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INFLAMMATION, GENOTOXICITY AND CELL PROLIFERATION IN NASAL LAVAGE AND NASAL EPITHELIUM OF PEOPLE EXPOSED TO URBAN POLLUTION

DISSERTATION

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List of Abbreviations:

NAL	Nasal Lavage
NALF	Nasal Lavage Fluid
PMN	Polymorphonuclear leucocytes
IL-8	Interleukin -8
IL-6	Interleukin- 6
IL-1β	Interleukin 1 ßeta
IL-5	Interleukin 5
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
PGE_2	Prostaglandin E_2
TNF-α	Tumor Necrosis Factor Alpha
TXB_2	Thromboxane B_2
URI	Upper respiratory illness
FEV_1	Forced expired volume in 1 second
FVC	Forced vital capacity
PEF	Peak expiratory flow
DEP	Diesel Exhaust Particles
PM	Particulate matter
PM_{10}	Particulate matter with 50% cut-off aerodynamic diameter of ≤ 10 μm
$PM_{2.5}$	Fine particulate matter
$PM_{0.1}$	Ultra fine particulate matter
MMAD	Mass median aerodynamic diameter
NAAQS	National Ambient Air Quality Standards (in USA)

CHAPTER 1

1 Genaral Introduction

1.1 Ambient exposure and adverse health effects

A complex mixture of chemicals and particulate matter is present in the ambient air of both rural and industrial areas. There is increasing concern about adverse effects on the cardiopulmonary and host defence systems resulting from chronic exposures to air pollutants. Epidemiological studies strongly suggest that children and adults have increased mortality and morbidity from photochemical smog and particulate air pollution (Brunekreef et al., 2000; Roemer et al., 2000; Hiltermann et al., 1997; Pope et al., 1999; Dockery et al., 1992,1993).

One of the most dramatic examples of the effects of air pollution on health was seen in an episode that started on 4 December 1952 in London and resulted in 4000 excess deaths in that month (HMSO, 1954; Logan, 1953). It was public concern about this, rather than pressure from the medical profession, that led to the first clean air legislation in Britain (Bates, 1994). Since then drastic efforts have been made to reduce air pollution.

The main air pollutants measured in the earlier years were sulphur dioxide (SO_2) and particulate matter (PM) in the form of black smoke from the burning of domestic coal. Early animal-based studies investigated the effects of relatively high doses (e.g. 50-400 ppm) of inhaled SO_2 on airway epithelium and demonstrated ulceration of large airway epithelium, increases of epithelial cell mitoses, and a goblet cell hyperplasia and increase in gland size which mimicked the changes observed in human bronchitis (Lamb and Reid, 1968). However as a result of national and international measures directed to control the most important sources of emission (i.e. power stations and refineries), SO_2 levels have decreased in several countries in Europe. Instead, in developed countries a new form of pollution has emerged resulting from increased use of liquid petroleum gas or kerosine in industry and increased use of motor vehicles. In

these settings ozone and respirable particular matter (e.g.PM_{10}) often exceed recommended standards and new threats include, nitrogen (NO_x) and diesel exhaust.
World-wide, about 480 million people are being exposed to increased levels of ozone (Schwela, 1996), of which at least 150 million are in Europe (Sivertsen and Clench-Aas, 1996). Air pollution in Western Europe is mainly traffic-related, and therefore ozone and NO_x are particularly important. In Eastern Europe pollution is mainly related to the combustion of fossil fuels, and so PM and SO_2 are the key constituents.
The US Environmental Protection Agency cited in the 1987 revision of the particulate air pollution standards, the results of three epidemiological studies as providing the key supporting evidence for the proposed particle standard (US-EPA, 1987). Since then there has been a burst of epidemiologic studies of the health effects of particulate air pollution in the succeeding ten years and the Environmental Protection Agency , in promulgating a revised particulate air pollution standard in 1997, cited more than 80 epidemiologic studies (US -EDA,1997). Nowadays there are at least more than 140 published epidemiologic studies of the health effects of particulate air pollution (Pope and Dockery, 1999).

The link between cardiopulmonary disease and extremely high concentrations of particulate and SO_2 pollution has been established since the 1970s. In the 1970s and 1980s some studies suggested that there might be important health effects at relatively low concentrations (Bates, 1980; Ware et al., 1981). However in an extensive research review several prominent British scientists (Holland et al., 1979) supported the common view that particulate air pollution at high levels posed a hazard to human health, but that the evidence was weak for health effects at the lower concentrations which existed in the USA and Britain by the 1970s. In the 1970s through the mid-1980s a small number of original studies suggested adverse health effects at contemporary pollution levels (notably Dockery et al., 1982; Lave and Seskin, 1970; Ostro, 1983; Özkaynak and Thurston, 1987; Samet et al., 1981; Whittemore and Koren, 1980). Many population based mortality studies have suggested mortality effects of chronic exposure to air pollution. In 1964 Martin reported that overall annual respiratory mortality (as opposed to episodic mortality) in the Greater London region was significantly related to smoke (or particulate pollution) levels. In 1970 Lave and Seskin reported the results of one of the first serious attempts to measure long-term mortality effects of air pollution in the

USA. They observed significant associations between annual mortality rates and particulate air pollution across US metropolitan areas. The work of Lave and Seskin was quite controversial, and later similar studies were conducted to replicate or refine their use of population-based cross-sectional study designs (Dockery et al., 1993; Pope et al., 1995; Woodruff et al., 1997). Most of these studies observed that mortality rates tended to be higher in cities with higher fine or sulphate particulate pollution levels. Regression techniques were used to evaluate cross-sectional differences in air pollution and mortality and to control for other ecological variables.

