A genetic epidemiological study of prevalence and association of genetic polymorphisms in asthma related phenotypes among children in Durban, Kwazulu-Natal.

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I, Michelle Makamure, declare that this is my own unaided work. This work has not been submitted previously to this or any other University.

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Abstract

Several genes are associated with an increased susceptibility to respiratory diseases, including asthma, which may be exacerbated by ambient air pollution. These genes include the Tumour Necrosis Factor alpha (TNFα) gene and the Cluster of Differentiation 14 (CD14) gene

A total of 104 schoolchildren from seven primary schools in a heavily industrialized region of south Durban participated in the study. For the purpose of this study, DNA was extracted from whole blood using the GENTRA Puregene kit. Genotyping for the TNF-308α G/A polymorphism was conducted using restriction fragment length polymorphism (RFLP) polymerase chain reaction (PCR). The CD14 (-159) C/T genotype was determined using real time PCR and Taqman probes (Applied Biosystems). Multiple logistic models and Pearson's chi-squared tests were used to evaluate the association between any asthma, BHR, atopy, persistent asthma and genotype. Covariate-adjusted generalised estimating equations (Martin *et al.*) with lags of 1-5 days were used to evaluate genotype effect modification of exposure-response.

The TNF-308α variant A allele was quite common in the population, it was detected in more than forty percent of the population and with an allelic frequency of 0.24. Similarly almost 38% of the population carried the variant CD14 (-159) T allele, with an allelic frequency of 0.24. TNF-308α G/A and CD14 (-159) C/T polymorphisms were not associated significantly with asthma, and its related respiratory phenotypes. In addition there was no association detected between any of the gene polymorphisms and the levels of their respective cytokine proteins. Increased TNFα levels were associated with persistent asthma. On the other hand lower sCD14 levels were associated with

atopy in children. There was a significant relationship between TNF- α levels and acute asthma (p=0.03) and sCD14 levels and atopy (p=0.04)

GEE models showed that the TNF- 308-α A allele carriers had a greater deterioration of lung function post pollution exposure to SO_2 (intraday variability FEV_1 readings lag 2) $_{\beta}$= 2.62, CI (0.51, 4.71) p= 0.02 and p (interaction=0.03). There was a statistically significant gene environment interaction with NO in individuals who were carriers of the TNF- A allele (Nadir of PF readings lag 2: β= -12.3, CI (-22.09, -2.51), p=0.01 p (interaction) =0.03.and 5 day average β= -42.83, CI (-70.11,-15.55), p≤0.005 and p (interaction) =0.01). With analysis of the CD14 gene polymorphism gene environment interaction, adverse effects of SO_2 were limited to individuals carrying the C allele of this polymorphism, β= -1.50, CI (-0.36, 3.37), p=0.01, p (interaction) =0.01. Carriers of the T allele seemed to have a protective effect with NO_2 and NO exposure. Intraday variability of FEV_1 improved 5 days post exposure to NO_2 β= -4.02, CI (-6.52,-1.53), p=0, p (interact) =0.05. There was also improvement five days post exposure to NO β= -9.42, CI (-12.45, -6.03), p= p≤0.005, p (interact) ≤0.005

There was no association of co-inheritance of the 2 gene polymorphisms, CD14 (-159) C/T and TNF-308α G/A, and protein expression or respiratory phenotype. The GEE model results were consistent with modification of air pollutant-pulmonary function relationships by proinflammatory cytokine associated genotypes.

Results indicate that genetic susceptibility combined with exposure to pollutants causes adverse respiratory effects. This study supports the importance of further investigation on these and other genotype variants involved in inflammation and respiratory linked phenotypes in larger cohorts.

Dedication

For the Children of South Africa and the world.

They deserve to live healthy lives free from pollution and disease

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Allele:	alternative forms of a gene or locus marker due to changes at the level of the DNA
Allergy:	is an immunological state where ubiquitous harmless substances are recognized as allergens by the immune system leading to complex defense mechanisms of chronic inflammation
ATS:	American Thoracic Society
BHR:	Bronchial Hyperresponsiveness
CD14:	Cluster of Differentiation 14: a pattern recognition receptor that can interact with a variety of bacterial ligands
sCD14:	soluble Cluster of Differentiation 14
CI:	Confidence interval
CO:	Carbon monoxide
COPD:	Chronic Obstructive Pulmonary Disease
DEAT	South African Department of Environmental Affairs and Tourism
DEPs:	Diesel Exhaust Particles
EDTA:	Ethylene diamine tetraacetic acid
EPA:	Environmental Protection Agency
ETS:	passive smoking or exposure to secondhand smoke is defined as the exposure of a nonsmoking person to tobacco combustion products from smoking by others
Exposure:	Exposure is defined as the “contact between a target organism and a pollutant at the outer boundary of the organism”, which is specifically the inhalation route in this study. Exposure may be quantified as the amount of pollutant available at the boundary of the receptor organism per specified time period.

FEV_1: Forced expiratory volume in one second

FVC: Forced Vital Capacity

GEE: Generalised estimating equations

Gene: an individual unit of hereditary. It is a specific instruction that directs the synthesis of a protein or ribonuclease acid product. Each gene is located at a specific site (locus) on a chromosome.

Genetic Model: The overall specification of how the diseases alleles act to influence the disease

Genome: the sum of all genetic information of an organism.

Genotype: Refers to the precise allelic makeup of an organism or cell. In reference to a specific gene, the genotype is the pair of alleles that occur at the chromosomal site of the gene, which may be the same (homozygous) or different (heterozygous).

GM-CSF: Granulocyte macrophage colony stimulating factor

Heterozygous: The alleles at a genetic locus are different from one another on the two partners of a chromosome pair.

Homozygous: the alleles at a genetic locus are identified on the two partners of a chromosome pair.

IgE: Immunoglobin E

Interquartile range: The interquartile range (IQR) is the distance between the 75^{th} percentile and the 25^{th} percentile. The IQR is essentially the range of the middle 50% of the data. Because it uses the middle 50%, the IQR is not affected by outliers or extreme values.

ISAAC: International Study of Asthma and Allergies in Childhood

NHANES III: The third national health and nutrition examination survey

NO: Nitrogen oxide

NO_2: Nitrogen Dioxide

O_3: Ozone

OR: Odds ratio

PC_{20}: Dose of methacholine causing a 20% drop in baseline FEV_1

PCR: Polymerase Chain Reaction

PF: Peak Expiratory Flow

Phenotype: Refers to the observed attribute, in this case, asthma or a component of asthma such as bronchial hyperresponsiveness.

PM_{10}: Respirable particulate matter less than 10 microns

Polymorphism: A tendency for a gene to exist in more than one form, or the specific alleles thereof.

ppb: parts per billion

SDHS: South Durban Health Study

SDIB: South Durban Industrial Basin

SNP: Single Nucleotide Polymorphism

SO_2: Sulphur Dioxide

Susceptibilty: the degree to which a person or a population is sensitive to either adverse or protective exposures in developing asthma.

TNFα: Tumor necrosis Factor alpha. TNF-α is a 157 amino acid pro-inflammatory cytokine protein manufactured by white blood cells to stimulate and activate the immune system in response to cancer, infection, exposure to endotoxin, or other products of bacterial, viral, parasitic or inflammatory origin

TNF-308α G/A: a promoter gene polymorphism of the TNFα gene

VOCs: Volatile organic compounds

WHO World Health Organization

Chapter 1 Introduction

1.1 Background

Asthma is complex disease characterized by airway inflammation, bronchial hyperresponsiveness and airflow obstruction. It is the most common chronic disease of childhood, and has increased in prevalence over the last 3-4 decades (Custovic *et al.* 2003). Studies suggest that the effects of air pollution vary among individuals because of the variation in individual genetic susceptibility (Kleeberger 2003). There are two major reasons to investigate genetic susceptibility to air pollution effects in humans. The first is that the effects of air pollution on respiratory outcomes are modest in the general population because the population includes individuals relatively resistant to air pollution. Thus, the ability to detect subtle effects of air pollution may depend on the ability to identify susceptible subpopulations. Second, susceptible groups might experience health effects at levels below current exposure standards. An essential question is: are environmental influences associated with asthma more likely to affect people with certain genetic profiles? It is therefore necessary to simultaneously study genetic variants in association with asthma related phenotypes and environmental exposures.

Understanding the biology of diseases may potentially lead to the development of strategies based on mass intervention or target risk intervention approaches. This could facilitate the setting up of a genetic risk profile for the development of asthma which would enable us to take preventative measures early in life for children with an increased genetic risk to allergic diseases. It could also allow the development of secondary preventative measures (the development of primary tests for population wide screening) and therapy (choosing among

alternative interventions or assist in the design of new drugs which are more specific, effective and safe).

Many genes have been implicated in asthma pathogenesis, but only those with a functional and biological relevance were chosen for this study. Since 2005, Single nucleotide polymorphisms (SNPs) in Cluster of differentiation 14 SNP C 159T [CD14 (-159) C/T] and Tumour necrosis factor α (TNF-308α) have been implicated in asthma pathogenesis.

The South Durban Industrial Basin (SDIB) is situated on the east coast of South Africa and has one of the highest concentrations of industrial activity in Africa. Discriminatory land use planning during the Apartheid era had placed a large residential community very close to industry and community in this region had previously requested an independent investigation into the air quality and health status in the SDIB. A completed study in Durban done in 2004-2005 involved repeated measures of pollutant exposures, across different seasons, near the vicinity of a cohort of schoolchildren aged 9-11 years old. This study called South Durban Health Study (SDHS) measured the health effects of pollutant exposures on children resident in the basin.

We conducted a retrospective study using stored bloods and health-environmental exposure data generated by the SDHS. The blood was collected from two areas of Durban one in the south and one in the north. In this South African population of children, the frequency of each these genetic polymorphisms and the risk conferred by that polymorphism on respiratory outcomes, was determined. We determined whether the relationship between exposure (to ambient air pollutants) and respiratory outcome was modified by genotype. A search of current literature revealed that there are no studies from

the African subcontinent on these genes in relation to environmental exposures and the asthma outcomes discussed in this thesis.

1.2 Research Problem

The increase in the prevalence of asthma during the past decades can only be explained by changes in the environment. But the question may arise “Why are some individuals and some families affected more easily than others? “ The answer may lie in the inter-individual genetic variation in response to environmental agents.

1.3 Aim

The aim of this study was to investigate the prevalence and association of single nucleotide polymorphisms (SNPs) CD14 (-159) C/T and TNF-308α G/A with the respiratory phenotype among children in Durban, KwaZulu-Natal and to evaluate the gene environment effect on respiratory outcomes.

1.3.1 Research Aims

The following were the aims of this study:

Research Aims

- To assess the frequency of gene variants CD14 (-159) C/T and TNF-308 (G/A) among schoolchildren from 2 areas in Durban.
- To evaluate the contribution of the two gene variants to various respiratory phenotypes
- To investigate fluctuations in lung function measures (FEV_1 and PF) in relation to daily averages in ambient air pollutants ($S0_2$, $N0_2$, NO, and PM_{10}) using genotypes as an effect modifier among the population of schoolchildren

Chapter 2 Literature Review

2.1 South Durban Industrial Basin (SDIB)

The South Durban Basin (SDB) extends from the Durban Central business district southward to Umbogintwini. The land use in the basin is mainly residential and industrial. This area is one of the most highly industrialized and most heavily polluted areas in Southern Africa and Africa as a whole. The basin contains two large petroleum refineries, a paper mill, a large chemical tank farm, land fill sites, incinerators, processing and manufacturing industries, major trucking, harbor and rail facilities, and other industries. Exacerbating the problem is the convergence of major traffic routes and geographical factors (metrology and complex topography) which predispose the area to poor pollution dispersion potential particularly during winter. There are some 200 000 people living in the suburbs of the Bluff, (including Bayhead and island View), Clairwood, Jacobs, Mobeni, Wentworth, Merebank, Merewent, Prospecton, Isipingo and Lamontville, around the Basin (Guastelle and Knudsen 2007). The siting of communities on the doorstep of industry was a legacy of poor apartheid planning practices dating back to the late 1950s to locate working class communities close to the workplace (Guastelle and Knudsen 2007).

2.1.1 Pollution in the Basin

The major sources of pollution in the South Durban basin are industry, transport, domestic fuel burning, biomass burning and pollutants transported via regional air movements (e.g. from the interior of the country). Communities in the SDB started to express concern about deteriorating air quality as far back as the 1960's, and efforts intensified in the 1980's and 1990's as air quality deteriorated

even further. This led to the formulation of the South Durban Multi-point Plan November 2000 which in turn led to the South Durban Health study (Naidoo *et al.* 2007)

2.1.2 South Durban Health Study (SDHS)

The South Durban Health Study (SDHS) was designed in response to the Multipoint Plan which was proposed at governmental level to understand the state of pollution in the South Durban Area, to develop a system to monitor fluctuations in pollution levels and to determine the extent to which pollution adversely impact on the health of the community (Naidoo *et al.* 2007). A longitudinal study was conducted from May 2004 to February 2005. It was the first study on the African continent to involve repeated measures of pollutant exposures, across different seasons, among a cohort of schoolchildren. In order to measure the health effects of exposure, daily lung function measures, Forced expiratory volume (FEV_1)(the maximal amount of air that can be exhaled in a period of time, usually one second) and peak flow (PF) (the maximum flow rate of air breathed out during forced expiration) were taken daily for a period of 3 weeks in each of 4 intensive phases. The participants were selected from four schools in south Durban and three in north Durban (Table 2.1).

Table 2.1: Schools enrolled in the South Durban Health study and their location

North Durban	South Durban
Briardale School (Newlands West)	Assegai School (Wentworth/Austerville)
Ferndale School (Newlands East)	Nizam School (Merebank)
Ngazana School (KwaMashu)	DirkieUys School (Bluff)
	Entuthukweni School (Lamontville)

Each child underwent baseline assessments, genetic profiling and allergen testing. Interviews were conducted with the child and the caregiver. The study

consisted of four intensive 3-week phases where air pollutant exposures were monitored either continuously or integrated over 24-hour periods, with simultaneous bihourly assessment of students while at school. The four study periods were scheduled to ensure seasonal variability was accounted for. The bi-hourly assessments consisted of lung function tests, including peak expiratory flow and forced expiratory volume in one second. A symptom and activity log was also completed for the preceding one and-a-half hour period (Naidoo *et al.* 2007).

The monitoring system that was implemented in the south Durban study provided information on a wide range of pollutants. The study found that the major sources of nitrogen dioxide (NO_2) pollution in South Durban are traffic and industry. This is due to the convergence of major traffic routes in the vicinity of the basin. It was also found that the annual guideline value for NO_2 of 21.0 parts-per-billion (ppb) had been exceeded at the Warwick and Ganges stations for all years(2004 to 2006) and at the City Hall station for 2004. These results were primarily all representative of vehicular traffic. Exceedances of the one-hour NO_2 guideline of 106 ppb have fluctuated from 40 in 2004, to 13 in 2005 to 84 in 2006. Particulate Matter of 10 micrometres or less (PM_{10}) concentrations were mainly influenced by traffic, industry, regional biomass burning, dust and salt. Annual average PM_{10} concentrations limit was exceeded at the Ganges station during 2004 and 2005. The epidemiological component of the study found that moderate ambient concentrations of NO_2, NO, and SO_2 were significantly associated with reduced lung function among children with persistent asthma. It was also found that modest increase in air pollution adversely affected pulmonary function of sensitive subpopulations. Attending a primary school in South Durban as compared to schools in the north of the city was significantly associated with an increased risk for persistent asthma and for marked airway hyper-reactivity. This study had several important advantages.

First, the study population of children exposed to ambient pollutants was confined to defined areas, each area with its own monitoring sites allowing a more precise estimation of exposure. Second, the pollutants were analysed in a systematic manner over the duration of the study, which allowed the correlation between increases in exposure and decrements in lung function measures. Thirdly, repeated measures of lung function and reported symptoms over a year provided additional power to identify specific impacts on susceptible groups (Naidoo *et al.* 2007; Reddy 2007).

The current study contributes to the genetic component of the SDHS which involves the investigation of TNF-308α and CD14 (-159) gene-disease-exposure relationship. The study focused on these genes because both CD14 and TNF-308α proteins have both been found to be elevated in the lungs and other tissues in the body as a result of inflammation infection or allergen provocation (Roitt and Delves 1992; Martinez 2007). Numerous studies have shown that the variant alleles of these genes have an impact on cytokine production and subsequently the respiratory phenotype.

An increase in the body of knowledge of asthma may potentially lead to the development of strategies based on mass intervention or target risk intervention approaches, this could facilitate the setting up of a genetic risk profile for the development of asthma that would enable us, for the first time, to take preventative early in life for children with an increased genetic risk to allergic diseases, secondary prevention (such as the development of primary tests for population wide screening) and therapy (such as choosing among alternative interventions or assist in the design of new drugs which are more specific, effective and safe (Reddy 2007).

This information will contribute in answering the question “Why are some individuals and some families affected more easily than others despite living in similar environments? “ The answer may lie in the inter-individual genetic variation in response to environmental agents.

However there are few studies that examine the relationship between genetic risk factors and environmental exposures in the exacerbation of asthma. Most of these studies were conducted mainly in the Northern hemisphere with Caucasian, Hispanic and Asian populations. In order to compare asthma prevalence with environmental exposures, other authors have used mortality data, admission records, absenteeism and activity limitation with cross sectional designs. The advantage of this study is the use of a longitudinal cohort design with repeated measures of lung function(FEV_1 and PF) as markers of respiratory health and simultaneous detailed air pollutant monitoring as close to the experimental sites as possible. Other asthma related phenotypes investigated included BHR and atopy (defined as skin test responsiveness to common allergens) (Naidoo *et al.* 2007).

2.2 Asthma and related phenotypes

Asthma is defined as a chronic disorder of the airways in which inflammatory symptoms are usually associated with widespread but variable airflow obstruction and may increase airway response to a variety of stimuli. Asthma is characterized by inflammation and air way constriction (Waldron 2007). The inflammation of the airways leads to episodes of wheezing and other respiratory symptoms that are associated with reversible airflow limitation. These symptoms are at least partly reversible either spontaneously or with treatment (Nadel and Busse 1998).

Asthma is also known to be associated with atopy which is the production of large amount of immunoglobin E (IgE) in response to a common antigen (Waldron 2007), usually proteins, and to develop typical symptoms such as asthma, rhino conjunctivitis, or eczema/dermatitis. Evidence has shown that both particulate and gaseous pollutants can act on the upper and lower airways to initiate and exacerbate cellular inflammation (Saxon and Diaz-Sanchez 2005).

Despite substantial research, it is still not clearly understood why some people get asthma and some do not. We do know that it is more common in individuals who have a family history of asthma or related atopic conditions such as eczema or allergic rhinitis, and also that people who have these allergic type diseases themselves are more likely to suffer from asthma (Waldron 2007). Genetic predisposition is therefore a contributing factor in the etiology of asthma.

2.2.1 Asthma Prevalence

The International Study of Asthma and Allergies in Childhood (ISAAC) was established in 1991 because of a concern that asthma and allergies were increasing in prevalence and severity, but little was known about neither the scale of the problem worldwide nor the factors affecting prevalence. A survey conducted by ISAAC found that African countries showed the following frequencies of asthma prevalence; Kenya (13.9%), Morocco (7.8%), Nigeria (10.7%), South Africa (16.1%) and Algeria (5.9%). Developed countries conversely showed markedly higher frequencies with UK (18.4%), New Zealand (29.7%), Ireland (31%) and USA (22.9%). ISAAC reports that the time trends in the prevalence of allergic symptoms indicate an increase for most of the African centres that were studied in comparison to the more affluent developed countries (Asher *et al.* 2006).

It is estimated that about 300 million people worldwide are living with asthma. The disease is more prevalent in childhood. In the United Kingdom (UK) it is thought that one in 10 children has a diagnosis of asthma. Asthma is very difficult to diagnose since there is no consistent diagnostic tool to detect asthma (Waldron 2007).

Childhood asthma is not distributed evenly throughout the population, and children who grow up in crowded urban neighbourhoods have higher rates of asthma and experience greater morbidity because of asthma. There are several environmental and lifestyle factors associated with urban living that are suspected to promote the development of asthma, particularly in the first few years of life (Gern 2010). In South Africa, relatively few studies have been conducted. A study published in 2003 compared traditional rural Xhosa children, recently urbanized Xhosa children and established city white children, and considered factors that may account for the observed increase in all of these groups. As determined by bronchial challenge with histamine, 17% of rural and 34.4% of recently urbanized Xhosa children had increased BHR, a marked increase from the 0.03% and 3.17% prevalence of increased BHR previously found. The prevalence of increased BHR in white urban children was 33% (Steinman *et al.* 2003). Table 2.2 shows a series of studies conducted in South Africa showing the trend in asthma prevalence in children and adolescents various regions of the country. Self-reported asthma prevalence ranged between 10 and 13%, relatively consistently among the different studies (Ehrlich and Jithoo 2006)

Table 2.2: Prevalence of reported asthma and recent wheezing in child and adolescent populations in South Africa (Ehrlich and Jithoo 2006)

Study year published	*Population N(age range)*	*Outcome measure*	*Prevalence (%)*
Burr (1994)	Southern Suburbs, Cape Town (1180, 12yrs)	Wheeze past 12 months, Asthma Ever	17.8 11.5
Ehrlich (1995)	Mitchel's Plain, Cape Town (1 955, 6-10 yr.)	Wheeze past 12 months Asthma ever	26.8 10.8
Nriagu (1999)	South-central Durban* (367, < 17 yr.).	Shortness of breath with wheeze past 12 months Asthma ever	16.0 10.0
Poyser (2000)	Cape Town (5 178, 13-14 yr.)	Wheeze past 12 months Wheeze past 12 months Asthma ever	16.0 6.4 13.3
Pather (2002)	Mitchell's Plain, Cape Town (17 446, 2-15 yr.)	Wheeze past 12 months 2-6 yr. Asthma ever 7-12 yr. 2-6 yr. 7-12 yr.	36.7 27.5 13.1 11.2
White (2003)	Northwest suburbs, Cape Town*(3 162, 9-15 yr.)	Wheeze past 12 months Wheeze past 12 months Asthma ever	33.0 18.3 23.7
Obihara (2005)	Low-income area, Cape Town (861, 6-14 yr.)	Asthma ever	12.3

* Residential areas in close proximity to petrochemical refineries and other industry

The participants in the Settlers school health study conducted in 2002 included 248 students and 25 teachers. The study found that 52% of the students in Grades 3 and 6 had any kind of asthma and 26% of the students were reported

to have persistent asthma (defined as asthma that causes symptoms more than twice a week). These results were staggering and higher than some of the most developed countries in the world like the USA with a frequency of 22.9% (Asher *et al.* 2006). Even though at the time of conducting the Settlers school health study the measured levels of air pollutants were low as compared both to international and South African standards and guidelines, the study results suggested that exposure to air pollutants was associated with more symptoms and poorer lung function in children with persistent asthma. The fact that air pollution levels during the study were lower than the average levels over the past several years may mean that health problems associated with air pollution during the months and years before were still greater than that found during the study period (Kistnasamy 2002).

2.2.2 Risk factors

The relative risk for allergic disease and the tendency to develop allergic disease are both strongly influenced by genetic factors, age and the conditions under which exposure takes place. The likelihood of developing allergic disease in an individual is not constant over time. For example it has been found that early feeding with foreign proteins, a protein that differs from those normally found in humans, is associated with an increased risk for allergic disease, but mostly so in genetically susceptible individuals (Bjorksten 1996). The issue of whether low rates of breast feeding are associated with asthma, wheezing and other manifestations of allergy, has been long debated (Brand *et al.* 2008). In older children, allergy is a common cause of asthma in affluent but not in non-affluent countries (Björksten 1998).

There are many well established risk factors for asthma. Infections are known to trigger and aggravate asthma in already sensitised individuals and infections

increase bronchial hyper reactivity. The presence and absence of infections may therefore explain variations over time in susceptibility to asthma in an individual. Various air pollutants may trigger an attack of asthma in asthmatic and hyper reactive individuals. Allergic asthma is more easily triggered when the allergen exposure takes place in an environment with air pollution. Factors known to both enhance sensitisation and trigger asthma include tobacco smoke; Nitrogen dioxide (NO_2), Sulphur dioxide (SO_2), ozone (O_3) and diesel exhaust particles (DEPs). Passive smoking is by far the most commonly identified trigger of asthma and risk factor for sensitisation (Björksten 1998).

2.2.2.1 Allergens

Exposure to allergens has been identified as the most important factor in the development of asthma. It is therefore important to measure allergens in the environment to establish dose–response relationships between exposures on the one hand and sensitization and clinical allergy on the other hand. In Africa, allergen-specific serum IgE levels are frequently poor predictors for clinical allergy so it is important to establish whether there is a correlation between allergen exposure and sensitization (Björksten 1998).

2.2.2.2 Diet

Lifestyle has changed greatly since the last century. Industrialization and the global market economy have played a role in influencing this shift leading to a wider range of foods, food additives, foods processed by industry, altered intestinal microbial flora and even diets and foods introduced from other areas to new areas, The type of foods eaten has changed dramatically with the introduction of new industrial products with numerous additives. Even fresh food

items differ in many respects from those available only a few decades ago. For example, the use of genetic modification of many crops and various chemical compounds, like sulphur dioxide, has prolonged the storage time of food e.g. apples for months with very limited knowledge of the side effects and future impacts (Björksten 1998). Diet plays a role in the development of asthma atopy and BHR, either protective or as a trigger. The most common trigger foods are dairy products, shell fish and nuts (van Ree and Yazdanbakhsh 2007).

Factors such as dietary fatty acid, number of older siblings, lack of breast-feeding, and atopy, have all been associated with asthma in older children (Haby, 2001). Human milk has been identified as a source of immunological information that may be transferred from the mother to her offspring. Breast feeding is essential for protection against infections in babies living under poor hygienic conditions in developing countries. Furthermore, asthma symptoms are most commonly caused by infections especially in young children (Björkstén *et al.* 2011). The hygiene hypothesis suggests that childhood asthma develops as a result of decreased exposure to infectious disease and other stimuli. As Westernization occurs, the environment becomes cleaner, and we then lack the immunologic and infectious stimuli needed for our immune systems to shift from the helper T-cells Th2 to Th1 response. This hypothesis claims that improved hygiene has removed a protective influence against atopy and asthma that was once provided by exposure to infections in early life. However, the hypothesis has been questioned in the US, where allergic asthma since the 1970s has increased in minorities living in poverty and with suboptimal hygiene conditions ("inner-city asthma") (Tang 2005).

.2.2.2.3 Pollution

There is a wide range of pollutants present in the indoor and outdoor air. They include many types of particulates, nitrogen oxides (NOx), carbon monoxide (CO), sulphur oxides (SO_2), ozone (O_3), photochemical oxidants, lead (Li Kam *et al.*), other heavy metals and a variety of volatile organic compounds. The major sources of the pollutants are combustion of fuels for electricity generation and transportation, industry, heating and cooking. In addition, reactions in the atmosphere among air pollutants may produce a number of secondary pollutants. Most air pollutants are a local phenomenon varying with local geography, emission rates and meteorological dispersion (McGranahan and Murray 2003).

Most people do not have the luxury of choosing the air they breathe, more especially so children, the elderly and the less privileged in society. Within this group, sensitive subpopulations have been identified:

- Very young children whose respiratory and circulatory systems are still maturing.
- The elderly whose respiratory and circulatory system function poorly.
- Persons with asthma and emphysema and heart disease (Turner *et al.* 1994)

Outdoor air pollution has been studied extensively as a potential risk factor for (exacerbations of) asthma and to a lesser extent of rhino-conjunctivitis (Nicolai *et al.* 2003; Heinrich and Wichmann 2004). Among those considered detrimental to health are the criteria pollutants including, but not limited to nitrogen compounds (NOx) sulphur dioxide (SO_2) and particulate matter (PM_{10})

2.2.2.3.1 Nitrogen Compounds

Nitrogen compounds are present in the atmosphere in both oxidized and reduced form. The reduced forms include ammonia (NH_3) and ammonium (NH_4). The oxidized forms in turn include nitric oxide (N_2O), nitrogen oxide (NO), nitrogen dioxide (NO_2), nitrous acid (HNO_2), nitric acid (HNO_3), peroxyacetylnitrate (PAN) and particulate nitrates (NO_3). Motor vehicles - both diesel and gasoline together with coal and petroleum burning energy plants are the main source of nitrogen oxides. Nitrogen dioxide (NO_2) is a brownish-red and toxic gas responsible for the photochemical haze known as smog (Dávila *et al.* 2007).

High levels of traffic-related emissions have been correlated to increased prevalence of respiratory allergies (Nicolai *et al.* 2003; Heinrich and Wichmann 2004). NO_2 has been reported to cause bronchitis and pneumonia, as well as increase susceptibility to respiratory infections (McGranahan and Murray 2003). Some epidemiological studies have associated NO levels to visits to the emergency service due to asthma attacks (Sunyer *et al.* 1997) or NO_2 indoor concentrations to asthma exacerbation (Nitschke *et al.* 2006).

However, there is a disparity of results in the literature (Dávila *et al.* 2007). The interdependence between NO_2 and other pollutants suggests that that the observed health effects could be due to an interplay among contaminants from combustion sources (McGranahan and Murray 2003).

2.2.2.3.2 Particulate Matter (PM)

Both organic and inorganic materials are found in what is referred to as particulate matter (PM). The health effects of particulate matter are significant

for short-term and long-term exposures, particularly those containing several metals and silicate-derived constituents that can be cytotoxic to lung cells Particles larger than 10µm generally get caught in the nose and throat, never entering the lungs. Particles smaller than 10µm can get into the large upper branches just below the throat where they are caught and removed (by coughing and spitting or by swallowing), particles smaller than 5µm can get into the bronchial tubes at the top of the lungs, and particles smaller than 2.5µm in diameter can penetrate the deepest (alveolar) portions of the lung. If these particles are soluble in water, they pass directly into the blood in the alveolar capillaries and if they are not soluble in water, they are retained in the deep lung for long periods. About 60% of PM_{10} particles (by weight) have a diameter of 2.5µm or less (Yang and Omaye 2009). Particulate matter less than 10 µm and less than 2.5 µm in size have been linked to allergic responses, asthma exacerbation, wheezing and lung development in children (Saxon and Diaz-Sanchez 2005). A recent study found a 10 mg/m^3 increase in PM_{10} as associated with a 2.54% increase in the number of pediatric asthma hospital admissions (Samoli *et al.* 2011).

In a study of 152 U.S. metropolitan areas, it was confirmed that there is a clear relationship between fine-particle air pollution and human deaths. Particulate matter, through an oxidant mechanism causes a sevenfold increase in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in human airway epithelial cells. NFκB is a transcription factor that can induce gene transcription in a variety of pro inflammatory cytokines, enzymes that generate mediators of inflammation and immune receptors oxidative stress mediated by particulate matter may result from direct generation of ROS from the surface of particles, where transition metals or even organic compounds impact on mitochondrial function of inflammatory cells. Also, the subsequent

damage to DNA may indicate how increased cancer risk is induced by particulate matter (Yang and Omaye 2009).

2.2.2.3.3 Sulphur Dioxide (SO_2)

The main sources of sulphur dioxide are the combustion of fossil fuels and industrial refining of sulphur containing ores. This gas reacts with other pollutants to produce sulphur trioxide, sulphuric acid and sulphates (McGranahan and Murray 2003). A recent study in Athens, Greece over the period 2001–2004 found a 10 mg/m^3 increase in SO_2 was associated with a 5.98% increase (95% CI: 0.88%, 11.33%) in the number of pediatric asthma hospital admissions(Samoli *et al.* 2011). SO_2 and its hydrolysis products have been associated with both acute bronchoconstriction and elevated morbidity and mortality rates. SO_2 is predominantly an upper airway irritant, producing bronchoconstriction and mucus. Asthmatics and others affected with hyper-reactive airways are most sensitive to acute exposures to SO_2 than healthy persons. In a study in Spain that enrolled 20 455 schoolchildren aged between 6 and 7 years, from 2002 to 2003, the annual average concentration of SO_2 showed a significant association with a higher prevalence of recent severe asthma (adjusted odds ratio [OR] between level-1 and level-3 pollution, 1.32; 95% confidence interval [CI], 1.01–1.73). Their findings suggested that air pollutants such as SO_2 and CO increase the risk of recent symptoms of asthma and allergic rhinitis in schoolchildren aged between 6 and 7 years in Spain (Arnedo-Pena *et al.* 2009).

Some of the effects associated with specific pollutants are presented in Table 2.3.

2.3 Genetic Predisposition to Asthma

Since its earliest descriptions, asthma has been recognised as a heritable disorder. Overall, estimates for heritability of asthma suggest that 40–60% of asthma risk is attributable to genetic factors (Manian 1997). The exact mode of

Table 1.3: Specific air pollutants and associated health effects (Turner *et al.* 1994)

Pollutant	*Effects*
CO(Carbon Monoxide)	Reduction of the ability of the circulatory system to transport O_2 Impairment of performance on tasks requiring vigilance Aggravation of cardiovascular disease
NO_2	Increased susceptibility to respiratory pathogens
O3	Decrement in pulmonary function Coughing and chest discomfort Increased Asthma attacks
Lead	Neurocognitive and neuro motor impairment Haem synthesis and hematologic alterations
Peroxyacyl nitrates, aldehydes	Eye irritation
SO_2 Particulate matter	Increased prevalence of chronic respiratory disease Increased risk of acute respiratory disease

inheritance remains elusive. Studies in monozygotic twins have found that genetic factors, whilst important, are not the sole arbiter of disease expression. The genetic and environmental factors that mediate asthma risk and expression are intricately linked. Environmental and genetic factors interact pre- and post-natally to determine phenotype in the child as shown in Figure 2.1 (Carroll 2005; Miller and Ho 2008).

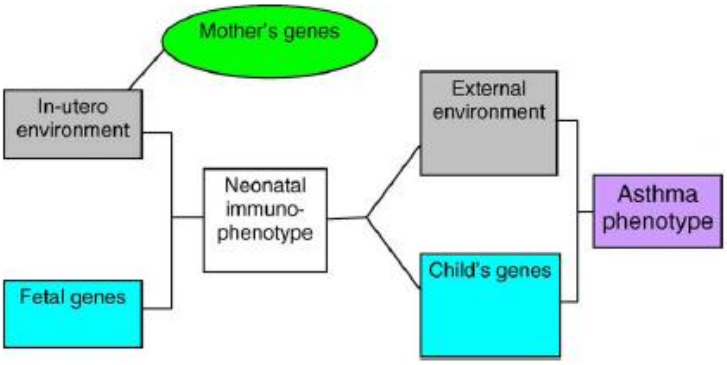


Figure 2.1. Proposed model of gene-environment interactions in determining asthma phenotype (Carroll 2005).

In twin studies, resemblance between identical (monozygotic) is compared with fraternal (dizygotic) twins. Monozygotic twins share 100 % of their genetic make-up and dizygotic twins share on average 50 % of their genes like other siblings. If genes influence a particular trait monozygotic twins should resemble each other to a greater extent than dizygotic twins. Another important goal in the study of twins is the estimation of the effects of environmental influences, because resemblance between twins can be due to environmental influences that they have in common: shared environment (e.g. parental influences, school). Environmental influences which are specific for a person (within family or unique environment) will contribute to differences between individuals with the same genetic make-up if they are reared in the same family (Los *et al.* 2001). The results from numerous twin studies have shown significant differences between monozygotic and dizygotic twin pairs, providing evidence of genetic inheritance of asthma (Chadwick and Gail 1996).

2.3.1 Asthma Genetics

The search for asthma susceptibility genes has been an area of intense investigation since the early 1900s (Carroll 2005). Two general approaches have been widely used to study the genetics of asthma: candidate gene association studies and, genome wide linkage or association studies followed by positional cloning. Using candidate gene association studies, more than 100 candidate genes have been studied because their biological function suggests that they could play a role in the pathogenesis of asthma. A summary is shown in Tables 2.4 and 2.5 (Vercelli 2008; Hizawa 2009).

Allergic diseases are complex genetic diseases resulting from the effect of multiple genetic and interacting environmental factors on their pathophysiology. Recent years have seen considerable progress in unravelling the contribution of these factors to an individual subject's susceptibility to, subsequent development of, and severity of disease. This has resulted in increasing insight into novel areas of allergic disease pathophysiology (Holloway *et al.* 2010).

2.3.2 Candidate gene association studies

The primary motivation of the candidate gene study is that the candidate gene might be in a pathway involved in asthma pathogenesis and might have a functional polymorphism that can be tested for association with asthma in a population. In this approach, a series of case and control subjects can be used to test for association of the functional variant with disease or, in a family context, evidence for linkage of the polymorphism (Steinke *et al.* 2008). Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (Adenine-A, Thiamine- T, Cytosine-C, or Guanine-G) in the genome sequence is altered. For a variation to be considered an SNP it

must occur in at least one per cent of the population (Pongracz and Keen 2009). SNPs in the promoter and coding regions of a wide range of candidate genes have been studied for association with a range of atopy-related phenotypes

Candidate genes are selected for analysis based on a wide range of evidence, such as biological function, differential expression in disease, involvement in other diseases with phenotypic overlap, affected tissues, cell type or types involved, and findings from animal models (Holloway et al. 2010).

Following candidate gene identification, it is important to explore the functional consequences of the associated genetic variation. It is likely that many of the genetic variants associated with asthma do not have functional consequences but are simply in Linkage disequilibrium (LD) with the causal gene (Zhang et al. 2008). LD is based on the observation that common genetic variants in individuals who carry a particular polymorphism at one site, often predictably carry other specific polymorphisms at other nearby variant sites (Pongracz and Keen 2009). In the context of genetic association study, this means that the measured risk allele may be acting only as a marker for another nearby disease susceptibility locus. For SNPs in coding regions it is possible to predict the consequences for protein structure and function, although such predictions have to be empirically verified. However, most SNPs in the human genome are found in non-coding DNA. Variants located in promoter regions may change gene expression by altering transcription factor binding sites or by other more subtle mechanisms (Zhang et al. 2008).

