system complications of HIV-1 infection: A correlation with blood-brain-barrier dysfunction. J Neurol Sci 156:53–58. doi: 10.1016/S0022-510X(98)00021-5

24. Torre D, Ferrario G, Speranza F, et al (1996) Serum concentrations of nitrite in patients with HIV-1 infection. J Clin Pathol 49:574–576. doi: 10.1136/jcp.49.7.574

25. Calabrese V, Cornelius C, Rizzarelli E, et al (2009) Nitric Oxide in Cell Survival: A Janus Molecule. Antioxid Redox Signal 11:2717–2739. doi: 10.1089/ars.2009.2721

26. Castiglione N, Rinaldo S, Giardina G, et al (2012) Nitrite and Nitrite Reductases: From Molecular Mechanisms to Significance in Human Health and Disease. Antioxid Redox Signal 17:684–716. doi: 10.1089/ars.2011.4196

27. Ivanov A, Values-Elliston VT, Ivanova ON, et al (2016) Oxidative Stress During HIV Infection: Mechanisms and Consequences. Oxid Med Cell Longev 2016:1–18. doi: 10.1155/2016/8910396

28. Darrow LA, Klein M, Strickland MJ, et al (2011) Ambient air pollution and birth weight in full-term infants in Atlanta, 1994-2004. Environ Health Perspect 119:731–737. doi: 10.1289/ehp.1002785

29. Niture SK, Khatri R, Jaiswal AK (2013) Regulation of Nrf2–An update. Free Radic Biol Med 66:36–44. doi: 10.1016/j.freeradbiomed.2013.02.008

30. Sangokoya C, Telen MJ, Chi J, Dc W (2010) microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. Blood 116:4338–48. doi: 10.1182/blood-2009-04-214817

31. Yamamoto M, Singh A, Sava F, et al (2013) MicroRNA expression in response to controlled exposure to diesel exhaust: attenuation by the antioxidant N-acetylcysteine in a randomized crossover study. Environ Health Perspect 121:670–5. doi:

10.1289/ehp.1205963

32. Yang M, Yao Y, Eades G, et al (2011) MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. Breast Cancer Res Treat 129:983–91. doi: 10.1007/s10549-011-1604-1

33. Cao SS, Kaufman RJ (2012) Unfolded protein response. Curr Biol 22:R622-6. doi: 10.1016/j.cub.2012.07.004

34. Malhotra JD, Kaufman RJ (2007) The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol 18:716–731.

APPENDIX 1

Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

22 April 2013

Dr. Rajen Naidoo
Discipline of Occupational and Environmental Health
University of KwaZulu-Natal
Howard College
Durban
4041

PROTOCOL: The Mother and Child in the Environment (THE MACE STUDY). REF: BF263/12.

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was provisionally approved by a quorate meeting of BREC on 09 October 2012 pending appropriate responses to queries raised. Your responses dated 12 April 2013 to queries raised on 06 March 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 22 April 2013.

This approval is valid for one year from **22 April 2013.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx. BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

The following Committee members were present at the meeting that took place on 09 October 2012:

Prof D Wassenaar	Chair
Prof V Rambiritch	Pharmacology
Prof A Coutsoudis	Paediatrics & Child Health
Dr T Crankshaw	External - Public Health
Mr Q Dlamini	Law
Dr U Govind	Private Pract. - Gen. Practitioner
Dr T Hardcastle	Surgery - Trauma
Dr Z Khumalo	KZN Health (External) - General Medicine
Prof TE Madiba	General Surgery
Dr R Maharaj	Obstetrics & Gynaecology
Dr K Naidoo	Family Medicine
Ms N Mhlongo	External - Lay member
Dr S Paruk	Psychiatry
Prof DJ Pudifin	Medicine
Prof C Rout	Anaesthetics
Dr MA Sathar	Medicine
Dr D Singh	Critical Care
Prof J Tsoka-Gwegweni	Public Health

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

PROFESSOR D R WASSENAAR
Chair: Biomedical Research Ethics Committee

APPENDIX 2

Determination of Single Nucleotide Polymorphism Genotypes

As described within the methodology sections of chapter 2 and 3, the genotypes of SNPs were determined using the differential PCR or PCR-RFLP. The visualisation of the amplification products after electrophoresis was done using the Bio-Rad ChemiDoc™ XRS+ System and Image Lab™ Software. The following band images were obtained to represent the genotypes of the SNPs investigated:

Chapter 2 – The effect of NOx pollution on oxidative stress in pregnant women living in Duran, South Africa