Then, during the relatively short time period of 1989-92, results of three loosely connected epidemiological research efforts from the USA were reported. These efforts included: (1) the Harvard Six Cities prospective cohort study that observed that long – term particulate exposure was associated with increased risk of respiratory illness in children (Dockery et al., 1989) and with increased risk of cardiopulmonary mortality (Dockery et al., 1993); (2) a serie of studies of in Utah Valley observed that particulate pollution was associated with a wide range of morbidity health end-points including respiratory hospitalisations (Pope, 1989, 1991), lung function and respiratory symptoms (Pope and Dockery, 1992; Pope et al., 1991), school absences (Ransom and Pope, 1992) and mortality (Archer, 1990; Pope et al., 1992); and (3) also a serie of studies that observed associations between daily changes in particulate air pollution and daily mortality (see reviews by Dockery and Pope, 1994; Ostro, 1993; Schwartz, 1994; Thurston, 1996). Also during this time period, results of other studies from the USA (Ostro et al., 1991; Thurston et al., 1992), Germany (Wichmann et al., 1989), Canada (Bates and Sizto, 1989), Finland (Pönkä, 1991) and the Czech Republic (Bobak and Leon, 1992) were published. The major outcome of these studies were unexpectedly large health effects of relatively low concentrations of particulate air pollution. Many scientists found that these studies were controversial, but the convergence of their reported results in such short time period resulted in a critical mass of evidence that prompted serious reconsideration of the contribution of particulate air pollution on human health (reviews by Dockery and Pope, 1996; and recent re-analysis by HEI, 2001), however, extension of the studies (Samet et al., 2001) have shown that the findings are statically robust and consistent.

1.1.1 The effects of air pollution on children

Harmful effects of air pollution are typically observed in the most sensitive subjects in the population such as asthmatics or patients with chronic obstructive pulmonary disease (Lebowitz, 1996), but also healthy persons may experience adverse health effects (Bascom et al., 1996). One of the most sensitive groups are children as shown by studies on the increased acute respiratory illness. A study in Provo (Utah) found that hospital admissions of children for acute respiratory disease were closely associated with levels of fine particulate pollution (particles less than 10 microns in size or PM_{10}) (Pope, 1991). In southern Ontario, hospital admissions for children in the summer were associated with ambient ozone and sulphate levels (Bates and Sizto, 1987). Burnett et al. published data reported on hospital admissions for 168 hospitals in Ontario over a 6-year period. They noted that in the summer respiratory admissions were closely associated with ozone levels, and they observed that among infants 15% of summer admissions were pollutant associated, whereas this proportion among the elderly was only 4%. Studies of hospital admissions in Toronto indicated that, in addition to ozone and sulfates, the aerosol hydrogen ion level and the PM_{10} are associated with increased admissions (Thurston et al., 1994). An environmental study in five cities in Germany found an association between chronic cough and levels of NO_2 (Schwartz et al., 1991)

Lung function is an objective and potentially sensitive indicator of acute response to air pollution. Two studies in the 1970s postulated that episodes of air pollution would lead to reversible deficits in lung function of children in Pittsburgh following a major air pollution episode had produced lower lung functions that would return to normal in the weeks following exposure. These data were suggestive of such a recovery, but were not very convincing as they lacked lung function measurements before or during the episode.

In 1978, Dockery and colleagues undertook a prospective study of lung function in Steubenville, Ohio, an area with frequent air pollution episodes. Baseline lung function of children in four schools was measured. Upon the declaration of an air pollution alert by the local air pollution control agency, lung function measurements were repeated. The three weekly follow-up measurements assessed recovery. These panels of elementary school children were monitored during four episode periods in 1978 through

1980 (Dockery et al., 1982). Declines in forced expired volume in 0.75 s ($FEV_{0.75}$) were observed following these episodes. While designed as a simple episode study, regression analyses suggested declines in lung function across the full range of exposures. This study first applied random effects models to assess the effects of air pollution exposures on individual children. This design and these analytic methods have been applied in many studies of repeated lung function measurements around the world to evaluate potential effects of particulate air pollution (Hoek and Brunekreef, 1993; Roemer et al., 1993; Pope and Dockery, 1992).

In a re-analysis of the Steubenville data, Brunekreef et al. (1991) found the strongest association with the mean particle concentrations over the previous 5 days. Similar decreases in forced expired volume in 1 s (FEV_1) were observed in school children following a particulate and SO_2 pollution episode in January 1985 in the Netherlands (Dassen et al., 1986). Subsequent panel studies of school children in the Netherlands with weekly lung function measurements (Hoek and Brunekreef, 1993,1994) have also shown decreased FEV_1 associated with daily PM_{10} concentrations. Effects of exposures lagged by up 7 days were observed. Studies in Montana (Johnson et al., 1982, 1990) observed similar declines in lung function associated with abnormal urban air pollution episodes, but comparable declines were not associated with a volcanic ash episode.