The advantage of this approach is that candidate genes have biological plausibility and often display known functional consequences that have potentially important implications for the disease of interest. Disadvantages are the limitation to genes of known or postulated involvement in the disease,

Table 2.4: Susceptibility genes for asthma and asthma-related traits (Hizawa 2009)

Gene	Chromosome	Function and pathway	Common variants	Number of positive association reports
				0 5 10 15 20 25 30 35 40
GSTM1	1p13.3	Environmental and oxidative stress detoxification	+/null	
FLG	1q21.3	Epithelial barrier integrity	Arg510X, 2282del4	
IL10	1q31-q32	Immunoregulation	-1082A/G, -571C/A	
CTLA4	2q33	T-cell-response inhibition and Immunoregulation	-318C/T, 49A/G	
IL13	5q31	TH2 effector functions	-1112C/T, Arg130Gln	
IL4	5q31.1	TH2 differentiation and IgE induction	-589C/T, +33C/T	
CD14	5q31.1	Innate immunity, microbial recognition	-1721G/A, -260C/T	
SPINK5	5q32	Epithelial serine protease inhibitor	Glu420Lys	
ADRB2	5q31-q32	Bronchial smooth-muscle relaxation	Arg16Gly, Gln27Glu	
HAVCR1	5q33.2	T-cell response regulation, HAV receptor	5383_5397del	
LTC4S	5q35	Cysteinyl leukotriene biosynthesis, inflammation	-444A/C	
LTA	6p21.3	Inflammation	NcoI (intron1)	
TNF-	6p21.3	Inflammation	-308G/A, -857C/T	
HLA-DRB1	6p21	Antigen presentation	Multi-SNP alleles	
HLA-DQB1	6p21	Antigen presentation	Multi-SNP alleles	
HLA-DPB1	6p21	Antigen presentation	Multi-SNP alleles	
GPRA	7p14.3	Regulation of cell growth and neural mechanisms	Haplotypes	
NAT2	8p22	Detoxification of drugs and carcinogens	Slow acetylation SNPs	
FCERIB	11q13	High-affinity Fc receptor for IgE	Ile181Leu, Gly237Glu	
CC16	11q12.3-q13.1	Epithelium-derived anti-inflammatory protein	38A/G	
GSTP1	11q13	Environmental and oxidative stress, detoxification	Ile105Val	
IL18	11q22.2-q22.3	Induction of IFNγ and TNF-	-656T/G, -137G/C	
STAT6	12q13	IL-4 and IL-13 signalling	2964G/A, (GT)n exon1	
NOS1	12q24.2-q24.31	Nitric oxide synthesis, cell-cell communication	3391C/T, 5266C/T	
CMA1	14q11.2	Mast-cell chymotryptic serine protease	BstX1, -1903G/A	
IL4R	16p12.1-p12.2	α-chain of the IL-4 and IL-13 receptors	Ile50Val, Glu551Arg	
CCL11	17q21.1-q21.2	Epithelium-derived eosinophil chemo attractant	Ala23Thr, -1328G/A	
CCL5	17q11.2-q12	Monocyte, T-cell and eosinophil chemo attractant	-403A/G, -28C/G	
ACE	17q23.3	Inactivation of inflammatory mediators	In/del	
TBXA2R	19p13.3	Smooth-muscle contraction, inflammation	924T/C, 795T/C	
TGFB1	19q13.1	Immunoregulation, cell proliferation	-509C/T	
ADAM33	20p13	Cell-cell and cell-matrix interactions	Multiple SNPs	
GSTT1	22q11.23	Environmental and oxidative stress, detoxification	A/null	

thereby excluding the discovery of novel genes that influence the diseases. There are almost 1,000 studies published that examine polymorphisms in several hundred genes for association with asthma and allergy phenotypes (Kabesch 2010). Table 2.5 shows some novel candidate genes or novel roles of known genes as of 2007 (Vercelli 2008).

Genetic studies of allergic disease have shown that susceptibility to allergic disease is likely to result from the inheritance of many mutant genes. Unfortunately, as in many other complex disorders, in allergic diseases any specific biochemical defect or defects at the cellular level that cause the disease are unknown, even though considerable knowledge has been accrued on molecular pathways involved in pathogenesis. By undertaking research into the genetic basis of these conditions, these mutant genes and their abnormal gene products can be identified solely by the anomalous phenotypes they produce. Identifying the genes that produce these disease phenotypes has provided a greater understanding of the fundamental mechanisms of these disorders. The results of studies of the genetic basis of allergic disease have increased our understanding of these conditions in a number of ways. There is now greater understanding of disease pathogenesis. This has led to the identification of novel genes and pathways leading to new pharmacologic targets for developing therapeutics. We can also identify environmental factors that interact with a subject's genetic makeup to initiate disease and confirmation of causality of environmental factors through Mendelian randomization. Due to this development, prevention of disease can be carried out by environmental modification, identification of susceptible subjects, and early-in-life screening and targeting of preventative therapies to at-risk subjects to prevent disease (Zhang *et al.* 2008).

Therapies can also be targeted depending on the genotype of an individual. There can be sub-classification of disease on the basis of genetics and targeting of specific therapies based on this classification, identification of subjects at risk of severe disease and targeting of preventative treatments can be carried out.

Table 2.5: Novel Asthma / allergy candidate genes and roles of known genes (Vercelli 2008)

Reference	*Finding*
(Hong *et al.* 2007)	A TNF-A promoter polymorphism (308G/A) appears to be associated with severe bronchial hyperresponsiveness in Korean children with asthma, possibly in synergism with CD14/ 159C/T
(Bae *et al.* 2007)	A SNP in the FceRIa promoter appears to be associated with aspirin-intolerant chronic urticaria and increased gene expression in mast cells.
(Acevedo *et al.* 2007)	Leukotriene C4 synthase (LTC4S) 2444AC is associated with IgE antibodies to Dermatophagoidespteronyssinus in a Colombian population.
(Thompson *et al.* 2007)	Genetic variation in integrin b3 (ITGB3)/CD61 affects asthma susceptibility and allergic sensitization, beginning early in life. Interestingly, different SNPs in the gene are associated with asthma and IgE.
(Ungvari *et al.* 2007)	Chronic Mycoplasma pneumoniae infection appears to be associated with physician-diagnosed asthma and the defective chemokine (C-C motif) receptor 5(CCR5) variant CCR5D32.
(Wu, H *et al.* 2007)	SNPs in S-nitrosoglutathione reductase (GSNOR) modulated asthma susceptibility in children from Mexico City.
(Raby *et al.* 2007)	A common mitochondrial haplogroup is associated with increased total serum IgE levels in white children participating in the Childhood Asthma Management Program.
(Koehm *et al.* 2007)	HLA-DRB1 alleles control allergic bronchopulmonary aspergillosis–like pulmonary responses in humanized transgenic mice.
(Dhiman *et al.* 2007)	SNPs in both the signalling lymphocytic activation molecule (SLAM/CD150) and CD46 genes are associated with measurable and significant variations in antibody response after measles vaccination.
(Yang, KD *et al.* 2007)	Infant frequent wheezing is associated with Clara cell protein 10 (CC10) 138GA and lower CC10 levels, but not allergic sensitization, in a perinatal cohort study
(Schulz *et al.* 2007)	A common IL31 haplotype is associated with increased IL31 expression and non-atopic eczema.
(Lee *et al.* 2007)	Variants in chemokine (C-C motif) receptor 3 (CCR3) are associated with eosinophil counts, particularly in combination with IL-5 receptor a (IL5RA) polymorphisms.
(Tantisira *et al.* 2007).	FCER2, which encodes the low-affinity IgE receptor, predicts the likelihood of treatment success in asthmatic children. The associations of FCER2/2206 TC with IgE level, severe exacerbations and FCER2 expression might provide a mechanistic basis for these findings
(Ueta *et al.* 2008).	Polymorphisms in IL4R appear to be associated with Stevens-Johnson syndrome in Japanese patients

In addition one may determine how a subject will respond to a particular therapy (pharmacogenetics) and individualized treatment plans may be developed (Holloway *et al.* 2010).

The genes implicated by genome-wide association studies (GWAS), genome-wide linkage studies, and candidate gene studies can be broadly divided into 4 themes: epithelial barrier function, environmental sensing and immune detection, tissue response, and TH2 cell polarization and response. A summary of these genes is shown in Figure 2.2. It is likely that future studies of the genes identified as susceptibility loci will show that some have roles more consistent with protection from rather than susceptibility to asthma, at which point those genes would be re-categorised (March *et al.* 2011).

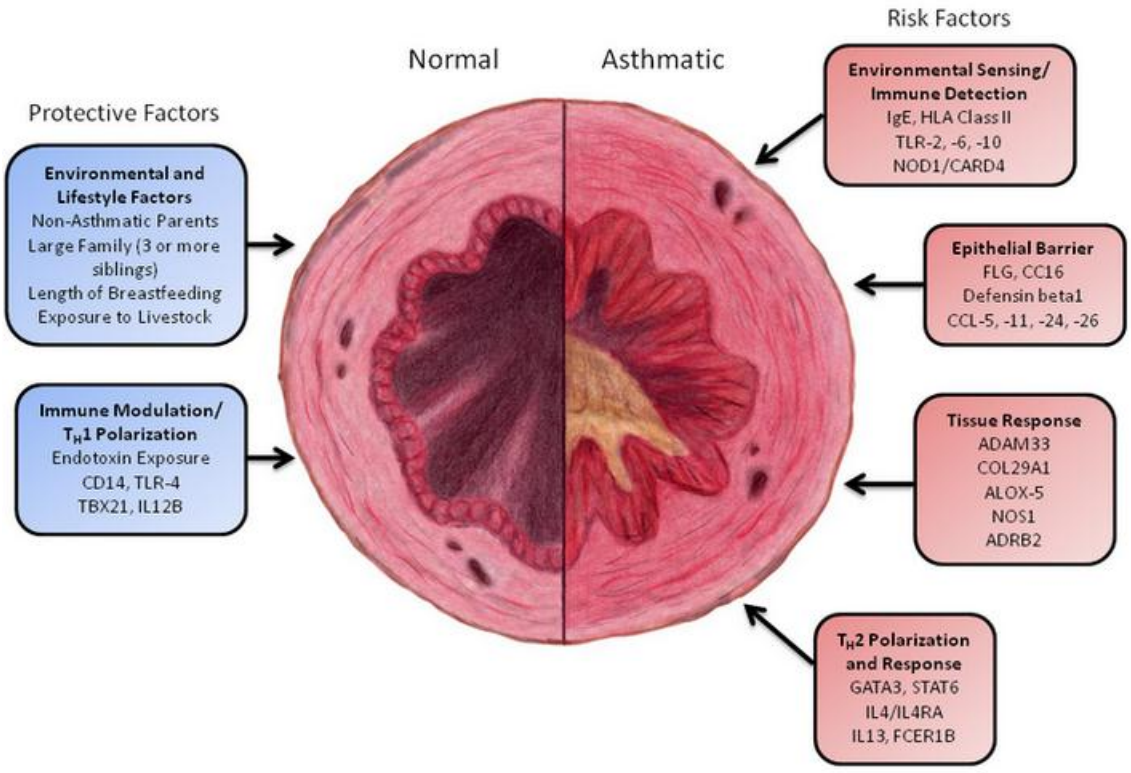


Figure 2.2. Risk vs. Protective Factors in Asthma (March *et al.* 2011)

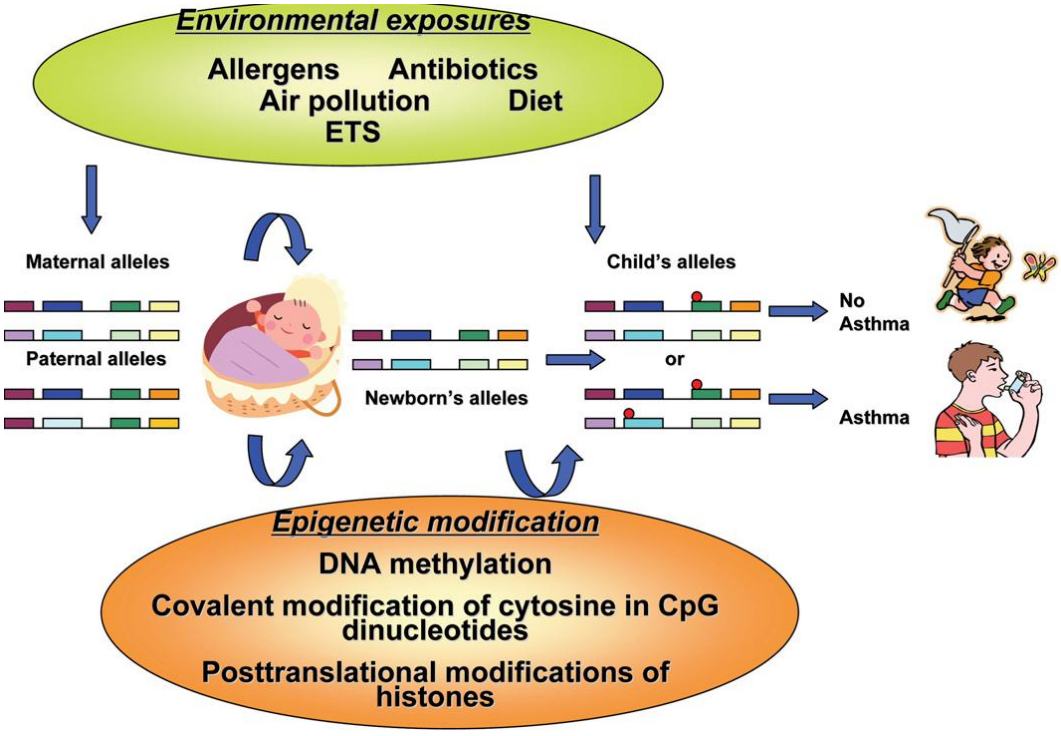
2.3.3 Epigenetics and Asthma

Epigenetics is the study of heritable changes in gene expression that occur in the absence of alterations in DNA sequences (Miller and Ho 2008). Epigenetic modifications may be passed on to daughter cells and offspring. Recent studies suggest that epigenetic regulation may in part mediate the complex gene-by-environment asthma (i.e., incidence and remission of symptoms) may be a result of epigenetic changes, such as DNA methylation, covalent histone modifications, micro RNA changes, and chromatin alterations, after early or later environmental exposures (Miller and Ho 2008). Some individuals are affected as young children, others as adults. Furthermore, some develop asthma or wheezing illness as a result of occupational exposures, whereas others may become affected after exposure to urban air pollution (Waldron 2007).

Traditional models for gene-by environment interactions proposed in research studies have not always matched observations by physicians. (Miller and Ho 2008). New developments in the past decade have dispelled the belief that a person is born with one set of genes and mutations. We also believed that these traits could not be changed over a life time. The ability to manipulate gene expression has led to the ground breaking field of epigenetics. Known or suspected drivers of epigenetic processes include many agents, such as heavy metals, pesticides, diesel exhaust, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radio activity, viruses, bacteria and basic nutrients (Koppelman and Nawijn 2011; Reddy 2011).

Among these drivers of epigenetic changes are known asthma risk factors. Several studies have shown that air pollution can alter the epigenetic state of the genome and may play an important role in the regulation of asthma associated genes. However, the proof that these epigenetic changes are causal of disease

in man is still awaited (Durham *et al.* 2010). Figures 2.3 and 2.4 show the proposed effects that pollution and external factors may have on the epigenetic state of individuals.



Key: ETS- Environmental Tobacco Smoke

- **Red dots- Refer to sites of epigenetic changes**

Figure 2.3. Environmental epigenetics and asthma the proposed effects that pollution allergens, antibiotics, diet and ETS can have on the epigenetic state of an individual (Miller and Ho 2008)

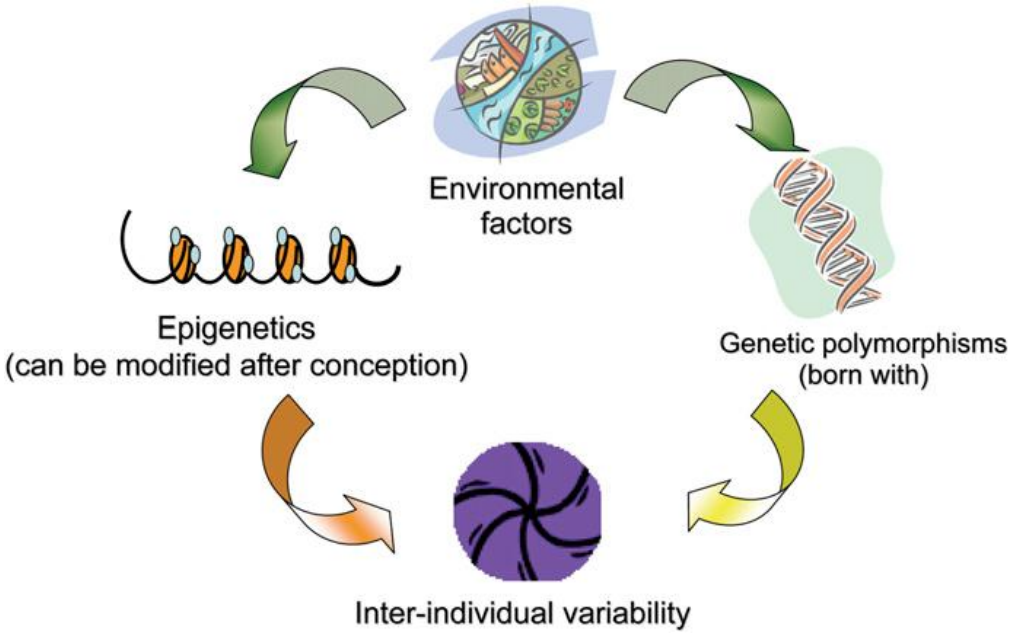


Figure 2.4. Epigenetic and genetic factors influence inter individual asthma risk (Miller and Ho 2008)

2.4 CD14 (-159) C/T and TNF-308α G/A

CD14 and TNFα genes were chosen for this study because they have common functional variants which have been implicated in possible gene-environment interactions in the context of respiratory diseases. A search through the current literature revealed that there are no studies from the African subcontinent on these gene polymorphisms in relation to environmental exposures and the asthma outcomes discussed. The following is a review of the reported effects of CD14 (-159) C/T and TNF-308α G/A on respiratory disease.

2.4.1 CD14 (-159) C/T

Genetic factors are critical determinants of lung inflammation and pulmonary function (Rylander 2002). It is hypothesized that genetic differences that affect responsiveness to endotoxin underlie the variability in airway responsiveness. Chemically, endotoxins are liposaccharides (Burki *et al.*). These are macromolecules consisting of two parts: a hydrophilic sugar part, the polysaccharide covalently linked to a hydrophobic lipid known as the Lipid A (Roitt and Delves 1992). CD14 is a high-affinity receptor for endotoxin (Wright *et al.* 1990). CD14 acts by transferring endotoxin and other bacterial ligands from circulating lipopolysaccharide-binding protein to the toll-like receptor 4 (TLR4)/MD-2 signalling complex. Engagement of this complex results in the activation of innate host defence mechanisms such as release of proinflammatory cytokines. Soluble CD14 (sCD14) is found in normal plasma at microgram concentrations (Martinez *et al*. 1998) and has been shown to be elevated in the lung as a result of inflammation , infection, or allergen provocation. The gene encoding CD14 is localized on chromosome 5q31.1 (Goyert *et al.* 1988); a region that has been linked to asthma (Martinez 1998; Postma 1995) and pulmonary function (LeVan *et al.* 2005) so this is a good choice for a candidate gene in the context of respiratory phenotypes.

The CD 14 polymorphism is located in the 5' flanking region of the CD14 gene at position 159 from the transcription start site. This polymorphism is frequent among both Hispanic and non-Hispanic white subjects, with approximately half of all chromosomes carrying the T allele and half the C allele (Baldini *et al.* 1999). CD14 (-159) C/T is one of the most widely tested genetic variations in relation to asthma and associated traits. The results of these studies have shown a remarkable, statistically significant heterogeneity, with some studies

indicating the T-allele as a risk factor, others the C-allele, and some studies finding no association. The most recent meta-analysis concluded that there is a protective dose-response relationship between the CD14 -260T allele and atopic asthma (Zhao and Bracken 2011). Recent studies in which exposure to house-dust endotoxin or to domestic sources of microbial exposure were assessed concomitantly with CD14 (-159) C/T have shown a consistent, replicable gene-environment interaction. Specifically, results suggest that the C-allele is a risk factor for allergic phenotypes at low levels of exposure, whereas the T-allele is a risk factor at high levels of exposure (Martinez 2007a)

Scientists have hypothesized that decreased endotoxin exposure and reduced innate immune responses have contributed to the increased sensitivity to allergens (Steinke *et al.* 2008). In 1999, Baldini and co-workers showed that polymorphisms in the CD14 gene led to a functional change in the expression of the gene. Studies examining the CD14 C-260T (same as CD14 (159) C/T) promoter polymorphism have provided insight into a partial explanation of this gene-environment interaction. Individuals homozygous for the T allele are protected against the development of asthma in houses with low endotoxin exposure; however, in houses with high endotoxin exposure, this genotype was associated with a higher risk for asthma (Zambelli-Weiner *et al.* 2005; Steinke *et al.* 2008).

In case-control and family-based studies, Smite and partners (2009) found that in atopic subjects, the presence of SNPs in the CD14, TLR4, and other TLR genes modified the association with the risk of asthma, particularly in the presence of country living. In a study on farm living in 2007, Bieli and partners also observed that certain alleles in the CD14 promoter region might be associated with protection against asthma and allergic disease in the presence of farm milk consumption (Bieli *et al.* 2007).

In Chinese children, higher sCD14 levels were found in CD14 (-159) TT homozygotes than in carriers of the other two genotypes (Leung *et al.* 2003). They found no association between allergic sensitization and CD14 (-159) CT but among atopic children, homozygotes for the T allele had higher total serum IgE than carriers of the other two genotypes (Leung *et al.* 2003). Among Czech children, carriers of the C allele were more likely than their peers to be sensitized to moulds (Buckova *et al.* 2003).In a study of German children no association was found between allergies and the CD14(-159) C/T polymorphism (Sengler *et al.* 2003). Hong and colleagues reported that, among Korean children with asthma, there was an interaction between CD14 (-159) C/T and a SNP in the tumour necrosis factor (TNF-) gene, TNF- 308, with only subjects with the risk alleles for each SNP (A for TNF- 308 and C for CD14 (-159) C/T showing increased rates of bronchial hyper responsiveness (Hong *et al.* 2007). One of the largest published studies to date on the CD14 (-159) C/T polymorphism was conducted in Australia. The study had 1000 subjects, of which, 55% of them had asthma. No association was found between CD14 (-159) C/T and asthma. A weak association was detected with atopy. Carriers of the T allele showed increased risk for asthma (Kedda *et al.* 2005).

At first glance, these results suggest a disappointing inconsistency. A far more complex picture emerged from further studies (Martinez 2007a). In a founder population in the United States of America (USA) findings suggested that there could be an antagonistic interaction between environmental conditions and CD14 (-159) C/T as determinants of allergic sensitization: the T allele could be either protective or a risk factor, depending on the degree of exposure to environmental microbial products (Ober, C *et al.* 2000), which could be present indifferent concentrations in urban and rural settings (Von Mutius, E *et al.* 2000). In support of this argument, four studies showed consistently replicated

evidence of a pattern of gene–environment interactions. These results suggest that the often encountered, limited replicability of genetic associations may, at least in part, be due to complex interactions between genes and environment in determining asthma-related outcomes (Martinez 2007a). Finally these findings are supported by Zhao and partners who concluded that carriers of the TT and CT genotypes were about 33% less likely (OR = 0.67, 95% CI: 0.54-0.84) and about 20% less likely (OR = 0.80, 95% CI: 0.66-0.95), respectively, to present with atopic asthma compared to carriers of the CC genotype. This finding was only obtained after restricting analysis to studies using atopic asthma and non-atopic non-asthma case-control phenotypes and excluding studies influencing heterogeneity, the genotype-specific odds ratios (ORs) suggested a codominant model These results demonstrate the importance of precisely specified case-control groups as well as the need to assess environmental interactions in the investigation of complex diseases such as asthma (Zhao and Bracken 2011).

In essence, the CD14 gene may contribute to the variation in lung function. It has been implicated in the pathobiology of respiratory diseases based on its role as a proinflammatory cytokine in asthmatics and atopic individuals. The conflicting results also provide the impetus to test for a gene environment interaction of this gene polymorphism in this population in Durban South, since an African population has not been studied in this context. The environments different populations live in are unique. The genetic epidemiology of disease may differ from one population to the next. Hence it is worth the effort to either challenge or corroborate the association of these genes to respiratory disease in different populations. The data generated will be specific to a local environment and a local gene pool. It may also reflect the impact the environmental pollution load has had on these communities and their genetic profiles, or conversely the impact their genetic profile has had on their phenotypes or whether it is a combination of both or more factors.

2.4.2 Tumour necrosis factor α Polymorphism (TNF-308α G/A)

TNF-α is a 157 amino acid pro-inflammatory cytokine protein manufactured mainly by white blood cells to stimulate and activate the immune system in response to cancer, infection, exposure to endotoxin, or other products of bacterial, viral, parasitic or inflammatory origin (Roitt and Delves 1992).

Inflammation occurs after most kinds of tissue injuries, infections or immunologic stimulation as a defence against foreign or altered endogenous substances. A pro-inflammatory cytokine accelerates inflammation and it also regulates inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types (*Cytokines & Cells Online Pathfinder Encyclopedia* 2011). Cytokines interact with cells of the immune system in order to regulate the body's response to disease and infection. Cytokines also mediate normal cellular processes in the body. Cytokines are released by cells into the circulation or directly into tissue. The cytokines locate target immune cells and interact with receptors on the target immune cells by binding to them. The interaction triggers or stimulates specific responses by the target cells. Overproduction or inappropriate production of certain cytokines by the body can result in disease. For example, it has been found that interleukin-1 (IL-1) and TNF-α are produced in excess in rheumatoid arthritis where they are involved in inflammation and tissue destruction (Eustice 2008).

TNF-α has been implicated in many aspects of the airway pathology in asthma. TNF-α was found in elevated concentrations in sputum, bronchoalveolar lavage (BAL), and lung biopsy samples from asthmatic patients (Kips 2001). It plays a

central role in the initiation of airway inflammation and the generation of airway hyperreactivity (Zhang *et al.* 2011). Inhaled TNF-α increases airway responsiveness to methacholine in normal and asthmatic subjects associated with a sputum neutrophilia. Additional data indicate that TNF-α can upregulate adhesion molecules, facilitate the immigration of inflammatory cells into the airway wall and activate pro-fibrotic mechanisms in the sub-epithelium. These data suggest that TNF-α plays a role in the initiation of allergic asthmatic airway inflammation and the generation of airway hyper-reactivity (Thomas, P. S. 2001b).

The gene encoding TNF-α is located on the short arm of chromosome 6 within the major histocompatibility (MHC) complex, where genetic alterations in the TNF-α locus are now known to be involved directly in high TNF-α production (Roitt and Delves 1992). The location of its gene within the major histocompatibility complex and biological activities has raised the possibility that polymorphisms within this locus may contribute to the pathogenesis of wide range of autoimmune and infectious diseases. For example, a bi-allelic single nucleotide substitution of G allele with A allele polymorphism at −308 nucleotides upstream from the transcription initiation site in the TNF-α promoter is associated with elevated TNF-α levels and disease susceptibilities (Elahi *et al.* 2009).

Several polymorphisms have been identified inside the TNF-α promoter positioned at (relative to the transcription start site) −1031 (T→C), −863 (C→A), −857 (C→A), −851 (C→T), −419 (G→C), −376 (G→A), −308 (G→A), −238 (G→A), −162 (G→A), and −49 (G→A), although those at positions −419, −163, −49 are rare in Caucasians. Among these variants, a polymorphism that directly affects TNF-α expression is located at nucleotide position−308. A single-base polymorphism within the promoter of the gene for TNF-α results in 2 allelic

forms, one in which guanine defines the common allele and the other in which guanine is substituted (Elahi *et al.* 2009). TNF-α-308 A allele is associated with increased levels of TNF-α in plasma and bronchoalveolar lavage fluid from asthmatic airways (Gao *et al.* 2006).

Cellular inflammatory responses to ozone have been mapped to chromosome 17 bringing renewed attention to TNF- which is increased after ozone exposure in bronchoalveolar lavage fluid in human, mouse and rat models and after direct exposure of human nasal epithelia or blood to ozone. Pre-treatment of rats with antibody to TNF-α can reduce ozone-induced inflammation and lung damage, and acute ozone-induced airway hyper-reactivity is reduced in TNF- receptor–deficient mice. The TNF-308α G/A polymorphism was found to be associated with lung function changes after ozone exposure (although actual amounts of TNF-α were not reported in bronchoalveolar lavage fluid) in a German population (Yang *et al.* 2005). Epidemiological studies also associate this polymorphism with an increased risk of both asthma and wheezing and have suggested that ozone can modify this effect (Saxon and Diaz-Sanchez 2005).

Pollution exposure studies have found increased amounts of proinflammatory cytokines, chemokines and adhesion molecules in bronchial lavage fluid and nasal washes (Saxon and Diaz-Sanchez 2005) and in serum (Roitt and Delves 1992). *In vitro* studies have demonstrated a direct effect of pollutants on the expression of these factors. O_3, SO_2 and Diesel exhaust Particles (DEPs) can all cause release of soluble intercellular adhesion molecule and proinflammatory cytokines such as GM-CSF and interleukin 8 (IL-8) from human normal bronchial epithelial cells *in vitro*. DEPs and their chemical constituents can also have similar effects on transformed bronchial and nasal polyps–derived upper airway epithelial cells. The production of GM-CSF and IL-8 by airway epithelial cells can be regulated by the cytokines IL-1 and TNF-α, whereas both DEPs and DEP extracts can induce in vitro production of these cytokines from pulmonary

alveolar macrophages. Thus, there is abundant evidence that pollution can stimulate an inflammatory response through interaction with the innate immune system (Saxon and Diaz-Sanchez 2005).

The most recent meta-analysis on TNF-308α found that the variant A allele carriers had a 38% increased risk of asthma, when compared with the homozygote GG (odds ratio (OR) =1.40, 95% confidence interval (CI), 1.13–1.68 for AA+AG vs. GG). This meta-analysis included a total of 4717 cases and 5012 controls in 29 case–control studies. The results showed significant elevated risks were associated with A allele carriers in Asians (OR=1.53, 95% CI=1.17–2.01 and p=0.002) but not in Caucasians (OR=1.06, 95% CI=0.75–1.50 and p=0.73). In the subgroup analysis by age, significant elevated risks were associated with A allele carriers in adults (OR=1.44, 95% CI=1.14–1.81, and p=0.002) and children (OR=1.37, 95% CI=1.03–1.82, and p=0.003). In the subgroup analysis by atopic status, significant elevated risks of asthma were associated with A-allele carriers in atopic population (OR=1.68, 95% CI=1.34–2.10, and p< 0.00001) but not in non-atopic population (OR=0.98, 95% CI=0.58–1.68, and p=0.95) (Zhang *et al.* 2011).

These findings indicate that this polymorphism may contribute to asthma pathogenesis and help to explain individual differences of host susceptibility to asthma. It is possible that different genetic backgrounds may account for these differences. In addition, asthma is a sophisticated disease which is also related to environmental factors. It is possible that these differences of genetic susceptibility might be also affected by exposure to various environmental factors. Thus, further studies are necessary to assess the effect of gene–environment interactions in different ethnicities and to validate these findings.

There is a need to generate information on the frequency of these gene polymorphisms in local populations in South Africa. This information will be

crucial in assessing any gene environment effects in the Durban South population and thereby planning public health interventions for identified high risk groups. A review of studies conducted in South Africa and Africa which have investigated the frequency of these polymorphisms in other diseases will be presented in the following section.

2.4.3 CD 14 and TNF- 308 gene polymorphisms in the African and South African Context

To our knowledge only one study has to date published the effects of CD14 polymorphism or TNF- 308 on an asthmatic African population. The study on CD14 evaluated the association of with asthma or atopy with 210 asthmatic children, 224 controls and 80 families in Tunisia. Six single nucleotide polymorphisms including CD14 (-159 C/T) were genotyped using polymerase chain reaction followed by restriction fragment length polymorphism in the case-control and family study. The CD14 (-159) C allele was found to be significantly higher in the asthmatic group when compared with controls ($p \leq 0.005$). Transmission disequilibrium test of 80 asthmatic families showed significant inheritance of the CD14 (-159) C allele to asthma-affected offspring. It was concluded that TLR9 and CD14 gene polymorphisms may contribute to an inherited predisposition to asthma in Tunisian children (Lachheb *et al.* 2008).

A study conducted with Egyptian children investigated the association of polymorphisms related to cytokine genes with susceptibility and severity of bronchial asthma. Asthmatic children showed a significantly higher frequency of IL-10−1082 G/G homozygosity genotype ($p < 0.001$; odds ratio [OR] = 7) with lower frequency of G/A heterozygosity genotype among cases. This was also found in cases with persistent asthma and eczema. These cases showed significantly lower frequency of TNF-308α G/A heterozygosity ($p < 0.05$; OR =

0.44). Also, male cases, cases with positive family history, and those patients with persistent types of asthma showed a higher frequency of TNF- 308α G/G homozygosity. This study concluded that IL-10−1082 G/G and TNF- 308α GG genotypes may be contributing factors in susceptibility as well as in severity of asthma among Egyptian children (Zedan *et al.* 2008).

Additionally studies on the African continent have been published on the effects of TNF-308α G/A polymorphism on tuberculosis, liver fibrosis, psoriasis, brain tumours, type 1 diabetes mellitus, Graves' disease, iron deficiency anaemia, brain tumours, rheumatic heart disease, severe sequelae of ocular chlamydia trachomatis, cervical cancer, malaria, autoimmune thyroid diseases, type 2 diabetes and obesity, periportal fibrosis and silicosis. There is published data on the association or effects of TNF-308α G/A nor CD14 (-159) C/T polymorphism on asthma phenotypes to date in South Africa. However, data has been published on the association of TNF-α with cervical cancer, silicosis HIV and systemic lupus erythematosus (SLE). There was no association was found between TNF-308α G/A polymorphism and the risk of cervical cancer among two South African ethnic population groups. However, the frequency of the A-allele was significantly lower in the South African population when compared to Caucasians and Chinese population groups suggesting that ethnic disparity may influence the levels of TNF-α (Govan *et al.* 2006). The genotype distribution of the TNF-308α GA polymorphism for the South African cancer cases were different to those reported in a Korean population group (see Table 2.6) (homozygous GG 71% vs. 90%; and heterozygous AG 26% vs. 6% respectively). The genotype distribution for GG and AA -308α genotypes for South African data were similar to those reported in Zimbabwean, Chinese, Italian, and Korean populations (Table 2.7). There was an increased frequency of the -308 TNF-α A-allele and decrease in the G allele in the British Caucasoid

group when compared to the South African population group (Govan *et al.* 2006).

Polymorphisms at positions -308, -238, and -376 in the TNF-α promoter region were compared in nine patients with severe silicosis, 112 patients with less severe silicosis, and 120 black South African gold miners without silicosis in an age-frequency-matched case- control study. The association remained significant (Fisher's exact p values = 0.011 and 0.011, respectively) when analysis was limited to the majority tribe (Basotho), which included all subjects with severe silicosis. Subjects with severe silicosis were also significantly more likely to have the TNF-308α A-allele (Fisher's exact p value = 0.034), but this result was confounded by ethnicity and was not significant within Basotho tribe members (Fisher's exact p value = 0.15). This study found that TNF-α promoter polymorphisms are associated with severe, but not “less severe”, silicosis in this population. A predominant effect on disease severity, rather than on disease frequency, appears to be a general feature of TNF-308α G/A promoter polymorphism (Corbett *et al.* 2002).

2.5 Gene-environment interactions

A recurrent problem with the genetic study of asthma is that many of the associations have not been replicated in multiple populations. This might be due to the fact that most studies do not take into account the contribution from the environment or other genes on expression of a given genetic variant or allele. Factors such as age, gender and diet influence the relationships between genetic variation and disease susceptibility (Stewart *et al.* 2007).

Studies have demonstrated up to a 50% contribution of the environment, this could have a dramatic confounding effect, especially in multicentre studies with

differential exposures. Some of the environmental factors that might contribute to the underlying genetic susceptibilities include endotoxin exposure (Vercelli 2008), diesel exposure, tobacco smoke, inhalant aeroallergens, diet, exposure to viral infections, and *in utero* factors during pregnancy. Incorporation of these risk factors into genetic studies allows the interplay between the gene and the environment to be investigated (Steinke *et al.* 2008). Gene- environment studies have focused on functional SNPs in candidate genes that are predicted to play a role in sensing these environmental agents and mediating the effects of exposure. To this end, the study of gene-environment interactions enables us to further understand the pathogenesis of an allergic disease such as asthma and the determinants of its severity and progression (Holloway *et al.* 2010).

Although these studies are yet to be replicated, they provide initial evidence of gene-environment interaction with allergens. The effects of air pollution on asthma susceptibility are also likely to be modified by SNPs in genes encoding inflammatory cytokines and metabolizing enzymes (Yang, IA *et al.* 2008). Although data are constantly emerging for gene-environment effects in asthma, the translational research challenge now is to integrate molecular, clinical, and epidemiologic studies of asthma to discover robust mechanisms of gene–environment interaction that would facilitate personalized interventions for persons with asthma. Furthermore, the use of genetic epidemiology is likely to present real opportunities for solving problems of casual inference in observational epidemiology. Epidemiologic studies of environmental exposures might identify spurious causes of disease caused by confounding by behavioural, physiologic, and socioeconomic factors related both to exposures and to disease end points (Holloway *et al.* 2010).

The first studies describing gene-environment interactions in childhood asthma only appeared in literature after 2000 (Leynaert *et al.* 2006). Those that have

been extensively studied can essentially be grouped into those associated with oxidative stress and interactions in which genes influence response to microbial organisms (McLeish and Turner 2007)

Table 2.6: Distribution (%) of -308 TNF-α genotypes in women with cervical cancer among different population groups (Govan *et al.* 2006).

Genotype	*South African n=244 (%)*	*Korean (South) n= 51 (%)*	*USA n = 127 (%)*	*Zimbabwe n = 103 (%)*
GG	174 (71)	46 (90)	91 (72)	74 (72)
GA	62 (26)	3 (6)	27 (21)	28 (27)
AA	8 (3)	2 (4)	9 (7)	1 (1)

Table 2.7 Distribution (%) of -308 TNF-α genotypes in the combined South African control population groups and other populations (Govan *et al.* 2006).

Genotype	*South African n=228 (%)*	*China (North and Ellis) n=300 (%)*	*Italy (North and Ellis) n= 216 (%)*	*Korea (South) n = 92 (%)*	*Manchester n = 106 (%)*	*Zimbabwe n = 101 (%)*
GG	172 (78)	274 (91)	172 (80)	85 (92.4)	65 (61)	81 (80)
AG	46 (20)	24 (8)	41 (19)	7 (7.6)	24 (23)	18 (18)
AA	10 (4)	2 (0.7)	3 (1)	0	17 (16)	2 (2)

Large cohort studies on children's' respiratory health have been (Ober, CCN and Abney 1998) done in several countries in the developed world, e.g. in the US (Ober, CCN and Abney 1998), England (Custovic *et al.* 2002), Europe (Wjst *et al.* 1998), Australia (Phelan *et al*. 2002), but to date there has been no similar work done on the African continent.

Several models of gene-environment interactions have been suggested. First, both the susceptible genotype and environmental exposure are required to produce a disease phenotype. Secondly, the environmental exposure causes increased risk of disease in everyone but a much greater effect in individuals with a susceptible genotype. Thirdly, the environmental exposure will only increase the risk of disease in people with a susceptible genotype. Fourth, both the environment and genotype produce excess risk (Wiesh and Meyer, 2000).
Different strategies have been used to assess the association of asthma and genetic variants. The candidate gene approach, selecting genes which have been shown in previous studies to be associated with asthma or some other respiratory phenotype, is at present, the most effective available to us in the South African context for studying complex diseases at the population level because it allows testing the interaction of a relatively small number of selected genes with other genes and environmental factors (Castro-Giner *et al* 2006). Of the approximately 500 association studies conducted with asthma (with simple association between gene and asthma outcome and no exposure), 60% of all studies were done on Caucasians, 28% on Asians and only 3% on populations of African ancestry (African American, African Caribbean, and 2 populations from West Africa). In most of these studies, statistical power was an issue, because less than 100 subjects were used. As of 2008 only 10 genes have been associated with asthma or atopy in more than 10 studies each (IL4, IL4RA, IL13, ADRB2, TNF-, HLA-DRB1, HLA-DQB1, FCER1B, CD14 and ADAM33)

(Yang *et al.* 2007), 15 have been replicated in 6 to 10 asthma studies (Steinke *et al.* 2008).