GSTM1 SNP genotypes

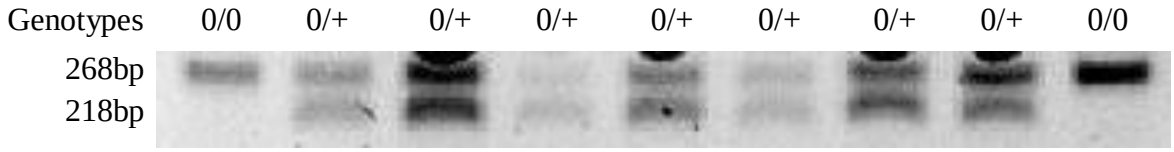

Figure A2.1 Visualisation of GSTM1differential PCR amplicons after electrophoresis, representing the genotypes of the GSTM1 SNP. The 0/+ genotype is represented by two bands, a 268bp and 218bp amplicon. The 0/0 genotype is represented by one band, the 268bp amplicon.

GSTP1 SNP Genotypes

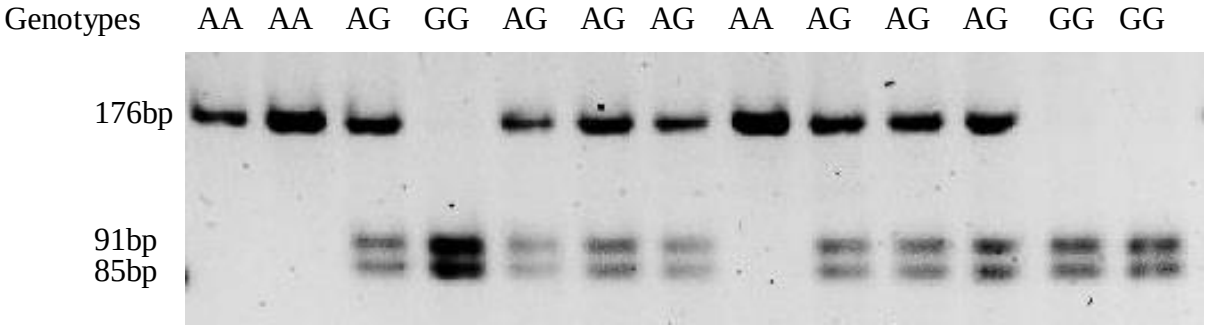

Figure A2.2 Visualisation of GSTP1 PCR-RFLP amplicons after electrophoresis, representing the genotypes of the GSTP1 SNP. The homozygous AA genotype is represented by a single band (176bp), the heterozygous AG genotype is represented by three bands (176bp, 91bp and 85bp), whilst the homozygous GG genotype is represented by two bands (91bp and 85bp).

Chapter 3 – OGG1 Ser326Cys polymorphism, HIV, obesity and air pollution exposure influences adverse birth outcome susceptibility, within South African Women

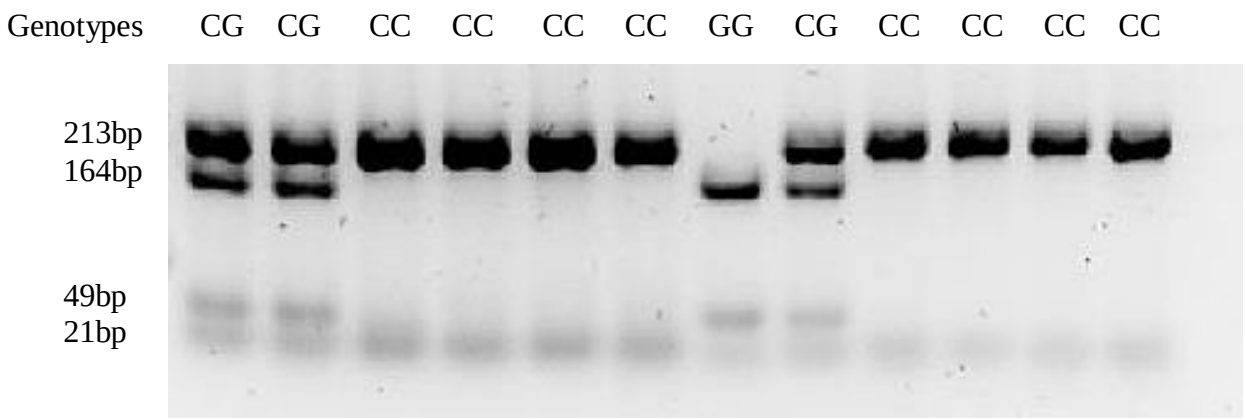

Figure A2.3 Visualisation of the OGG1 PCR-RFLP amplicons after electrophoresis, representing the genotypes of the OGG1 SNP. All genotypes have a 21bp amplicon due to a *Sat1* invariant site induced by the mismatch. The homozygous CC genotype is represented by a one band (213bp), the heterozygous CG genotype is represented by three bands (213bp, 164bp and 49bp), whilst the homozygous GG genotype is represented by two bands (164bp and 49bp).