Koenig and colleagues (1993) studied the lung function (forced vital capacity (FVC) and FEV_1) of children in Seattle, Washington, with relatively low particulate air pollution levels. Lung function declines were associated with fine particulate air pollution for asthmatic children, but not for non-asthmatic children. Overall, these studies generally observed a decrease of less than 0.35% in FEV_1 associated with each $10\mu g/m^3$ increase in daily mean PM_{10}. The first study to show the importance of particle numbers was Peters et al (1997) reporting an association between PEF decrease and particle counts in asthmatics. Several studies have used peak expiratory flow (PEF) measurements as an indicator of acute changes in lung function, including studies in the Netherlands, Utah Valley, Utah Uniontown and State College , Pennsylvania, Mexico City, and Port Alberni, British Colombia (Hoek and Brunekreef, 1993; Pope and Dockery, 1992; Neas et al., 1995, 1996; Vedal et al., 1998). In these studies, a $10\mu g/m^3$ increase in PM_{10} was associated with very small, but often statistically significant, decreases in peak flow measurements. As with FEV_1, the strongest associations with

peak flow included particulate pollution over the previous several days, allowing for a lag in effect.

Several studies that have evaluated associations between particulate air pollution and chronic respiratory symptoms and disease. These include the Harvard Six Cities Study's (Dockery et al., 1989) analysis of bronchitis in children, and analysis of the symptoms data from the 24-cities study of school children (Dockery et al., 1996). The effects of air pollution on respiratory disease or symptoms were estimated while adjusting for individual differences in various other risk factors. In all of these studies, statistical significant positive associations were observed between particulate air pollution and respiratory symptoms. Chronic cough, bronchitis and chest illness (but not asthma) were associated with various measures of particulate air pollution, suggesting that particulate air pollution was most consistently associated with a 5-25% increase in bronchitis or chronic cough.

In Western Europe , summer O_3 concentrations frequently exceed the NAAQS and there is concern about the health effects for exposed populations, specifically school children. Frischer et al. (1993) studied upper airway inflammation in 44 children using repeated nasal lavage (NAL) from May to October 1991. Days with high (>180 ppb) and low (<140 ppb) ozone exposures were compared. There was a significant increase in intra-individual mean PMN counts on high ozone days and linear regression analysis of log-PMN counts yielded a significant effect for ozone. The same group (Frischer et al., 1997) explored the in vivo hydroxyl radical attack in nasal lavage samples from the 44 children studied in the previous paper. The focus of the paper was based on historical data showing that ozone reacts with water and gives rise to reactive hydroxyl radicals capable of oxidising a wide range of biomolecules. Based on their findings, Frischer et al., (1997) suggested that hydroxyl radical attack subsequent to ambient ozone takes place in the upper airways of healthy children, and relates to lung function decrements.

Calderon- Garciduenas et al. (1992) characterised nasal and changes in short-term (<30 days) and long-term (>60 days) residents of Mexico City in Residents of high-ozone areas had loss of cilia, basal cell hyperplasia, squamous metaplasia and submucosal vascular proliferation. Similar changes, as well as infiltration of epithelium by neutrophils, were seen in a subsequent study of preadolescent children from the same high-pollution area (Calderon- Garciduenas et al., 1995). These studies demonstrated

the relevance of nasal tissues as sentinels of exposure to polluted urban environments, as well as attesting to the feasibility of field studies in paediatric populations.

Certain common respiratory viruses, including respiratory syncytial virus (RSV), rhinovirus and parainfluenza virus, can directly stimulate human airway epithelial cells to produce cytokines and chemokines (Becker et al., 1991). To determine whether similar cytokine induction might occur in vivo in childhood infections, Noah et al. (1995a,b) performed serial nasal lavages and superficial subturbinate epithelial biopsies in infants and young children in a day-care center, around the time of naturally acquired viral upper respiratory illness (URI). Concentrations of cytokines in nasal lavage fluid (NALF) were measured and significant increases in NAL levels of cytokines IL-1β, IL-6, IL-8, and TNF-α were noted during acute URI compared with pre-illness baselines in the same children. At follow-up 2-4 weeks after the onset of symptoms, levels of most cytokines had fallen back to near baseline. Paired superficial nasal epithelial biopsies were obtained for some children during acute upper respiratory illness and at follow-up. Most subjects had increases in epithelial mRNA abundance for IL-1β, IL-6, IL-8 during acute URI, suggesting that the epithelium was at least one cellular source of the increases in these cytokines noted in NAL fluid. Noah et al. (1995a,b, 1997) have also used NAL to characterise baseline levels of inflammation and cytokines in children with asthma and cystic fibrosis. In a study of school-age children, subjects were characterised on the basis of history, immediate hypersensitivity allergy skin tests, airway reactivity, and lung function as being either non-allergic/non-asthmatic, allergic but non-asthmatic, or allergic and asthmatic (Noah et al., 1995a,b). These children underwent a single nasal lavage during an asymptotic period in the summer. NAL levels of IL-8 and ECP were greater in asthmatics compared with levels in the other two groups. This findings is consistent with activation of epithelial IL-8 as either a contributing cause or a consequence of the eosinophils-dominated inflammation characteristic of asthmatic airways.