However, there are few examples from the literature of specific gene-environment interactions in relation to asthma (Tables 2.8 and 2.9). The clearest examples of genetic interactions for inhaled pollutants exist for ozone, environmental tobacco smoke (ETS) and endotoxin (London 2007).

The example of complex interactions between environmental exposures and polymorphisms in the CD14 gene in predisposing for allergy-related conditions offers a good indication of the complexity of the mechanisms that determine susceptibility to these conditions (Martinez 2005). Numerous studies have examined gene environment effects with asthma ETS, endotoxin, and pollution. In a study of Puerto Rican and Mexican families, patients with asthma with the CD14 (-159) TT genotype and exposure to ETS had lower serum IgE levels (Choudhry *et al.* 2005). In 596 nuclear families consisting of patients with asthma 4 to 17 years of age and their parents in Mexico City, the A allele of the TNF-308 SNP was associated with increased risk of asthma, especially among children of non-smoking parents (p_{int} =0 09)(Wu *et al.* 2007).

The CD14 (-159) T-allele, was protective against allergy, with a stronger effect in subjects with a farm childhood. In a French population, the CD14 (-159) TT genotype was associated with lowered risks of atopy and nasal allergy in subjects exposed to farm residence early in life (Leynaert *et al.* 2006). Similarly a study from Denmark showed that the CD14 (-159) T-allele was associated with a lower prevalence of allergy, and this tendency was stronger in subjects with a farm childhood (Smit *et al.* 2007).

The C-allele was found to be a risk factor for allergic phenotypes at low levels of exposure, whereas the T-allele was a risk factor at high levels of microbial exposure (Wu, H *et al.* 2007). The effect of exposure to day care on early life immune responses and atopy, respectively, has been shown to be modified by CD14 polymorphisms.

In an acute exposure study, 51 individuals undergoing ozone challenge were genotyped for 4 polymorphisms across the TNF gene (Yang *et al.* 2005). Mean reduction in FEV1 with ozone challenge was greatest in TNF-308α GG individuals compared with the other genotypes. A potential role for TNF polymorphism in susceptibility to childhood asthma has also been studied in the large Child's Health Study in the United States. Children with TNF-308α GG had decreased risk of asthma and lifetime wheezing. The protective effects of the GG genotype on wheezing outcomes were of greater magnitude in lower than higher ozone communities. The reduction of the protective effect from the -308 GG was most marked in the GSTM1-null and GSTP1 Ile/Ile groups (Cameron *et al.* 2006).

There is convincing evidence that air pollutants, in particular fine particles and ozone, exacerbate existing asthma, increase the risk for asthma hospitalization and emergency department visits, and impair lung function. Few studies have addressed interactions between air pollutants and genetics in relation to asthma (von Mutius, Erika 2009). Thus the consideration of environmental factors into genetic analyses of CD14 (-159) C/T and TNF-308α G/A may help to reveal some genetic effects that are masked by stronger environmental exposures. Finally, the analysis of gene-environment interactions may result in a reconciliation of seemingly contradictory findings with these SNPs.

Table 2.8: Gene environment association studies of the CD14(-159) C/T and asthma and related phenotypes based on a review of published articles in PubMed and a review by (Martinez, FD 2005) and (Székely and Pataki 2009)

Author	*Study*
(Bottema *et al.* 2008)	Study indicated that atopy is importantly influenced by interleukin 13 at age 1–8 years and by CD14 in interaction with pet exposure at ages 4 and 8 yrs. Additionally, pooled data improved effect estimates and genetic effects could be detected in interaction with important environmental factors.
(Bernstein *et al.* 2006)	No associations were found with individual SNPs and Diisocyanate asthma (DA). When stratified according to specific diisocyanate exposure, a significant association was found between the IL4RA (I50V) II and CD14 (C159T) CT genotype combination and the triple genotype combination IL4RA (I50V) II, IL-13 (R110Q) RR, and CD14 (C159T) CT was significantly associated with DA in HDI-exposed workers. Gene-environmental interactions may contribute to the pathogenesis of (DA), and gene-gene interactions may modulate this relationship.
(Choudhry *et al.* 2005)	Data suggested a gene-by-environment interaction between CD14 genotypes and environmental tobacco smoke among Latinos with asthma.
(Pacheco *et al.* 2010)	Laboratory animal allergy, murine allergen exposure, CD14/−159 or −550 genotype, did not predict changes in lung function. This shows that significant gene-environment interaction affects airways function in laboratory animal workers.
(Smit, LA *et al.* 2011)	The association between occupational endotoxin exposure and wheeze in agricultural workers was significantly modified by genetic variants in CD14 and MD2. The study suggests that carriers of the functional CD14/-260 C allele are more responsive to endotoxin exposure than T allele homozygotes.
(Svendsen *et al.* 2007)	Children without measurable CD14 expression on circulating neutrophils had significantly reduced pulmonary function unlike children with measurable CD14 expression this study concluded that asthmatic children with muted surface expression of CD14 on circulating neutrophils may have a decreased capacity to respond to bacterial components of PM.

Table 2.9: Gene environment association studies of the CD14(-159) C/T and asthma and related phenotypes based on a review of published articles in PubMed and a review by (Martinez, FD 2005) and (Székely and Pataki 2009)

Author	*Study*
(Smit, LAM *et al.* 2009)	TLR2 and CD14 SNPs were associated with asthma and atopic asthma respectively. In addition, CD14, TLR2, TLR4, and TLR9 SNPs modified associations between country living and asthma.
(Zambelli-Weiner *et al.* 2005)	The TT genotype was found in the study to might protect against asthma for individuals with low house dust endotoxin (HDE) but may was found to be a possible risk factor for individuals with high HDE suggesting a gene-environment interaction.
(Zhang, G *et al.* 2009)	Finnish and Russian Karelians are of the same ethnic group but the earlier have a higher prevalence of allergic disease than Russian Karelians. The Karelian population offers a unique opportunity to analyse genetic and allergic disease interactions between 'Western' and 'Eastern' environment. The study concluded that an Eastern versus Western environment appears to exert an effect via opposite alleles on risk of allergic diseases in adult women.
(Zhao and Bracken 2011)	A protective dose-response relationship between the CD14 -260T allele and atopic asthma susceptibility was observed
(Keskin *et al.* 2006)	C/T Polymorphism in position 159 in the promoter region of the polysaccharide promoter gene (Coded CD14) in Peripheral blood mononuclear cells (PBMCs) taken from asthmatic children. PBMCs of Asthmatic children with TT Genotype generate more IL- 1β and IL-10 upon endotoxin stimulation
(Buckova, D *et al.* 2006)	Positive association of IgE mediated allergic diseases (positive skin prick test)with the C allele of the CD14/ 159C/T polymorphism
(Le Souëf 2006)	This polymorphism is a predisposing factor to asthma in children but not in young adults

Table 2.9 (continued): Gene environment association studies between TNF- α 308 (G>A) polymorphism and asthma and its related phenotypes based on (Székely and Pataki 2009) and (Gao *et al.* 2006)

Author	*Study*
Gao et. al, 2006)	TNF-2 allele confers significant risk to asthma
(Zhu *et al.* 2000)	TNF-[α]-308*, is not a risk factor for the development of atopy, asthma, and rhinitis by 12 months of age.
(Winchester *et al.* 2000)	TNF- -α-308 allele 2 (−308A) was significantly associated with self-reported childhood asthma in the UK/Irish population.
(Wang *et al.* 2004)	Concluded that TNF-α-308 may be a risk factor for atopic asthma, whereas the LT-α-NcoI polymorphism may modify risk to atopic asthma with TNF-α-308
(Sandford *et al.*)	The A allele of the TNF-A-α G-308A polymorphism was a risk factor for asthma-related phenotypes in girls but not boys.
(Moffatt and Cookson 1997)	The association was confined to the LTαNcoI*1/TNF-308*2 haplotype, so that it was not possible to differentiate between the effects of LTαNcoI and TNF-308 alleles.
(Louis *et al.* 2000)	The polymorphism -308 in the promoter of the TNF-α gene does not confer a susceptibility to develop asthma nor to grade its severity
(Li Kam *et al.* 1999)	Found no independent association of TNF- alleles with BHR the authors concluded that the − 308 TNF-2 promoter polymorphism may form a component of the genetic predisposition to BHR in asthma
(Witte 2002)	The TNF- -α -308*2 allele increases risk of asthma.
(Di Somma *et al.* 2003)	Association of asthma to TNF-2 reflects linkage disequilibrium with genes influencing specific immune response.
(Chagani *et al.* 1999)	TNF-α 308 polymorphism may be a risk factor for asthma but does not increase the risk of a fatal or a near-fatal asthma attack
(Bilolikar *et al.* 2005)	Significantly lower nasal TNF-α levels were found in the presence of one or two TNF-α 308A alleles. TNF-α 308 and LT-α 252 genotype combinations had a significant influence on nasal TNF-α levels
(Beghe *et al.* 2004)	These results suggest that HLA class I antigens and TNF-α A-308G are not associated with susceptibility or resistance to the development of Toluene diisocyanate (TDI)-induced asthma.

The challenge in gene-environment studies is to use genetic biomarkers to establish associations between exposure and human disease in epidemiological studies and then to use the knowledge to design and conduct appropriate preventative interventions in high risk populations such as children, the elderly and the immune compromised (Suk *et al.* 2003).

There are two major reasons to investigate genetic susceptibility to air pollution effects in humans. The first is that the effects of air pollution on respiratory outcomes are modest in the general population because the population includes individuals relatively resistant to air pollution. Thus, the ability to detect subtle effects of air pollution may depend on the ability to identify susceptible subpopulations. Second, susceptible groups might experience health effects at levels below current exposure standards. An essential question is: are environmental influences associated with asthma more likely to affect people with certain genetic profiles? It is therefore necessary to simultaneously study genetic variants in association with asthma related phenotypes and environmental exposures.

Understanding the biology of diseases may potentially lead to the development of strategies based on mass intervention or target risk intervention approaches. This could facilitate the setting up of a genetic risk profile for the development of asthma which would enable us to take preventative measures early in life for children with an increased genetic risk to allergic diseases. It could also allow the development of secondary preventative measures (the development of primary tests for population wide screening) and therapy (choosing among alternative interventions or assist in the design of new drugs which are more specific, effective and safe) (Reddy 2007).

Many genes have been implicated in asthma pathogenesis, but only those with a functional and biological relevance were chosen for this study. Since 2005, SNPs in CD14 and TNF- 308 have been implicated in asthma pathogenesis. A retrospective study was conducted using stored bloods and health-environmental exposure data generated by the South Durban Health Study (SDHS). In this South African population of black African children, the frequency of each these genetic polymorphisms and the risk conferred by that polymorphism on respiratory outcomes, was determined. It was also determined whether the relationship between exposure (to ambient air pollutants) and respiratory outcome was modified by genotype. A search of current literature revealed that there were only one study on each of these polymorphisms on the African subcontinent in relation to environmental exposures and asthma phenotypes. One study was conducted in Tunisia and another in Egypt (Lachheb *et al.* 2008; Zedan *et al.* 2008)

The following were the aims of this study:

Research Aims

- To assess the frequency of gene variants CD14 (-159) C/T and TNF-308 (G/A) among schoolchildren from 2 areas in Durban.
- To evaluate the contribution of the two gene variants to various respiratory phenotypes
- To investigate fluctuations in lung function measures (FEV_1 and PF) in relation to daily averages in ambient air pollutants ($S0_2$, $N0_2$, NO, and PM_{10}) using genotypes as an effect modifier among the population of schoolchildren

Chapter 3: Methodology

Genetic and biochemical data on the TNF-308α G/A and CD14 (-159) C/T polymorphism was collected and used with retrospective data collected as outlined below.

The SDHS was completed in 2004-2005 and it involved repeated measures of pollutant exposures, across different seasons, near the vicinity of a cohort of schoolchildren aged 9-11 years old. The South Durban Health Study (SDHS) measured the health effects of pollutant exposures on the children using lung function measures, FEV_1 and PF, which were taken daily for a period of 3 weeks in each of 4 intensive phases.

Data produced from the SDHS is used in the current research. This included respiratory data using a validated questionnaire from the British Medical Research Council and the American Thoracic Society that assessed the presence and severity of asthma among participants. Information was gathered on wheezing, coughing, chest tightness, and shortness of breath, bronchitis, hay fever, activity limitations and medication use, health services utilization and a variety of covariates. The following data was also generated. Baseline BHR was established with methacholine challenge testing and allergic skin prick testing (8 different antigens were tested). Acute respiratory outcomes was correlated with daily changes in levels of air pollution i.e. changes in lung function measures, i.e., FEV_1, and peak expiratory flow (PEF) in relation to daily fluctuations in SO_2, NO, NO_2, and PM_{10} over a period of a year.

Stored biological samples (whole bloods) collected during the SDHS were used for DNA analysis in this current study and genetic polymorphism results were

statistically modelled with the results produced from the SDHS study which includes phenotypic markers such as asthma, atopy and bronchial hyper responsiveness and air pollutant data for levels of PM_{10}, SO_2, NO and NO_2. The sampling strategy and protocols for the collection of respiratory and clinical data and pollutant monitoring is included in Appendices 1, 2, 3, and 4.

The current study investigated two genetic polymorphisms which are known to contribute to differing susceptibilities to respiratory outcomes when exposed to varying environmental conditions. For each of these polymorphisms, it was determined whether the relationship between exposure and respiratory outcome was modified by genotype. DNA analysis was carried out on frozen blood samples obtained from the SDHS. The samples were stored at -80ºC. For the genetic study the sample group comprised of 104 African children.

3.1 Selection of Communities/Sampling strategy

In order to properly characterise exposure and health outcomes, a broad geographical coverage of the Durban South basin was necessary. The following residential areas were selected In the Durban South: (a) Merebank, (b) Wentworth/Austerville; (c) Bluff and (d) Lamontville. Comparison communities in the northern residential areas of the Metropolitan boundaries selected were: (a) Newlands East; (b) Newlands West and (c) KwaMashu. The latter communities were selected because of: their proximity to each other; having a similar socio-economic profile as communities in the Durban South and having relatively little industrial exposure

3.2 Ethical Considerations

3.2.1 Ethics Approval

The research project obtained expedited ethical approval from the Ethics Committee of the University of KwaZulu-Natal (Ref: BE156/09) (Appendix 5). Permission for use of the SDHS samples and study results was granted by the Principal Investigator (Appendix 6).

3.2.2 Individual informed consent

Individual informed consent was obtained for all participants. In the case of children, this was obtained from their parent or guardian (Appendix 7). The children themselves were given an informed assent form (Appendix 8). In this instance each participant was given a comprehensive explanation in the language of their choice (Appendix 9). The content of these forms included the aims of the research, the purpose of the interview, the tests that would be conducted on them, use of their data and the confidentiality of all results. It was emphasised that participation was voluntary and withdrawal at any time was permitted without penalty. No financial incentives were provided for participation in the study.

3.2.3 Participants' confidentiality

All information obtained during the study from interviews and genetic assessments were treated in a strictly confidential manner and were only accessible to the research team. These results would be released to any clinician/guardian/agency if this was desired by the individual participant.

3.2.4 Reporting and publication of results and reports

In the publication of research results and reports, all data will be treated as grouped, thus no individual will be identified from such documentation. As per the required guidelines of the University of KwaZulu-Natal, the final content of the articles submitted to peer review scientific journals will be the responsibility of the researchers

3.3 DNA Extraction

Genomic DNA was extracted from stored blood using a PUREGENE DNA isolation kit (cat #D5000; GENTRA, Minneapolis, MN). DNA was quantified using the Nanodrop ND 1000 Spectrophotometer. DNA samples were then aliquoted and stored at –70°C. Working stocks were kept at -20°C at a concentration of 100 ng/µl and 30 ng/µl. All genotyping assays were conducted by a researcher who was blinded to child ID and status.

3.4 TNF-308α G/A Polymorphism

3.4.1 Genotyping of TNF-308α G/A

For the detection of TNF- 308 the DNA was standardized to 3ng/µl. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism(PCR-PFLP) method was used to detect the TNF- 308 genotype DNA (9ng) was added to a PCR mix containing 40 pmol of forward primer,(5'-AGGCAATAGGTTTTGAGGGCCAT-3') and reverse primer (5'-TCCTCCCTGCTCCGATTCCG-3) in a 25 µl reaction containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1X Green GoTaq Flexi buffer (Promega), 1.25U GoTaq DNA polymerase (Promega) (see Table 3.1).

The reaction mixture was run in a Gene Amp® PCR system 9700 thermal cycler for an initial denaturation step at 96°C for 12 min, amplification was carried out by 35 cycles of denaturation at 94°C for 3 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min (Wilson *et al.* 1992).

Table 3.1: TNF-308α G/A PCR Reaction Mastermix

Component	*Concentration*	*1x PCR*
DNTPs	0.2mM	2µl
Forward Primer	20pMol/µl	2 µl
Reverse Primer	20pMol/µl	2 µl
Buffer	1x	5 µl
MgCl2	1.5mM	1.5 µl
Taq	1.25U	0.25 µl
Nuclease free H20	9.25µl	9.25 µl
DNA		9µg

The PCR product was then digested by restriction endonuclease (NcoI; Fermentas). Overnight digestion at 37°C was performed in a total volume of 29.5 µl, containing 10 µl of the PCR product, 2 µl Buffer Tango (33 mM Tris-acetate (pH7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) and 1.5 µl (15U) Nco1 restriction endonuclease. Restriction fragments were electrophoresed on a 3% agarose gel containing 0.5 mg/ml ethidium bromide and visualized by UV light and digitally photographed using a gel documentation system (Chemi-Doc XRS; Bio-Rad).

- G allele 87 and 20 base pair products
- A allele 107base pair product

3.4.2 Analysis of plasma TNF-α concentration

Plasma TNF-α levels were measured using the human TNF-α Max Standard™ Enzyme-linked immuno-absorbent assay (ELISA) kit (Biolegend) following the manufacturer's instructions. Briefly, high-affinity microtitre plates were coated with 100 µl TNF-α capture antibody (18 hours at 4°C). Plates were washed and treated with 200 µl assay diluent. Thereafter, 100 µl standards and samples were added. Biotinylated anti-human TNF-α detection antibody and avidin-horseradish peroxidase were then added, followed by the TMB substrate and stop solution. Absorbance was measured at 450nm (570nm reference) (Bio-Tek µQuant ELISA plate reader). Plasma concentrations of TNF-α were calculated by extrapolation from a standard curve.

3.5 CD14 (-159) C/T Polymorphism

3.5.1 Genotyping of CD 14(-159) C/T

The CD14 genotypes were determined by Taqman Assays (Applied Biosystem, Foster City, CA). This polymorphism was done using Assay- by-Design (Taqman® SNP Genotyping Assays) assay mix. The primer and probe sequences are shown below in Table 3.2.

Table 3.2: CD14 Primer and Probe Sequences for Taqman PCR (Barber *et al*. 2007)

Forward Primer : ACCCTAGATGCCCTGCAGAA
Reverse Primer : GGAAATATTGCAATGAAGGATGTTT
Taq Man probes
Fam : TTCCTGTTACGGCCC
Vic : CTTCCTGTTACGGTCC

The PCR mastermix was made up as below in Table 3.3. Wet DNA was used for the PCR. The reaction mixture was incubated at 50°C for 2 mins, then 95°C for 10 mins. This was followed by annealing and primer extension at 60°C for 1 min. The fluorescence of PCR products was detected by the ABI Prism ®7000 sequence detection system (SDS) and was analysed by the on board software (Applied Biosystems). Allelic discrimination plots were used to determine genotype. Each PCR plate (96 well) had at least 2 no template controls. There was 100 % matching of these controls.

Table 3.3: CD14 PCR Reaction Preparation

	1x Mix
Taq ® Universal PCR Mastermix, No Amperase ® UNG (2X) (ABI 4331349)	12.5µl
40X Assay Mix	0.625µl
Nuclease Free H_2O	8.875 µl
DNA	3 µl

3.5.2 Analysis of serum CD14 concentration

Plasma CD14 levels were measured using the Quantikine Human sCD14 Immunoassay ™ (R & D SYSTEMS Minneapolis, MN) following manufacturer's instructions. Briefly, the assay employs the quantitative sandwich enzyme immune assay technique. A monoclonal antibody specific for sCD14 was precoated onto a microplate. Standards and samples were pipetted (100µl) into the respective wells and any sCD14 present would bind to the immobilized antibody. After washing away any unbound substances with a buffer (400µl), an enzyme linked polyclonal antibody specific for sCD14 was added to the wells. After washing, to remove any unbound antibody-enzyme reagent, a substrate solution (200µl) was added to the wells and colour developed in proportion to the amount of sCD14 bound in the initial step. The colour development was

stopped by adding a stop solution (50µl) and the intensity of the colour was measured at 450nm (570nm reference) (Bio-Tek µQuant ELISA plate reader). Serum concentrations of sCD14 were calculated by extrapolation from the standard curve. The data was first linearized by plotting the log of sCD14 concentrations versus the log of the absorbance and the best fit line was determined by regression analysis.

3.5.3 Statistical Analysis

All data was initially captured using Microsoft Excel Software and subsequent analyses were done using STATA (version 9, College Station, TX, USA). CD14 and TNF- 308 polymorphisms were categorized into two groups, based on the absence or presence of the polymorphic allele (wild type homozygous versus the combined heterozygous plus the variant homozygous genotype), using the dominant model ensured increased statistical power. Frequency distributions of categorical variables and means, standard deviation and ranges of continuous variables were calculated. Continuous variables included age, weight, height, levels of environmental pollutants and levels cytokine proteins in plasma. Categorical variables include genotype, age, gender, region (north/south Durban) and respiratory disease outcomes namely asthma BHR and atopy. The respiratory outcomes were defined as discussed below.

3.5.3.1 Bronchial Hyperresponsiveness

BHR was defined as follows:

1 = Marked BHR: dose ≤ 4 and 20% or more drop on FEV_1 (compared to maximum (saline) or 20% increase on bronchodilator (compared to baseline)

2= Probable BHR: 4< dose ≤ 8 and 20% or more drop on FEV_1 (compared to maximum (saline)) or 20% increase on bronchodilator (compared to baseline)

3 = Borderline/ Possible BHR: 8 < dose ≤ 16 and 20% or more drop on FEV_1 (compared to maximum saline) or 20% increase on bronchodilator (compared to baseline)
4 = Normal Airway reactivity: dose > 16 and drop less than 20% with any other dose.

Categories 1, 2 and 3 were collapsed to define the variable “positive evidence of airway hyperreactivity” while category 4 defined “normal airway reactivity”

3.5.3.2 Atopy

Atopy was defined as having a positive reaction to the skin prick test greater than that of the response to the histamine for any of the following allergens: house dust mite, cat, dog cockroach, cladosporium, grass and mould. A greater than 3mm difference in mean diameter between allergen and control wheal was considered positive. Atopy was considered as a dichotomous variable i.e. atopic or non-atopic.

3.5.3.3 Asthma Severity

In this study, asthma severity was categorized in two ways: probable (or known) asthma of any severity (designated “Any Asthma”); and probable (or known) persistent asthma, including mild and moderate to severe persistent asthma (designated “Persistent Asthma”). These categories were determined by the responses from the screening questionnaire (which was completed by either the caregiver or head of household) modeled on the ATS and BRMC questionnaires

3.5.3.4 Any Asthma

A child was considered **to have probable (or known) asthma (of any severity)** if any of the following were true:

(a) Three or more of the six non-exercise related symptoms (i.e., questions S22, S23, S24, S25, S28 and S29) were reported (at any frequency or level greater than ”never”).

S22. In the past 12 months, how often has your child had a **cough that won't go away**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year□ □6 never
S23. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) **with a cold**?	□1 more than 1 time per month □2 3 to 12 times in the whole year □3 1 or 2 times in the whole year □4 never
S24. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) ***without* a cold**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S25. In the past 12 months, how often has your child had an attack of **wheezing** that made it **hard to breathe or catch his or her breath**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S28. In the past 12 months, how often has your child complained that his or her **chest felt tight or heavy**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S29. In the past 12 months, how often has your child's **sleep been disturbed due to wheezing, coughing, chest tightness or shortness of breath**?	□1 most nights □2 more than 1 time per week □3 more than 2 times per month □4 more than 1 time per month □5 3 to 12 times in the whole year □6 1 or 2 times in the whole year □7 never

Either exercise symptoms (i.e., S26 and S27) was reported with frequency of three times or more in the past year i.e. S26 (1, 2, 3, or 4); S27 (1, 2, 3, or 4)

S26. In the past 12 months, how often has your child **wheezed while exercising, running or playing?**	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S27. In the past 12 months, how often has your child **coughed while exercising, running or playing**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never

3.5.3.5 Persistent Asthma

A child was considered to have **probable (or known) mild persistent asthma** if, firstly, the child meets the diagnostic criteria for asthma above, and secondly, any of the following are true:

a) any daytime symptom (i.e., questions S22 through S28) is reported as being present “every day”

S22. In the past 12 months, how often has your child had a **cough that won’t go away**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year□ □6 never
S23. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) **with a cold**?	□1 more than 1 time per month □2 3 to 12 times in the whole year □3 1 or 2 times in the whole year □4 never
S24. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) ***without* a cold**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S25. In the past 12 months, how often has your child had an attack of **wheezing** that made it **hard to**	□1 every day □2 more than 2 times per week □3 more than 1 time per month

breathe or catch his or her breath?	□4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S26. In the past 12 months, how often has your child **wheezed while exercising, running or playing?**	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S27. In the past 12 months, how often has your child **coughed while exercising, running or playing**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S28. In the past 12 months, how often has your child complained that his or her **chest felt tight or heavy**?	□1 every day □2 more than 2 times per week □3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never

b) sleep disturbance (question S29) is reported "more than one time per week" or "most nights" i.e 1 or 2

S29. In the past 12 months, how often has your child's **sleep been disturbed due to wheezing, coughing, chest tightness or shortness of breath**?	□1 most nights □2 more than 1 time per week □3 more than 2 times per month □4 more than 1 time per month □5 3 to 12 times in the whole year □6 1 or 2 times in the whole year □7 never

c) Daily use of doctor-prescribed medicine (i.e., "yes" on question S32) with any daytime symptom reported as being present "more than two times per week" i.e. option 2 for Questions S22-S28 above.

S32. Does your child take **any of these doctor-prescribed medications every day**, even when he/she is **not** having trouble breathing?	□1 Yes □2 No □8 Does not apply

d) One or more daytime symptoms are reported as being present "more than 2 times per week" i.e. option 2 for Questions S22-S28 above.

e) Sleep disturbance reported is reported “more than 2 times per month” i.e option 2 on S29.

f) Daily use of doctor-prescribed medicine (i.e., “yes” on question S32).

Bivariate associations between genotype and outcomes, such as asthma BHR and atopy, were evaluated. Genotype was investigated as a predictor of outcome/response and as a modifier of the main effects of environmental pollution. Logistic models were developed for binary outcome variables and adjusted for relevant covariates. Effect modifications were examined by including interaction terms in the models. Odds ratios and confidence intervals of 95% were calculated and p values < 0.05 were considered statistically significant.

Data produced by the SDHS was made available for the purposes of this current study. This data included:

- Substantial repeated measures of pulmonary function tests (PFTs). On each child 4 Pulmonary Function Tests (PFTs) were conducted during a school day, this was done every day for three consecutive weeks, once every season.

- Simultaneously, daily hourly measurements of the criteria pollutants (SO_2, NO, NO_2 and PM_{10}) were collected continuously monitored for the duration of the SDHS (Appendix 2).

The longitudinal design of this study allowed the investigation of how daily and bihourly fluctuations in outdoor contaminant levels affected potential fluctuations in pulmonary function measures. Linear regression models were fitted using generalized estimating equations (GEEs, assuming normal distribution with identity link) using PROC GENMOD for SAS to accommodate the correlation structure arising from repeated measurements on the same individual. An

exchangeable correlation working structure was used.

Within-day variability for FEV_1 was defined as:

100 x (the maximum best FEV1- minimum best FEV1)/maximum best FEV1

where the “best FEV1” is the highest valid value for the specific time of day (08h00, 09h45, and 11h30, 13h20), thus providing a single summary lung function measurement per child, per day. Within-day variability for PF is defined analogously to within-day variability for FEV_1.

Covariates used in the GEE models included race, school, caregiver smoking, caregiver education, household income and season. Effect modification was examined by including genotype and pollutant product terms in the models. The gene-environment-interaction was assessed for associations of exposure to SO_2, NO, NO_2 and PM_{10} with nadir and intraday variability of peak expiratory flow (PF) and forced expiratory volume in one second (FEV_1), using CD14 (-159) C/T and TNF-308α G/A genotypes as the effect modifiers. Nadir FEV_1 or Nadir PF was the daily lowest valid value (i.e the minimum best of all the lung function values taken for a particular day).

Daily exposures estimates were based on the child's school: PM_{10} and SO_2 used school-based measurements; and NO and NO_2 used the spatial average across either north (3 schools) or south (4 schools) Durban, as not all schools had NO_x monitors. Multiple imputation procedures (repeated five times for each exposure parameter) were used to obtain a complete data set. Lag effects were modeled to account for both acute and prior exposure effects, and included lags of 1 to 5 days as well as the a 5-day average. The percent change in within-day variability in FEV1 were estimated for an increase of one interquartile range in

each pollutant (NO2: 8.19 ppb, NO: 29.7 ppb, PM10: 29.4 ug/m^{-3} and SO2: 9.8 ppb). The interquartile range was calculated as the 75th - 25th percentile value concentrations, using all of the concentration measurements obtained in the study. The use of the IQR for a specific pollutant, when multiplied by the corresponding estimated coefficient and transformed appropriately (since logarithms are typically used), allows a direct comparison of the effect size among the pollutants used in the study, and it assures the magnitude of the change in exposure being examined for effects is relevant to the study population. This approach also accounts for the differences among concentrations units. An adverse effect in lung function would be denoted by an increase in the estimate for intraday variability in FEV_1. Analyses used SAS (Version 9.1) and STATA (version 9, College Station, TX, USA).

Chapter 4: Results

4.1 Demographic Characterization of study Population

A sample of indigenous, South African children was genotyped for polymorphic variants of TNF-308α G/A and CD14 (-159) C/T. The demographic, phenotypic and genotypic characteristics of the population sample are summarized in Table 4.1. A random sample of 149 African children was used. However, only 104 of the samples were included in the study. This was because some of the stored samples were insufficient for DNA extraction and genotyping. The average age was 10.3 years and a larger proportion of the children were female (71%). Half the children in the sample were exposed to tobacco smoke in their households. Approximately 40% of the study population carried the TNF variant (GA/AA), while 36% carried the CD14 polymorphic variant (CT/TT). This genotype distribution will be discussed further in the section below. A relatively high proportion, almost 40%, of the study population was reported as having any asthma. Nearly a third of the population was atopic (27.4%). These statistics are a reflection of the community's concern over the environment in the South Durban basin.

4.2 TNF- 308 α and Respiratory Outcomes

4.2.1 Genotyping of TNF- 308α G/A

Polymorphic variation at position 308 in TNF-α promoter gene was investigated in a random sample of 104 indigenous African children. PCR-RFLP analysis was used to genotype the samples. The reaction produced a 107 base pair product (lane 9) (Figure 4.1) which was then digested with *NcoI* restriction

endonuclease. Samples which were homozygous for the A allele did not contain the restriction endonuclease consensus sequence and remained undigested,

Table 4.1: Demographic, phenotypic and genotypic characteristics of study population (n=104)

Categories	n=104 (%)
Average Age	10,3
Sex	
Male	33 (29,8)
Female	71 (68,3)
Prevalence of asthma and related phenotypes	
Any Asthma (n=90)	35 (38,9)
Persistent Asthma (n=90)	14 (15,6)
Atopy (n=95)	26 (27,4)
Bronchial Hyper responsiveness (n=80)	15 (18,8)
Environmental Exposures	
Exposure to Environmental Tobacco Smoke (n=96)	48 (50)
Genotyping	
TNF- (308α) G/A	
TNF- 308 α wild type (GG)	62 (59,6)
TNF- 308 α heterozygote (GA)	34 (32,7)
TNF- 308 α homozygote (AA)	8 (7,7)
TNF- 308 α variant dominant model (GA/ AA)	42 (40,3)
CD14 (159)C/T	
CD 14 (159) wild type (CC)	45 (63,4)
CD 14 (159) heterozygote (CT)	20 (28,2%)
CD 14 (159) homozygote (TT)	6 (8,5%)
CD 14 variant dominant model (CT/TT)	26 (36,6)

therefore producing no restriction fragments (Lane 3 and 8) (Figure 4.1). Amplification products from samples homozygous for the G allele were completely digested to two fragments of 20 bp and 87 (lanes1, 2, 4, 5, 6) (20 bp

band not shown). Digestion of heterozygous samples resulted in three restriction fragments of 107 bp, 87 bp and 20 bp (lane 7). A high proportion of the population was homozygous for the wild-type TNF-308α GG (59.6%). The TNF-308α AA genotype was relatively rare with only eight participants carrying this genotype representing 7.7% of the study population. Despite this, the AA genotype frequency in the Durban population is higher compared with other published results of the polymorphism in other African populations. The frequency of the GG genotype is reduced in the Durban population compared to other African countries. The frequency of the heterozygotes GA and the AA in this Durban population are strikingly similar to that in the USA and in Manchester England (Table 2.6 and 2.7).

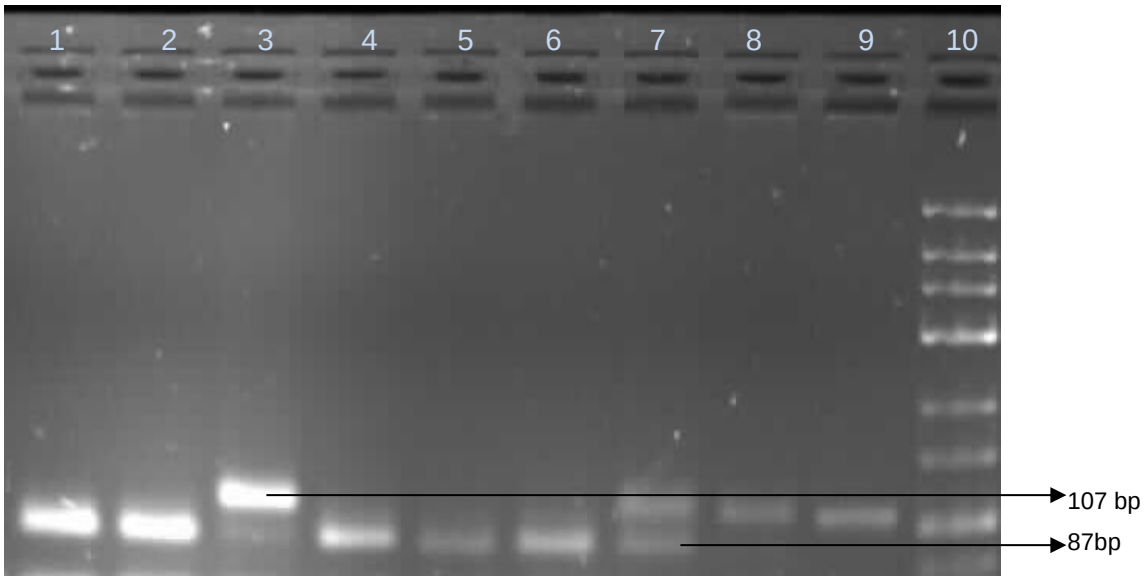


Figure 4.1. PCR RFLP analysis of TNF- 308 gene polymorphism. Agarose gel picture showing TNF- 308 genotyping results. Lanes 1, 2,4,5,6 GG, Lane 7 GA, Lanes 3 and 8 AA, lane 10 DNA ruler.

4.2.2 Bivariate Analysis of Genotype and Respiratory Outcome

Genotype frequencies observed in the study population did not deviate from those predicted by the Hardy Weinberg Equilibrium (p=0.29) (Table 4.2). The Pearson's chi squared test was used to evaluate whether a particular respiratory outcome was associated with genotype. Tables 4.3 and 4.4 below show the frequencies of the TNF- 308 genotypes in relation to persistent asthma, any asthma, doctor diagnosed asthma, atopy and BHR. Bivariate tests indicated no statistically significant association between the TNF-α-308 G/A promoter polymorphism with asthma related phenotypes such as bronchial hyper responsiveness and atopy using both the dominant model and the recessive model. However with the recessive model the relationship between bronchial hyper responsiveness and genotype almost reached significance (p=0.06) (Table 4.4). Twenty percent of the participants who were BHR cases had the AA genotype that represented half of the participants who were AA genotype. The A allele has been linked to more severe disease and increased levels of TNFα cytokine in literature. The highest proportion of GG patients (71.4%) had persistent asthma (p= 0.47).

4.2.3 Association of TNF-308α gene polymorphism with TNF- α protein levels

The mean level of TNF- α protein was 7.30pg/ml±3.48pg/ml for AA, 14.69 pg/ml ± 17.67 pg/ml for AG and 12.15 pg/ml ± 9.76 pg/ml for GG. This range of concentrations is shown in Figure 4.2 The median readings of the TNF-α protein were similar for all three genotypes. The highest reading was a 100pg/ml in GG individual though this reading was considered an outlier. The highest TNF-α

level in a GA individual was similar at 92.06pg/ml. In AA genotype participants the minimum reading of cytokine was 3.82pg/ml and the maximum was 11.76pg/ml. Carriers of the A allele have been found in literature to be associated with higher TNFα production. There were no significant differences in TNF-α levels between the genotypes in both the dominant model p=0.66 and the recessive model p= 0.40 (Figure 4.2 and 4.3).