APPENDIX 3

Quantification of 8-oxoguanosine – Standard curve for Chapter 2

The OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs. Inc.) was used to quantify the concentration of 8-OHdG within maternal serum samples. Ten 8-OHdG standards were prepared through serial dilution, where 10µL of the 8-OHdG was diluted in 990µL of assay diluent, followed by 8 dilutions of 500µL assay diluent. The last standard would contain only assay diluent which would set up 10 standards of 8-OHdG with concentrations ranging from 0-20ng/mL. A standard curve was set up following the completed assay as per manufacturer's instructions (Figure A3.1). This was used to generate a logarithmic equation for the best fit line which was used to extrapolate the concentrations of the unknown samples.

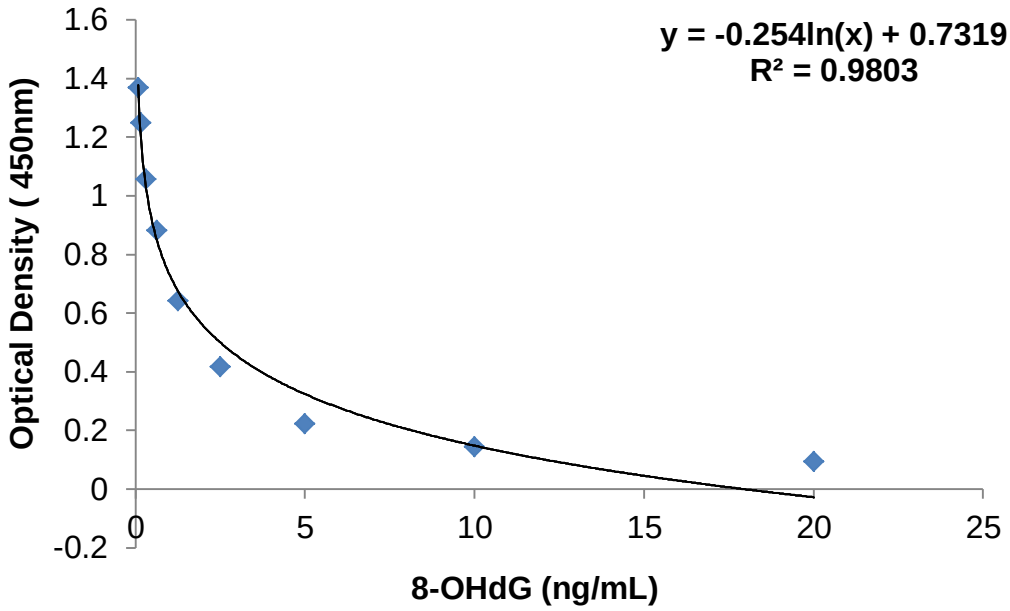

Figure A3.1 Standard curve using known 8-OHdG concentrations (in duplicate) to extrapolate the 8-OHdG concentration within unknown serum samples using the OxiSelect™ Oxidative DNA Damage ELISA.

APPENDIX 4

Chapter 3 – OGG1 Ser326Cys polymorphism, HIV, obesity and air pollution exposure influences adverse birth outcome susceptibility, within South African Women – Supplementary Material

Table A4.1 OGG1 Genotypic and obesity frequency distributions among patients delivering LBW or NBW neonates, at term (TB) or pre-term (PTB) that are HIV+ and HIV- (n=302)

	Birth Weight (g)			Gestational Age (weeks)		
	NBW n(%)	LBW n(%)	p-value	TB n(%)	PTB n(%)	p-value
Non-obese, CC genotype						
T3						
				56 (90.32)	6 (9.68)	***0.024**
				17 (70.83)	7 (29.17)	
Obese, CC genotype						
T3						
HIV-	44 (93.62)	3 (6.38)	***0.029**			
HIV+	39 (78)	11 (22)				
Obese, South living						
T1						
HIV-	45 (97.83)	1 (2.17)	***0.022**			
HIV+	19 (82.61)	4 (17.39)				

n=sample size, NBW= normal birth weight, LBW= low birth weight, TB=term birth, PTB= pre-term birth
NBW (>2500g), LBW (<2500g), TB (>37 weeks), PTB (<37 weeks)
Statistical significance: *$p<0.05$*