These studies illustrate that both acute and chronic effects can be measured in children due to ambient air pollution. Acute effects suggest that upper airway inflammation is a primary response that can be used to study (i) different pollutants, and (ii) to study early responses in exposed subjects.

1.1.2 Airway Inflammation and Ambient Exposure

As demonstrated above, many studies have shown associations between exacerbation of respiratory disease and air pollution (Calderon et al., 1999). In these studies several components of ambient air pollution have been demonstrated to cause acute and a chronic airway inflammation. The most potent actions seem to be induced by ozone ambient and particulate matter (PM). Therefore the following paragraphs review the toxicity of these two major air pollutants .

1.1.2.1 Ozone

Ozone (O_3) is mainly produced in the troposphere by a series of sunlight-driven reactions involving nitric oxides and volatile organic compounds arising largely from human activities of combustion (Bascom et al., 1996; Sandastrom, 1995). During hot summers, ground-level concentrations of ozone may exceed 0.2 ppm in Central Europe and other areas of the world, such as California (Sandstrom, 1995). Ozone may also be produced in high concentrations in workplaces such as welding plants paper mills. Ozone is virtually insoluble in water and, unlike NO_2, is deposited along the entire airway. In resting subjects, approximately 90% of inspired O_3 is absorbed with substantial uptake and effects in both the upper and lower airways (Gerrity et al., 1988; Hiltermann et al., 1998; Hu et al., 1992). Mathematical models have suggested that the tissue dose of inhaled O_3 is greatest at the position of the bronchoalveolar junction (Miller et al., 1985; Overton et al., 1987), and this region has been shown to be very sensitive to O_3 induced damage, as shown by histopathological studies in animals (Barry et al., 1988; Carlsson et al., 1996). However, as O_3 is highly reactive and reacts directly with alveolar epithelium (Langford et al., 1995; Pryor, 1992). The majority of the effects of O_3 are probably mediated by a cascade of secondary free-radical-derived products and cellular damage occurs when antioxidant defences are overwhelmed (Kelly et al., 1995; Mustafa, 1990). Is toxic effects include stimulation of airway irritant receptors, alterations of epithelial permeability, ciliary damage, alterations to Clara cells (responsible for the detoxification of many pollutants), epithelial denutation and fibrosis

each of these very much dependent on dose (Bhalla and Crocker, 1986; Bhalla et al., 1990; Schwartz et al., 1976).

In normal subjects, ozone has two principal effects. One is a reproducible decrease of both forced vital capacity (FVC) and FEV_1 (with increased non-specific bronchial responsiveness and substernal discomfort when taking a deep breath) (Anon, 1996; Bates and Hazucha, 1973; Koren and Bromberg, 1995; Peden, 1997). The second is the development of neutrophilic inflammation as quickly as 1 h after exposure (Anon, 1996; Koren et al., 1991; Peden, 1997). Interestingly, these two effects are not correlated with each other, suggesting that separate mechanisms mediate these changes.

The effect of ozone on lung mechanics depends on the concentration of ozone, duration of exposure and the level of exercise (with corresponding increases in minute ventilation) (McDonnel et al., 1983). Indeed, exposures to ozone without exercise generally reveal no effect on lung function at levels below 0.50 ppm (Anon, 1996; Folinsbee et al., 1994; McDonnel et al., 1983). With exercise, ozone induces increases in respiratory frequency and the level of ozone required to cause decreases in FEV_1, FVC and airway resistance can be much lower (McDonnel et al., 1991).

Examination of the action of a variety of pharmacological agents on the effect of ozone on respiratory mechanics has given some insight into the mechanisms of this effect. Atropine inhibits ozone-related decreases in airway resistance (though not spirometry), indicating that vagal mechanisms are involved in the respiratory response to ozone (Widdicombe et al., 1988; Widdicombe, 1994; Beckett et al., 1985). Ozone also could directly increase smooth muscle sensitivity to acetylcholine, consistent with the observation that sensitivity to inhaled methacholine is increased after ozone exposure (Beckett et al., 1985; Holtzman et al., 1983). Ozone also effects eicosanoid responses in the airway, thus altering airway mechanics. Levels of PGE_2 bronchoalveolar lavage (BAL) recovered from normal subjects after O_3 exposure correlate with observed lung function decrements (Koren et al., 1991). Additionally, several laboratories have shown that cyclooxygenase inhibitors, such as ibuprofen and indomethacin can inhibit ozone induced decreases in spirometry, although they have little effect on inflammatory responses to ozone (Eschenbacher et al., 1989; Hazucha et al., 1996; Schelegle et al., 1987). Airway C fibres can be stimulated by PGE_2, suggesting a mechanism by which ozone-related increases in PGE_2 also may account for changes in lung function due

ozone (Coleridge et al., 1993). Interestingly, β-agonist have little influence on the immediate effect of ozone on lung function. While there is one report that β-agonist can block some ozone-induced decreases in lung function, most studies indicate that β-agonist have no effect on the deleterious effect of ozone on lung function (Folinsbee, 1993; Gong et al., 1988).