Table 4.2: Genotypic and allelic frequencies of the TNF-308α G/A polymorphism

Genotype Frequencies	*Sample (n=104)*
GG	62 (59.6%)
GA	34 (32.7%)
AA	8 (7.7%)
Allele Frequencies	
G	76%
A	24%

Table 4.3: Frequencies of TNF-308α G/A Polymorphic genotypes stratified by respiratory outcomes using the Dominant Model

Genotype	*Any Asthma N=90 (%)*		*Persistent Asthma N=90%*		*BHR N= 80 (%)*		*Atopy N= 95 (%)*	
	Non Case	**Case**	**Non Case**	**Case**	**Non Case**	**Case**	**Non Case**	**Case**
GG	31 (57.4)	23 (42.6)	44 (81.5)	10 (18.5)	40 (80)	10 (20)	44 (74.6)	15 (25.4)
GA/AA	24 (66.7)	12 (33.3)	32 (88.9)	4 (11.1)	25 (83.3)	5 (16.67)	25 (69.4)	11 (30.6)
P-value	0.377		0.390		0.477		0.568	

Table 4.4: Frequencies of TNF-308α G/A Polymorphic genotypes stratified by respiratory outcomes using the Recessive Model

Genotype	*Any Asthma N=90 (%)*		*Persistent Asthma N=90%*		*BHR N= 80 (%)*		*Atopy N= 95 (%)*	
	Non Case	**Case**	**Non Case**	**Case**	**Non Case**	**Case**	**Non Case**	**Case**
GG	31 (57.4)	23 (42.6)	44 (81.5)	10 (18.5)	40 (80)	10 (20)	44 (74.6)	15 (25.4)
GA	20 (64.5)	11 (35.5)	28 (90.3)	3 (9.7)	22 (91.7)	2 (8.3)	19 (65.5)	10 (34.5)
AA	4 (80)	1 (20)	4 (80)	1 (20)	3 (50)	3 (50)	6 (85.7)	1 (14.3)
P-value	0.64		0.47		0.06		0.54	

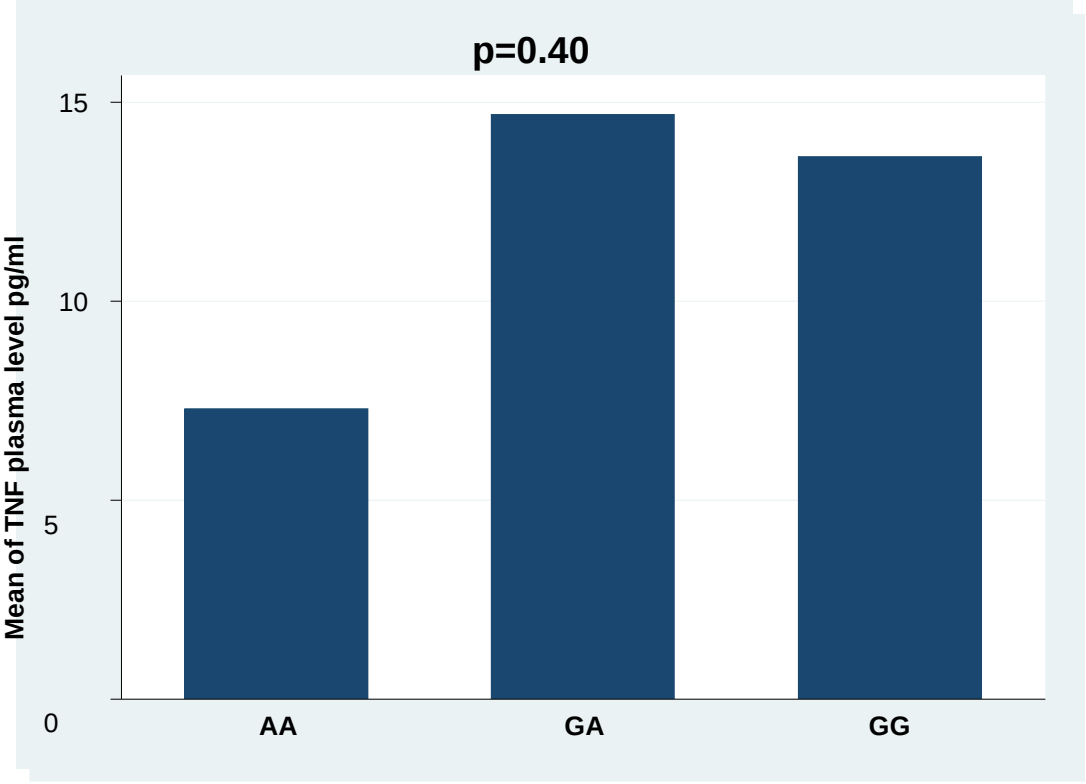


Figure 4.2. TNF plasma levels in all participants stratified by TNF α 308 gene polymorphisms n= (84)

4.2.4 Concentration of TNF-α protein compared with Respiratory phenotypes

The participants in the study had a mean TNF- α plasma level of 12.68 pg/ml ± SD 12.85 pg/ml. When comparing participants with or without any asthma there was no significant difference in TNF-α levels in participants with any category of asthma and those without any category of asthma. However one participant with asthma had a TNF-α concentration of 92.06pg/ml. The median reading in asthma cases was slightly higher (11.76pg/ml compared to 9.12pg/ml) than non-cases (Figure 4.3). Similarly TNF- levels were generally higher in atopic than in non-atopic participants (Figure 4.5). The mean for atopic participants was 15.81±20.93pg/ml and non-atopic 12.19±9.90pg/ml. The maximum TNF-α level

in atopic patients was 92.06pg/ml compared 44.71pg/ml in the non-atopic subjects. The median TNF- level was also slightly higher in atopic participants. However there was no significant difference in TNF- α levels among atopic and non-atopic participants.

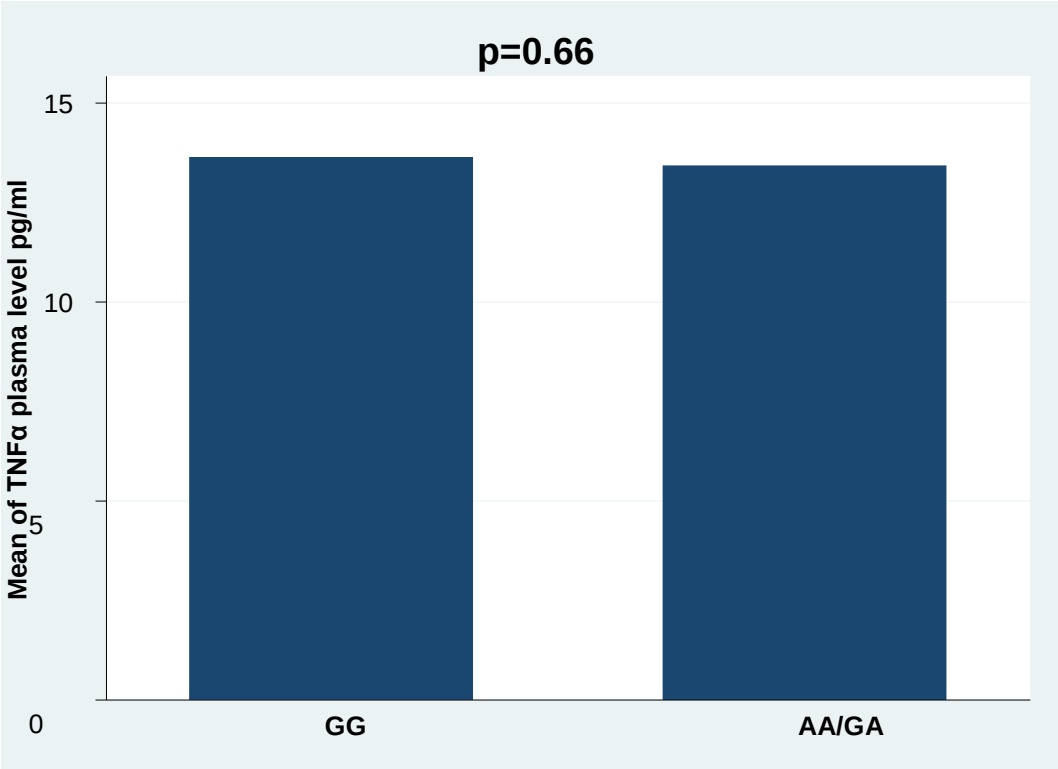


Figure 4.3. TNF plasma levels in participants stratified according to the Dominant Model n= (84)

TNF- α levels were unexpectedly higher in participants without bronchial hyper responsiveness, although this difference was not statistically significant p=0.18 (Figure 4.6). The mean and median TNF- level in participants without BHR was almost double the TNF- level in BHR cases. Also the highest reading 90.06pg/ml was a participant without BHR.

Participants with persistent asthma showed a significant difference in TNF- α levels in subjects with persistent asthma and those without (p=0.03) (Figure 4.7). The mean TNF-α level was significantly higher in persistent asthma cases (20.94±23.52 pg/ml). Notably the highest TNF- α level was 92.6pg/ml from a participant with persistent asthma and with the GA polymorphic genotype.

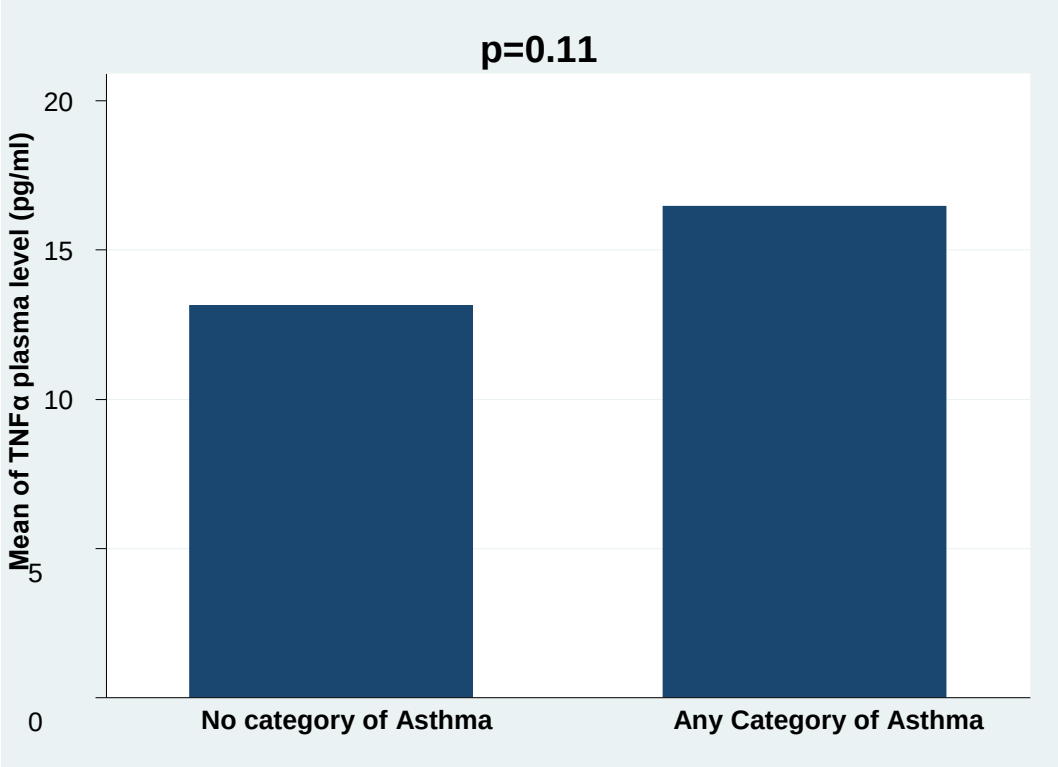


Figure 4.4. TNF α protein level in participants with Any Category of asthma and those without Asthma n= (74)

4.2.5 Multiple Logistic Regression Models

Associations of genetic variables with respiratory outcomes of interest (atopy, any asthma, persistent asthma and bronchial hyper responsiveness) were examined using multivariate logistic regression models. The models were

adjusted for sex and age. These covariates were chosen *apriori.* Other potential covariates such as education, smoking and breastfeeding were eliminated in a backwards stepwise regression model as non-significant. No statistically significant associations were detected with respect to any of the respiratory outcomes examined. In each of the models the respiratory outcome was the dependant variable and genotype was independent variable (Tables 4.5 and 4.6).

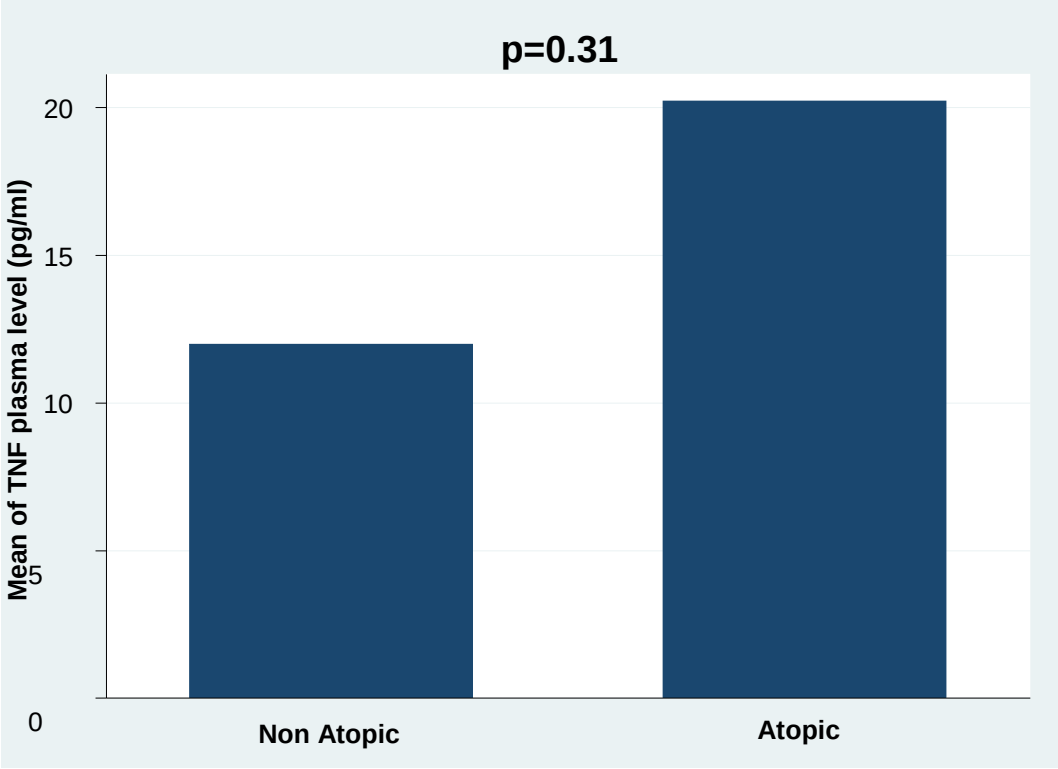


Figure 4.5. TNF α levels in Atopic and Non Atopic participants n= (78)

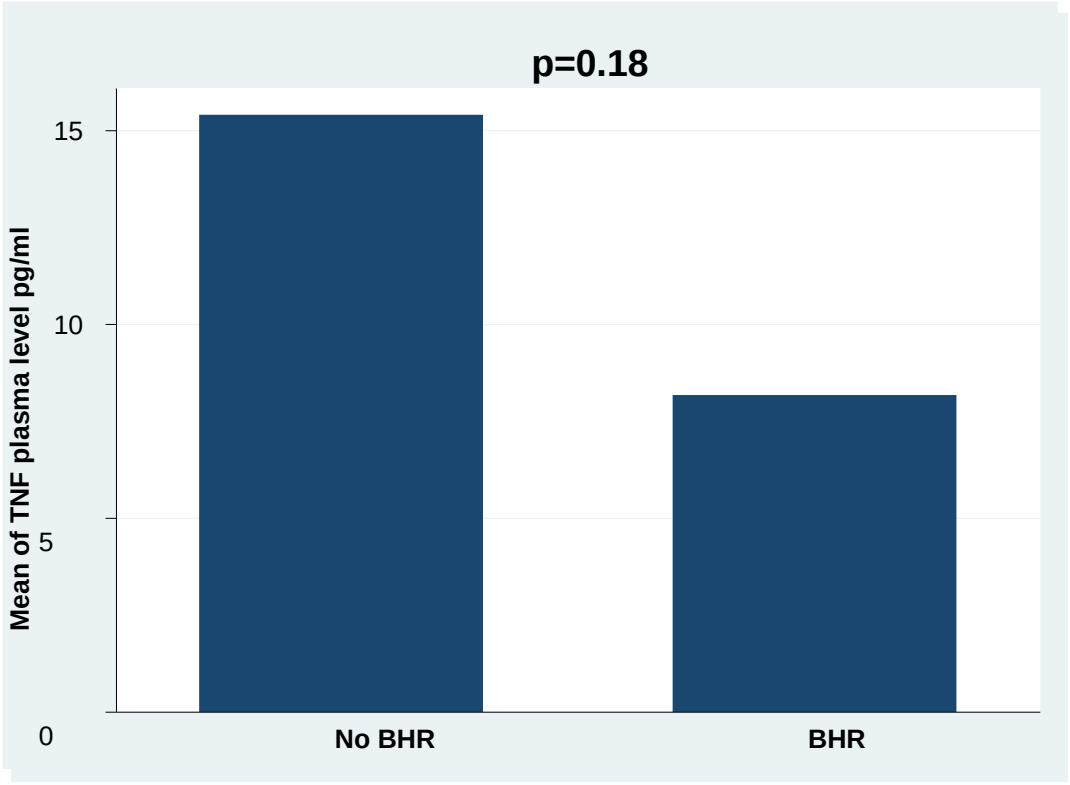


Figure 4.6. TNF α levels in participants with and without bronchial hyperresponsiveness n= (66)

4.2.6 Regression Models of ambient exposure and lung function measures using Generalised Estimating Equations

The gene environment interaction was assessed by including a product term (genotype X pollutant) in linear regression models. These models assessed the relationship between SO_2, NO, NO_2 and PM_{10} exposure and changes in lung function tests (FEV_1 and PF) using TNF- 308 polymorphic genotype as an effect modifier (Tables 4.7 and 4.8). In these models the estimate is the expected change in lung function associated with an increase in interquartile range in ambient pollutant. There were a few significant gene environment interactions with TNF- 308-α polymorphisms and SO_2, NO_2, PM_{10} and NO.

Tables 4.7 and 4.8 present models addressing potential effect modification by genotype on the association between air pollutant exposures and changes in lung function (FEV_1 and PF intraday variability). In each of the tables the estimate is a measure of the change in lung function. If the estimate of intraday variability of FEV_1 or PEF increases this indicates deterioration or worsening of lung function. The nadir of PEF and FEV_1 shows the lowest or minimum lung function reading. A decrease in the estimate of nadir of lung function readings shows a lower lung function or deteriorating lung function. In essence increased

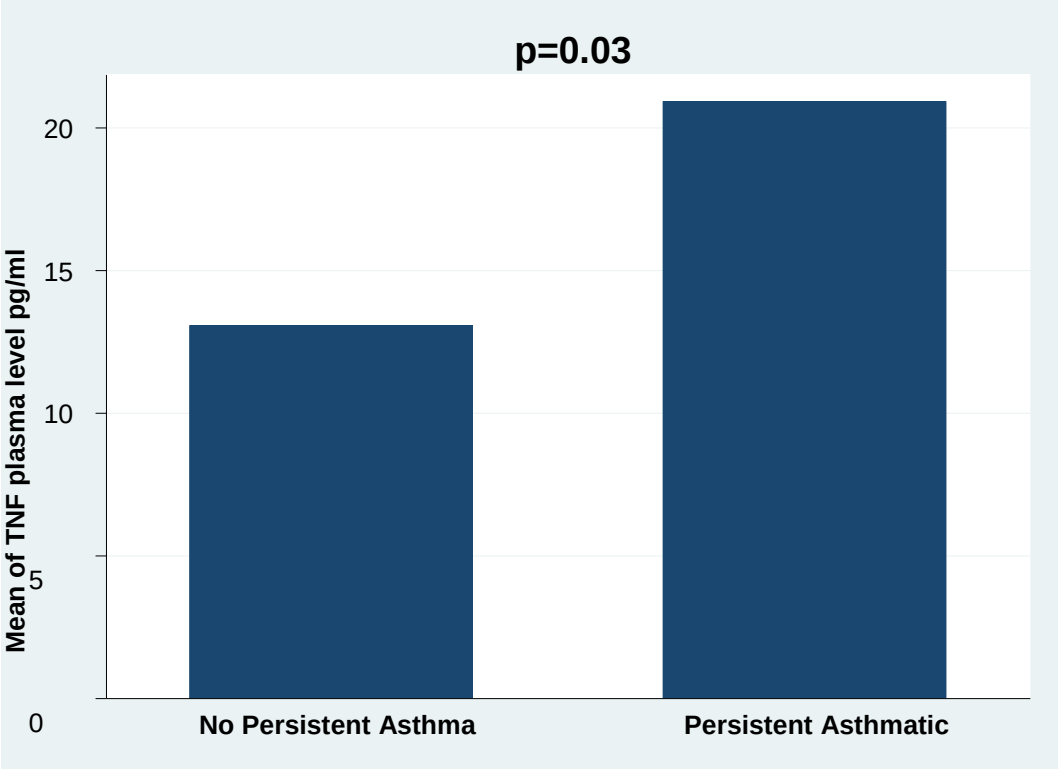


Figure 4.7. TNF alpha levels in participants with and without persistent asthma n= (74)

Table 4.5: Association of TNF-308α gene polymorphisms with Any Asthma and Persistent Asthma

Genotypes	Any Asthma N=90			Persistent Asthma N=90		
	Adj OR	95% CI	p value	Adj OR	95% CI	p value
GG	1,00			1,00		
AG/ AA	0,65	0,27;1,59	0,35	0,46	0,12;1,69	0,24

Logistic regression models adjusted for age and gender

Table 4.6: Association of TNF-308α gene polymorphisms with Atopy and Bronchial Hyper responsiveness

Genotypes	Atopy N=95			Bronchial Hyperresponsiveness N=80		
	Adj OR	95% CI	p value	Adj OR	95% CI	p value
GG	1.00			1,00		
AG/ AA	1,31	0,52;3,33	0,56	0,84	0,25;2,79	0,77

Logistic regression models adjusted for age and gender

intraday variability and lower nadir estimates are markers of adverse lung function. The estimate of the change in lung function was measured at various lags post exposure. Lag 1 is one day post exposure and Lag 2 is two days post exposure. An average of 5 days of exposure was also included.

Among TNF- GG positive children there were two instances of a statistically significant association with increased exposures to PM_{10} with FEV_1 intraday variability. No significant gene environment interaction was seen with PM_{10}. At 2 days post exposure (lag 2) there was a significant association between lung function (FEV_1 intraday variability) and PM_{10} in GG individuals β= 1.35, CI

(0.12,2.58), p=0.03 (Table 4.7). A statistically significant relationship was also shown with nadir of FEV_1 and PM_{10} at lag 2 again β= -0.02, CI (-0.04, 0), p=0.05.

The estimates for FEV_1 and PEF variability generally increased with increasing pollutant lagged exposure both with TNF- GG and in the TNF- GA/ AA participants showing the adverse cumulative effect of the pollutants on the children (Table 4.7). The effect estimates of SO_2 on children who were carriers of the A allele were generally higher even reaching a significant gene pollutant interaction with intraday variability in FEV_1 at lag 2 β= 2.62, CI (0.51, 4.71), p=0.02 [p (interaction) =0.03] (Table 4.7). This indicates a greater magnitude of deterioration of lung function post pollution exposure in individuals who are carriers of the A allele. This allele has been associated with higher levels of TNF-α production which has been found in large amounts in bronchial fluids during asthma attacks.

In addition children who were carriers of the A allele showed statistically significant adverse effect modification when exposed to increasing levels of NO using Nadir of PEF as an outcome at lag 1 β= -9.00, CI(-16.68,-1.31), p=0.04, Lag 2 β= -12.3, CI (-22.09,-2.51), p=0.01, p(interaction)=0.03 and 5 days average β= -42.83, CI (-70.11,15.55), p=0, p(interaction)=0.01 and Nadir of FEV_1 as an outcome 5 days post exposure β= -0.17, CI (-0.32,-0.01), p=0.040) (Table 4.8). This again confirmed that there was a significant deterioration of lung function in carriers of the A allele when exposed to increasing levels of NO. The gene environment interaction was significant at 2 days post exposure (p=0.03) and 5 days post exposure (p=0.01). There were also some conflicting results showing a significant association in the ‘‘unexpected’’ protective direction (i.e., higher exposure to NO and NO_2 associated with decreased Intraday variability in FEV_1). This could be due to small sample sizes as the effect

estimates generally showed the adverse relationship between increased pollutant exposure and lung function and carrying the A allele(Table 4.8). No statistically significant gene environment interaction was seen with PM_{10} in all the regression models (Table 4.7)

Table 4.7: Effect of pollutant exposure stratified by TNF-308α gene polymorphisms. Percent change[1] in intraday variability[2] of FEV_1, Intraday Variability of PEF[3], Nadir of FEV_1[4] and Nadir of PEF[5] associated with ambient levels[6] of pollutants (SO_2 and PM_{10}) from single pollutant linear regression models using generalized estimating equations.

Lung Function Outcome	Genotype	Lag	SO_2				PM_{10}			
			β	CI	p	[7]p (int)	β	CI	p	P (int)
Intraday Variability FEV_1	**TNF-AA/GA**	**Lag 1**	2.28	(-0.28,4.85)	0.08	0.25	0.4	(-0.93,1.73)	0.56	0.69
	TNF- GG		0.81	(-1.29,2.90)	0.45		0.06	(-0.88,1.00)	0.9	
	TNF-AA/GA	**Lag2**	**2.62**	**(0.51,4.71)**	**0.02**	**0.03**	0.88	(-1.31,3.07)	0.43	0.71
	TNF- GG		0.24	(-1.17,1.65)	0.74		**1.35**	**(0.12,2.58)**	**0.03**	
	TNF-AA/GA	**5 days**	3.5	(-1.63,8.62)	0.18	0.12	1.91	(-1.70,5.52)	0.3	0.96
	TNF- GG		1.02	(-3.67,5.70)	0.67		2.02	(-0.47,4.50)	0.11	
Intraday Variability PEF	**TNF-AA/GA**	**Lag 1**	1	(-1.52,3.52)	0.44	0.43	-0.05	(-0.99,0.9)	0.92	0.87
	TNF- GG		0.14	(-1.58,1.86)	0.88		-0.15	(-0.81,0.51)	0.66	
	TNF-AA/GA	**Lag2**	1.54	(-0.44,3.52)	0.13	**0.04**	-0.64	(-1.92,0.65)	0.33	0.26
	TNF- GG		-0.43	(-2.06,1.19)	0.6		0.25	(-0.58,1.09)	0.55	
	TNF-AA/GA	**5 days**	3.4	(-1.59,8.39)	0.18	0.31	-0.03	(-2.51,2.45)	0.98	0.64
	TNF- GG		2.03	(-1.97,6.04)	0.32		0.73	(-1.29,2.74)	0.48	
Nadir of FEV_1	**TNF-AA/GA**	**Lag 1**	0.05	(-0.003,0.11)	0.06	0.57	0	(-0.03,0.04)	0.77	0.51
	TNF- GG		0.03	(-0.01,0.08)	0.16		0.02	(-0.001,0.03)	0.07	
	TNF-AA/GA	**Lag2**	0.01	(-0.04,0.06)	0.65	0.64	-0.03	(-0.07,0.01)	0.18	0.75
	TNF- GG		0	(-0.05,0.04)	0.91		**-0.02**	**(-0.044,0)**	**0.05**	
	TNF-AA/GA	**5 days**	0.02	(-0.18,0.22)	0.83	0.88	-0.05	(-0.13,0.04)	0.3	0.65
	TNF- GG		0.01	(-0.14,0.16)	0.9		-0.02	(-0.072,0.03)	0.37	
Nadir of PEF	**TNF-AA/GA**	**Lag 1**	0.54	(-11.24,12.31)	0.93	0.55	-0.09	(-3.78,3.61)	0.96	0.56
	TNF- GG		4.36	(-4.36,13.08)	0.33		1.24	(-1.30,3.78)	0.34	
	TNF-AA/GA	**Lag2**	-3.78	(-12.12,4.56)	0.37	0.73	-4.64	(-10.54,1.26)	0.12	0.19
	TNF- GG		-1.87	(-13.23,9.49)	0.75		-0.35	(-2.99,2.29)	0.8	
	TNF-AA/GA	**5 days**	-9.62	(-45.13,25.89)	0.6	0.9	-7.64	(-16.72,1.44)	0.1	0.46
	TNF- GG		-10.93	(-43.82,21.97)	0.52		-2.75	(-12.19,6.69)	0.57	

[1] the percent change value shown is for an increase of one interquartile range in each respective pollutant: PM_{10}: 29.4 ug m^{-3} and SO_2: 9.8 ppb.
[2] intraday variability for FEV_1 is defined as : 100 (maximum best FEV_1-minimum best FEV_1)/maximum best **FEV_1** where the "best FEV_1" is the highest valid, error-free value for the specific time of day (08h00, 09h45, 11h30, 13h20).
[3]Intraday variability for PEF is defined analogously to within-day variability for FEV_1.
[4]Nadir **FEV_1** is defined as the minimum of the (up to 4) best FEV1s on a given day,
[5] Nadir PF is defined analogously
[6] pollution levels used in regression models are the 5-day average.
Covariates in each model: race, school, caregiver smokes, asthma severity, interactions between asthma severity and exposure
[5]Interaction p-value

Table 4.8: Effect of pollutant exposure stratified by TNF-308α gene polymorphisms. Percent change[1] in intraday variability[2] of FEV_1, Intraday Variability of PEF[3], Nadir of FEV_1[4] and Nadir of PEF[5] associated with ambient levels[6] of pollutants (NO_2 and NO) from single pollutant linear regression models using generalized estimating equations.

Lung Function Outcome	Genotype	Lag	NO_2				NO			
			β	CI	p	p (int)[7]	β	CI	p	P (int)
Intraday Variability FEV	**TNF- AA/GA**	**Lag 1**	**-1.85**	**(-3.36,-0.35)**	**0.02**	**0.03**	-2.44	(-5.28,0.41)	0.09	
	TNF- GG		1.38	(-1.21,3.98)	0.3	**0.03**	-0.63	(-2.29,1.04)	0.46	0.27
	TNF- AA/GA	**Lag2**	-1.86	(-4.34,0.62)	0.14	0.11	**-3.28**	**(-5.89,-0.68)**	**0.01**	
	TNF- GG		0.7	(-1.21,2.61)	0.47	0.11	0.33	(-1.59,2.25)	0.74	**0.03**
	TNF- AA/GA	**5 days**	-1.4	(-5.01,2.22)	0.45	0.12	-1.38	(-7.60,4.84)	0.66	
	TNF- GG		2.39	(-0.79,5.57)	0.14	0.12	1.96	(-2.37,6.28)	0.38	0.37
Intraday Variability PEF	**TNF- AA/GA**	**Lag 1**	-1	(-3.01,1.02)	0.33	0.21	-0.12	(-2.57,2.32)	0.92	
	TNF- GG		0.54	(-0.71,1.78)	0.4	0.21	0.11	(-1.11,1.32)	0.87	0.87
	TNF- AA/GA	**Lag2**	0.24	(-2.81,3.29)	0.88	0.99	-0.59	(-3.45,2.27)	0.69	
	TNF- GG		0.23	(-1.14,1.59)	0.75	0.99	-0.47	(-1.87,0.92)	0.51	0.94
	TNF- AA/GA	**5 days**	0.6	(-4.62,5.82)	0.82	0.64	2.44	(-3.38,8.27)	0.41	
	TNF- GG		1.97	(-0.36,4.3)	0.1	0.64	1.51	(-1.46,4.48)	0.32	0.77
Nadir of FEV_1	**TNF- AA/GA**	**Lag 1**	-0.03	(-0.10,0.05)	0.48	0.78	-0.03	(-0.09,0.04)	0.42	
	TNF- GG		-0.01	(-0.06,0.03)	0.56	0.78	0	(-0.03,0.03)	0.91	0.5
	TNF- AA/GA	**Lag2**	-0.05	(-0.12,0.02)	0.14	0.53	0.01	(-0.07,0.08)	0.89	
	TNF- GG		-0.03	(-0.06,0.01)	0.16	0.53	-0.02	(-0.05,0.02)	0.26	0.57
	TNF- AA/GA	**5 days**	-0.13	(-0.33,0.08)	0.22	0.58	**-0.17**	**(-0.32,-0.01)**	**0.04**	
	TNF- GG		-0.07	(-0.14,0.01)	0.09	0.58	-0.07	(-0.17,0.02)	0.14	0.34
Nadir of PEF	**TNF- AA/GA**	**Lag 1**	**-12.89**	**(-22.96,-2.83)**	**0.01**	0.08	**-9.00**	**(-16.68,-1.31)**	**0.02**	
	TNF- GG		-2.22	(-8.31,3.88)	0.48	0.08	-0.46	(-5.69,4.78)	0.86	0.07
	TNF- AA/GA	**Lag2**	-10.97	(-22.88,0.96)	0.07	0.09	**-12.3**	**(-22.09,-2.51)**	**0.01**	
	TNF- GG		0.48	(-4.82,5.77)	0.86	0.09	0.67	(-5.32,6.66)	0.83	**0.03**
	TNF- AA/GA	**5 days**	-21.43	(-58.13,15.27)	0.25	0.39	**-42.83**	**(-70.11,-15.55)**	**0**	
	TNF- GG		-4.76	(-15.31,5.8)	0.38	0.39	-3.63	(-17.98,10.72)	0.62	**0.01**

[1] the percent change value shown is for an increase of one interquartile range in each respective pollutant: NO_2: 8.19 ppb and NO: 29.7 ppb.
[2] ntraday variability for FEV_1 is defined as : 100 (maximum best FEV_1-minimum best FEV_1)/maximum best **FEV_1**; where the "best FEV_1" is the highest valid, error-free value for the specific time of day (08h00, 09h45, 11h30, 13h20).
[3] Intraday variability for PF is defined analogously to within-day variability for FEV_1.
[4] Nadir **FEV_1** is defined as the minimum of the (up to 4) best FEV1s on a given day,
[5] Nadir PF is defined analogously
[6] pollution levels used in regression models are the 5-day average
Covariates in each model: race, school, caregiver smokes, asthma severity, interactions between asthma severity and exposure
[7] Interaction p-value

4.3 CD14 (159) C/T polymorphism and Respiratory Outcomes

4.3.1 Genotyping

The SNP in CD 14 (159) was genotyped by Custom TaqMan® SNP Genotyping Assays (Applied Biosystems Foster City). Multiplex real time PCR technology was used to detect the CD14 (159) polymorphism. The genotype frequencies in this sample were CC 63%, CT 28% and TT 9%. (Table 4.9) Allelic discrimination plots were used to identify the genotype of each sample. The notation used was Allele 1 homozygous CC, Allele 2 homozygous TT and heterozygous CT. 3% of the samples were not successfully genotyped. The observed genotype frequencies did not show a significant departure from Hardy Weinberg equilibrium (p= 0.10)

Pearson's chi squared test was performed to evaluate whether a particular respiratory outcome was associated with genotype. Table 4.10 and 4.11 below show the frequencies of the CD14 (159) C/T polymorphism genotypes in relation to persistent asthma, any asthma, doctor diagnosed asthma, atopy and BHR. There was no statistically significant association between the CD 14 (159) C/T polymorphism with asthma related phenotypes such as bronchial hyper responsiveness and atopy using both the dominant and recessive model. In the Any asthma category, 61.5% of the CC genotype patients were non cases. A similar trend is seen in the other outcomes such as persistent asthma 62.3%, BHR 71.7%, and atopy (71.7%). Individuals who were carriers of the TT genotype on the other hand, tended to report having more respiratory symptoms. Those who reported having any asthma, Persistent Asthma, BHR and Atopy were 9.5%, 28.6%, 10% and 11% respectively. With all the respiratory outcomes, the proportion of cases with the TT genotype were higher than the non-cases (Table not shown).

Table 4.9: Genotypic and allelic frequencies of the CD 14 (159) C>T polymorphism

Genotype Frequencies	*Sample (n=104)*
CC	45 (63.4 %)
CT	20 (28.2%)
TT	6 (8.5%)
Allele Frequencies	
C	0.77
T	0.23

Table 4.10: Frequencies of CD 14 Polymorphic genotypes stratified by respiratory outcomes using the Dominant Model

Genotype	*Any Asthma N=60 (%)*		*Persistent Asthma N=60%*		*BHR N= 56 (%)*		*Atopy N= 64 (%)*	
	Non Case	**Case**	**Non Case**	**Case**	**Non Case**	**Case**	**Non Case**	**Case**
CC	24 (66,7)	12 (33.3)	33 (91.7)	3(8.3)	33(86.8)	5(13.2)	33(75)	11(25)
CT/TT	15 (62.5)	9 (37.5)	20 (83.3)	4 (16.7)	13 (72.2)	5 (27.8)	13 (65)	7 (35)
P-value	0.7		0.3		0.2		0.4	

Table 4.11: Frequencies of CD14 Polymorphic genotypes stratified by respiratory outcomes using the Recessive Model

Genotype	*Any Asthma N=60 (%)*		*Persistent Asthma N=60%*		*BHR N= 56 (%)*		*Atopy N= 64 (%)*	
	Non Case	**Case**	**Non Case**	**Case**	**Non Case**	**Case**	**Non Case**	**Case**
CC	24(66.7)	12 (33.3)	33(91.7)	3 (8.3)	33 (86.8)	5 (13.2)	33(75)	11 (25)
CT	12 (63.2)	7 (36.6)	17 (89.5)	2 (10.5)	9 (69.2)	4 (30.8)	10 (66.7)	5 (33.3)
TT	3 (60)	2 (40)	3 (60)	2 (40)	4 (80)	1 (20)	3 (60)	2 (40)
P-value	0.9		0.2		0.3		0.6	

4.3.2 Association of CD 14 (159) gene polymorphism and sCD 14 levels

Plasma sCD14 levels were measured in 54 samples. There was no significant difference between genotypes, of the mean sCD14 levels (p=0.47). The mean sCD14 level for CC genotype was 8.8ng/ml±4.1ng/ml and for CT and TT combined was 7.8ng/ml±4.7ng/ml (Figure 4.8).

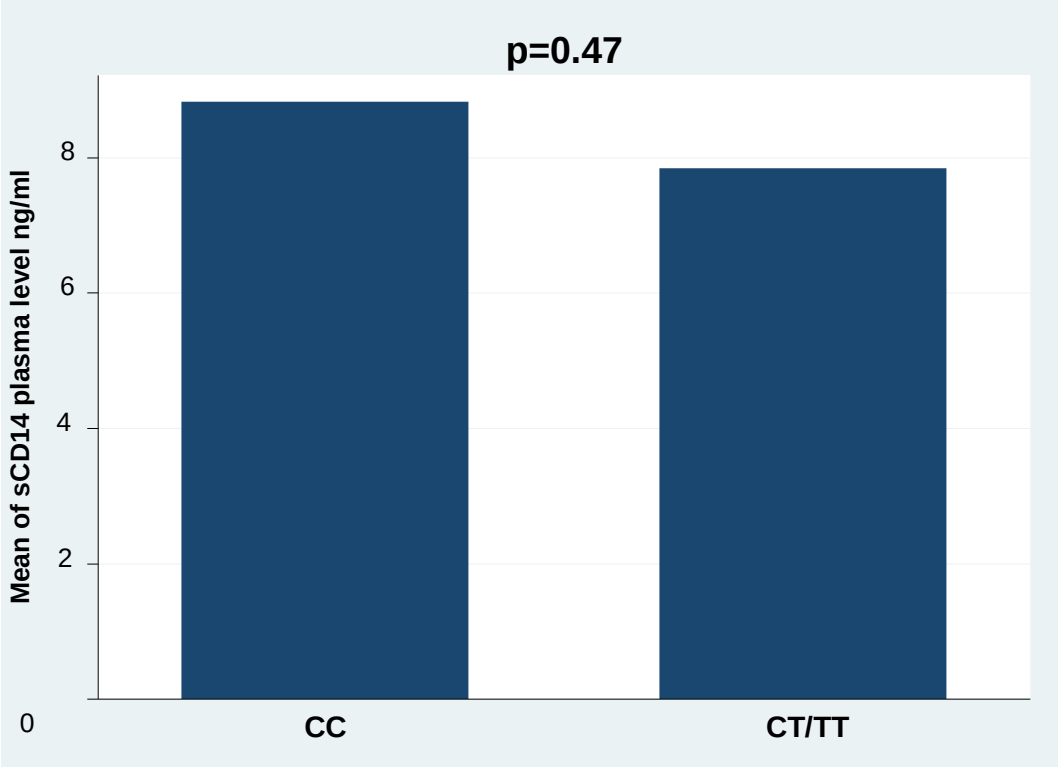


Figure 4.8. The levels of soluble CD 14 protein in plasma samples stratified by the CD14 (-159) C/T genotype n= (46)

The median sCD14 levels were higher in the CC genotype individuals (11.0ng/ml as compared to 8.9ng/ml and 3.4ng/ml in the CT and TT individuals). The mean levels of sCD14 were similar across the three genotypes.