Table A4.2 Impact of maternal clinical characteristics on the risk of low birth weight – a multi-variate logistic regression analysis (n=128)

Variable	OR[a] (95%CI)	p-value
	Low Birth Weight	
HIV	0.844 (0.045, 15.74)	0.910
Maternal age	1.10 (0.783, 1.55)	0.578
BMI	1.00 (0.999, 1.00)	0.482
Hb	1.01 (0.960, 1.06)	0.746
Iron	0.995 (0.956, 1.04)	0.836
BP systolic	1.00 (0.999, 1.00)	0.770
BP diastolic	0.999 (0.996, 1.00)	0.135
OGG1 (CC genotype)	0.407 (0.013, 12.99)	0.611
Area (South)	0.959 (0.028, 33.34)	0.982
NOx levels	1.23 (0.990, 1.54)	0.061
	R^2 = 0.7930 (**p<0.0001)	

Results described: OR, (95%CI), p-value with regression model R^2-value and p-value
n= sample size, OR= odds ratio, CI= confidence interval, HIV= HIV positive, BMI= body mass index, Hb= haemoglobin, BP= blood pressure, OGG1 represents CC genotype, Area represents heavily polluted south
[a]Adjusted for maternal characteristics: HIV status, BMI, area, NOx levels, maternal age, haemoglobin, iron, blood pressure systolic and diastolic, OGG1 CC SNP and neonatal characteristics: low birth weight, neonate gender, parity, body: brain index, ponderal index, Apgar scores 1min and 5min (head circumference and neonatal length weren't adjusted for because of collinearity)
Statistical significance: **p<0.01, *p<0.05*

Table A4.3 OGG1 Genotypic frequency distributions among patients delivering male and female neonates that are NBW/LBW, at TB/PTB that are HIV+ and HIV- (n=302)

	Birth Weight (g)			Gestational Age (weeks)		
	NBW n(%)	LBW n(%)	p-value	TB n(%)	PTB n(%)	p-value
Male						
HIV-	128 (88.28)	17 (11.72)	0.417	137 (94.48)	8 (5.52)	*0.024
HIV+	71 (84.52)	13 (15.48)		72 (85.71)	12 (14.29)	
Female						
HIV-	132 (88.59)	17 (11.41)	0.073	133 (89.86)	15 (10.14)	0.813
HIV+	54 (79.41)	14 (14.29)		60 (90.91)	6 (9.09)	
Male CC						
HIV-	53 (88.33)	7 (11.67)	0.440	56 (93.33)	4 (6.67)	*0.05
HIV+	34 (82.93)	7 (17.07)		33 (80.49)	8 (19.51)	
Male CG+GG						
HIV-	29 (82.86)	6 (17.14)	0.726	33 (94.29)	2 (5.71)	0.322
HIV+	11 (78.57)	3 (21.43)		12 (85.71)	2 (14.29)	
Female CC						
HIV-	45 (91.84)	4 (8.16)	*0.024	44 (89.80)	5 (10.20)	0.325
HIV+	25 (73.53)	9 (26.47)		28 (82.35)	6 (17.65)	
Female CG+GG						
HIV-	33 (89.19)	4 (10.81)	0.890	33 (89.19)	4 (10.81)	0.362
HIV+	7 (87.50)	1 (12.50)		7 (100)	0 (0)	

n=sample size, NBW= normal birth weight, LBW= low birth weight, TB=term birth, PTB= pre-term birth
NBW (>2500g), LBW (<2500g), TB (>37 weeks), PTB (<37 weeks)
Statistical significance: *$p<0.05$*

APPENDIX 5

Quantification of nitrites and nitrates – Standard curve for Chapter 4

An in-house method for the quantification of NO metabolites (nitrites/nitrates) was used to determine the concentration of NO within maternal serum samples. Nine sodium nitrate standards were prepared through serial dilution. The last standard would contain only dH_2O which would set up 9 standards of known nitrate concentration ranging from 0-200µM. After the assay was performed, as described in chapter 4 methodology section, a standard curve was set up (Figure A5.1). This was used to generate a linear equation for the best fit line which was used to extrapolate the concentrations of the unknown serum samples.