Regarding ozone and airway inflammation , ozone induces neutrophilic influx into the airway in normal subjects, as revealed in analysis of BAL fluid and in bronchial mucosal biopsies following exposures ranging from 0.10 to 0.4 ppm. This neutrophilic influx appears to be maximal between 1 and 6 h after exposure and persists to 18 h (Folinsbee, 1993; Koren et al., 1991). Direct comparison of the time course of mediator response to ozone indicates that changes in IL-6 and PGE_2 peak 1 h after exposure whereas fibronectin and plasminogen activator are higher 18 h after exposure (Koren et al., 1991).

The epithelium is thought to be very important in airway response to ozone. Epithelial cell lines exposed to ozone generate the eicosanoid PGE_2, TXB_2, PGF_{2a}, and the proinflammatory cytokines IL-6 and IL-8, as well as the proinflammatory protein fibronectin (McKinnon et al., 1992, 1993). Nasal epithelial cells placed into culture respond to high level (0.5 ppm) ozone by generating ICAM-1, the major adhesion molecule for neutrophils and eosinophils in the airway, as well as the cytokines IL-1, IL-6 and TNF-α (Beck et al., 1994). Thus effect of ozone on epithelial cells probably plays a major role in the inflammatory response to this pollutant, whereas neural responses (C fiber, pain fibers and possibly cholinergic fibers) probably exert the major effect on lung function changes (Widdicombe, 1988).

With the exception of PGE_2 none of the markers of inflammation noted above correlate with immediate changes in the lung function of normal subjects (Anon, 1996; Peden, 1997). Nor has an association been made between ozone-induced inflammation and airway responsiveness in normal subjects. Thus, the clinical significance of ozone-induced inflammation in normal subjects remains unclear. It has been suggested that this proinflammatory effect of ozone could have a greater significance in asthma, a disorder characterised in part by an eosinophilic inflammation of lower airways (Ball et al., 1996; Molfino et al., 1991).

In persons with allergic rhinitis or asthma, ozone exposure appears to induce both eosinophil and PMN influx. This was initially observed by Bascom and colleagues, who reported an increase in both neutrophils and eosinophils in nasal lavage fluid obtained from allergic rhinitis after a 4-h exposure to 0.5 ppm ozone (Bascom et al., 1990). This was later shown to be true in mite–sensitive asthmatics as well, following exposure to 0.4 ppm ozone for 2 h. In the later study, ozone also caused increases in eosinophil cationic protein, suggesting that eosinophils may have been activated (Peden et al., 1995).

1.1.2.2 Particulate matter (PM)

Ambient particulate matter (PM) is the term used to define a complex mixture of anthropogenic and naturally occurring airborne particles, described under total suspended particles (TSP), PM10, fine ($PM_{2.5}$) and ultra fine ($PM_{0.1}$) particles. European and US regulatory agencies are considering more stringent air quality standards for airborne PM, largely based on epidemiological studies showing associations between mortality from respiratory and cardiovascular diseases and PM (Dockery et al., 1993; WHO., 1999; Samet et al., 2000). From these studies it is estimated that per 10 $\mu g/m^3$ increase in the annual concentration of $PM_{2.5}$, mortality increases with 1.4 %, while respiratory disease such as bronchitis or asthma exacerbation's increase by as much as 4 % (WHO., 1999). It is by no means clear how exposure to PM, typically as low as 30 $\mu g/m^3$, can produce these health effects observed in epidemiology studies and which components of PM mediate these effects. Although epidemiological and toxicological evidence suggest that it is the fine ($PM_{2.5}$) and even the ultra fine ($PM_{0.1}$) fraction that is responsible, there is no general agreement (Oberdorster et al., 1994; Wichmann et al., 2000). The wide number of endpoints suggests that more than one component may be driving the health effects (Donaldson et al., 1998; Dreher et al., 2000).

Leading hypotheses regarding the agents or particle-properties mediating the pulmonary effects include transition metal content (Dreher et al., 2000), particle size and surface (Donaldson et al., 1998) or endotoxin contamination (Monn et al., 1999). In addition particles can carry or present other compounds such as polycyclic aromatic hydrocarbons (PAHs) or proteins which could lead to an increased allergic or inflammatory response (van Zijverden et al., 2000; Nel et al., 1999). Local effects in the lung appear to be driven by increased production of inflammatory mediators through oxidative stress-sensitive pathways. Transition metals play a key-role in this process due to their ability to react with superoxide or H_2O_2, which is called Fenton-reactivity.

The metals in air pollution particles originate from natural sources and vary with the source of the particles (Schroeder et al., 1987). Those metals in highest concentrations in crustal dust are iron, manganese, zinc, lead, vanadium, chromium, nickel, copper, cobalt, mercury and cadmium. Metals are more frequently associated with air pollution particles of anthropogenic origin. These typically have values of mass median aerodynamic diameter (MMAD) less than 2.5 μm (Schroeder et al., 1987). Iron is the

metal present in greatest concentration in most emission source air pollution particles. An exception is oil fly ash, which contain significant concentrations of vanadium and nickel compounds (Henry and Knapp, 1980). This fly ash is emitted by power plants and other industries that burn oil. Fly ash from coal-fired power plants also has high concentrations of iron, zinc, lead, vanadium, manganese, chromium, copper, nickel, arsenic, cobalt, cadmium, antimony and mercury (Schroeder et al., 1987). The concentration of iron in the atmosphere is several-fold greater than that of all other metals. This metal concentrates in the fog water of urban settings, in cloud droplets and in aerosol particles (Jacob et al., 1989) and is common on PM_{10}.