No significant difference was found when using the recessive model for analysis p=0.68 (Figure 4.9). Subjects with the TT genotype had the lowest mean plasma levels (6.7±6.3ng/ml), the lowest plasma level reading (2.6ng/ml) and the lowest median plasma sCD14 level (3.4ng/ml). Participants with the CT genotype had the lowest maximum reading compared to the other genotypes (13.9ng/ml) and the subject with the lowest sCD14 reading in the whole study group was a CT genotype participant (2.6ng/ml).

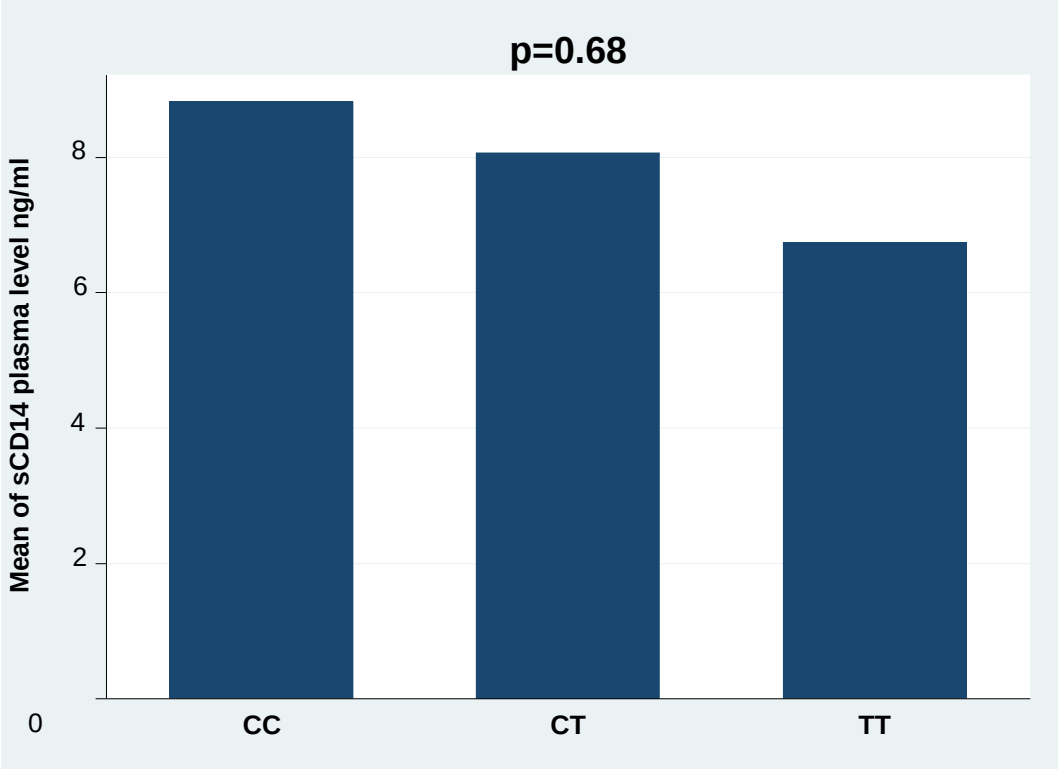


Figure 4.9. The levels of soluble CD 14 protein in plasma samples stratified by the CD14 (-159) C/T n= (46)

4.3.3 Concentration of sCD14 plasma protein compared with respiratory phenotypes

There was no statistically significant difference in the protein levels in patients with any category of asthma, BHR and persistent asthma. Figures 4.10, and 4.11 show that subjects who reported having a respiratory phenotype such as asthma and bronchial hyperresponsiveness had higher sCD14 levels than those who were not cases although the differences were not statistically significant. Participants stratified by atopic status showed a statistically significant difference in sCD14 levels among atopic and non-atopic subjects. The difference was protective with non-atopic patients having the higher sCD14 protein levels (p=0.04).

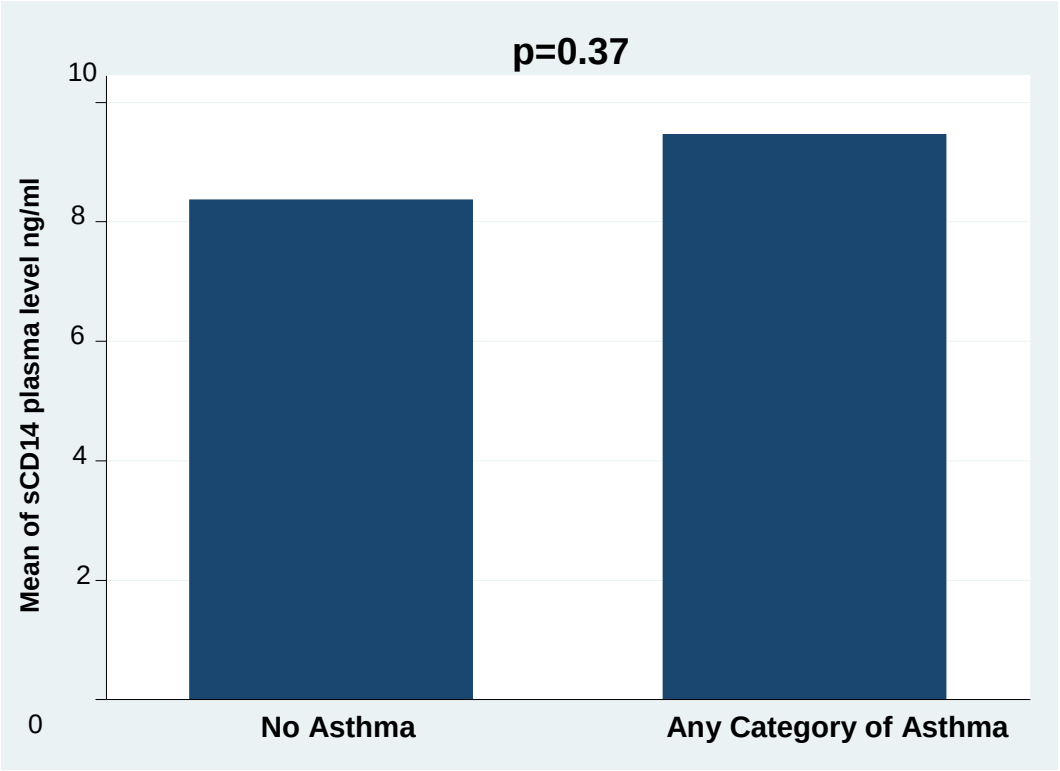


Figure 4.10. Plasma levels of sCD14 protein in participants with any category of Asthma and those without Asthma n= (49)

Participants who were reported as having any asthma had a higher mean (11.0ng/ml±4.2ng/ml) as compared to those without asthma (8.7ng/ml±4.1ng/ml) (Fig 4.10). The median reading was also higher in asthmatics as compared to non-asthmatics (11ng/ml and 8.7ng/ml respectively). In contrast, the maximum reading of the non-asthmatics (14.2ng/ml) was higher than that of the asthmatics (13.9ng/ml). Similarly the minimum reading of non-asthmatics (2.7ng/ml) was higher than that of the cases (2.6ng/ml). These differences did not reach statistical significance (p=0.37).

There was a difference in sCD14 levels of participants with BHR compared with non-cases (Figure 4.11). Participants with BHR had higher mean level of sCD14 (10.1ng/ml±4.2 ng/ml) in their plasma than those without BHR (7.8 ng/ml±4.2ng/ml). The median reading of the BHR cases (12.3ng/ml) was higher than that of non BHR-cases (8.3ng/ml). The highest reading of sCD14 was unexpectedly from a child without BHR (14.2ng/ml). These differences were not statistically significant (p=0.20)

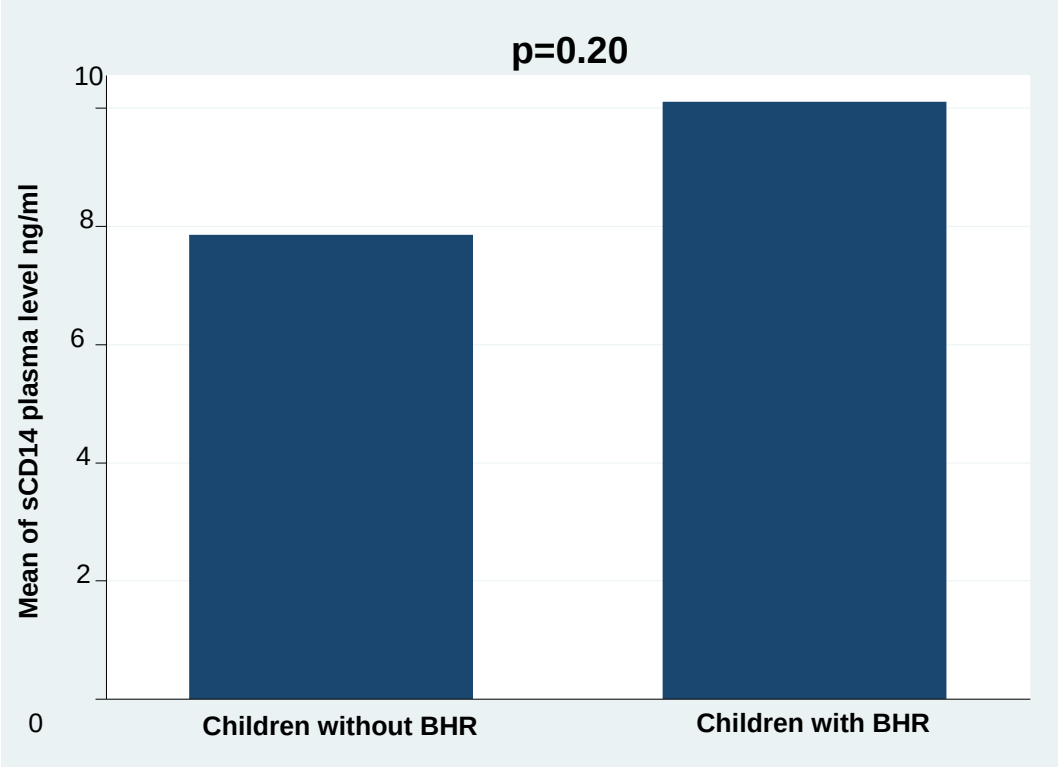


Figure 4.11. sCD14 protein levels in participants stratified by bronchial hyper responsiveness (BHR) n= (43)

There was a statistically significant difference in sCD14 levels among participants who were atopic and those who were not (p=0.04). Unexpectedly atopic patients had a lower mean sCD14 level (6.8 ng/ml ±4.5ng/ml) compared to non-atopic patients (9.5 ng/ml±3.9 ng/ml). The median reading in atopic patients was similarly low (3.6 ng/ml) as compared to non-atopic patients (11.3 ng/ml) (Figure 4.12).

When participants were stratified by the presence or absence of persistent asthma, there was no significant difference the levels of sCD14. Figure 4.13 clearly shows similar distribution of the readings. The median of both cases and non-case were similar (10.4ng/ml and 10.3ng/ml respectively). The means were

similar in non-case (8.9 ng/ml±4.0 ng/ml) and in the persistent asthma cases (8.4 ng/ml±4.9 ng/ml).

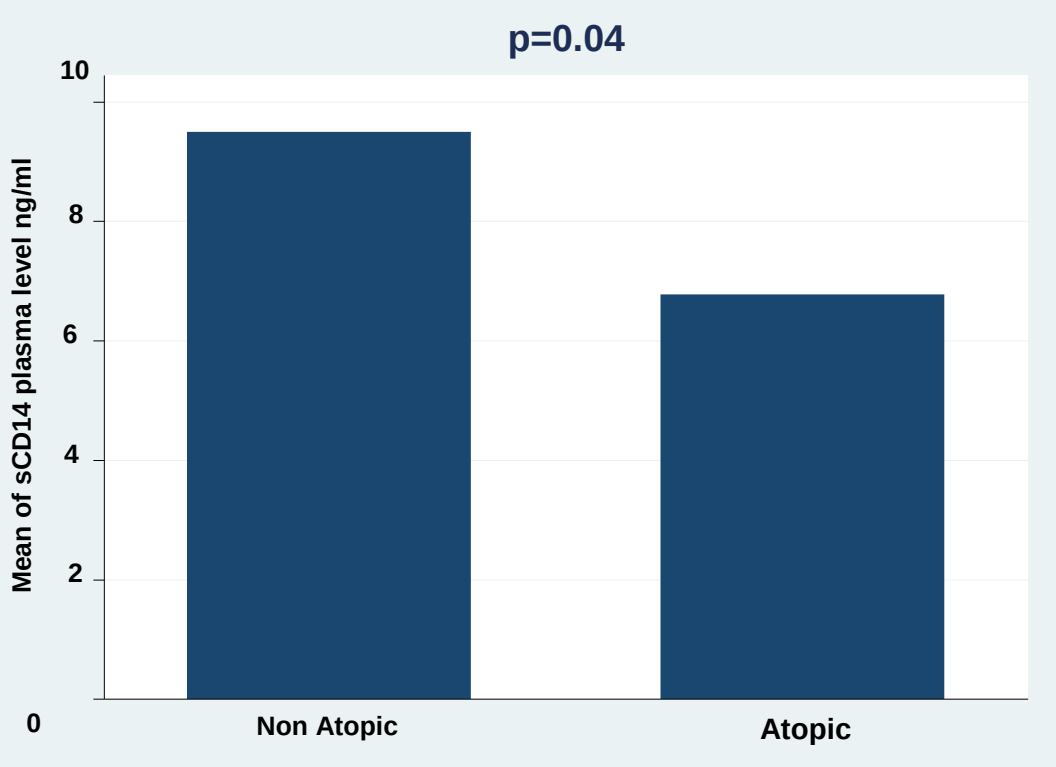


Figure 4.12. Plasma sCD14 levels in participants stratified by atopy n= (49)

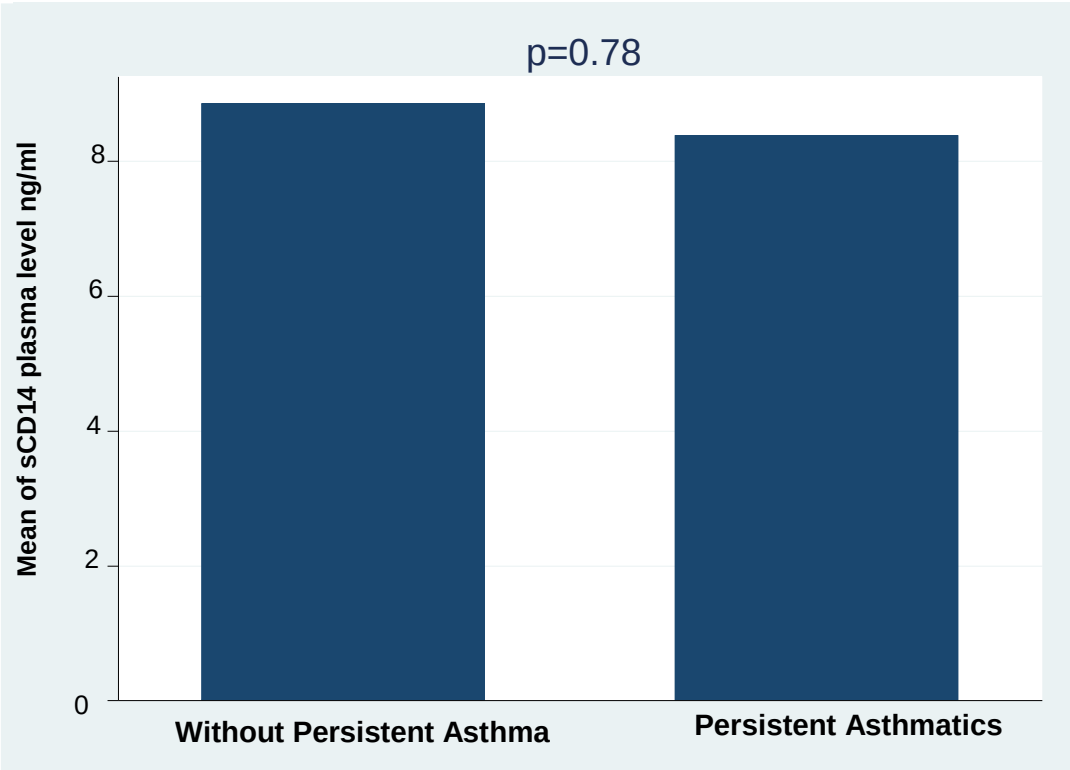


Figure 4.13. Plasma sCD14 levels in participants stratified by persistent asthma n= (49)

4.3.4 Multiple Logistic Regression Models

Associations of genetic variables with respiratory outcomes of interest (atopy, any asthma, persistent asthma and bronchial hyper responsiveness) were examined using multivariate logistic regression models. The models were adjusted for sex and age. These covariates were chosen *apriori*. Other potential covariates such as education, smoking and breastfeeding were eliminated as non-significant in a backwards stepwise regression model. No significant associations were detected with respect to any of the respiratory outcomes examined. In each of the models, the respiratory outcome was the dependant variable and genotype was independent.

Table 4.12: Association of CD14 (-159) gene polymorphism with any asthma and persistent asthma

Genotypes	*Any Asthma N=60*			*Persistent Asthma N=60*		
	Adj OR	**95% CI**	**P value**	**Adj OR**	**95% CI**	**P value**
CD14 CC	1.00			1.00		
CD14 CT/TT	1.29	0 .40; 4.18	0.67	2.06	0.36; 11.95	0.42

Logistic regression models adjusted for age and gender

Table 4.13: Association of CD14 (-159) gene polymorphisms with atopy and bronchial hyperresponsiveness

Genotypes	**Atopy N=64**			***BHR N=56***		
	Adj OR	**95% CI**	**P value**	**Adj OR**	**95% CI**	**P value**
CD14 CC	1.00			1.00		
CD14 CT/TT	1.62	0.51; 5.20	0.41	3.22	0.74; 13.99	0.118

Logistic regression models adjusted for age and gender

4.3.5 Regression Models of ambient exposure and lung function measures using Generalised Estimating Equations

The gene environment interaction was assessed by including a product term (genotype X pollutant) in the linear regression models. These models assessed the relationship between SO_2, NO, NO_2 and PM_{10} exposure and changes in lung function tests (FEV_1 and PEF) using CD14 (-159) C/T polymorphic genotype as effect modifier (Tables 4.14 and 4.15). In these models the estimate is the expected change in lung function associated with an increase in interquartile range in ambient pollutant. There were a few significant gene environment interactions with CD14 gene polymorphism and ambient pollutants

Tables 4.14 and 4.15 present models addressing potential effect modification by the CD14 (-159) C/T gene polymorphism on the association between air

pollutant exposures and lung function outcomes. The lung function outcomes measured were FEV_1 and PEF intraday variability. In each of the tables the estimate is a measure of the change in lung function. If the estimate of intraday variability of FEV_1 or PEF increases this indicates deterioration or worsening of lung function. The nadir of PEF and FEV_1 shows the lowest or minimum lung function reading. A decrease in the estimate of nadir of lung function readings shows a lower lung function or deteriorating lung function. Increased Intraday variability and lower nadir estimates are markers of worsening lung function. The estimate of the change in lung function was measured at various lags post exposure. Lag 1 is one day post exposure and Lag 2 is two days post exposure.

In seven exposure-response models, individuals with the CD14 (-159) CT and TT polymorphic genotypes showed statistically significant associations of exposure with FEV_1 intraday variability (Table 4.14 and 4.15). In six of these models (NO_2 lag 1 (β= -2.72 CI (-4.17,-1.27), p=0, p(interact)=0.05), lag 2(β= -3.38 CI(-4.69,-2.06) p=0.00, p(interact)=0.03), Average for 5 days (β-4.02, CI(-6.52,-1.53), p=0.00, p(interact)=0.05); NO lag 2(β-4.5, CI(-6.02,-2,98) p=0.00, p(interact)=0.00) and Average of 5 days (β= -9.24, CI (-12.45,-6.028), p=0.00, p(interact)=0.00) SO_2 lag 1(β= -3.56, CI (-6.96,-0.17), p=0.04 p(interact)=0.01), the CT and TT genotypes modified the association between exposure and decreased FEV_1 intraday variability significantly compared to children with the CC genotype. The CT/TT genotype appeared to be protective to increased exposure to pollutants NO_2, NO, and SO_2.

In three instances children with the CD14 (-159) CC genotype showed statistically significant decrement in lung function when modeled with exposure. In Table 4.14 one day post exposure (Lag 1) to SO_2 (p=0.01) was associated with increased FEV_1 intraday variability β= -1.50, CI (-0.36, 3.37), p=0.01, p (interaction) =0.01. This was the only significant gene environment interaction

for the CD14 CC genotype. Two days post exposure to PM_{10} (p=0.05) was also associated with increased FEV_1 intraday variability β= -1.26, CI (0.02, 2.50), p=0.05. Five days post exposure to NO_2 was associated with increased intraday variability of PEF β= -0.07, CI (-0.13, 0) (p=0.05) (Table 4.15).

No significant gene environment interactions were seen with $PM_{10,}$ although there were two instances of a statistically significant association between increased exposures to PM_{10} and decreased lung function. First with FEV_1 intraday variability at lag 2 β=1.26, CI (0.02, 2.50), p=0.05 and another association between PM_{10}.and nadir of FEV_1 at lag1 β= 0.03, CI (0.01, 0.06), p=0.02 (Table 4.14).

Table 4.14: Effect of pollutant exposure stratified by CD14 gene polymorphisms. Percent change[1] in intraday variability of FEV_1[2], Intraday Variability of PEF[3], Nadir of FEV_1[4] and Nadir of PEF[5] associated with ambient levels[6] of pollutants (SO_2 and PM_{10}) from single pollutant linear regression models using generalized estimating equations.

Lung Function Outcome	Genotype	Lag	SO_2				PM_{10}			
			β	CI	p	[7]p (int)	β	CI	p	[7]p(int)
Intraday Variability FEV_1	**CD14 CC**	**Lag 1**	**1.50**	**(-0.36,3.37)**	**0.01**	**0.01**	0.24	(-0.63,1.10)	0.59	0.48
	CD14CT/TT		**-3.56**	**(-6.96,-0.17)**	**0.04**		-0.42	(-2.13,1.31)	0.64	
	CD14 CC	**Lag2**	0.53	(-0.99,2.05)	0.49	0.06	**1.26**	**(0.02,2.50)**	**0.05**	0.98
	CD14CT/TT		-3.21	(-6.89,0.47)	0.09		1.23	(-0.76,3.22)	0.23	
	CD14 CC	**5 days**	0.39	(-3.24,4.02)	0.83	0.43	2.11	(-0.62,4.85)	0.13	0.26
	CD14CT/TT		-2.83	(-11.55,5.88)	0.52		0.53	(-2.51,3.56)	0.73	
Intraday Variability PEF	**CD14 CC**	**Lag 1**	0.03	(-0.02,0.08)	0.22	0.35	0.01	(-0.01,0.03)	0.34	0.73
	CD14CT/TT		-1.05	(-3.66,1.56)	0.43		-0.26	(-1.31,0.78)	0.62	
	CD14 CC	**Lag2**	0.00	(-0.04,0.050)	0.84	0.63	-0.02	(-0.03,0.003)	0.11	0.97
	CD14CT/TT		-0.91	(-3.88,2.06)	0.55		0.07	(-0.72,0.86)	0.87	
	CD14 CC	**5 days**	0.06	(-0.09,0.20)	0.44	0.42	-0.03	(-0.08,0.01)	0.14	0.72
	CD14CT/TT		-0.40	(-6.74,5.94)	0.90		-0.46	(-2.77,1.85)	0.70	
Nadir of FEV_1	**CD14 CC**	**Lag 1**	0.47	(-1.41,2.35)	0.63	0.46	-0.05	(-0.70,0.60)	0.87	0.15
	CD14CT/TT		0.06	(-0.01,0.14)	0.08		**0.03**	**(0.01,0.06)**	**0.02**	
	CD14 CC	**Lag2**	-0.10	(-2.02,1.82)	0.92	0.64	0.09	(-0.80,0.98)	0.84	0.56
	CD14CT/TT		0.04	(-0.10,0.17)	0.58		-0.04	(-0.10,0.03)	0.30	
	CD14 CC	**5 days**	1.11	(-2.22,4.44)	0.51	0.42	0.51	(-1.40,2.41)	0.60	0.72
	CD14CT/TT		-0.11	(-0.51,0.28)	0.58		-0.01	(-0.12,0.09)	0.80	
Nadir of PEF	**CD14 CC**	**Lag 1**	1.33	(-6.16,8.81)	0.73	0.87	0.23	(-2.25,2.71)	0.86	0.37
	CD14CT/TT		2.58	(-10.88,16.05)	0.71		2.46	(-1.69,6.60)	0.25	
	CD14 CC	**Lag2**	-3.35	(-14.07,7.37)	0.54	0.38	-0.03	(-2.90,2.84)	0.98	0.29
	CD14CT/TT		7.07	(-13.83,27.98)	0.51		-4.44	(-12.16,3.29)	0.26	
	CD14 CC	**5 days**	-8.62	(-43.42,26.18)	0.63	0.71	-4.97	(-13.69,3.75)	0.26	0.60
	CD14CT/TT		2.31	(-55.84,60.45)	0.94		-1.51	(-12.66,9.63)	0.79	

[1] the percent change value shown is for an increase of one interquartile range in each respective pollutant: PM_{10}: 29.4 ug m^{-3} and SO_2: 9.8 ppb.
[2] intraday variability for FEV_1 is defined as : 100 (maximum best FEV_1-minimum best FEV_1)/maximum best FEV1; where the "best FEV_1" is the highest valid, error-free value for the specific time of day (08h00, 09h45, 11h30, 13h20).
[3]Intraday variability for PEF is defined analogously to within-day variability for FEV_1.
[4]Nadir FEV_1 is defined as the minimum of the (up to 4) best FEV1s on a given day,
[5] Nadir PF is defined analogously
[6] pollution levels used in regression models are the 5-day average.
Covariates in each model: race, school, caregiver smokes, asthma severity, interactions between asthma severity and exposure
[7]nteraction p-value

Table 4.15: Effect of pollutant exposure stratified by CD14 gene polymorphism. Percent change[1] in intraday variability of FEV_1[2], Intraday Variability of PEF[3], Nadir of FEV_1[4] and Nadir of PEF[5] associated with ambient levels[6] of pollutants (NO_2 and NO) from single pollutant linear regression models using generalized estimating equations.

Lung Function Outcome	Genotype	Lag	NO_2				NO			
			β	CI	p	[7]p (int)	β	CI	p	P (int)
Intraday Variability FEV_1	CD14CT/TT	Lag 1	**-2.72**	**(-4.17,-1.27)**	**0.00**	**0.05**	**-2.8**	**(-5.041,-0.565)**	**0.01**	0.41
	CD14 CC		0.29	(-2.45,3.03)	0.84		-1.42	(-3.88,1.05)	0.26	
	CD14 CT/TT	Lag2	**-3.38**	**(-4.69,-2.06)**	**0.00**	**0.03**	**-4.5**	**(-6.016,-2.979)**	**0.00**	**0.00**
	CD14 CC		-0.68	(-2.71,1.35)	0.51		-0.16	(-2.25,1.93)	0.88	
	CD14 CT/TT	5 days	**-4.02**	**(-6.52,-1.53)**	**0.00**	**0.05**	**-9.24**	**(-12.448,-6.028)**	**0.00**	**0.00**
	CD14 CC		-0.16	(-3.33,3.00)	0.92		0.44	(-4.38,5.26)	0.86	
Intraday Variability PEF	CD14 CT/TT	Lag 1	-1.5	(-3.22,0.23)	0.09	0.19	-1.78	(-4.249,0.69)	0.16	0.2
	CD14 CC		-0.02	(-0.06,0.02)	0.31		-0.02	(-0.06,0.02)	0.3	
	CD14 CT/TT	Lag2	-1.39	(-3.71,0.93)	0.24	0.76	-1.43	(-4.891,2.031)	0.42	0.83
	CD14 CC		-0.02	(-0.06,0.01)	0.21		-0.03	(-0.057,0.01)	0.12	
	CD14 CT/TT	5 days	-2.25	(-5.69,1.19)	0.2	0.77	-4.33	(-10.006,1.35)	0.14	0.95
	CD14 CC		**-0.07**	**(-0.13,0)**	**0.05**		-0.1	(-0.20,0.01)	0.08	
Nadir of FEV_1	CD14 CT/TT	Lag 1	0	(-0.06,0.05)	0.94	0.58	-0.02	(-0.08,0.036)	0.46	0.93
	CD14 CC		0.04	(-1.52,1.60)	0.96		0.12	(-1.38,1.62)	0.88	
	CD14 CT/TT	Lag2	-0.02	(-0.09,0.05)	0.58	0.93	0.01	(-0.051,0.073)	0.73	0.31
	CD14 CC		-0.95	(-2.56,0.65)	0.24		-1.02	(-2.45,0.41)	0.16	
	CD14 CT/TT	5 days	-0.1	(-0.32,0.12)	0.37	0.77	-0.09	(-0.255,0.078)	0.3	0.95
	CD14 CC		-0.09	(-2.68,2.50)	0.94		0.23	(-2.99,3.45)	0.89	
Nadir of PEF	CD14 CT/TT	Lag 1	-4.4	(-18.91,10.1)	0.55	0.92	-6.55	(-14.988,1.885)	0.13	0.42
	CD14 CC		-5.21	(-10.80,0.38)	0.07		-2.33	(-8.67,4.02)	0.47	
	CD14 CT/TT	Lag2	-3.71	(-21.07,13.65)	0.68	0.64	-2.76	(-21.305,15.794)	0.77	0.97
	CD14 CC		0.7	(-5.18,6.57)	0.82		-2.38	(-7.75,2.99)	0.39	
	CD14 CT/TT	5 days	-10.37	(-54.91,34.17)	0.65	0.83	-18.95	(-64.807,26.9)	0.42	0.66
	CD14 CC		-5.34	(-15.15,4.48)	0.29		-8.02	(-24.97,8.94)	0.35	

[1] the percent change value shown is for an increase of one interquartile range in each respective pollutant: NO_2: 8.19 ppb and NO: 29.7 ppb.
[2] intraday variability for FEV_1 is defined as : 100 (maximum best FEV_1-minimum best FEV_1)/maximum best FEV1; where the "best FEV_1" is the highest valid, error-free value for the specific time of day (08h00, 09h45, 11h30, 13h20).
[3]Intraday variability for PEF is defined analogously to within-day variability for FEV_1.
[4]Nadir FEV1 is defined as the minimum of the (up to 4) best FEV1s on a given day,
[5] Nadir PF is defined analogously
[6]pollution levels used in regression models are the 5-day average
Covariates in each model: race, school, caregiver smokes, asthma severity, interactions between asthma severity and exposure
[7]Interaction p-value

4.4 Coinheritance of CD14 and TNF- α

An investigation was done on whether the coinheritance of the different combination of CD14 and TNF-α allele has an association with protein expression and respiratory phenotype outcome. The largest proportion of participants were carriers of both wild types (Group 1). Group one participants had the highest mean sCD14 levels (9.27ng/ml±.10ng/ml). Group 3 (carriers of the TNF- wild type and the CD 14 polymorphism) had the highest TNF- α protein level. Analysis was done on the mean protein level of each genotype combination. There was no statistically significant difference between any of the gene combinations and plasma protein level ANOVA p= 0.48 for sCD14 and p=0.58 for TNF- α (Table 4.16). Chi squared tests were carried out to see if there was any association between respiratory outcomes and any of the gene combinations.

Table 4.16: Association between the combined effect of TNF-308 G/A and CD14 159 C/T and the cytokine protein levels

Gene combination	***N***	***sCD14 Levels***	***TNF- α levels***
TNF-α and CD14		**Mean (ng/ml)**	**Mean (pg/ml)**
Group 1 (TNF- GG +CD14 CC)	29	9.27±4.10	12.40±11.99
Group 2 (TNF- AA/GA + CD14 CT/TT)	13	8.99±4.67	10.36±7.76
Group 3 (TNF- GG +CD 14 CT/TT)	13	6.53±4.72	18.23±26.51
Group 4 (TNF- AA/GA + CD14 CC)	16	7.97±4.32	11.64±10.26
P value (Anova)		0.48	0.58

There were no statistically significant associations between any of the gene combinations and the studied respiratory outcomes (Table 4.17 and 4.18).

Table 4.17: Frequencies of participants with gene combinations of CD14 and TNF- 308α Polymorphic genotypes stratified by respiratory outcomes Any Asthma and Persistent Asthma

Gene combination	***Any Asthma***	***Persistent Asthma***
TNF-α and CD14	***N=60 (%)***	***N=60%***

	n	Case	Non Case	n	Case	Non Case
Group 1 (TNF- GG +CD14 CC)	24	8 (33,3)	16 (66,7)	24	2 (8,3)	22 (91,7)
P value		0,83			0,41	
Group 2 (TNF- AA/GA + CD14 CT/TT)	13	3 (23,1)	10 (77,0)	13	1 (1)	12 (92.3)
P value		0.25			0.52	
Group 3 (TNF- GG +CD 14 CT/TT)	11	6 (54,5)	5 (45,5)	11	3 (27,3)	8 (72,7)
P value		0.13			0.11	
Group 4 (TNF- AA/GA + CD14 CC)	12	4 (33,3)	8 (66,7)	12	1 (8,3)	11 (91,7)
P value		0.59			0.57	

Table 4.18: Frequencies of CD 14 Polymorphic genotypes stratified by respiratory outcomes BHR and Atopy

Gene combination TNF-α and CD14	***BHR N= 56 (%)***			***Atopy N= 64 (%)***		
	n	**Case**	**Non Case**	**n**	**Case**	**Non Case**
Group 1 (TNF- GG +CD14 CC)	24	8 (33,3)	16 (66,7)	28	5 (17,9)	23 (82,0)
P value		0,51			0,11	
Group 2 TNF- AA/GA + CD14 CT/TT)	8	2 (25)	6 (75,0)	8	1 (12,5)	7 (87,5)
P value		0,44			0,27	
Group 3 (TNF- GG +CD 14 CT/TT)	10	3 (30,0	7 (70,0)	12	6 (50)	6 (50)
P value		0,25			0,06	
Group 4 (TNF- AA/GA + CD14 CC)	13	1 (7,7)	12 (92,3)	16	6 (37,5)	10 (72,5)
P value		0,26			0,34	

Chapter 5: Discussion

Two gene polymorphism namely TNF-308α G/A and CD14 (-159) C/T were investigated in the children in Durban. The TNF308α variant A allele was quite common in the population. It was detected in more than 40% of the population

with an allelic frequency of 0.24. Similarly almost 38% of the population carried the variant CD14 (-159) T allele also with an allelic frequency of 0.24. The average age of the children was ten years and almost 50% of them reported to have been exposed to tobacco smoke in addition to living in a high pollution area. Almost 39% of the population reported to have any category of asthma. In association studies of the gene polymorphisms and their impact on phenotype TNF-308α G/A and CD14 (-159) C/T polymorphisms were not associated significantly with asthma, and its related respiratory phenotypes. In addition there was no association detected between any of the gene polymorphisms and the levels of their respective cytokine proteins Association was detected with two respiratory phenotypes namely acute asthma and atopy with levels of circulating cytokines of TNFα and sCD14 respectively. There was a significant relationship between TNF- α levels and acute asthma (p=0.03) and sCD14 levels and atopy (p=0.04). Participants with persistent asthma had significantly higher TNF-α levels compared to non-cases. Participants that were not atopic had significantly higher sCD14 levels than atopic subjects.

GEE models to investigate the gene- exposure interaction showed that the TNF-308-α A allele carriers had a greater magnitude of deterioration of lung function post pollution exposure to SO_2 β= -2.62, CI (0.51,4.71) p= 0.02 and p(interaction=0.03 There was a statistically significant gene environment interaction with NO in individuals who are carriers of the TNF- A allele (Nadir of PF readings lag 2: β= -12.3, CI (-22.09, -2.51), p=0.01 p(interaction)=0.03.and 5 day average β= -42.83, CI (-70.11,-15.55), p=0 and p (interaction)=0.01). In analysis of the CD14 gene polymorphism gene environment interaction adverse effects of SO_2 were limited to individuals carrying the C allele of this polymorphism β= -1.50, CI (-0.36, 3.37), p=0.01, p (interaction) =0.01. Carriers of the T allele seemed to have a protective effect with NO_2 and NO exposure. Intraday variability of FEV_1 improved when exposed to NO_2 after five days β= -4.02, CI (-6.52,-1.53), p=0, p (interact) =0.05. There was also improvement five days post exposure to NO β= -9.42, CI (-12.45, -6.03), p=0.00, p (interact) =0.00

In analysing the coinheritance of the two gene polymorphisms (CD14 159-C/T and TNF- 308α G/A), to assess their impact on protein expression we found no association between the genotype combinations and either the CD14 and TNF- α cytokine levels.

Our results for the frequency of the variant A allele in the Durban African population sample are similar to other African populations with different diseases and the results are comparable to other studies on the continent on TNF-α-308 SNP. In a study of cervical cancer the genotype distribution of the TNF-308α GA polymorphism for the South African cancer cases was GG 71%, GA 26% and AA 3% (Govan *et al.* 2006). In a study of silicosis in gold miners in the Free State, the genotype frequencies were as follows for moderate silicosis patients GG 63%, GA 31%, AA 5% (Corbett *et al.* 2002). In HIV patients recruited from a clinic at King Edward VIII Hospital in Durban the genotype frequencies were GG 60% GA 37.3%, AA 2.7%.

The A allele seems rarer in African populations than in Caucasian population. The frequency of the AA genotype in our study population was higher than all three of the above studies (7.7%) . Govan and partners found that the genotype distribution for GG and AA -308α genotypes for South African data were similar to those reported in Zimbabwean, Chinese, Italian, and Korean populations (Table 2.6 and 2.7). There was an increased frequency of the -308 TNF-α A-allele and decrease in the G allele in the British Caucasian group when compared to the South African population group. These data highlight the possible variability of cytokine gene frequencies in different population groups.

In our study population, 50% of BHR patients were AA genotype which was the highest proportion of carriers of this genotype across all the respiratory phenotypes (p=0.06) (Table 4.4). This finding is similar to that of Hong *et al*. (2007) who found the TNF-308α A allele was strongly associated with severe

BHR in Korean children with severe asthma. Participants with persistent asthma had significantly higher TNFα levels (p=0.03). This is consistent with studies by Witte *et al*. who reported that the relationship between asthma and the high TNF-308α producing allele A, was further strengthened when restricting cases to those individuals reporting acute asthma. Notably the highest TNF-α level was in a participant with persistent asthma and of the GA genotype (Witte 2002).

Zhang et al recently found that the A variant allele carriers had a 53% increased risk of asthma in Asians OR=1.53, 95% CI=1.17–2.01 and p=0.002, but not in Caucasians (OR=1.06, 95% CI=0.75–1.50 and p=0.73). They noted that this could be attributed to genetic backgrounds; in addition the environment could play a major role. It is possible that these differences in genetic susceptibility may be affected by exposure to various environmental factors (Zhang *et al.* 2011). Asian countries are amongst the fastest growing economies of the world leading to more industrial processing thereby higher pollution levels. The environment may be a trigger for adverse respiratory outcomes. This could account for differing results in differing populations.