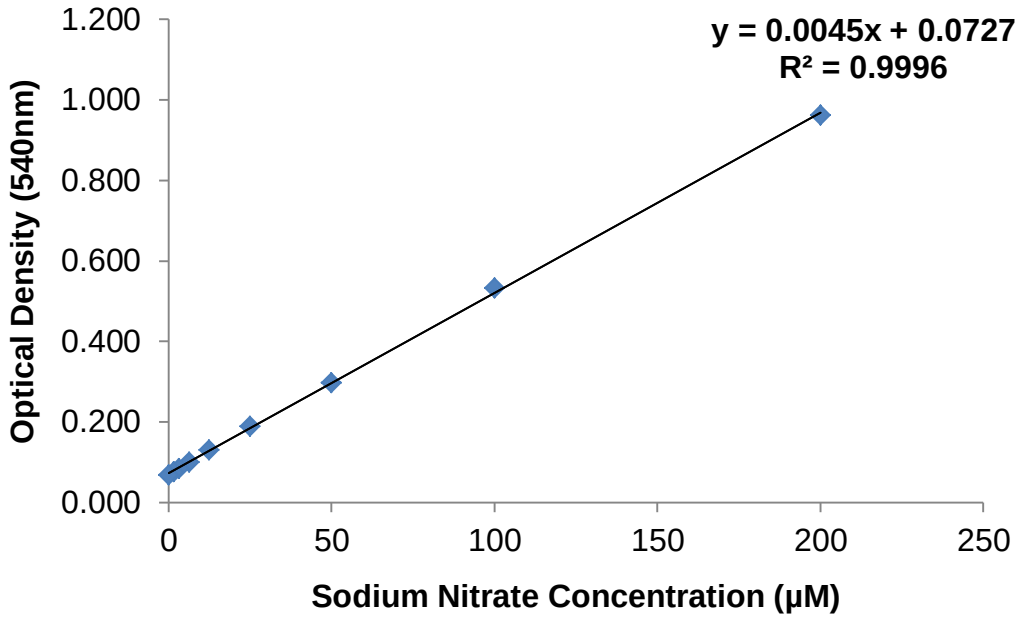

Figure A5.1 Standard curve set up with known sodium nitrate concentrations (in duplicate) to extrapolate the NO concentration within unknown serum samples.

APPENDIX 6

Chapter 4 – HIV induces nitric oxide and lipid peroxidation, which influences neonatal birthweight in a South African population – Supplementary Material

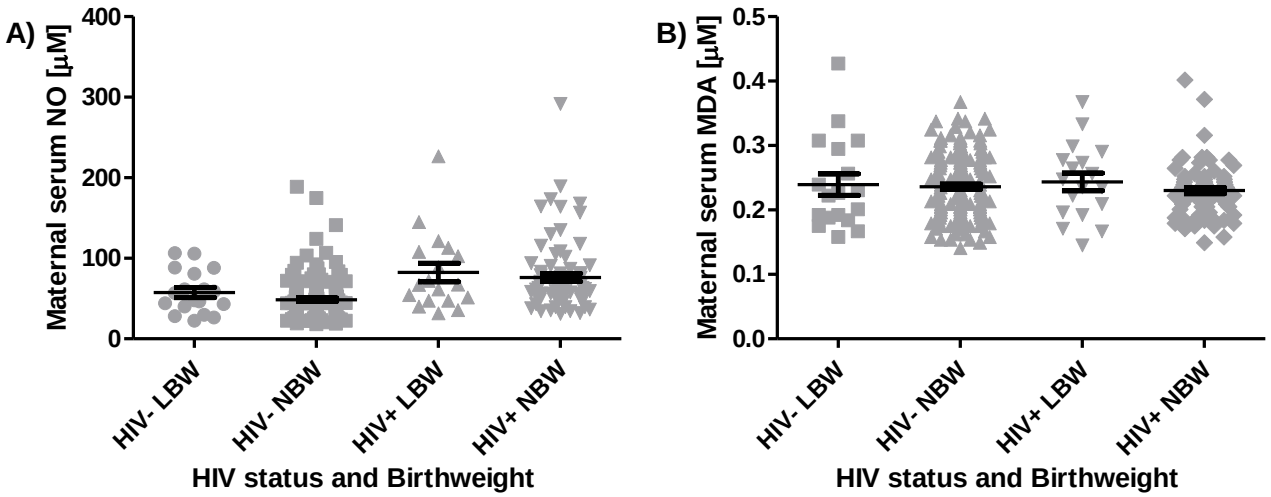

Figure A6.1 Maternal serum NO (µM) (A) and MDA (µM) (B) levels between HIV+ and HIV- mothers who deliver low birthweight (LBW) (<2500g) and normal birthweight (NBW) (>2500g) neonates.