There is a vast array of particles which have been identified and shown to have biological effects in animal and in vitro studies. Active agents in particles include silica, metal ions (such as iron, vanadium, nickel and copper), organic residues such as PAHs found on diesel exhaust particles, acid aerosols and biological contaminants such as endotoxins (Anon, 1996). Although currently human exposure studies with PM are ongoing at the Particle Center in Rochester (NY) and at US-EPA, not many data are available (Ghio and Devlin, 2001). In this connection we would like to discuss as inflammatory effects of diesel exhaust particles on respiratory tract as an example.

1.1.2.2.1 Diesel exhaust

Diesel exhaust is a major contributor to atmospheric pollution by particulate matter, including ultra fine particles (<100nm). The main gases which also result from combustion of diesel fuel are carbon monoxide (CO), oxides of nitrogen (NO, NO_2) and SO_2, while a small but significant percentage of the fuel is polymerised, pyrolysed, cracked, oxidised, sulfonated and nitrated into several hundred of compounds (Scheepers and Bos, 1992, I). Ultra fine particles such as in DEP can be found in urban atmosphere in very large numbers (>0.5 x 10^5 particles/ cm^3), can be highly reactive (Oberdorster et al., 1996) and may penetrate through airway epithelium and vascular walls.

In humans, challenge studies examining the effect of DEP on allergic inflammation have typically employed a nasal challenge model. An initial report of the effect of DEP

on IgE production in humans was published by Saxon and colleagues (1994), who reported that exposure of human volunteers (four atopic and seven non-atopic) to DEP yields remarkable increases in nasal IgE production 4 days after DEP challenge, without any effect on IgG, IgA or IgM production (Diaz-Sanchez et al., 1994). These findings were reported with doses of 0.3 mg DEP. No such effect was observed with doses of 0.1 or 1.0 mg. The same study reported that DEP challenge of nasal mucosa also results in increased cytokine production by cells recovered in lavage fluid. In this study, subjects underwent nasal lavage before and after challenge with 0.3 mg of DEP. The recovered cells were then processed and analysed for mRNA analysis. Cells recovered before challenge were generally found to have detectable mRNA levels for γ-interferon, IL-2 and IL-13. In contrast, DEP challenge yielded cells which had detectable levels of many cytokines, including IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and γ-interferon. IL-4 protein also was measured in postchallenge lavage(Diaz-Sanchez et al., 1997, see also Table 1). Recent studies of Salvi and colleagues cited by Blomberg (1997) have demonstrated that exposure of normal subjects to 300 $\mu g/m^3$ diesel exhaust in the presence of 1.6 ppm NO_2 for 1 h produces a pronounced inflammatory response in the airways. In bronchial biopsies taken 6 h after cessation of exposure there was a significant increase in the number of adhesion molecules, LFA-1+ cells (e.g. neutrophils) and a trend towards an increase of VLA4+ cells (e.g. eosinophils). There was also a T cell response with CD4+ (T helper) cells predominating and increased expression of mRNA for IL-8 and a similar trend for IL-5.

In **animal studies** DEPs aggravate ovalbumin-induced airway inflammation in sensitised mice characterised by infiltration of eosinophils and lymphocytes and increases in the number of goblet cells in the bronchial (Takano et al., 1997; van Zijverden et al., 2000). There is also enhanced local expression of IL-5, Il-4, GM-CSF and IL-2 in both lung tissue and BAL. These experimental findings support the hypothesis that DEPs enhance the manifestations of allergic asthma. Taken together, these experimental and human studies suggest that DEPs have the capacity to enhance allergic inflammation and may also play a part in contributing to the increasing incidence of allergy and asthma seen in the developed Western world (Diaz Sanchez, 1997; Diaz Sanchez et al., 1996).

1.1.2.2.2 Ultrafine particles

Some researchers have developed the hypothesis that ultrafine particles (< 0.1µm) are responsible for the association between particles and health effects with the present low concentrations of outside air particles (Oberdorster et al., 2001 Seaton et al., 1995). Several mechanisms could contribute to a increased toxicity of ultra fine particles:

1) For a given aerosol mass, ultra fine particles have a much higher number of particles and surface compared with larger particles. Fine and ultra fine particles can carry into the deep respiratory system adsorbed reactive gases, radicals, transition metals or organic components; the larger surface of ultra fine particles can absorb more toxic surfaces material transport than the larger particles with a smaller surface.
2) The deposition efficiency of inhaled ultra fine particles in the respiratory tract is very high. It is estimated that 50% of the inhaled particles with a diameter of 20nm are deposited in the alveolar region of the human lung, together with a somewhat lower quantity, which is deposited in the lower airways (Kim et al., 2001).
3) Particles, which do not immediately dissolve in the epithelial liquid, represent the interface between held back particles and the cells, liquids and lung tissue. Therefore the very large surface of ultra fine particles can cause surface-dependent reactions.
4) The protection of the lung tissue by active phagocytosis of particles by alveolar macrophages is impaired, since ultra fine particles are less well detected by these cells. Additionally, ultrafine particles impair the clearance of fine particles (Renwick et al., 2001).
5) After deposition ultrafine particles penetrate faster in the interstitial lung tissue than larger particles (Ferin et al., 1992). There is provisional evidence that ultra fine particles can be transported from there into distant organs like the liver and the heart. Newer studies with healthy and susceptible laboratory animals showed that the inhalation of particles can lead to modifications in the cardiac rhythm or repolarisation but the implication of these results for the mechanisms with humans is unclear. It was suggested that the inflammation due to PM could lead to a transient rise of the blood clotting as part of the acute phase reaction accompanied