Tumour necrosis factor-α increases the expression of cellular adhesion molecules and facilitates the passage of leucocytes into the airway in response to allergen and to bacterial products. In addition, it would appear to increase airway smooth muscle cell contractility and expression of eotaxin, and also to increase IL-5 secretion. In asthma, as in other situations, TNF-α may have apoptotic activity (programmed cell death), although this specific question has not been addressed within the airway, but perhaps it could be responsible for airway epithelial shedding. There are also data to implicate TNF-α in airway remodelling and fibrosis (Thomas 2001). Gene polymorphisms in the promoter region of the TNFα gene may exert a large degree of transcriptional control over cytokine production. These effects, however, have not been comprehensively investigated in the context of infection. The precise mechanisms of genotypic influences on transcriptional regulation are currently unknown. However, it is

thought that the G to A transition at the -308 locus is associated with conformational changes that increase binding affinity of transcription factors such as nuclear factor-kappa B (NF-κB) (Wilson *et al.* 1992). Although in the current study we did not detect any association between the respiratory outcomes and the TNF-α-308G/A polymorphism, we did find an association between elevated plasma TNF-α levels and persistent asthma (p=0.03).

Different studies have used different approaches, thus making it difficult to draw a general conclusion. So far, evidence suggests that circulating TNF-α levels do not seem to correspond with the TNF-α-308 promoter polymorphisms. However, although circulating TNF-α level might be under a multifactorial regulatory process, local TNF-α concentration might be of greater importance and under increased control by specific polymorphisms. The associations between TNF-α genotype and disease are not absolute as suggested by numerous conflicting studies. Nevertheless, it is clear that the genetic regulation of TNF-α at sites of inflammation is important. Under circumstances where the release of TNF-α has been triggered by the genetically endowed capacity for greater TNF-α production, this leads to more severe inflammatory reactions (Elahi *et al.* 2009). In our study we did not detect an association between the TNF-308α G/A polymorphism and respiratory outcomes. This finding is supported by a functional genomics study that examined the association between TNFα mRNA, protein level expression, and TNFα polymorphisms. This study was conducted on healthy subjects, studied either independently or in haplotype combination. Using a meta-analysis for the TNF-308α G/A polymorphism, they confirmed the absence of any association between TNF-α mRNA and protein levels, and TNF-308α G/A genotypes. This study and meta-analysis of the literature confirmed the absence of any functional consequences of the TNF-308α G/A promoter polymorphism, either alone, or in various haplotype combinations in healthy subjects (Mekinian *et al.* 2011).

Nevertheless we did detect an association between TNF-concentration and acute or persistent disease. In a meta-analysis paper that reviewed the role the TNFα gene polymorphisms in cytokine production it was similarly noted that *in vitro* stimulation of TNFα production by cells from G/G homozygous individuals and G/A heterozygote individuals have produced conflicting results. Some have reported higher TNF-α production by cells from G/A donors than by G/G cells Louis *et al.* 1998). Others studies have reported no significant effect (O' Donnell *et al.* 2004; Zhang *et al.* 2011). However, it is interesting to note that these studies used different liposaccharide concentrations and the number of individuals with the G/A genotype studied was, in most studies small. Thus decreasing the power of the study to detect any significant difference between genotypes (Elahi *et al.* 2009). It is plausible that the inconsistency of results could be due to differing methodologies and sample sizes. It has also been suggested that the association of asthma to TNF-308α A allele reflects linkage disequilibrium with genes influencing specific immune response (Di Somma *et al.* 2003).

The current study found gene environment interactions between the TNF-308α A allele and exposure to SO_2, NO_2 and NO. The A allele has been associated with higher levels of TNFα production in several studies as mentioned earlier and TNFα has been found in large amounts in bronchial fluids during asthma attacks (Witte 2002). It is tempting to suggest that an association between these biomarkers and the hypothesis that a functional consequence of TNFα promoter polymorphisms could be a variation in the levels of TNFα production. Many studies have examined the functional consequence of the TNF-308α G/A SNP on TNFα production, but the results remain controversial (Mekinian *et al*. 2011).

The effect estimates of SO_2 on children who were carriers of the A allele were generally higher and a significant gene pollutant interaction was observed with intraday variability of FEV_1 (β= -2.62, 95% CI (0.51, 4.71), p=0.02 p_{int}=0.03) at lag 2 Table 4.7 There were 2 statistically significant gene environment

interaction with NO at 2 days post exposure (β= -12.3, 95%CI (-22.09,-2.51), p=0.01, p (interact) =0.03) and 5 days post exposure (β= -42.83, 95%CI (-70.11,-15.55), p=0.00, p(interact)=0.01) in individuals who are carriers of the TNF- A allele. This supports the notion that the A allele may increase susceptibility to the adverse effects of air pollutants (Witte 2002; Zhang *et al.* 2011). This data also shows that asthma is a complex disease which cannot be explained by simple genetics. Several different genes or control mechanisms are likely to contribute to expression of the asthma phenotype (Reddy *et al.* 2012).

Our data shows that, under conditions of increasing exposure to SO_2, NO_2 and NO, participants with the TNF-308α A allele have decreased lung function. Generally the effect estimates showed the adverse effects of increased pollutant exposure and lung function among those carrying the A allele (Table 4.7 and 4.8).

Primary sources of Nitrogen oxides emissions include motor vehicles on the various motorways which converge in the South Durban basin (Naidoo *et al.* 2007). Nitrogen oxides react with other pollutants to form ground-level ozone. This irritates the nose and throat, especially in people with asthma, and appears to increase susceptibility to respiratory infections. Ground-level ozone may also, reduce lung function (Turner *et al.* 1994). Generally, air concentrations of SO_2 are highest near large industrial complexes. In the Durban South industrial basin the oil refineries, paper producer and sugar refinery are responsible for 80% of the SO_2 pollution load (eThekwini Health and Norwegian Institute for Air Research 2007). SO_2 will cause heightened sensitivity to allergens that commonly trigger asthma attacks, narrowing of the airways and cause breathing problems for children and adults who have asthma and are physically active outdoors. SO_2 causes wheezing, chest tightness and shortness of breath even among healthy people who do not have asthma. This combination of factors, the environment and genetic, certainly add to the biology of asthma (Turner *et al.*

1994).

Some results in the gene environment analysis, showed a significant association in the ‘‘unexpected’’ protective direction (i.e., higher exposure to NO and NO_2 associated with decreased Intraday variability in FEV_1). This may be attributed to the fact that the effects of the four pollutants were studied independently, using the approach of one gene with one exposure. This model does not account for interaction of pollutants and modifying the effect on lung function (Reddy *et al.* 2012).

The second SNP we studied was CD14 (-159) C/T (rs256190 also reported as CD14, -260C>T). This SNP is also found in the promoter region of the CD14 gene. This SNP encodes for a receptor protein that binds liposaccharide and its expression may be partially regulated at the genetic level (Dressing *et al*.2007). Liposaccharide is the principal component of endotoxin and it induces lung inflammation and originates from the outer membrane of Gram negative bacteria. Ligand binding activates innate immune system pathways that may trigger atopic asthma (Dobrovolskaia and Vogel 2002). Atopic asthmatic subjects show increased expression of CD14 after acute allergen provocation (Virchow, JCJ *et al.* 1998) and LPS inhalation (Alexis *et al.* 2001).

The frequency of the CC, CT, ant TT genotypes was 63.4%, 28.2%, and 8.5% respectively**.** The frequency of the C and T alleles was 0.77 and 0.23 respectively. The frequencies we obtained in our persistent asthmatics CC, CT, and TT, 42.9%, 28.6% and 28.6% respectively (p=0.2) are different to those in the only other study on CD14(-159) C/T gene polymorphism on the African continent, a Tunisian population of children. The genotype frequencies in asthmatic children in the Tunisian population were, for CC, CT and TT respectively, 22%, 43%, 35%. With our population in Durban, a markedly higher number of children with asthma that had the CC genotype (57%) p=1.0 was seen (compared to 22% in the Tunisian population (chi square 12.06 p=0.002). The

frequency of the variant T allele was notably lower in the children in Durban South at 23% in the whole study population as compared to 68% in asthmatic Tunisian children. It should be noted that the Tunisian consisted of Caucasians (Lachheb *et al.* 2008). This indicates that the T allele may be rarer in African populations as compared to other populations (East Asian 57.5% Indian 62.1%, European 45.7%). The Yoruba in Ibadan Nigeria are said to have an allele frequency of 29.3% which is closest to the frequency here in Durban (Zhao and Bracken 2011). More studies will have to be done to build data on the frequencies in this polymorphism among African populations.

We found that CD14 (-159) C/T polymorphism was not significantly associated with asthma, and its related respiratory phenotypes such as atopy and BHR. These findings are similar to those by Zhao *et al*. (2011) who published a meta-analysis of 23 studies which yielded a non-significant overall association between the CD14(-159) C/T gene polymorphism and asthma. The authors observed that there was high heterogeneity across studies. Heterogeneity in meta-analysis refers to variation in study outcomes between studies. After restricting analysis to studies using atopic asthma and non-atopic non-asthma, case-control phenotypes and excluding studies influencing heterogeneity, the genotype-specific odds ratios (ORs) suggested a codominant model. Carriers of the TT and CT genotypes were about 33% less likely (OR = 0.67, 95% CI: 0.54-0.84) and about 20% less likely (OR = 0.80, 95% CI: 0.66-0.95), respectively, to have atopic asthma compared to carriers of the CC genotype. Zhao and partners then concluded that there was a protective dose-response relationship between the CD14 (-159) T allele and atopic asthma susceptibility. These results demonstrate the importance of precisely specified case-control groups as well as the need to assess interactions in the investigation of complex diseases such as asthma (Zhao and Bracken 2011).

Association was detected between persistent asthma and CD14. There was a significant relationship between sCD14 levels and atopy (p=0.04). Participants

who were not atopic had significantly higher sCD14 levels than atopic subjects. Still, no association was detected between any of the gene polymorphisms and the levels of their respective cytokine proteins.

Participants stratified by atopic status showed a statistically significant difference in sCD14 levels among atopic and non-atopic subjects. The difference was protective with non-atopic patients having the higher sCD14 protein levels ($p=0.04$). This relationship with atopy is similar to results obtained by Levan and partners in 2008. They found that sCD14 levels at baseline were modulated by the atopic status of the individual. They observed lowered sCD14 levels among atopic subjects than non-atopic subjects before endotoxin inhalation. This supports our research findings (LeVan *et al.* 2008).

Haplotype studies have suggested that the CD14 159 C/T polymorphism alone is not sufficient to modulate sCD14 levels, and that combined effects of different SNPs should be taken into account (LeVan *et al.* 2008). These conflicting results could be due to lack of power, linkage disequilibrium, genetic heterogeneity, gene interactions, gene environment interactions or population based differences in CD14 haplotypes (Hong *et al.* 2007) and age (Munthe-Kaas *et al.* 2010)

Another plausible reason why there was lack of association between the genetic level and the phenotype may be epigenetic changes. A reduced effect of CD14 polymorphisms on sCD14 levels through childhood was observed in a recent study. At birth Munthe-Kaas et al. (2010), detected an association in 3 CD14 polymorphisms rs2569191, rs5744455 and rs2569190 (-159 C/T). By age 10 only rs5744455 was still associated with sCD14 and CD14 methylation increased during the same period. Their study implied that epigenetic changes indicated by gene environment interaction in early life may explain reported inconsistencies in the role of CD14 polymorphisms and the risk of atopic disease in childhood.

The study population we studied in Durban had an average age of 10.3 years. Gene methylation might be a plausible explanation for the lack of association of the gene polymorphism with sCD14 levels considering the long history of environmental pollution in Durban south. In addition a study by Hollingsworth *et al.* (2008)showed that a folate rich diet during pregnancy resulted in increased methylation of several specific gene promoters in mice offspring, resulting in decreased gene expression, and enhanced allergic immune responses in the mice (Prescott and Allen 2011).

Participants stratified by atopic status showed a statistically significant difference in sCD14 levels among atopic and non-atopic subjects. The difference was protective with non-atopic patients having the higher sCD14 protein levels (p=0.04). This finding is supported by Jones *et al* (2002). They sought to determine whether infant and maternal levels of soluble CD14 (sCD14) are associated with the atopic outcomes of infants. There was no difference in plasma sCD14 levels at birth of children with a high compared with those with a low risk of development of atopy. Amniotic fluid sCD14 levels at midgestation (16-17 weeks) were significantly lower when the child was subsequently atopic ($P < 0.05$). Soluble CD14 levels in breast milk collected 3 months postpartum were significantly lower in children with eczema at 6 months of age, irrespective of whether they were atopic ($p = 0.003$). They then concluded that exposure to reduced levels of sCD14 in the foetal and neonatal gastrointestinal tract is associated with the development of atopy, eczema, or both. Thus the exogenous supply of sCD14 might influence immunologic reactivity both locally and systemically in early life and thereby influence disease outcome (Jones *et al.* 2002).

CD14 is a pattern recognition receptor that can interact with a variety of bacterial ligands. During gram-negative infection, CD14 plays an important role in the induction of a protective immune response by virtue of its capacity to recognize lipopolysaccharide in the bacterial cell wall. Knowledge of the contribution of

CD14 to host defence against gram-positive infections is limited (Dessing *et al.* 2007). Activation of macrophages represents one of the initial events in the innate immunity to intracellular infections. CD14 is expressed principally by cells of monocyte/macrophage lineage and plays a pivotal role in the innate recognition of bacterial cell wall components. The binding of a microbial component to CD14, as an accessory receptor for toll-like receptor (TLR), results in cellular activation and initiates a variety of effector functions including cytokine secretion and proliferation (Anas *et al.* 2010). CD14 exists in both membrane-bound and soluble forms. Soluble CD14 (sCD14) is present in the circulation and other body fluids, and its levels in plasma increase during inflammation and infection (Ayaslioglu *et al*. 2012)

The hygiene hypothesis may explain why non atopic patients had higher sCD14 plasma levels. Their immune systems may be functioning optimally and reacting appropriately to diseases and infection. It is possible that the atopic children have immature immune systems that overreact to allergens. Exposure to microbial products such as lipopolysaccharide or other endotoxins during childhood appears to stimulate CD14 (an endotoxin coreceptor, along with toll-like receptor [TLR] 4) and could activate maturation of type 1 helper T cells (TH1), thereby suppressing the TH2 response and leading to atopy (Kavut *et al.* 2012).

TLR are members of a family of pattern-recognition receptors, which recognize molecular structures of bacteria, viruses, fungi and protozoa (pathogen-associated molecular patterns or PAMPs), as well as endogenous structures and proteins released during inflammation (damage/ danger-associated molecular patterns or DAMPs (Anas *et al.* 2010).

Decreasing family sizes and higher standards of personal hygiene, both of which result in a lower rate of cross-infection within households, have been suggested as explanations for the increase in allergic disease seen during the past 30 years

(Strachan 1989). While the frequency of asthma has increased, the percentage of young families in the United States with more than two children has declined, from 36% in 1970 to 21% in 1998. During this same period, day-care attendance has increased, involving 60% of preschool children in 1995. However, whereas 65% of four-year-old children attended a day-care centre in 1995, only 7% of infants less than a year old did so (Ball *et al*. 2000) Day-care attendance is also lowest among children from low-income families, among whom morbidity from asthma is high (Wissow *et al.* 1988).

The original population sample from which our sample was selected was from a relatively poor socioeconomic group. Although education levels among the caregivers were relatively high with 42.8% having matriculated from high school, there was a significant disparity with income. Approximately 20% of all caregivers earned R 10 000 or less per annum, while 48% earned between R 10 000-R75 000 per annum (Reddy 2007). We currently have no data to confirm the microbial exposure individuals in this population had in infancy

Despite the probability of immature immune systems in this community of children, there is still very high pollution exposure in the communities in South Durban (Naidoo *et al.* 2007). In addition there is a high traffic volume which has been associated with asthma, cough and wheeze, and in children additionally exposed to environmental tobacco smoke, with allergic sensitisation. However, effects of socioeconomic factors associated with living close to busy roads cannot be ruled out (Nicolai *et al.* 2003)

In addition, current research findings support a role for epigenetics in the development and persistence of allergic disease (North and Ellis 2011). Considering the level of pollution in the vicinity of our study population there is a probability of DNA damage methylation and histone modification by suspected drivers of epigenetic processes heavy metals, pesticides, diesel exhaust, tobacco

smoke, polycyclic aromatic hydrocarbons, hormones, radio activity, viruses, bacteria and basic nutrients (Koppelman and Nawijn 2011; Reddy 2011)**.**

Despite not detecting an association between the CD14 gene polymorphism with respiratory outcome or protein expression we did detect significant gene environment interaction between NO_2 NO and SO_2 and the CD14 (-159) C/T gene exposure and increased FEV_1 ($p<.0.05$) in the protective direction i.e. variability of FEV_1 became smaller. Our observation is in agreement with a recent meta-analysis that found a significant decrease in atopic asthma risk for the TT and CT genotypes as compared with the CC genotype (Zhao and Bracken 2011).

Children carrying the CC genotype, presented with an increase in intraday variability of FEV_1 and adverse effect on lung function at (NO lags1, 2 &5 days and NO_2 lag1, 2 & 5 days). In these instances adverse effects of NO, SO_2 and NO_2 were limited to individuals carrying the C allele of this polymorphism. This finding shows that that SNP may act as a modifier of asthma risk in individuals with differing degrees of environmental endotoxin exposure. T allele carriers have been found to have higher serum CD14 levels than carriers of the C allele (Martinez 2007). Functional genomic studies have supported this finding by showing increased transcriptional activity of the CD14 (-159) T allele (Simpson *et al.* 2006a). Homozygotes of the T allele appear to be protective for asthma at low levels of endotoxin exposure, but may increase asthma risk at high levels of endotoxin exposure (Martinez 2007).

The findings of the current study are consistent, with the most current meta-analysis on atopic asthma versus non-asthma. These results may allude that higher CD14 expression in TT homozygotes increased sensitivity to protective effects of low level endotoxin exposure compared to carriers of other genotypes. At higher levels of endotoxin exposure, however, induced CD14 expression could

be increased in carriers of the C allele showing a reversed protective effect. This hypothesis was developed by Martinez (Martinez 2007)

The current study has several strengths. The study population exposed to ambient pollutants was confined to defined areas with its own monitoring site, allowing a more precise estimation of exposure. Secondly, the initial study on which this current study was based on analysed pollution data systematically, allowing correlation between increases in exposure and lung function measures. A limitation of the current study is small sample size, although power was enhanced by characterization of individual exposure and repeated measures over time. There were missing samples in the study so a complete set of genotype data could not be obtained from all the originally selected participants. We therefore reduced the sample size to have a complete data set (n=104). The sample size tested for CD14 plasma levels (n=49) polymorphism was small this was due to the prohibitive cost of the cytokine detection reagents.

The increasing prevalence of asthma and other related respiratory diseases is an important public health concern. Studies such as ours, although preliminary, that evaluate the functional significance of particular polymorphisms according to whether their molecular actions are influenced by environmental exposures, are important. With increasing industrialization, one can reason that genetically vulnerable high risk populations are being increasingly exposed 'to environmental influences that have altered in recent decades. In the near future, an understanding of the biology of candidate genes and gene environment interaction may lead to development of more effective strategies to prevent or treat complex respiratory diseases.

Chapter 6 Conclusion

Significant gene environment interaction was shown between the TNF- 308 α promoter polymorphism and the CD14 promoter polymorphism in a population of children in Durban South Africa. This was despite lack of association detected between the gene polymorphisms with the proteins they code for or the various respiratory outcomes. These results show that gene polymorphisms may be insufficient to cause disease when taken individually. However they might induce significant modulation of regulatory pathways when taking into account gene by gene interactions, gene by environment interaction or both. Thus complex diphenotypes, polygenic diseases would not be expected to be an all or non-event but rather to result in a combination of small quantitative effects (LeVan *et al.* 2008).

We have also demonstrated that exposure to NO was associated with poorer lung function in TNF-308α A allele carriers and SO_2, was associated with poorer lung function in TNF-308α A allele and CD14 (-159) C allele carrier. This association is significantly modified by an individual's genotype. These genes polymorphisms were detected in the general population, and they may have important implications for public health. This suggests a significant gene-environment interaction between the TNF-308α and CD14 (-159) genotypes and air pollution.

Public health interventions to improve air quality can improve health at the population level. However, these improvements don't necessarily always translate into reduced exposure for all residents. First, control strategies are aimed at criteria pollutants and it is not known whether levels of other pollutants have also decreased over time. Second, changing demographics and increasing urbanisation and rapid industrialisation negatively impact long-term environmental planning.

In our study, it was disturbing to note that relatively modest increases in the concentrations of ambient pollutants affect respiratory health. In light of the substantial and consistent associations between ambient concentrations of the 4 pollutants assessed and adverse effects on lung function among children who are genetically predisposed, strategies for reducing ambient environmental pollution should be urgently considered. Current legal standards and more importantly, enforcement of these standards should be reviewed. Our results emphasize the importance of considering genetically susceptible populations when setting standards. By presenting the distribution of risks across the population, risk assessment can be far more effective in shaping public policy that is both preventative and fair. However, there is controversy around the ethical, economic, and legal ramifications of the use of genetic information.

The data on genetic susceptibility does not support a policy of large-scale individual screening, because there are too many polymorphisms involved that contribute to asthma risk and the costs would be prohibitive. Although genetic testing is not feasible or desirable, disease prediction might become feasible in the future. Although predictive testing for single gene disorders (e.g. cancers) is useful, predicted health gains for multifactorial diseases such as asthma are greater from those strategies directed at the whole population rather than targeted at a high risk group. Also, resource allocation to support genomics technologies is a problem in developing countries, where funding to treat disease epidemics such as HIV and TB is more important than allocating money to research in genetics. Therefore the gap is growing between those countries that can use this technology and those that cannot.

Public health associated benefits linked to genetic epidemiological information include tailored treatment regimens, prevention and management of disease. Genetic information may be used presymptomatically for targeted interventions

including diet, medication or lifestyle modifications. Increased risk may advocate certain behavioral changes.

The increasing prevalence of asthma and other related respiratory diseases is an important public health concern. Studies such as ours, although preliminary, that evaluate the functional significance of particular polymorphisms according to whether their molecular actions are influenced by environmental exposures, are important. With increasing industralisation, one can reason that genetically vulnerable high risk populations are being increasingly exposed to environmental influences that have altered in recent decades. For this “population-at-risk”, standard approaches of health promotion and health protection strategies do not apply. Public health strategies should aim to protect these individuals and by protecting the most susceptible members of our population, we invariably protect everyone. In the near future, an understanding of the biology of candidate genes and gene-environment interaction may lead to development of more effective strategies to prevent or treat complex respiratory diseases.

Further research on the impact of environmental factors such as pollution, diet and microbial exposure on DNA regulation and function, is required to obtain a clearer picture of disease risk, prevention and intervention in vulnerable populations like children in our local populations

The example of complex interactions between environmental exposures and polymorphisms in the CD14 and TNF gene in predisposing for allergy-related conditions offers a good indication of the complexity of the mechanisms that determine susceptibility to these conditions. Contrary to what has been the rule for monogenic diseases, the association between genetic variations and polygenic conditions such as asthma and allergies may not always be unidirectional; that is, not always will the same alleles be associated with the conditions under study. Concepts of penetrance of genetic variations that ignore these nonlinear influences (which may affect gene-gene and gene-environment

interactions) may hinder a better understanding of mechanisms of disease involved, and therefore may delay the development of preventive strategies for these common conditions. Discrepancies between well-designed genetic studies of asthma and allergies, therefore, may be suggesting something fundamental about how these diseases develop and how it will be possible to abolish them in the future (Martinez 2005).

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3. APPENDIX 1

SDHS methodology for the collection of epidemiological data including lung function measures, symptoms and allergy status

- **1.1 Collection of symptom logs and bihourly lung function data**

A central aspect of the health data collection was bihourly symptom logs and the measures of lung function collected five days per week over three week period in each of four seasons. This was the data used to determine whether there was an association between daily fluctuations in ambient air pollution levels and fluctuations in health status.

1.2 Bihourly measures of pulmonary function during the schoolday

The AirWatch® (iMetrikus, Carlsbad, California, USA) brand airway monitor was used to monitor fluctuations in peak expiratory flow (PEF) and forced expiratory volume at one second (FEV_1) of each participant. This portable, hand-held device has a number of distinct advantages over methods used previously to obtain repeated measures of a forced expiratory manoeuvre in field studies. First, unlike the case with traditional peak flow meters, the FEV_1 is also obtainable. FEV_1 has inherently greater reproducibility than PEF and is a more clinically relevant measure (Thiadens *et al.,* 1999). Second, results of up to 500 expiratory manoeuvres was digitally stored in each Air Watch. A unique patient identifier and the time and date of each expiratory manoeuvre was manually downloaded into a data base. Each participant received his/her own peak flow device, which were kept at the school, and was clearly labeled with the participant's full name to avoid inadvertent exchange of devices.

The quality of such peak flow and FEV1 measures collected in the field tend to be quite variable, but is responsive to focused training of participants in good technique with frequent reinforcement. An intensive training session was conducted at the school with the participants in the proper performance of peak flow maneuvers. As part of this training each participant was individually coached and observed by field supervisors to ensure his or her ability to perform valid and reproducible expiratory maneuvers. In addition, during the actual intensive phases of data collection when participants used the peak flow meters, supervisors observed expiratory maneuvers to ensure proper technique. Participants were retrained at the beginning of each of the four-week intensive data collection periods.

On each of the five schooldays during the week, participants were be asked to perform a session of three consecutive maneuvers every one and a half to two hours (four times per 5.5 hour schoolday: approximately 08h00, 09h45, 11h30 and 13h20), and immediately prior to completion of the bihourly logs described above. The highest PEF and highest FEV_1 from each session, even if from different maneuvers, was used in data analyses. All schools were studied simultaneously.

- **1.3 Collection of baseline pulmonary data :Baseline spirometric assessments and**

- **1.3.1 methacholine challenge tests**

Baseline spirometric assessments and methacholine challenge tests was conducted on all schoolchildren participating in the three-week intensive data collection sessions. All American Thoracic Society (ATS) guidelines for conducting spirometry were followed (ATS, 1995). Spirometers were calibrated at least twice a day with a three-liter syringe. Technologists who had undergone training in standard technique conducted spirometry, which was performed in a sitting position without nose clips. The lung function indices of primary interest included forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1). Special instructions were given to participants to ensure that tested individuals did not take any anti-asthmatic inhalers (12 hours before) or oral asthma medications (48 hours before) prior to the test. Participants with an obstructive pattern at baseline ($FEV_1/FVC < 0.75$) were administered an inhaled bronchodilator and had testing repeated. Those without a baseline obstructive pattern underwent methacholine or histamine nonspecific challenge testing according to an abbreviated protocol used in epidemiological surveys (Yan *et al.,* 1983). Special precautionary measures included having readily available oxygen and B_2-adrenergic agents for nebulization. Additionally, emergency medical personnel were either physically on site or within quick access at all times during nonspecific challenge testing. All students were assessed during school hours (Naidoo *et al.,* 2006)

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- **1.3.2 Collection of questionnaire data**

Carefully selected interviewers drawn from the communities or from students at the involved universities and Technikons were trained and supervised to conduct baseline interviews with participants and their caregivers. Training included techniques and practice in conducting interviews in a consistent and neutral fashion. Components of this questionnaire included demographic information; assessment of presence and severity of respiratory and other relevant symptoms using standardized validated questions from sources such as the British Medical Research Council and American Thoracic Society; validated questions to specifically address the presence and severity of asthma among participants including information concerning wheezing, coughing, chest tightness, shortness of breath, activity limitations, and medication use; health services utilization; quality of life measures; perinatal history; place of birth and residential history; potential confounding factors such as exercise, viral respiratory infections, exposure to cigarette smoke, pre-existing medical conditions. (Annexure1: Child screening questionnaire). All questionnaires were available in English, isiZulu and Afrikaans, and was conducted in the language of choice of the interviewee

by an interviewer fluent in that language. Child participants were interviewed at school and their caregivers at the homes of the participants.

- ### 1.3.3 Assessment of allergic status

All pupils who participated in the three-week intensive data collection study were requested to participate in skin prick testing. Antigens tested included mixed cockroach, mixed dust mite, mould mix (*Aspergillus*, *Cladosporium* and *Penicillium*), cat, dog, mouse, rat, ragweed, mixed grasses, plus histamine as a positive control and saline as a negative control. These tests were conducted at school on a different day than the methacholine challenge testing. Participants were informed to stop any antihistamines and any other reactive medication (H_2 antagonists, tricyclic antidepressants, corticosteroids etc) at least 24 – 72 hours pre-test. The test was applied to the volar surface of the forearm, and read approximately 15 – 20 minutes later. The wheal and erythema were read and measured according to a standardised method, and an outline of the wheal and erythema was recorded on see through tape for a permanent record. A greater than 2 mm difference in mean diameter between allergen and control wheal was considered as positive. Emergency health personnel were on site to clear each participant receiving skin testing and were equipped with proper medications and resuscitation equipment in the unlikely event that any individual had a severe reaction to a skin test. Collection of this data allowed for the assessment of whether skin test positivity is associated with genotype.

4. APPENDIX 2

- **ENVIRONMENTAL MONITORING OF AMBIENT POLLUTANTS**

5.

2.1 Conventional Pollutants

- ***2.1.1 Nitrogen Dioxide (NO_2)***

Monitoring. This pollutant was sampled continuously at 8 monitoring sites that can be grouped into 3 categories:

- Lower DSIB with 3 monitoring sites (Southern Works, Jacobs, Wentworth) that capture industrial sources.
- Central DSIB/traffic sites with 4 monitoring sites (Warwick, City Hall, King Edward, Ganges) that reflect primarily vehicular sources.
- Northern area with 1 monitoring (Ferndale) which is some distance from major roads and industry.

Monitoring of NO_2 (and NO) used conventional continuous gas-phase chemiluminescence's detection (Monitor Europe, model ML 9841 B, set to operate in a 0 to 1000 ppb range). These monitors are designated under US EPA regulations as an equivalent method.

Data processing/quality/status. At each site, data were collected as 5-min averages, which were processed to 1 hr averages if at least half of the data for that hour were available. The 1-hr averages were processed to 24 hr averages, from noon to noon, if at least half of the hourly data in the period were available. Data capture rates were good, e.g., for the period 2.1.04 through 1.10.05, the overall capture rate for valid 24-hour observations was 83.4% (range from 76% at King Edward to 94% at Ganges). The distribution plots do not show strikingly high statistical outliers, though a number of higher observations are seen at Ferndale, City Hall, Warwick, King Edward, etc.

Spatial variation. Concentrations across the 8 monitoring sites show that the lowest levels are in the north (11 ppb), highest concentrations in the center city and industrial areas (19 - 24 ppb), and somewhat lower levels at Southern Works and Wentworth in the south (12 - 14 ppb). As expected, concentrations were generally highest at traffic-impacted sites (City Hall, Warwick, and King Edward) and some of the industrial sites (especially Jacobs).

Temporal variation. At all sites, concentrations show very strong seasonality with the highest levels in the winter period (March - August, roughly 20–25 ppb), and the lowest levels in summer (October – February, 16-17 ppb).
Across much of the region, daily levels were moderately to highly correlated, e.g., correlation coefficients range from 0.51 to 0.84 among the lower basin monitors. Concentrations at the northern site are lower and have only low-to-moderate correlations with the other sites (0.13 to 0.53). Some of the highest

concentrations at all sites (except the northern site) were seen on July 21-23, 2004, a period that bears more investigation.
Autocorrelation was high, about 0.7 for 1 day lags. All sites show small day-of-week effects, with levels about 10% lower on the weekends. Distributions at each site show several (1 to 6) 24-hour observations that might be considered "modest" statistical outliers. Traffic impacted sites (City Hall, Ganges, Warwick) had more statistical outliers.

Exposure estimates. This pollutant was not monitored at the school sites. To reflect a mixture of industrial and vehicular sources and derive population-oriented exposure estimates, several options were considered for the southern Durban area:

a) Averaging concentrations at the central and lower basin sites (7 monitoring locations).

b) Using the more representative lower basin sites (Wentworth, Jacobs, Southern Works and Ganges), excluding downtown and highly traffic impacted sites (Warwick, City Hall, King Edward). Although Ganges was originally considered to be traffic-impacted site, traffic influence was gauged to be only moderate and thus was included in the southern average.

Given the similar levels, high correlation, and the advantage of additional observations that can increase the representativeness of the data, we opted to use option b. This is supported by trends that show that the industrial sites appear to be occasionally influenced by local sources. Averaging across the 5 monitoring sites will diminish such effects.
For the northern Durban area, the northern site (Ferndale) was used to estimate exposures.

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- ***2.2.2 Nitrogen Oxide (NO)***

Monitoring. This pollutant was sampled continuously at 8 monitoring sites as described for NO_2. (The same equipment is used to monitor NO and NO_2.) Many of the same results and conclusions apply for these closely related pollutants. This section discusses only significant differences.

Data processing/quality/status. At each site, data were collected as 5 min averages, which were processed to 1 hr averages if at least half of the data for that hour were available. The 1 hr averages were processed to 24 hr averages, from noon to noon, if at least half of the hourly data in the period were available. Data capture rates were good. For the period 2.1.04 through 1.1005, the overall capture rate for valid 24-hour observations was 83.4% (range from 76% at King Edward to 94% at Ganges).

Spatial variation. Based on averages, concentrations at Ganges and Warwick, the most traffic-impacted sites, were considerably higher than levels elsewhere, while levels at Wentworth and Southern Works, away from traffic but near industrial sources, were by far the lowest. High peak concentrations (> 200 ppb, 24-hr average maximum) were occasionally observed at City Hall and King Edward, in addition to Warwick and Ganges (where levels reached or exceed 300 ppb).
Larger spatial differences were seen for NO compared to NO_2, reflecting the influences of local sources and the short lifetime of NO. As seen for NO_2, Levels at the northern site (Ferndale) were considerably lower than levels measured at most of the southern sites.

Temporal variation. At all sites, concentrations show very strong seasonality with the highest levels in winter (March - August, but peaking in July at up to 140 ppb), and lowest levels in summer (October – February, generally below 20 ppb). Given that NO (and NO_2) emissions are likely relatively uniform over the year, this variation is likely to result from the poorer dispersion conditions occurring the winter (as seen for other pollutants), and from the shorter lifetime of NO in the summer (due to faster reaction including scavenging by O_3).
Across the region, levels were highly correlated at the 24-hr level, e.g., correlation coefficients ranged from 0.5 to 0.9 among the 8 monitoring sites. Unlike NO_2, NO concentrations at the northern site remained highly correlated with NO levels at the other sites, though concentrations were lower. As for NO_2, some of the highest concentrations at all sites (except the northern site) were seen on July 21 - 23, 2004.
Autocorrelation was moderate, about 0.5 for 1 day lags. All sites showed moderately strong day-of-week effects, and concentrations fell by ~30% on the weekends, except at Ferndale where changes were smaller. Distributions at each site show a few 24-hour values that might be considered “modest” statistical outliers; however, the data generally performed consistently. Overall, NO patterns are consistent with vehicular and industrial emission sources.

Exposure estimates. This pollutant was not monitored at the school sites. The same options as discussed for NO_2 are appropriate. In this case, however, distance to major roads will likely be even more important.

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- ***2.2.3 Sulfur Dioxide (SO_2)***

Monitoring. SO_2 was monitored continuously at 16 monitoring sites by ultraviolet fluorescence spectrometry using US EPA reference methods. Monitors were located at 7 schools:

1. Assegai Primary School initially used an API 100A but this was swapped out to an ML 2015 in May 2004 due to problems;
2. Dirkie Uys had a API 100A which failed and was replaced with a Dasibi 4108 in January 2005 (also problematic);

3. Nizam Primary School used an API 100A, which was replaced in January 2005 with a similar instrument (taken from Lamontville).
4. Lamontville (Entuthukweni) School used an API 100A, which failed, was repaired, and then moved to Nizam.
5. Briardale used a TECO 43A instrument.
6. Ferndale used a Monitor Labs ML 9850B instrument (serial no. M1873–M702)
7. Ngazana Primary School used a Monitor Labs ML 9850B instrument.

In addition, eThekwini Municipality monitored SO_2 at 9 stations in 2004, with the Settlers' monitor doubling as an H_2S analyzer (providing measurements every 10 min as compared to every 5 min at the other sites). Due to the need for H_2S data to resolve complaints in Merebank, the City Hall SO_2 analyser was moved to Southern Works in July 2004 to measure H_2S (eThekwini, 2004). Of the Municipality's 9 SO_2 analysers, 8 were relatively new (2003, Monitor Lab 9850B); the 9th instrument was from the Settlers School caravan (API 110A).

Data processing/quality/status. At each site (except one as noted above), data were collected as 5 min averages, which were processed to 1 hr averages if at least half of the data for that hour were available. The 1 hr averages were processed to 24 hr averages, from noon to noon, if at least half of the hourly data in the period were available.
Several of the monitors at the school sites experienced drift problems, probably a result of inadequate temperature control in the instrument or in the enclosure. Drift resulted in slowly varying negative or positive biases that was easily detected. This bias was corrected on a monitor-specific basis by subtracting the long term baseline, computed as a running average (typically considering a 400 h window) of low (1st) percentile hourly concentrations in a time window typically 68 hours before and 4 hours after the current value. Minima were allowed to vary only slowly (< 5 ppb) otherwise a new window was utilised. This approach is reasonable since background SO_2 values at all sites approached zero almost every day due to strong variation in source emissions and meteorology, and since background levels were negligible. Statistical and visual checks ensured that this approach yielded reasonable and robust values. Some small (< -1 ppb) negative values remain after this correction, a normal result for this measurement, even at monitors that do not experience excessive drift.
Overall, 6 324 valid 24-hr observations were collected across the 16 monitors for the period 1.1.04 through 6.10.05. The data capture rates varied across the monitoring sites. Due to equipment moves and instrument failures, SO_2 records are incomplete for certain periods at certain sites. For example, as few as 97 days of data were available for Lamontville, and 129 days at Briardale.

Spatial variation. Average concentrations across the 16 sites varied widely. SO_2 monitoring results are grouped into three categories:

- Low concentrations (1-3 ppb) at Briardale, Ferndale, Ngazana

- Medium concentrations (6-10 ppb) at Dirkie Uys, Nizam, Lamontville, City Hall, Grosvenor, and Prospecton
- High concentrations (12-20 ppb) at Assegai, Warwick, Jacobs, Settlers, Ganges, and Southern Works.

Southern Works had by far the highest concentrations, averaging 20 ppb with 24-hr peaks reaching 127 ppb, which shows the influence of nearby SO_2 sources (e.g., Mondi, Sapref and Engen). Overall, the spatial distribution reflects the distribution of industry in the South Basin.

Temporal variation. At all sites except Southern Works, concentrations show moderately strong seasonality with the highest levels in winter (March - August, but peaking in June/July) and the lowest levels in summer (December to February). Levels within an area showed low to moderate correlation. The highest intersite correlation was observed at the northern school sites (correlation coefficients of 0.5 – 0.7). In the Southern Basin, correlation coefficients were lower (typically 0.2 to 0.5). Correlations between Southern Works and the other monitoring sites were smaller (-0.1 to 0.5). Correlations were also low for the Settlers School and Prospecton sites with other sites.

Autocorrelation was low to moderate, ranging from 0.2 to 0.7. Day-of-week effects varied by site. Jacobs, Ganges and Southern Works showed the greatest variation, with weekend levels decreasing by 20 to 50% from weekday levels. However, weekend SO_2 concentrations increased by 25 to 35% at Dirkie Uys and Briardale. Insufficient data were available at Lamontville to quantify day-of-week patterns.

Time-of-day patterns often help to confirm the influence of local sources. Mornings have the highest concentrations at Dirkie Uys, Nizam, Wentworth, and Settlers.

- Afternoons have the highest concentrations at Assegai, Lamontville, Briardale, Ferndale, and Ngazana.

In part, this reflects the influence of the sea/shore breeze, e.g., the monitoring sites closest to the coast tend to be flushed with cleaner air in the afternoon, lowering concentrations. However, actual patterns are complex, and likely depend on season, the direction of local sources, the rotation of the wind field, and dispersion characteristics.

The distributions show that apparent statistical outliers occur at most of the monitoring sites, with the exception of Prospecton, which is some distance from large SO_2 sources.

Exposure estimates. While SO_2 was monitored at all schools, this pollutant shows considerable spatial variation. Moreover, large amounts of data were missing at several sites. We believe that the best approach to reflect spatial and temporal variation is to utilise school-based monitoring, after imputing missing data using SO2 concentrations (and other variables) collected across the entire network. With respect to averaging time, we considered using exposure

measures based on hourly peaks and possibly 15-min averages, however, this was not done for several reasons: (1) Symptom data was not always collected at the same time; (2) some of the symptom data represented the previous 24-hr period; (3) our previous experience indicated 1-hr and 24-hr data are highly correlated; (4) typically 24-hr data provide more reliable results in the health models; and (5) imputing very short-term data was both difficult and unreliable.

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- ### *2.2.4 Particulate Matter under 10 µm Diameter (PM_{10})*

Monitoring. PM_{10} was monitored using several types of monitoring systems.

- Five TEOM (tapered element oscillating microbalance, Rupprecht & Patashnick, 1400a) samplers, a US EPA-approved method, were used by the eThekwini Municipality at five stations (Warwick, Ferndale, City Hall, King Edward and Ganges). TEOMs provide continuous measurements.

Several types of gravimetric samplers were deployed. All were programmed to collect 24-hr samples from noon-to-noon.

- Three Thermo Sequential Medium Volume Samplers (FH 95 SEQ Sequential Particulate Samplers) were deployed at three sites (Ferndale, Briardale, Ngazana). This instrument operates at an air flow rate of 2.3 m^3/h and is designed for automatic sequential sampling on 16 filter cassettes held in a filter cassette magazine. The sampler was programmed to collect 24-hr samples from noon-to-noon, on 47 mm Millipore glass fibre filters. Inlets for the collocated TEOM and Thermo samplers at Ferndale were approximately 3 m apart horizontally and 1.2 m vertically (the TEOM being in a caravan and the Thermo mounted on the ground).

- Three Digitel DHA 80 High Volume Samplers were deployed at three sites (Assegai, Dirkie Uys, Lamontville). The sampler is capable of sampling from 100 to 1000 L/min (6 to 60 m^3/hr) and is designed for sequential sampling on 16 ring assemblies holding 150 mm diameter filters (Ederol Glass Fibre – microfilter 227/1/60). A flow rate of 30 m^3/hr was used.

- A R&P Partisol-FRM Model 2000 Air Sampler collected PM_{10} at Nizam. This single channel sampler meets US EPA specifications for both $PM_{2.5}$ and PM_{10} (RFPS-0498-117 and RFPS-1298-126, respectively). The sampler used a PM_{10} inlet, a flow rate of 16.7 L/min (1 m^3/h), and a filter holder/cassette containing a 47 mm dia glass fibre filter. System electronics maintain the volumetric flow rate, record the elapsed sampling time, and calculate the total sample volume.

For the metals analysis (described later), selected PM_{10} and $PM_{2.5}$ samplers were equipped with Teflon filters. For most of the samples, as mentioned above, glass fiber filters were utilised, given their availability for each of the sampler types and due to their lower cost. We did not observe any systematic biases between filter types in the collocation tests (described below).

Pre- and post-sampling weights were determined after conditioning filters for several days in a controlled temperature (25 ^{o}C) and humidity (50% RH) environment established at the Durban Institute of Technology (DIT). We could not consistently achieve a RH below 40%, as suggested by US EPA, however, 50% RH was maintained, and gravimetric measurements were reproducible and consistent, based on repeated measurements of a QA filter and replicates. Each filter was weighed three times using an analytical microbalance readable to 10 µg (Ohaus 11378-050). Daily QA checks included repeated weighings of a QA blank filter and field blank filter. Concentrations were calculated using the average weights and the average of pre- and post-sampling flow rates (where available) multiplied by the elapsed time. Concentration uncertainties were calculated using the standard deviation in the pre- and post-sampling measurements.

Collocation study 1. Prior to initiating the main study, a collocation study was conducted to compare the various performance of PM monitors and to refine protocols. (This study is being written up by Michael Van Der Merwe at the Durban Institute of Technology as part of his master's thesis.) A total of 12 samplers of 5 types were tested at the Wentworth site over a three week period. Due to space and power limitations, 6 to 8 samplers were tested simultaneously at this site. The Partisol 2025 sampler was used as a reference sampler and was operated continuously throughout this period. Concentrations obtained by the reference sample (average = 37.4 ± 11.6 µg m^{-3}) closely matched simultaneous 24-hr PM_{10} TEOM measurements obtained at three nearby sites operated by eThekwini Municipality (City Hall, King Edward, Ganges; average = 37.0 ± 11.5 µg m^{-3}). This suggests that the results of the reference monitor were accurate, assuming that PM_{10} gradients are small (as shown below) and that losses of volatile and semi-volatile PM components on the heated TEOM are small relative to gravimetric measurements. Previous studies have indicated that TEOM losses are generally below 10% for $PM_{2.5}$, and a considerably lower loses are expected for PM_{10}.

The collocation comparisons included 19 days in which 126 PM_{10} measurements were obtained (average of 10.5 measurements per sampler, 18 – 38 measurements per sampler type). Concentrations during this period spanned a large range (19 to 102 µg m^{-3} based on the reference sampler), thus providing an excellent test. Study results indicated that 97% of the samples fell within 20% of the reference sampler. (One sampler type, a Topaz, failed to operate properly and is excluded from this analysis.) Some small biases were identified by sampler type, as indicated by the regression results below:

C(Partisol 2000) = 1.01 C (Partisol 2005) R^2 = 0.91 n = 18

C(DHA 80 HiVol) = 0.919 C(Partisol 2005) R^2 = 0.84 n = 32

C(FG 95 MedVol) = 0.921 C(Partisol 2005) + INTERCEPT R^2 = 0.89 n = 38

Mean and absolute mean biases were 0.6 and 6 μg m^{-3}, respectively. Overall, the agreement, high correlation, and small biases, indicate that PM_{10} measurements obtained by the five types of samplers (Partisol 2000, Partisol 2005, DHA Hivol, FG MedVol, TEOM) were comparable.

Collocation study 2. A second "collocation" study was obtained by maintaining a TEOM monitor at Ferndale throughout the study, in addition to the filter-based method (low volume Thermo sampler) also used at the same site. Both TEOM and filter-based PM_{10} measurements were available for 129 days. Several statistical outliers, e.g., much higher PM_{10} concentrations obtained from the filter-based measurement, were noted on several days (e.g., 26.9.04 and 9.7.05). Comparing all available PM_{10} data (including these outliers), the correlation coefficient was 0.71, 84% of observations were within a factor of 2, the mean bias was 9 $\mu g/m^3$, and the relative bias was 11% (the filter-based method yielded higher concentrations). Overall, this performance does not match the performance obtained in the first collocation study. The poorer performance may have resulted from many possible reasons: (1) This distant site (Ferndale) involved the most sample transport which may have resulted in particle loss, and thus may represent a worst-case situation, though it is recognised that all of the school-based sites involved sample transport. (2) Losses of volatile and semi-volatile components from the TEOM measurements might cause differences from the gravimetric measurements, as mentioned above. Differences might be larger at this site since ambient temperatures are slightly cooler at the higher elevation of Ferndale (thus increasing the temperature difference between the TEOM and ambient air). Still, this seems unlikely to cause large differences. (3) The presence of strong localised sources that produce a range of particle sizes that are sampled differentially by the TEOM and the Thermo Low Volume samplers, a result of different sampling efficiencies or "cut-points." However, mean concentrations did not vary much between the TEOM and gravimetric measurements, suggesting this was not a major factor. (4) Experimental error, including sampler, operator and laboratory errors. We cannot definitively identify the error source, and all of these (and perhaps other) errors might be responsible. As discussed in the next section, we identified 5 outliers at this site, the removal of which improve results significantly, e.g., the correlation between TEOM and filter-based measurements at Ferndale increased to 0.79 (from 0.70).

Data processing/quality/status. Data capture rates varied across the monitoring sites and by monitoring type. The TEOMs had capture rates exceeding 80%. Filter-based samples at the schools were collected daily during intensives, and less frequently, on an every 6^{th} day schedule. The number of 24-hr filter samples at the sites ranged from 131 at Briardale to 168 at Nizam. The filter-based methods did not achieve the precisions of the TEOM measurements, a result of many factors, e.g., sampler errors, particle/fibre shedding during filter handling and transport, and errors in pre- and post-exposure weighing (that in turn are partly attributable to fluctuations in the weighing room temperature and humidity). Because the TEOM monitoring was continuous and filter-based methods focused

on the intensive periods, these monitoring types are analysed separately. TEOM measurements are not adjusted for possible losses of volatile and semi-volatile compounds. We also noted that while the TEOM PM_{10} levels were correlated across the region (though at times certain areas were elevated due to local influences as discussed below), a small number of the filter-based PM_{10} measurements were low, particularly at Ferndale where a few observations fell below the collocated TEOM PM_{10} measurements. The reasons for such discrepancies are unknown (these samples passed the normal QA checks) as discussed above. To identify such low values, we identified the 2nd lowest daily TEOM PM_{10} measurement in the network as a background concentration, which was decreased by 25% to account for possible experimental errors. Then, filter-based TEOM measurements falling below this value were considered to be erroneous and thus deleted. Over 21 months of daily data, this procedure removed only 16 observations (2 to 5 observations at each site). We also considered high-end outliers by visually examining the trend plots using both TEOM and filter data, and looked for filter-based measurements that were a factor of 2 or more above TEOM measurements. Five such observations were located, from 133 to 267 μg m^{-3}. These outliers quite clearly were erroneous and their removal greatly enhanced the performance of the imputation procedures, which provides additional support for the validity of this procedure.

Spatial variation. Based on averages, TEOM-based PM_{10} concentrations were nearly identical, 38 – 39 μm m^{-3}, at four sites, and slightly elevated, 46 μg m^{-3}, at Ganges. Concentrations at other percentiles were also closely matched. For the filter-based measurements, average concentrations at the 7 school sites were in the same range, 41 – 58 μg m^{-3}. Thus, only modest spatial differences are observed for average PM_{10} concentrations. Maximum 24-hr average concentrations approached or exceeded 150 μg m^{-3} at most sites. The highest concentrations were observed at Assegai (south) and Ngazana (north), two widely separated monitors. Assegai may be affected by a combination of industrial, vehicular and open burning emissions; Ngazana may be largely affected by open burning emissions.

Temporal variation. All sites showed very strong seasonality with the highest levels in winter (March – August), typically peaking in July (when 24-hr concentrations exceeded 150 μg m^{-3} at 11 sites), and the lowest levels in summer (December – April).

The five TEOM-based measurements tracked extremely closely ($r > 0.92$). This also applied to the TEOM PM_{10} concentrations at the northern site (Ferndale) when compared to levels at the four DSIB TEOM monitors, though Ferndale concentrations were slightly lower. The TEOM measurements were moderately to highly correlated with the filter-based measurements, with correlation coefficients from 0.53 to 0.82. Thus, PM_{10} levels were moderately-to-highly correlated across all sites.

PM_{10} levels showed moderate to high autocorrelation, about 0.6 to 0.9 for 1 day lags. No day-of-week effects were indicated. As mentioned, distributions most

sites include several 24-hour values that might be considered modest statistical outliers.

Exposure estimates. This pollutant shows significant temporal variation, but relatively little spatial variation. There are several approaches:

a) Filter-based measurements are available at the 7 schools and could be used.

b) A Durban-wide PM_{10} measurement based on TEOMs could be derived, e.g., as a 5-site average.

c) A DSIB-wide average based on TEOMS could be derived, e.g., as a 4-site average, and the TEOM measurements at Ferndale would be used for the three northern schools.

d) Combined approach, e.g., using filter based measurements at the 7 school sites but imputed and bounded using TEOM data.

The TEOM-based approaches would provide similar results, given the high correlation, but would offer the advantage that TEOMs provide more reliable and continuous coverage. On the other hand, the filter-based methods may reflect local spatial gradients at the schools. Also, the filter-based methods collected samples from noon-to-noon. The TEOM data are hourly, and thus can be manipulated to obtain 24-hr average values for various start/stop times. We selected approach d, which relied upon school-based filter-measurements with imputation and validation checks that used, among other, information provided by the TEOM measurements.

Results for Air Pollutant Monitoring

Compliance with guidelines and standards
Below are the monitoring results and exposure assessment approach for pollutants monitored in this study. The WHO guidelines are used with several "lower" target levels. Analysis included measurements collected at permanent sites in the eThekwini monitoring network and monitoring conducted in the SDHS conducted in schools and elsewhere.

- Average SO2 values in 2005 achieved the limit value of 19 ppb at all sites. The Southern Works site was the network's hotspot with a value of 22 ppb in 2004 and 16 ppb in 2005. The same site exceeded the daily limit value (48 ppb) 34 times in 2004 and 1 times in 2005, and the 10 min value (191 ppb) 796 times in 2004 and 240 times n 2005. The 2005 reductions are attributed to the installations of SO2 scrubbers at Mondi in May 2005. There were additional exceedances of the 10-min and 24-hr limit values at Settlers, Ganges (not in 2005), Grosvenor, Wentworth and Jacobs, but not at outlying sites (Prospection and Ferndale). In the school based monitoring, no sites exceeded the annual limit value, and two exceedances of the 24 hr limit value were noted at Assegai and Lamontville, 1 each).

- Average PM10 concentrations at at Ganges (46 and 43 ug/m3 in 2004 and 2005, respectively) exceeded the annual limit (40 ug/m3) in 2005. The annual PM10 target value (30ug/m3) was exceeded at all 5 PM10 monitoring sites. The 24-hr limit values (75 ug/m3) was exceeded 18-36 times at each of the five sites monitoring PM10. The number of exceedances of the 24-hr PM10 target value (50ug/m3) was not specified, however it was calculated that all sites had multiple exceedances ranging from 34 (King Edward) to 64 (Ganges) times for about 570 study days at each site. In the school based monitoring, all 7 sites also exceeded the annual limit value, and all sites also exceeded the 24-hr limit multiple times, from 2 (Nizam) to 36 (Assegai) times over the studt period (based on 130 daily samples at each site).
- Average NO2 at Warwick and Ganges (21 ppb) exceeded or attained the annual limit value (21 ppb) in 2005. The 1-hr limit (106 ppb) was exceeded from 0-5 times at the 8 sites measuring this pollutant in 2005, down from 0-9 times per site in 2004.

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19. APPENDIX 3

- ***Child Screening Questionnaire***

South/North Durban Health Study

S1.

Date:____/____/____

Day Month

Year

S2. Study Identification No. | | | | - | | | | - | | | |

Screening Questionnaire

- ***Screening Questionnaire about***

_____ ***[* full *name of child must be printed here by study staff or teacher before the questionnaire is brought home]***

(This questionnaire is available in isiZulu. Please request this version if this is your preference)

This questionnaire should be completed by the person who most often takes care of the child.

Please put a tick ☑ in the correct box for each question.

- **BACKGROUND INFORMATION**

S3. Child's full name **[should be filled in before child brings this home]**	_____ First _____ Middle _____ Surname

S4. Child's current grade in school	____
S5. Child's date of birth	_____/_____/_____ day month year
S6. Is the child:	□1 Male □2 Female
S7. Usual language spoken at home:	□1 English □2 Zulu □3 Xhosa □9 Other (Specify:_____)
S8. Your name:	_____ _____ First Middle _____ Surname
S9. Your telephone numbers:	home: _____ work: _____ cell: _____ or □1 I do not have a telephone

We may need to contact you again to obtain additional information. Please give me the name, address and telephone number of two relatives or friends who would know where you could be reached in case we have difficulty in contacting you.

S10. Name of first contact person:

_____

S11. Telephone number of first contact person: _____

S12. Address of first contact person:

_____

_____

House No. Road/Street

_____

_____

Suburb/Township

Postal Code

S13. Relationship of contact person to you:

_____

S14. Name of second contact person:

_____

S15. Telephone number of second contact person: _____

S16. Address of second contact person:

_____

House No. Road/Street

_____ _____

Suburb/Township **Postal Code**

S17. Relationship of contact person to you:

_____

S18. What is the complete address of the household where the child sleeps most often ?	_____ House No. _____ _____ Road/Street _____ _____ Suburb/Township _____ Postal Code
S19. How does the child usually get to school?	□1 walks □2 driven in a private vehicle □3 driven in a taxi □4 takes a bus □9 Other (Specify:_____)
S20. Are you the main person who takes	□1 Yes □2 No

care of this child?	
S21. How are you related to **this child**?	☐1 Mother ☐2 Father ☐3 Grandmother ☐4 Grandfather ☐5 Aunt ☐6 Uncle ☐9 Other (**specify:** _____)

A. CHILD'S SYMPTOMS INFORMATION

S22. In the past 12 months, how often has your child had a **cough that won't go away**?	☐1 every day ☐2 more than 2 times per week ☐3 more than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year☐ ☐6 never
S23. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) **with a cold**?	☐1 more than 1 time per month ☐2 3 to 12 times in the whole year ☐3 1 or 2 times in the whole year ☐4 never
S24. In the past 12 months, how often has your child had **wheezing** (a whistling sound from the chest) ***without* a cold**?	☐1 every day ☐2 more than 2 times per week ☐3 more than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 never
S25. In the past 12 months, how often has your child had an attack of **wheezing** that made it **hard to breathe or catch his or her breath**?	☐1 every day ☐2 more than 2 times per week ☐3 more than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 never
S26. In the past 12 months, how often has your child **wheezed while exercising, running or playing?**	☐1 every day ☐2 more than 2 times per week ☐3 more than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 never
S27. In the past 12 months, how often has your child **coughed while exercising, running or playing**?	☐1 every day ☐2 more than 2 times per week ☐3 more than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 never
S28. In the past 12 months, how often has your child complained that his or her **chest**	☐1 every day ☐2 more than 2 times per week

felt tight or heavy?	□3 more than 1 time per month □4 3 to 12 times in the whole year □5 1 or 2 times in the whole year □6 never
S29. In the past 12 months, how often has your child's **sleep been disturbed due to wheezing, coughing, chest tightness or shortness of breath**?	□1 most nights □2 more than 1 time per week □3 more than 2 times per month □4 more than 1 time per month □5 3 to 12 times in the whole year □6 1 or 2 times in the whole year □7 never
S30. Has a doctor or nurse **EVER** said that your child had: (check ALL that apply)	□1 Asthma □2 Bronchitis or Bronchiolitis □3 Reactive Airway Disease (RAD) □4 Pneumonia □5 Asthmatic Bronchitis
S31. In the past 12 months has your child **taken any medications, nebulisers, or inhalers (pumps) prescribed by a doctor** for any of the conditions listed above?	□1 Yes □2 No
S32. Does your child take **any of these doctor-prescribed medications every day**, even when he/she is **not** having trouble breathing?	□1 Yes □2 No □8 Does not apply
S33. In the past 12 months how many times has your child **had to make an unplanned visit to a doctor's office for breathing problems?**	□1 0 times □2 1 time □3 2 times □4 3 or 4 times □5 5 or 6 times □6 7 times or more
S34. In the past 12 months how many times has your child **been to the emergency room** (but not stayed overnight in the hospital) **for breathing problems?**	□1 0 times □2 1 time □3 2 times □4 3 or 4 times □5 5 or 6 times □6 7 times or more
S35. In the past 12 months how many times has your child **had to stay in the hospital for one night or more because of breathing problems?**	□1 0 times □2 1 time □3 2 times □4 3 or 4 times □5 5 or 6 times

	□6 7 times or more

B. MEMBERS OF YOUR HOUSEHOLD

S36. How many people live in your household? (Include the child, yourself, and all other adults and children whether related to you or not)	_____ (write in number)
S37. Write in the name of the person who you consider to be the head of the household -- this will usually be the person who owns or rents this home	**Head of Household** _____ _____ _____ **first name middle name surname** **OR**□□□□1 I am the Head of Household
S38. Does this person (or you, if you are the head of the household) have a husband or wife who also lives in this household?	□1 Yes □2 No
S39. If "yes", write in the name of this person	**Husband or Wife of Head of Household** _____ _____ _____ **first name middle name surname** **OR**□□□□1 I am the husband or wife of the Head of Household
S40. Write in the name of the person who most often takes care of the child.	**Person who most often takes care of the child** _____ _____ _____ **first name middle name surname** **OR**□□□□1 I am the person who most often takes care of the child

TABLE 1: TABLE OF PERSONS LIVING IN YOUR HOUSEHOLD – LIST <u>EVERYONE</u> WHO USUALLY SLEEPS AT YOUR HOUSEHOLD – BOTH ADULTS AND CHILDREN. INCLUDE PERSONS WHO ARE NOT RELATED TO THE HEAD OF THE HOUSEHOLD, AS WELL AS FAMILY MEMBERS. START WITH THE THE CHILD, FOLLOWED BY YOURSELF.

[PLEASE PRINT CLEARLY!]

S41. FIRST NAME	**S42.** MIDDLE NAME	**S43.** SURNAME	**S44.** RELATIONSHIP TO HEAD OF HOUSEHOLD	**S45.** AGE OF PERSON IN YEARS	**S46.** SEX OF PERSON	
a.	**a.**	**a.**	**a.**	**a.**	□1 Male	□2 Female
b.	**b.**	**b.**	**b.**	**b.**	□1 Male	□2 Female
c.	**c.**	**c.**	**c.**	**c.**	□1 Male	□2 Female
d.	**d.**	**d.**	**d.**	**d.**	□1 Male	□2 Female
e.	**e.**	**e.**	**e.**	**e.**	□1 Male	□2 Female
f.	**f.**	**f.**	**f.**	**f.**	□1 Male	□2 Female
g.	**g.**	**g.**	**g.**	**g.**	□1 Male	□2 Female
h.	**h.**	**h.**	**h.**	**h.**	□1 Male	□2 Female
i.	**i.**	**i.**	**i.**	**i.**	□1 Male	□2 Female
j.	**j.**	**j.**	**j.**	**j.**	□1 Male	□2 Female
k.	**k.**	**k.**	**k.**	**k.**	□1 Male	□2 Female
l.	**l.**	**l.**	**l.**	**l.**	□1 Male	□2 Female
m.	**m.**	**m.**	**m.**	**m.**	□1 Male	□2 Female
n.	**n.**	**n.**	**n.**	**n.**	□1 Male	□2 Female

S47. Do you have any comments about the project or the questionnaire?

_____
_____
_____
_____
_____
_____

THANK YOU FOR COMPLETING THIS QUESTIONNAIRE

Please have your child return it by _____, _____ to his or her teacher.

20. APPENDIX 4

Child caregiver baseline survey

South/North Durban Health Study

G1.

Date:____/____/____

Day Month

Year

G2. Study Identification No. | | | | - | | | | - | | | |

Child Caregiver Baseline Survey

Cover Sheet

G3. Name of respondent:	_____ First _____ Middle _____ Surname
G4. Relationship to Head of Household:	_____
G5. Phone numbers:	**home:** _____ **work:** _____ **cell:** _____
G6. Name of child participating in the study:	_____ First _____

	Middle _____ Surname
G7. Child's birth date:	_____/_____/_____ Day Month Year
G8. Child's sex:	☐$_1$ Male ☐$_2$ Female
G9. Child's school:	_____
G10. What is the complete address of this household?	_____ House No. _____ Road/Street _____ City _____ Postal Code
G11. Interviewer's Name:	_____
G12. Interview time started:	Time: _ _:_ _ am/pm
G13. [INTERVIEWER: Enter gender of respondent]	☐$_1$ Male ☐$_2$ Female
G14. Who is the person most responsible for care of **[child]** or most familiar with any health problems (s)he has?	_____
[If answer is not "me" then assess informally whether the person knows enough to complete the questionnaire.]	
G15. How are you related to **[child]**?	☐$_1$ Mother ☐$_2$ Father ☐$_3$ Grandmother ☐$_4$ Grandfather ☐$_5$ Aunt ☐$_6$ Uncle ☐$_7$ Other (**SPECIFY:** _____)

[INTRODUCTION: INTERVIEWER READS TO RESPONDENT]

The purpose of this questionnaire is to collect information about your child's health status.

Your answers will help us figure out how to assist you and your child in protecting your child's health. If there is a question you do not want to answer, please let me know and we can skip it. All of your responses are confidential and will not shown to anyone outside the study team without your written consent.

A. BIRTH HISTORY

G16. In what country was **[CHILD]** born?	_____ country name
G17. What is the highest grade or year of regular school has **[CHILD]** completed?	Grade_____
G18. How old was the biological mother of **[CHILD]** when **[CHILD]** was born?	_____ years age
G19. Did **[CHILD]'s** biological mother smoke at anytime while she was pregnant with **[CHILD]?**	□1 Yes □2 No □8 Don't know
G20. At any time during the pregnancy did **[CHILD]'s** biological mother quit or refrain from smoking for the rest of the pregnancy?	□1 Yes □2 No □8 Don't know
G21. Did **[CHILD]** receive any newborn care in an intensive care unit, premature nursery or any other type of special care facility?	□1 Yes **[if yes: How many days: _____ days]** □2 No
G22. How much did **[CHILD]** weigh at birth?	_____grams number □8 Don't know
G23. Did **[CHILD]** weigh more than 2.5 kg or less?	□1 more than 2.5 kg □2 2.5 kg or less □8 Don't know
G24. Did **[CHILD]** weigh more than 4.0 kg or less?	□1 more than 4.0 kg □2 4.0 kg or less □8 don't know

B. DIET

G25. How often does **[CHILD]** eat breakfast: everyday, on some days, rarely, never, or on weekends only?	□1 everyday □2 some days □3 rarely □4 never □5 weekends only
G26. During the past 12 months, has **[CHILD]** changed eating habits to try to lose weight?	□1 Yes □2 No
G27. During the past 12 months, has **[CHILD]** changed what you eat for any medical reason or health condition?	□1 Yes □2 No
G28. What was the medical reason or health condition that caused **[CHILD]** to change what he/she eats? **[Mark all that apply – USE HAND CARD CCG-1]**	☐1 High Blood Pressure/Hypertension ☐2 High Blood Cholesterol ☐3 Allergy ☐4 Diabetes ☐5 Formula-related reason ☐6 Due to infections ☐7 Gastro-intestinal Problems ☐8 Overweight/Obesity ☐9 Nutrition/Anemia ☐10 Other, specify: _____
G29. Do you consider **[CHILD]** to be overweight, underweight, or about the right weight?	□1 overweight □2 underweight □3 about the right weight

C. HEALTH SERVICES AND HEALTH IMPAIRMENT

G30. Would you say that **[CHILD]** s health in general is excellent, very good, good, fair, or poor?	□1 excellent □2 very good □3 good □4 fair □5 poor
G31. Is there a particular clinic, health center, doctor's office, or other place that you usually go to if **[CHILD]** is sick, or needs advice about health or for routine care?	□1 Yes □2 No
G32. Is there one particular doctor or health professional that **[CHILD]** usually	□1 Yes □2 No

sees?	
G33. About how long has it been since you or anyone last saw or talked to a medical doctor or other health professional about **[CHILD]** ? Include doctors seen while a patient in a hospital.	□1 less than 1 year □2 more than 1 year, but less than 2 years □3 more than 2 years, but less than 5 years □4 5 years or more □5 never □8 don't know
G34. Since **[CHILD]** was born, how many different times has **[CHILD]** stayed in the hospital overnight or longer? **Do not include the hospitalization when [child] was born**.	□0 none _____ times number
G35. Is **[CHILD]** able to take part at all in any of the usual kinds of activities done by most children of his/her age?	□1 Yes □2 No
G36. Is **[CHILD]** limited in the kind or amount of activities can do because of an impairment or health problem?	□1 Yes □2 No
G37. Does any impairment or health problem now keep **[CHILD]** from attending school?	□1 Yes □2 No
G38. Does **[CHILD]** need to attend a special school or special classes because of any impairment or health problem?	□1 Yes □2 No
G39. How long ago was the impairment or health problem first noticed?	_____ number □1 months □2 years □8 don't know
G40. Did a doctor ever say that **[CHILD]** had rheumatic fever/rheumatic heart disease?	□1 Yes □2 No **[GO TO G44]**
If yes:	
G41. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years

G42. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 Don't know
G43. Has **[CHILD]** ever been treated by a Doctor for this illness?	□1 Yes □2 No □8 Don't know
G44. Did a doctor ever say that **[CHILD]** had epilepsy/fit/convulsion?	□1 Yes □2 No **[GO TO G48]**
If yes:	
G45. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G46. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 Don't know
G47. Has **[CHILD]** ever been treated by a Doctor for this illness?	□1 Yes □2 No □8 Don't know
G48. Did a doctor ever say that **[CHILD]** had Cerebral palsy?	□1 Yes □2 No **[GO TO G52]**
If yes:	
G49. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G50. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 Don't know
G51. Has **[CHILD]** ever been treated by a Doctor for this illness?	□1 Yes □2 No □8 Don't know
G52. Did a doctor ever say that **[CHILD]** had mental retardation?	□1 Yes □2 No **[GO TO G56]**
If yes:	
G53. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G54. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G55. Has **[CHILD]** ever been treated	□1 Yes

by a Doctor for this illness?	□2 No □8 don't know
G56. Did a doctor ever say that **[CHILD]** had muscle weakness or paralysis of the arms?	□1 Yes □2 No **[GO TO G60]**
If yes:	
G57. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G58. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G59. Has **[CHILD]** ever been treated by a Doctor for this illness?	□1 Yes □2 No □8 don't know
G60. Did a doctor ever say that **[CHILD]** had muscle weakness or paralysis of the legs?	□1 Yes □2 No **[GO TO G64]**
If yes:	
G61. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G62. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G63. Has **[CHILD]** ever been treated by a Doctor for this illness?	□1 Yes □2 No □8 don't know
G64. Did a doctor ever say that **[CHILD]** had asthma?	□1 Yes □2 No **[GO TO G68]**
If yes:	
G65. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G66. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G67. Has **[CHILD]** ever been treated by a	□1 Yes □2 No

Doctor for this illness?	□8 don't know
G68. Did a doctor ever say that **[CHILD]** had chronic bronchitis?	□1 Yes □2 No **[GO TO G72]**
If yes:	
G69. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G70. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G71. Has **[CHILD]** ever been treated by a doctor for this illness?	□1 Yes □2 No □8 don't know
G72. Did a doctor ever say that **[CHILD]** had hay fever?	□1 Yes □2 No **[GO TO G76]**
If yes:	
G73. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G74. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G75. Has **[CHILD]** ever been treated by a doctor for this illness?	□1 Yes □2 No □8 don't know
G76. Did a doctor ever say that **[CHILD]** had hypertension or high blood pressure?	□1 Yes □2 No **[GO TO G80]**
If yes:	
G77. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G78. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G79. Has **[CHILD]** ever been treated	

by a doctor for this illness?	□1 Yes □2 No □8 don't know
G80. Did a doctor ever say that **[CHILD]** had high blood cholesterol?	□1 Yes □2 No **[GO TO G84]**
If yes:	
G81. How old was **[CHILD]** when he/she first had this illness?	_____ number □1 months □2 years
G82. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G83. Has **[CHILD]** ever been treated by a doctor for this illness?	□1 Yes □2 No □8 don't know
G84. Has **[CHILD]** ever seen a psychiatrist, psychologist, or psychoanalyst about any emotional, mental, or behavioral problems?	□1 Yes □2 No
G85. During the past 12 months, has **[CHILD]** taken any prescribed medicines or drugs to help control activity or behavior?	□1 Yes □2 No
G86. During the past 12 months, how often did **[CHILD]** complain of headaches? Would you say never, rarely, sometimes, frequently, or always?	□1 never □2 rarely □3 sometimes □4 frequently □5 always
G87. During the past 12 months, how often did **[CHILD]** complain of stomach aches? Would you say never, rarely, sometimes, frequently, or always? [**Do not include menstrual cramps]**	□1 never □2 rarely □3 sometimes □4 frequently □5 always
G88. Does **[CHILD]** have any speech defect, such as stuttering, stammering, or lisping?	□1 Yes □2 No
G89. Has **[CHILD]** ever had anemia, sometimes called "tired blood" or "low blood?"	□1 Yes □2 No □8 don't know

G90. Now I will ask about some immunizations that may have received. It may be easier to recall this information if you have a record of **[CHILD]**'s shots. Do you have a vaccinationrecord for **[CHILD]** that I can see?	□1 Vaccination record available □2 Vaccination record NOT available
G91. Has **[CHILD]** ever received a DPT or tetanus shot? A DPT shot is to prevent diphtheria, tetanus, and pertussis or whooping cough. **[Verify with vaccination record if available]**	□1 Yes □2 No **[GO TO G93]** □8 don't know **[GO TO G93]**

G92. How long ago was **[CHILD]** 's last DPT or tetanus shot?	_____ number □1 months □2 years
G93. During the past 12 months, how many times did **[CHILD]** have an accident, injury or poisoning, excluding lead poisoning, that required medical attention?	□0 None _____ number times □8 don't know

D. RESPIRATORY CONDITIONS AND ALLERGY

Cough	
G94. Does **[CHILD]** usually cough on most days for 3 consecutive months or more during the year?	□1 Yes □2 No
G95. Does **[CHILD]** usually cough first thing in the morning in the winter?	□1 Yes □2 No
if no to the above, SKIP to PHLEGM:	
G96. Does **[CHILD]** usually cough at all during the rest of the day?	□1 Yes □2 No
Ignore an occasional cough	
G97. For how many years has **[CHILD]** had this cough?	_____ Number years
Phlegm	
Count phlegm on first going outdoors. Exclude phlegm from the nose. Count swallowed phlegm.	
G98. Does **[CHILD]** usually bring up any phlegm/sputum/mucus from your chest first thing in the morning in the winter?	□1 Yes □2 No
G99. Does **[CHILD]** usually bring up any phlegm/sputum/mucus from his/her chest during the day in the winter?	□1 Yes □2 No
if no to above, SKIP to EPISODES OF COUGH and PHLEGM	
G100. Does **[CHILD]** bring up phlegm like this on most days for as much as	□1 Yes □2 No

three months each year?	
G101. Does **[CHILD]** usually bring up phlegm at all on getting up or first thing in the morning?	□1 Yes □2 No
G102. For how many years has **[CHILD]** had trouble with phlegm?	_____ years
G103. Has **[CHILD]** ever coughed up blood?	□1 Yes □2 No
G104. Was this in the past year?□□	□1 Yes □2 No
Episodes Of Cough And Phlegm	
G105. Has **[CHILD]** had periods or episodes of (increased) cough and phelgm lasting for 3 weeks or more each year?□	□1 Yes □2 No
Breathlessness	
G106. Is **[CHILD]** troubled by shortness of breath when hurrying on level ground?	□1 Yes □2 No
G107. Does **[CHILD]** get short of breath walking with other children of his/her own age on level ground?	□1 Yes □2 No
G108. Does **[CHILD]** have to stop for breath when walking at his/her own pace on level ground?	□1 Yes □2 No
G109. Is **[CHILD]** too breathless to leave the house or breathless on dressing or undressing?	□1 Yes □2 No
Wheezing	
G110. Does **[CHILD]** chest ever sound wheezy or whistling?	□1 Yes □2 No **[GO TO G125]**
[If no, GO to WEATHER] if yes to above, is it:	
G111. When **[CHILD]** has a cold?	□1 Yes

	□2 No
G112. Occasionally apart from colds?	□1 Yes □2 No
G113. Most days or nights?	□1 Yes □2 No
G114. For how many years has this been Present?	_____ years
G115. How many episodes of wheezing or Whistling has **[CHILD]** had in the past 12 Months?	_____ number
G116. How many times in the past 12 Months was **[CHILD]** hospitalised overnightfor these episodes of wheezing or whistling?	_____ number
G117. Can you estimate the total cost of all these hospitalizations for the past year? **[HELP RESPONDENT FIGURE OUT BY SUMMING ACROSS COST OF EACH HOSPITALIZATION]**	**R**_____**,**____ □8 Don't know
G118. How many times in the past 12 Months has **[CHILD]** gone to a doctor's Surgery or hospital emergency room for one of these episodes of wheezing or whistling?	_____ number
G119. Can you estimate the total cost of all these visits for the past year? **[HELP RESPONDENT FIGURE OUT BY SUMMING ACROSS COST OF EACH HOSPITALIZATION]**	**R**_____**,**____ □8 Don't know
G120. Has **[CHILD]** ever had an ATTACK of wheezing that has made him/her feel short of breath?	□1 Yes □2 No
if yes to above	
G121. How old was **[CHILD]** when	_____ Age in years

he/she had your first such attack?	
G122. Has **[CHILD]** had 2 or more such Episodes?	□1 Yes □2 No
G123. Has **[CHILD]** ever required medicine or treatment for the(se) attack(s)?	□1 Yes □2 No
G124. Is/Was **[CHILD]'s** breathing Absolutely normal between attacks?	□1 Yes □2 No
Weather	
G125. Does the weather affect **[CHILD]'s** chest?	□1 Yes □2 No
Only record "YES" if adverse weather definitely and regularly causes chest symptoms if yes to above	
G126. Does the weather make **[CHILD]** short of breath?	□1 Yes □2 No
G127. What kind of weather?	_____
21. Other Symptoms And Allergies	
During the past 12 months, has **[CHILD]** had any episodes of:	
G128. Stuffy, itchy, running nose?	□1 Yes □2 No
G129. Watery, itchy eyes?	□1 Yes □2 No
G130. During the past 12 months, how many episodes of stuffy, itchy, running nose or watery, itchy eyes has **[CHILD]** had?	□1 none □2 constantly/continuously _____ episodes
Are ANY of the above symptoms (wheezing, whistling, runny nose, watery eyes etc), brought on by:	
G131. Exercise or cold air?	□1 Yes □2 No

G132. Animals?	□1 Yes □2 No
G133. Housedust?	□1 Yes □2 No
G134. pollen?	□1 Yes □2 No
G135. Wool clothing	□1 Yes □2 No
G136. Cigarette smoke	□1 Yes □2 No
G137. Soaps, sprays or detergents	□1 Yes □2 No
G138. Colds or 'flu	□1 Yes □2 No
G139. Air pollution	□1 Yes □2 No
G140. Strong odours/smells	□1 Yes □2 No
G141. Other things	□1 Yes □2 No □9 please specify _____
G142. During which months of the year does pollen make **[CHILD]'s** symptoms worse? **[circle months that apply]**	□1 ALL months J F M A M J J A S O N D
ALLERGY	
G143. Within an hour after eating something, has [CHILD] ever had a severe reaction, such as itching all over, trouble breathing, flushing, or swelling of the hands and feet?	□1 Yes □2 No
G144. Within an hour after receiving allergy shots or allergy tests, has [CHILD] ever had a severe reaction, such as itching all	□1 Yes □2 No □3 Never had allergy shots or tests □8 Don't know

over, trouble breathing, flushing, or swelling of the hands and feet?	
G145. **Has [CHILD] ever given up or had to** **avoid a pet because of allergies?**	□1 Yes □2 No