with inflammation (Seaton et al., 1995). Recent experiments with hamsters show that particles can penetrate into the systemic circulation, after intratracheal instillation of particles (Nemery, 2001).

Currently, inflammation is regarded upon as a key-process for both acute respiratory and systemic effects. Although ultrafine particles have been shown to be very efficient on a mass basis to cause inflammation (Oberdorster, 2001) also other fractions of PM are able to modulate the inflammatory response. Both the soluble metal fraction (Kodavanti et al., 2001) and endotoxin (mainly on coarse particles) have been shown to either induce or prime the inflammatory response (Monn and Becker, 1999).

1.1.3 Inflammation, Genotoxicity and Proliferation

Inflammation has been recognised in both acute and chronic effects of air pollution, including genotoxic and mutagenic outcomes. In the lung the mechanism underlying this carcinogenic response is still unclear, although it is postulated that reactive oxygen species (ROS), derived from inflammatory neutrophils play a key role (Dungworth et al., 1994; Driscoll et al., 1997). Upon activation, neutrophils and other phagocytes generate superoxide (O2•-) by the reduction of oxygen, a reaction catalysed by the enzyme NADPH-oxidase. During this 'respiratory burst' the action of additional neutrophilic enzymes, such as superoxide dismutase, myeloperoxidase and nitric oxide synthase give rise to basically four oxidants: superoxide (O2•-), hydrogen peroxide (H_2O_2), nitric oxide (NO•) and hypochlorous acid (HOCl) (Babior, 2000), which are originally manufactured for killing invading micro-organisms. However, in the early eighties, studies performed by Weitzman and Weitberg showed that products of the respiratory burst of neutrophils caused both cytogenetic changes and malignant transformation in target cells, clearly contributing to the hypothesis that inflammation and carcinogenesis are linked via ROS-induced damage to target cellular DNA (Weitberg et al., 1983; Weitzman et al., 1984).

ROS are supposed to be involved in carcinogenesis by their capacity to induce genotoxic effects to cellular DNA (Wiseman et al., 1996). Apart from testing single reactive oxygen or nitrogen species, in vitro models have been used in which target cells are co-incubated with neutrophils. Early work using these models demonstrated that neutrophils were able to induce sister chromatin exchanges in co-cultured target cells (Weitberg et al., 1983). Shacter et al. (1988) showed that neutrophils induced strand breaks in neighbouring cells, and they suggested that hydrogen peroxide was the essential mediator of these genotoxic effects. More specifically, hydroxyl radicals (•OH), intracellularly produced from H_2O_2 by Fenton chemistry or by decomposition of peroxinitrite ($ONOO^-$, an product of the reaction of $O_2^{\bullet-}$ with NO•) are thought to be the most likely ROS to be responsible for these neutrophils-mediated DNA damaging effects (Schaufstätter et al., 1988). This is further confirmed by experiments performed in our laboratory, demonstrating that neutrophils caused the induction of the •OH specific DNA base lesion 8-Hydroxydeoxyguanosine (8-OHdG) in alveolar type II cells in vitro (Knaapen et al., 1999). These in vitro data are in line with observations in rat

lungs, where 8-OHdG was induced in epithelial cells during particle-elicited inflammation (Nehls et al., 1997). In rats, chronic exposure to particles can lead to tumour formation. Genetic alterations induced by neutrophil-derived oxidants, and increased cell proliferation are thought to be key factors in this mechanism. (Borm et al., 1997; Driscoll, 1997). However, apart from these studies, it is still speculative whether neutrophils directly contribute to genotoxicity in lung epithelial cells.

Cell proliferation is also a major mechanistic consideration in chemical carcinogenesis for both genotoxic and non-genotoxic agents (Swenberg et.al., 1993). Increased cell proliferation decreases the time that is available for DNA repair of adducts from exogenous and endogenous sources. DNA-reactive chemicals are far more effective mutagens and carcinogens when there is also increased cell turnover. DNA synthesis is involved in chromosomal aberrations, insertions, deletions and gene amplification, which in turn are important mechanisms in chemical carcinogenesis. Growth factors associated with tissue regeneration can provide a selective growth advantage to and there by clonally expand initiated cell population (Butterworth et. al.,1995). Formaldehyde- induced nasal cancer in rats illustrates the importance of cell turnover as a driving force in cancer. Although formaldehyde is a weak mutagen, the patterns of formaldehyde induced necrosis and regenerative cell proliferation determine the tumor induction and the site specificity of formaldehyde-induced squamous cell carcinomas in the nasal passages of rats (Morgan et. al., 1997). The concentration dependent increases in cell proliferation correlate strongly with the tumor response curve, supporting the concept that sustained increases in cell proliferation are crucial in formaldehyde carcinogenesis (Monticello et. al., 1994).