EAR INFECTION

G146. Did **[CHILD]** ever have an ear infection or an earache?	□1 Yes □2 No □8 don't know
G147. How many times has **[CHILD]** had an ear infection or an earache?	□0 never □1 once □2 twice □3 3 – 5 times □4 6 or more times □8 don't know
G148. How old was **[CHILD]** when had the first ear infection or earache?	□1 less than 1 year old ⇨ ___months age □2 1 year old or older ⇨ _____ years age
G149. Was **[CHILD]** ever treated by a doctor for ear infection(s) or earache(s)?	□1 Yes □2 No □8 don't know
G150. Has **[CHILD]** ever had trouble hearing with one or both ears? Do not include any problems which lasted just a short period of time such as during a cold.	□1 Yes □2 No □8 don't know
G151. Does **[CHILD]** still have trouble hearing with one or both ears?	□1 Yes □2 No □8 don't know
G152. Does **[CHILD]** use a hearing aid?	□1 Yes □2 No □8 don't know
G153. How long ago did **[CHILD]** last have hearing tested?	□1 never □2 6 months or less □3 more than 6 months, but less than 12 months □4 more than 12 months, less than 2 years □5 more than 2 years, less than 5 years □6 more than 5 years □8 don't know

SCHOOL ATTENDANCE

G154. During the past 12 months, about how many whole days was **[CHILD]** absent from school because of illness, playing truant, or for other reasons?	☐$_0$ none _____ days number ☐$_8$ don't know
G155. Has **[CHILD]** ever skipped any grades for any reason?	☐$_1$ Yes ☐$_2$ No
G156. Has **[CHILD]** repeated any grades for any reason?	☐$_1$ Yes ☐$_2$ No
G157. What grade did **[CHILD]** repeat?	1 2 3 4 5 6 7 8
Why did **[CHILD]** repeat the grade(s)? **[USE HAND CARD CCG-2]**	☐$_1$ Academic failure ☐$_2$ Immature/acted too young ☐$_3$ Frequently absent ☐$_4$ Moved into a more difficult school ☐$_5$ Language problem ☐$_6$ Learning/behavior problem ☐$_7$ Hearing/vision problem ☐$_8$ Health problem ☐$_9$ Relocation problem ☐$_{10}$ Language problem ☐$_{11}$ Other, specify: _____
G158. Has **[CHILD]** ever been suspended, excluded or expelled from school?	☐$_1$ Yes ☐$_2$ No
G159. How many times has **[CHILD]** been suspended, excluded or expelled from school?	_____ times number
G160. On the average during the school year, how many hours per week does **[CHILD]** work in a paid or unpaid job?	☐$_1$ none ☐$_2$ 5 or fewer hours ☐$_3$ 6-9 hours ☐$_4$ 10-14 hours ☐$_5$ 15-19 hours ☐$_6$ 20-24 hours ☐$_7$ 25 or more hours

ASTHMA SEVERITY

G161. Has a *doctor or nurse* ever told you that **[child]** has asthma?	☐$_1$ Yes **[GO TO G162]** ☐$_2$ No **[READ PASSAGE BELOW]** ☐$_8$ Don't know **[READ PASSAGE**

	BELOW]
If NO or DON'T KNOW say: From now on, when I say asthma, I will be talking about breathing problems such as episodes of wheezing, coughing, tightness of the chest, heaviness in the chest or shortness of breath that **[child]** may sometimes experiences. I understand that [he or she] may or may not be having any problems like this. Okay? **[GO TO G164]**	
G162. How old was **[child]** when a doctor or nurse told you that he/she had asthma?	____ years old
G163. Does **[CHILD]** still have this illness?	□1 Yes □2 No □8 don't know
G164. In the past 12 months, how often has your Child had a cough that won't go away? Would you say… **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G165. In the past 12 months, how often has your child had wheezing (a whistling sound from the chest) with a cold? **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to 12 times in whole year ☐5 1 or 2 times in the whole year ☐6 Never
G166. In the past 12 months, how often has your child had wheezing (a whistling sound from the chest) *without* a cold? **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G167. In the past 12 months, how often has your child had an attack of wheezing that made it hard for him or her to breathe or catch his or her breath**?** **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G168. In the past 12 months, how often has your child wheezed with exercise or running or playing hard? **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G169. In the past 12 months, how often has your child coughed with exercise or	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month

running or playing hard? **[USE HAND CARD CCG-3]**	☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G170. In the past 12 months, how often has your child complained that his or her chest felt tight or heavy? **[USE HAND CARD CCG-3]**	☐1 Every day ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G171. In the past 12 months, how often has your child's sleep been disturbed due to wheezing, coughing, chest tightness or shortness of breath?	☐1 Most nights ☐2 More than 2 times per week ☐3 More than 1 time per month ☐4 3 to 12 times in the whole year ☐5 1 or 2 times in the whole year ☐6 Never
G172. Are there any particular seasons or months when **[child's]** symptoms are worse?	☐1 YES **[GO TO G173]** ☐2 NO **[GO TO G174]**
G173. During which season (or months) does **[child]** have the most breathing problems? **[CHECK ALL THAT APPLY]**	☐1 Spring (September, October, November) ☐2 Summer (December, January, February) ☐3 Autumn (March, April, May) ☐4 Winter (June, July, August) ☐5 Never has breathing problems
G174. I am going to read a list of things that might bring on wheezing, tightness in the chest, cough, or shortness of breath in some children. I would like to know whether each of these things brings on these symptoms for **[child]**. **[CHECK ALL THE RESPONSES THAT R. MENTIONS, REMEMBER TO REPEAT QUESTION FROM TIME TO TIME]** **[USE HAND CARD CCG-4]**	☐1 Being active (running, playing, swimming, or exercising) ☐2 Sprays or strong smells (such as colognes, perfumes, or cleaning supplies) ☐3 Colds or flu ☐4 Cold air ☐5 Change in weather ☐6 Laughing or crying hard ☐7 Dust ☐8 Pets ☐9 Truck or car exhaust ☐10 Hot summer days ☐11 Pollen, trees, fresh cut grass ☐12 Mold and mildew ☐13 Smoke ☐14 Cockroaches ☐15 Certain foods ☐16 Nothing causes breathing problems ☐99 Other (**SPECIFY:** _____)
Has a doctor *ever* told you that **[child]** has.... **[READ ALL CHOICES]**	
G175. Allergies	☐1 Yes

	☐$_2$ No
G176. Eczema	☐$_1$ Yes ☐$_2$ No
G177. Reactive airway disease	☐$_1$ Yes ☐$_2$ No
G178. Asthmatic bronchitis	☐$_1$ Yes ☐$_2$ No
G179. Any other lung/breathing condition	☐$_1$ Yes (**SPECIFY:** _____) ☐$_2$ No

B. HEALTH SERVICES UTILIZATION

G180. Not including the emergency room, does **[child]** have a *regular* family doctor or health care provider that you usually go to for his/her health care?	☐$_1$ Yes What is Doctor or Clinic's name _____ ☐$_2$ No **[GO TO G182]**
G181. When is the last time you visited this doctor or clinic?	_____ month/year

ASTHMA MEDICATION

Can you bring all the medications in the home that **[child]** is has ever taken for asthma, wheezing, tightness in the chest, shortness of breath, or cough. This includes those medications that a doctor or clinic has prescribed and those that a doctor did not prescribe, for example, over-the-counter drugs or home remedies. [Ask respondent to bring you all containers of medication in that house that the child has used. Fill in names from containers] **[IF CHILD HAS NEVER TAKEN ANY MEDICATION FOR ASTHMA, SKIP TO ASTHMA HEALTH SERVICES UTILIZATION SECTION, Q 18]**

Medication Name	Code [**LEAVE BLANK**]	Is the container present and have you seen it...	Is this a. . .	Was this medicine prescribed by a doctor?	How often did the doctor say to take it or use it?		Can you tell me when [child] last used this medicine?	How much does a one month supply of the medication [if taken as prescribed] cost you?	Does **[child]** use this medication less often than needed or prescribed because of the cost?
G182.	**G183**	**G184.** ☐1 container seen ☐2 container not seen	**G185.** ☐1 pill ☐2 liquid (to swallow) ☐3 inhaler/pump ☐4 added to a breathing machine or nebulizer	**G186.** ☐1 Yes ☐2 No	**G187.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G188.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G189.** **R**_____,_____	**G190.** ☐1 Yes ☐2 No
G1	**G192**	**G193.**					**G197.**	**G198.**	**G199.**

91.	**.**	□1 container seen □2 container not seen	**G194.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G195.** ☐1 Yes ☐2 No	**G196.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**R**_____,_____	□1 Yes □2 No
G200.	**G201.**	**G202.** □1 container seen □2 container not seen	**G203.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G204.** ☐1 Yes ☐2 No	**G205.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G206.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G207.** **R**_____,_____	**G208.** □1 Yes □2 No
G209.	**G210.**	**G211.** □1 container seen □2 container not seen	**G212.** ☐1 pill ☐2 liquid ☐3 inhaler/puff	**G213.** ☐1 Yes ☐2 No	**G214.** PRN (as needed) ☐1	____ times/day or ____	**G215.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month	**G216.** **R**_____,_____	**G217.** □1 Yes □2 No

			er ☐4 breathing machine or nebulizer			puffs/day	☐5 more than 1 month ago		
G218.	**G219.**	**G220.** ☐1 container seen ☐2 container not seen	**G221.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G222.** ☐1 Yes ☐2 No	**G223.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G224.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G225.** **R_____,_____**	**G226.** ☐1 Yes ☐2 No

G227. Are there any other medications that **[child]** has taken in the last month for asthma that aren't here?	☐1 Yes (**GO TO G228)** ☐2 No
Complete chart below	

Medication Name	Code **[LEAVE BLANK]**	Is the container present and have you seen it...	Is this a. . .	Was this medicine prescribed by a doctor?		How often did the doctor say to take it or use it?	Can you tell me when [child] last used this medicine?	How much does a one month supply of the medication [if taken as prescribed] cost you?	Does **[child]** use this medication less often than needed or prescribed because of the cost?
G228.	**G229.**	**G230.** ☐1 container seen ☐2 container not seen	**G231.** ☐1 pill ☐2 liquid (to swallow) ☐3 inhaler/pump ☐4 added to a breathing machine or nebulizer	**G232.** ☐1 Yes ☐2 No	**G233.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G234.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G235.** **R**_____,___	**G236.** ☐1 Yes ☐2 No
G237.	**G238.**	**G239.** ☐1 container seen ☐2 container not seen	**G240.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G241.** ☐1 Yes ☐2 No	**G242.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G243.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G244.** **R**_____,___	
G246.	**G247.**	**G248.** ☐1 container seen ☐2 container not seen	**G249.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G250.** ☐1 Yes ☐2 No	**G251.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G252.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G253.** **R**_____,___	
G255.	**G256.**	**G257.** ☐1 container	**G258.**	**G259.**	**G260.**		**G261.**	**G262.**	**G263.**

		seen ☐2 container not seen	☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	☐1 Yes ☐2 No	PRN (as needed) ☐1	____ times/day or ____ puffs/day	☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**R**_____,___	☐1 Yes ☐2 No
G264.	**G265.**	**G266.** ☐1 container seen ☐2 container not seen	**G267.** ☐1 pill ☐2 liquid ☐3 inhaler/puffer ☐4 breathing machine or nebulizer	**G268.** ☐1 Yes ☐2 No	**G269.** PRN (as needed) ☐1	____ times/day or ____ puffs/day	**G270.** ☐1 today ☐2 yesterday ☐3 last week ☐4 last month ☐5 more than 1 month ago	**G271.** **R**_____,___	**G272.** ☐1 Yes ☐2 No

G273. Are there other medications that a doctor or clinic has prescribed for asthma, wheezing, tightness in the chest, shortness of breath, or cough, that you have not bought because of the cost?	☐1 Yes ☐2 No
G274. If yes, do you have the prescription still **[copy name and dose from prescription].**	a. _____ b. _____ c. _____ d. _____ e. _____
G275. If you don't have the prescription, do you remember the name of the medication(s)?	a. _____ b. _____ c. _____ d. _____ e. _____
G276. How much would a one-month supply **[of each medication**] cost?	a. R_____,_____ b. R_____,_____

	c. R_____,_____ d. R_____,_____ e. R_____,_____ □8 don't know

OTHER MEDICINAL USAGE

The following questions concern **[CHILD]'s** use of medicines, other than those for asthma which we just covered, and certain products the past month.

G277. Has **[CHILD]** taken or used any medicines for which a doctor's or dentist's prescription is needed, in the past month? This includes any products which cannot be obtained without a doctor's or dentist's prescription.	□1 Yes □2 No
IF ANY YES: May I see the containers for all of the prescription medicines **[CHILD]** took in the past month? [**Proceed to table on next page**]	

	MEDICATION 1	MEDICATION 2	MEDICATION 3	MEDICATION 4
Enter the complete name of the medication from the label or probe respondent	**G278.** _____ NAME	**G284.** _____ NAME	**G290.** _____ NAME	**G296.** _____ NAME
Check Item	**G279.** □1 container seen □2 container not seen, information furnished by respondent	**G285.** □1 container seen □2 container not seen, information furnished by respondent	**G291.** □1 container seen □2 container not seen, information furnished by respondent	**G297.** □1 container seen □2 container not seen, information furnished by respondent
What is the health problem **[CHILD]** had for which he/she took this medication?	**G280.** _____ CONDITION	**G286.** _____ CONDITION	**G292.** _____ CONDITION	**G298.** _____ CONDITION
For how long has **[CHILD]** been taking/using this type of product?	**G281.** _____ number □□ □1 days □2 weeks □3 months □4 years □8 don't know	**G287.** _____ number □□ □1 days □2 weeks □3 months □4 years □8 don't know	**G293.** _____ number □□ □1 days □2 weeks □3 months □4 years □8 don't know	**G299.** _____ number □□ □1 days □2 weeks □3 months □4 years □8 don't know
How much does a one month supply of [**NAME**] medication [if taken as prescribed] cost you?	**G282.** **R**_____,____	**G288.** **R**_____,____	**G294.** **R**_____,____	**G300.** **R**_____,____

Do you use [**NAME**] medication less often than needed or prescribed because of the cost?	**G283.** □1 Yes □2 No	**G289.** □1 Yes □2 No	**G295.** □1 Yes □2 No	**G301.** □1 Yes □2 No

	MEDICATION 5	MEDICATION 6	MEDICATION 7	MEDICATION 8
Enter the complete name of the medication from the label or probe respondent	**G302.** _____ NAME	**G308.** _____ NAME	**G314.** _____ NAME	**G320.** _____ NAME
Check Item	**G303.** □1 container seen □2 container not seen, information furnished by respondent	**G309.** □1 container seen □2 container not seen, information furnished by respondent	**G315.** □1 container seen □2 container not seen, information furnished by respondent	**G321.** □1 container seen □2 container not seen, information furnished by respondent
What is the health problem **[CHILD]** had for which he/she took this medication?	**G304.** _____ CONDITION	**G310.** _____ CONDITION	**G316.** _____ CONDITION	**G322.** _____ CONDITION
For how long has **[CHILD]** been taking/using this type of product?	**G305.** _____ number □□ weeks □1 days □2 □3 months □4 years □8 don't know	**G311.** _____ number □□ weeks □1 days □2 □3 months □4 years □8 don't know	**G317.** _____ number □□ weeks □1 days □2 □3 months □4 years □8 don't know	**G323.** _____ number □□ □1 days □2 weeks □3 months □4 years □8 don't know
How much does a one month supply of [**NAME**] medication [if taken as prescribed] cost you?	**G306.** **R**_____,_____	**G312.** **R**_____,_____	**G318.** **R**_____,_____	**G324.** **R**_____,_____

Do you use [**NAME**] medication less often than needed or prescribed because of the cost?	**G307.** □1 Yes □2 No	**G313.** □1 Yes □2 No	**G319.** □1 Yes □2 No	**G325.** □1 Yes □2 No

VITAMIN USAGE

G326. Has **[CHILD]** taken or used any vitamins in the past month? Please include those that are prescribed by a doctor or dentist and those that are not prescribed.	□1 Yes □2 No	
IF ANY YES: May I see the containers for all of the vitamins **[CHILD]** took in the past month? [**Proceed to table on next page**]		
	VITAMIN 1	VITAMIN 2
Enter the complete name of the vitamin from the label or probe respondent	**G327.** _____ NAME	**G333.** _____ NAME
Check Item	**G328.** □1 container seen □2 container not seen, information furnished by respondent □3 product name not on container	**G334.** □1 container seen □2 container not seen, information furnished by respondent □3 product name not on container
[Enter manufacturer's or distributor's name and address (city and province)]	**G329.** _____ NAME _____ CITY _____ PROVINCE	**G335.** _____ NAME _____ CITY _____ PROVINCE
How often did **[CHILD]** take **[product]** in the past month?	**G330.** _____ number of times □1 day □2 week □3 month □99 other specify: _____ □8 don't know	**G336.** _____ number of times □1 day □2 week □3 month □99 other specify: _____ □8 don't know

How much **[product]** did **[CHILD]** take each time she/he took it	**G331.** _____ number □1 capsules/tablets □2 teaspoons □3 tablespoons □4 ounces □5 drops □6 packets/packs □7 ml □9 other specify: _____ □8 variable amounts □88 don't know	**G337.** _____ number □1 capsules/tablets □2 teaspoons □3 tablespoons □4 ounces □5 drops □6 packets/packs □7 ml □9 other specify: _____ □8 variable amounts □88 don't know
For how long has **[child]** been taking this type of product	**G332.** _____ number of times month □1 less than one □2 months □3 years □9 other specify: _____ □8 don't know	**G338.** _____ number of times month □1 less than one □2 months □3 years □9 other specify: _____ □8 don't know
	VITAMIN 3	VITAMIN 4
Enter the complete name of the vitamin from the label or probe respondent	**G339.** _____ NAME	**G345.** _____ NAME
Check Item	**G340.** □1 container seen □2 container not seen, information furnished by respondent □3 product name not on container	**G346.** □1 container seen □2 container not seen, information furnished by respondent □3 product name not on container
[Enter manufacturer's or distributor's name and address (city and province)]	**G341.** _____ NAME _____ CITY _____ PROVINCE	**G347.** _____ NAME _____ CITY _____ PROVINCE

How often did **[CHILD]** take **[product]** in the past month?	**G342.** _____ number of times ☐1 day ☐2 week ☐3 month ☐99 other specify: _____ ☐8 don't know	**G348.** _____ number of times ☐1 day ☐2 week ☐3 month ☐99 other specify: _____ ☐8 don't know
How much **[product]** did **[CHILD]** take each time she/he took it	**G343.** ☐1capsules/tablets ☐2 teaspoons ☐3 tablespoons _____ number ☐4 ounces ☐5 drops ☐6 packets/packs ☐7 ml ☐9 other specify: _____ ☐8 variable amounts ☐88 don't know	**G349.** ☐1capsules/tablets ☐2 teaspoons ☐3 tablespoons _____ number ☐4 ounces ☐5 drops ☐6 packets/packs ☐7 ml ☐9 other specify: _____ ☐8 variable amounts ☐88 don't know
For how long has **[child]** been taking this type of product	**G344.** ☐1 less than one month _____ number of times ☐2 months ☐3 years ☐9 other specify: _____ ☐8 don't know	**G350.** ☐1 less than one month _____ number of times ☐2 months ☐3 years ☐9 other specify: _____ ☐8 don't know
	VITAMIN 5	VITAMIN 6
Enter the complete name of the vitamin from the label or probe respondent	**G351.** _____ NAME	**G357.** _____ NAME
Check Item	**G352.** ☐1 container seen ☐2 container not seen, information furnished by respondent ☐3 product name not on container	**G358.** ☐1 container seen ☐2 container not seen, information furnished by respondent ☐3 product name not on container

[Enter manufacturer's or distributor's name and address (city and province)]	**G353.** _____ NAME _____ CITY _____ PROVINCE	**G359.** _____ NAME _____ CITY _____ PROVINCE
How often did **[CHILD]** take **[product]** in the past month?	**G354.** _____ number of times ☐1 day ☐2 week ☐3 month ☐9 other specify: _____ ☐8 don't know	**G360.** _____ number of times ☐1 day ☐2 week ☐3 month ☐9 other specify: _____ ☐8 don't know
How much **[product]** did **[CHILD]** take each time she/he took it	**G355.** ☐1 capsules/tablets ☐2 teaspoons ☐3 tablespoons ☐4 ounces _____ number ☐5 drops ☐6 packets/packs ☐7 ml ☐9 other specify: _____ ☐8 variable amounts ☐8 don't know	**G361.** ☐1 capsules/tablets ☐2 teaspoons ☐3 tablespoons ☐4 ounces _____ number ☐5 drops ☐6 packets/packs ☐7 ml ☐9 other specify: _____ ☐8 variable amounts ☐8 don't know
For how long has **[child]** been taking this type of product	**G356.** ☐1 less than one month _____ number of times ☐2 months ☐3 years ☐9 other specify: _____ ☐8 don't know	**G362.** ☐1 less than one month _____ number of times ☐2 months ☐3 years ☐9 other specify: _____ ☐8 don't know

D. CAREGIVER'S QUALITY OF LIFE

Now, I am going to ask you some similar questions about how your child's asthma has affected you and also some questions about your health.

G363. In the past 3 months, how often did you wake up or lose sleep because of **[child's]** asthma? would you say you woke up or lost sleep……	☐$_1$ most nights ☐$_2$ more than 2 times per week ☐$_3$ more than 2 times per month ☐$_4$ 1 or 2 times per month ☐$_5$ no nights
G364. Is there a particular season or month when you wake up or lose sleep most because of **[child's]** asthma?	☐$_1$ YES ☐$_2$ NO **[SKIP TO G366]**
G365. During what season or month do you wake up or lose sleep most because of **[child's]** asthma? **[CHECK ONE]**	☐$_1$ Spring (September, October, November) ☐$_2$ Summer (December, January, February) ☐$_3$ Autumn (March, April, May) ☐$_4$ Winter (June, July, August)
G366. During the last **[USE ANSWER FROM G365]**, how often did you wake up or lose sleep because of **[child's]** asthma?	☐$_1$ Most nights ☐$_2$ More than 2 times per week ☐$_3$ More than 2 times per month ☐$_4$ 1 or 2 times per month ☐$_5$ No nights
G367. In the past 3 months, how often did you have to change your daytime or evening plans because of [**child's**] asthma? Would you say it was…..	☐$_1$ Most days/evenings ☐$_2$ More than 2 times per week ☐$_3$ More than 2 times per month ☐$_4$ 1 or 2 times per month ☐$_5$ No days/evenings
G368. Is there a particular season or month when you have to change your daytime or evening plans most because of **[child's]** asthma?	☐$_1$ YES ☐$_2$ NO **[SKIP TO G371]**
G369. During what season or month do you have to change your daytime or evening	☐$_1$ Spring (September, October, November) ☐$_2$ Summer (December, January, February)

plans most because of **[child's]** asthma? **[CHECK ONE]**	☐3 Autumn (March, April, May) ☐4 Winter (June, July, August)
G370. During the last **[USE ANSWER FROM G369]**, how many days or nights per week do you have to change your daytime or evening plans because of **[child's]** asthma? Would you say it was…	☐1 Most days/evenings ☐2 More than 2 times per week ☐3 More than 2 times per month ☐4 1 or 2 times per month ☐5 No days/evenings

E. HOUSEHOLD ENVIRONMENTAL CHECKLIST

G371. Do you rent or own your home or neither?	☐$_1$ rent ☐$_2$ own **[skip to G373]** ☐$_3$ neither **[skip G373]**
G372. In general, how easy or difficult would you say that it is to get your landlord to make repairs when they are needed? Would you say . . . **[READ CHOICES]**	☐$_1$ Very easy ☐$_2$ Somewhat easy ☐$_3$ Neither easy or difficult ☐$_4$ Somewhat difficult ☐$_5$ Very difficult
At any time during the year is there standing water or puddles located in. . . **[READ EACH CHOICE]**	
G373. [Child's] sleeping room?	☐$_1$ Yes ☐$_2$ No
G374. The sitting room?	☐$_1$ Yes☐$_2$ No ☐$_3$ No sitting room
G375. The kitchen?	☐$_1$ Yes☐$_2$ No
G376. Another place I did not specify?	☐$_1$ Yes (**specify**_____) ☐$_2$ No
In the past year have you had any other problem with water damage or leaking water in your home, such as from a leaking roof or leaky plumbing	
G377. [Child's] sleeping room?	☐$_1$ Yes ☐$_2$ No
G378. The sitting room?	☐$_1$ Yes☐$_2$ No ☐$_3$ No sitting room
G379. The kitchen?	☐$_1$ Yes☐$_2$ No
G380. Another place I did not specify?	☐$_1$ Yes (**specify**_____) ☐$_2$ No
G381. How many pets of each type come inside the home?	Dog? _____ Cat? _____ Other pets (**SPECIFY:** _____) ☐$_1$ No pets in the house **[GO TO G384]**

[IF ANY CATS OR DOGS OR OTHER PETS WITH FUR ARE PRESENT, ASK G382. AND C383.]	
G382. Do any of these pets spend any time in child's bedroom?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't Know
G383. Are the pets put out of the house at night?	☐$_1$ Yes ☐$_2$ No ☐$_3$ Sometimes ☐$_8$ Don't Know
G384. Are there cockroaches in your Home?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G385. Have you had any problems with cockroaches in your home during the past year?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G386. Have you or someone else (your landlord, another family member, a professional) treated your home for cockroaches in the past year?	☐$_1$ Yes ☐$_2$ No **[GO TO G389]** ☐$_8$ Don't know **[GO TO G389]**
G387. When was the last time it was treated ?	☐$_1$ Within last month ☐$_2$ 1 to 3 months ago ☐$_3$ 3 to 6 months ago ☐$_4$ 6 to 12 months ago ☐$_5$ More than 12 months ago ☐$_8$ Don't know
G388. What was used to treat your home for roaches? **[READ EACH CHOICE AND CHECK ALL THAT APPLY]**	☐$_1$ Dry powder ☐$_2$ Spraying ☐$_3$ Gel ☐$_4$ Roach bait trap (**SPECIFY:** _____) ☐$_5$ Boric acid ☐$_6$ Other (**SPECIFY:** _____) ☐$_8$ Don't know

G389. Have you had any problems with mice or rats in your home during the past year?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G390. Have you or someone else (your landlord, another family member, a professional) treated your home for rats or mice in the past year?	☐$_1$ Yes ☐$_2$ No **[GO TO G393]** ☐$_8$ Don't know **[GO TO G393]**
G391. When was the last time?	☐$_1$ Within last month ☐$_2$ Between 2 and 6 months ago ☐$_3$ Between 6 and 12 months ago ☐$_4$ More than 12 months ago ☐$_8$ Don't know
G392. How is your home treated for rats or mice? [**READ EACH CHOICE AND CHECK ALL THAT APPLY**]	☐$_1$ Spring traps ☐$_2$ Glue traps ☐$_3$ Poison ☐$_4$ Other (**SPECIFY:** _____) ☐$_8$ Don't know

F. BEHAVIOR CHANGE TO REDUCE ENVIRONMENTAL HAZARDS

The purpose of these questions is to look at the environment in your home and how it relates to your child's asthma as well as the health of other household members.

G393. Is there anyone whose paying job is working around chemicals (such as pesticides, paints) or dust living in the home?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G394. If yes, do they usually wear their work clothes home?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G395. Is there anyone whose informal job (includes working with chemicals) is in or near the home?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G396. During the last 2 weeks, how many times was the room in which **[child]** sleeps dusted?	☐$_1$ None ☐$_2$ 1 ☐$_3$ 2 ☐$_4$ 3 ☐$_5$ 4 or more ☐$_8$ Don't know
G397. What do you use when you dust?	☐$_1$ Dry cloth ☐$_2$ Damp cloth ☐$_9$ Other (**SPECIFY:** _____)
G398. During the last 2 weeks, how many times were other rooms in the house dusted?	☐$_1$ None ☐$_2$ 1 ☐$_3$ 2 ☐$_4$ 3 ☐$_5$ 4 or more ☐$_8$ Don't know

G399. How often do you change the **[child's]** Bedding? **[DO NOT READ RESPONSE CATEGORIES TO RESPONDENT; CHOOSE CATEGORY WHICH FITS RESPONSE]**	☐$_1$ once a week or more ☐$_2$ more than every two weeks ☐$_3$ more that once a month ☐$_4$ once a month or less ☐$_9$ Other (**SPECIFY:** _____) ☐$_8$ Don't know
G400. When the child's bedding are machine washed, what temperature is usually used for the wash cycle? **[WE ARE INTERESTED IN THE WASH CYCLE ONLY. THIS IS THE FIRST CYCLE OF THE WASHING MACHINE. EXAMPLE: SOMEONE USES THE WARM-COLD SETTING, YOU WOULD RECORD WARM.]**	☐$_1$ Hot ☐$_2$ Warm ☐$_3$ Cold ☐$_4$ Not applicable [**DO NOT READ**] ☐$_8$ Don't know
G401. When the child's bedding is hand washed, what temperature is usually used for the wash?	☐$_1$ Hot ☐$_2$ Warm ☐$_3$ Cold ☐$_4$ Not applicable [**DO NOT READ**] ☐$_8$ Don't know
G402. Do you or any member of your family add anything to the wash to help get rid of dust mites? **[PROMPT: "Such as eucalyptus oil."]**	☐$_1$ Yes (**SPECIFY:** _____) ☐$_2$ No ☐$_8$ Don't know
G403. How often does the cover on your child's bed get washed (i.e. bedspreads/comforters)? **[RECORD THE CATEGORY CLOSEST TO THE RESPONSE. IF RESPONDENT UNSURE, READ RESPONSES]**	☐$_1$ Once a week or more often ☐$_2$ More than once a month ☐$_3$ More often that every 3 months (4 times a year) ☐$_4$ More often that every six months (2 times a year) ☐$_5$ Less often than every six months ☐$_8$ Don't know

G404. Does **[child]** have stuffed animals in his or her bedroom?	☐$_1$ Yes ☐$_2$ No **[GO TO G407]** ☐$_8$ Don't know
G405. Do **[child's]** stuffed animals get washed?	☐$_1$ Yes ☐$_2$ No **[GO TO G407]** ☐$_8$ Don't know or not applicable **[GO TO G407]**
G406. How many times per year do **[child's]** stuffed animals get washed? **[DO NOT READ RESPONSE CATEGORIES TO RESPONDENT; CHOOSE CATEGORY WHICH FITS RESPONSE]**	☐$_1$ Once a week ☐$_2$ Once a month ☐$_2$ Every three months (4 times a year) ☐$_3$ Every six months (2 times a year) ☐$_4$ Less than once a year ☐$_5$ Once a year ☐$_9$ Other (**SPECIFY:** _____) ☐$_8$ Don't know
Have you done any of the following things around the house because of **[child's]** asthma?	
G407. Removed visible mold growth?	☐$_1$ Yes ☐$_2$ No
G408. Removed pets from the home?	☐$_1$ Yes ☐$_2$ No ☐$_3$ Never had pets
G409. Changed cigarette-smoking rules in the home?	☐$_1$ Yes, no one allowed to smoke ☐$_2$ Yes, reduced amount of smoking ☐$_3$ Yes, limited smoking to one room. ☐$_4$ No ☐$_5$ Never any smokers
G410. Attempted to control or eliminate cockroaches?	☐$_1$ Yes ☐$_2$ No ☐$_3$ Never a problem
G411. Attempted to control or eliminate mice or rats?	☐$_1$ Yes ☐$_2$ No ☐$_3$ Never a problem
G412. Did you do any other things around the house because of [**child's**] asthma?	(**SPECIFY:** _____)
Now I'm going to ask you a few questions about smoking. These questions concern smoking of cigarettes.	
G413. How many people who live in [child's]	

home smoke? **[INCLUDE RESPONDENT IF SMOKER.]**	_____ people ☐$_1$ None **[GO TO G418]**
G414. Do you smoke cigarettes, even occasionally?	☐$_1$ Yes ☐$_2$ No **[GO TO G418]**
G415. About how many cigarettes a day do you now smoke?	_____ cigarettes
G416. How often do you go outside the home to smoke?	☐$_1$ Always ☐$_2$ Sometimes ☐$_3$ Rarely ☐$_4$ Never ☐$_8$ Don't know
G417. Does **[child]** smoke cigarettes?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G418. Do any frequent visitors smoke?	☐$_1$ Yes ☐$_2$ No ☐$_8$ Don't know
G419. Many people have difficulties keeping their children away from cigarette smoke. Do you have problems keeping **[child]** away from people who are smoking?	☐$_1$ Yes ☐$_2$ No
G420. How frequently is your child around people who are smoking? Would you say. . . . **[READ CHOICES]**	☐$_1$ Daily ☐$_2$ Several times a week ☐$_3$ Several times a month ☐$_4$ Never ☐$_8$ Don't know

Thank you for your time for these questions.

We may need to contact you again to obtain additional information. Please give me the name, address and telephone number of two relatives or friends who would know where you could be reached in case we have difficulty in contacting you.

Name of first contact person:
_____

Telephone number of first contact person: _____

Address of first contact person:

_____
_____
House No. Road/Street

_____
Suburb/Township Postal Code

Relationship of contact person to you: _____

Name of second contact person:
_____

Telephone number of second contact person: _____

Address of second contact person:

_____ _____
House No. Road/Street

_____ _____
Suburb/Township Postal Code

Relationship of contact person to you: _____

THANK YOU FOR COMPLETING THIS QUESTIONNAIRE!

END: Thank you for helping us!

Interview completed at: Time: _ _:_ _ am / pm

Appendix 5

Appendix6

APPENDIX 7
Parent Informed Consent

Informed Consent Form for participant's parent / legal guardian

Please note: this form is to be read, or read to, and signed by the child's parent or legal guardian and an adult witness

Your consent is required so that your child
_____ can participate in a
FIRST NAME SURNAME
study of the health effects of air pollution at the _____School.

If you have any queries after reading this consent form, kindly call Ms. Poovie Reddy @ 204 2082 (b/h).

The study is being conducted in conjunction with the Nelson R. Mandela Medical School. The purpose of the study is to find out whether health problems (like asthma, bronchitis, reactive airway disease, pneumonia and asthmatic bronchitis) experienced by learners at the school, are caused by air pollution. This study will determine whether certain genes are involved in your child's response to air pollution. The study has the support of the school staff, community groups in South Durban and the City Health Department.

If you agree to participate, your child will be required to give a sample of blood which will be taken by trained medical personnel.

Risks:

⇒ There are no risks from taking the samples of blood, but should any medical emergency arise, there will be trained medical personnel on site to render assistance.

Confidentiality:
The results and information that we collect from you and your child is ***completely confidential***. Other than the study personnel, this information will never be seen by anyone without your written consent. The results of the overall study will be made available to the school and the community and will be presented so as to protect the identity of individual participants.

Costs to you resulting from participation in the study:

The study is offered at ***no cost*** to you. If a problem is discovered and you wish to consult a doctor, we will recommend a doctor. However, the study cannot pay for these additional medical visits or treatments.

Voluntary nature of participation:
You and your child are free to decline to participate or to withdraw from the study at any time without suffering any penalty or disadvantage.

Consent:
I understand the meaning of the information given above.

I hereby consent to having my child

_____
FIRST NAME **SURNAME**
participate in the study.

<u>Documentation of consent:</u>

The child's parent or legal guardian and an adult witness should sign and date both copies of this document. You or your child should return ONE copy to your child's teacher at his/her School by _____.

.

It will be given to the research staff and kept with the records of the study. The other copy is for you to keep.

_____ _____

Printed name of child's parent / legal guardian **Signature**

_____ _____

Printed name of witness **Signature**

Date: _____

APPENDIX 8

Child Informed Assent

IMPORTANT:

1) If you do not understand any words, please ask for an explanation before giving

INFORMED ASSENT FORM

Title of research project:
Epidemiologial and genetic risk factors associated with asthma among children in the South Durban Region, KwaZulu Natal.

Please circle the appropriate answer:

1. Have you understood the subject information sheet?
   YES/NO
2. Did you discuss the study with anyone? YES/NO
3. Who did you discuss it with? _____

4. Do you have any questions about the study or about your role in the study?
   YES/NO
5. Are you worried about any part of this study? YES/NO
6. Have you received enough information about this study?
   YES/NO
7. Do you understand how you will be involved in this study?
   YES/NO
8. Do you understand that you are free to withdraw from this study:
   (a) at any time and;
   (b) without having reason to withdraw
   YES/NO
9. Do you agree to voluntarily take part in this study?
   YES/NO

If you have answered **NO** to any of the above questions, please obtain the necessary information **BEFORE** signing

I, _____ hereby give assent for the proposed procedures to

SUBJECT'S NAME
be performed on me as part of the above mentioned project.

_____
_____
(PRINTED NAME OF WITNESS) **(SIGNATURE)**

APPENDIX 9

Information Sheet

IMPORTANT

1) If you do not understand any words, please ask for an explanation before giving

SUBJECT INFORMATION SHEET

Title of research project:
Epidemiological and genetic risk factors associated with asthma among children in South Durban Region, KwaZulu Natal.

I am requesting your permission to participate in a study of the health effects of air pollution in the eThekwini Municipality. I would like to describe to you the purpose of the study, what you would be asked to do if you agree to participate, and what the risks and benefits of participating in the study are. If you have any questions or if something I say is not clear to you, please stop me and let know right away.

The study has the support of the local industry, community groups concerned about these sorts of health problems, and the City Health Department. We are studying this community because of its location near sources of air pollutants such as oil refineries and because of health concerns expressed by teachers and students at the school and by the larger community. The purpose of the study is to figure out whether any health problems are being caused by air pollution in the community, and, if so, to make recommendations to improve the situation.

If you agree to participate, you and your parent will be interviewed, you will be asked to complete baseline breathing tests, and, each day for a period of two or three weeks, fill out a daily log about activities and any breathing problems, and blow into a handheld device several times a day to further test your breathing, and also have a blood test conducted.

Baseline breathing tests. You will be asked to blow several times into a machine which measures how well your lungs are working. You will be asked to repeat the breathing test after you first breathe in a small amount of a chemical subsistence (either methacholine or histamine). This test helps up find out if you

may have a breathing problem like asthma. You may be asked to breathe in this substance and then blow into the machine a few times.

Blood Tests. Trained technicians will take samples of blood from you. These tests will determine if you have genes that put you at greater risk of getting respiratory problems. Only specified tests will be conducted on this blood sample, and the remainder will be destroyed immediately after. You WILL NOT BE INJECTED WITH ANY SUBSTANCE/MEDICATION.

Risks. There are no risks from the interview, keeping the daily logs, blowing into the handheld device, or the baseline simple breathing test. The breathing test using the chemical substance can cause chest tightness, hoarse voice or a sore throat for a short time in some people. This can be treated immediately with a different medication, which you breathe in. You will only be given the chemical substance if the simple breathing test is normal. This greatly reduces the chance of having a serious problem.

Expected benefits to you and others. Your parents will be given a written copy of all your child's test results along with an explanation of what they mean. What we learn from this study may help to protect people in South Africa and other parts of the world from problems caused by air pollution.

Confidentiality. The interview, diary, and breathing test information we collect about you is completely confidential and will never be seen by anyone other than the personnel conducting the study without your without your written consent. The results of the overall study, which will be made available to the local government and the community, will be presented so as to protect the identity of individual participants.

Voluntary nature of participation. You are free to refuse to participate or to withdraw from the study at any one time without suffering any penalty or disadvantage.

Contact person. You may contact **MS POOVIE REDDY** (telephone no.: **2042082**) or **DR RAJEN NAIDOO** (telephone no.: **2604385**) for answers to further questions about the research.