Calderon and colleagues demonstrated that increased and sustained cell proliferation were present in the nasal respiratory epithelium of lifelong adults residents of Mexico City and in newly arrived adult individuals in a period as short as 1 week (Calderon-Garciduenas et al., 1999). The increase in nasal cell proliferation was evaluated as the percentage of cells in the replicative DNA synthesis phase of the cell cycle (S phase) was persistent through days with different levels of pollutants. This increase in cell proliferation is in agreement with other reports of induced pathological changes in the nasal passages of Mexico City travellers. These observations suggest an increased potential risk factor of developing nasal proliferative disease due air pollution including ozone and PM.

1.2 The nose as a target organ for environmental exposure

1.2.1 Nasal functional anatomy

The nasal cavity and mucosa both warm, moisten and filter incoming air and also contain receptors for olfaction. Together with the lower airways, they maintain an environment in which temperature and humidity are optimal for the main alveolar function : oxygen and carbon dioxide exchange (Proctor and Andersen, 1982). The nose conditions between 10 000 and 20 000 liters of inspired air per day and is the most important portal of entry for the gases, particulates, allergens, and micro-organisms present in ambient air (Cole, 1993; Henderson et al., 1993). The inspiratory passage of ambient air through a healthy nose is capable of eliminating a large portion of those health hazards. SO_2 is considerably more soluble than CO_2 and is clearly taken up in large airways (including nasopharynx) where it has irritant effects. Increased inspiratory flow allows deeper penetration of inhaled SO_2 to the lower airways and increased minute ventilation, increases total exposure dose. Interestingly, nasal breathing largely cancels the effect of SO_2 on pulmonary function. This may be due to absorption of the water-soluble gas by the nasal mucosa. Because most people shift from strictly nasal breathing to oronasal respiration with moderate exercise, increased levels of SO_2 may reach the bronchial airway in that fraction of inspired air which does not interact with the nasal mucosa during exercise. Asthmatics also are more likely to have nasal pathology as well (such as allergic rhinitis or sinusitis), which may further decrease nasal airflow during exercise and perhaps increase the percentage of inspired air that is not nasal tissue (Linn et al., 1983).

Fry and Black (1973) investigated the sites of particle deposition and the clearance rates in the human nose using monodispersed particles of polystyrene, labelled with ^{99m}Tc, and reported that at least 45% of the retained material was deposited in the anterior region of the nasal passages. The site of maximal deposition was 2-3 cm behind the tip of the nose. Particles deposited on the ciliated epithelium were rapidly removed by the mucociliary clearance mechanisms, while particles deposited in the unciliated region were removed relatively slowly (Hilding, 1963). Recent studies indicate that the major deposition site for the largest inhalable particles (>10μm aerodynamic diameter) as well

as for the ultra fine particle fraction (< 0.1μm). Soluble materials deposited on the nasal mucosa will be accessible to underlying cells if they can diffuse through the mucus prior to removal via mucociliary transport. Dissolved substances may be subsequently translocated to the bloodstream following movement within intercellular pathways between epithelial cell tight junctions or by active or passive transcellular transport mechanisms. The nasal passages have an extensive vasculature and therefore uptake into the blood from this region can occur rapidly (Rasmussen et al., 1990; Swift and Proctor, 1988; Swift et al., 1992).

The nasal cavity is lined by surface epithelial cell populations, each with specific roles in conducting and maintaining the normal functions of the nose. These cell populations include squamoas, transitional, respiratory and olfactory epithelium. The respiratory epithelium covers over 80% of the nasal airway and is pseudostratified and ciliated with mucus secreting cells. It is the primary cellular component in the dynamic mucociliary apparatus. This apparatus is the first line of defence for both the upper and lower respiratory tracts, eliminating potentially injurious, inorganic and organic particles and gases. Inhaled agents are deposited on the mucous layer, and are propelled by the synchronised cilia beating from the airway to the digestive tract, where the agents are eliminated from the body. For interactions to occur between inhaled materials and the epithelium, the materials must penetrate the airway lining fluid (ALF) (Leopold, 1995). ALF is a thin layer of a complex viscoelastic fluid composed of a mucus gel layer on the surface of a sol layer bathing the cilia; high molecular weight glycoconjugates are the major contributors to the high viscosity and the gel-like properties of the mucus. The main mucus glycoproteins are mucins, polyanions associated with cations and positively charged proteins. Their acidic character is due to the presence of sulphate groups, a feature observed in epithelial goblet cells which secrete more acidic mucin than their counterparts in submucosal glands (Stahl and Ellis, 1973). Four proteins compose 40-60% of the total protein in human nasal secretions: serum albumin, lysoszyme, immunglobin A and lactoferrin. The total protein content of human nasal mucus ranges from 1.6 to 2.5 mg/ml (Brofeldt et al., 1986). Lipids (predominantly cholesterol and lesser amounts of phospholipids), ions (sodium, chloride and potassium), small molecular weight organics, antioxidants, and inflammatory mediators also are present in the nasal fluids.

Antioxidants play a critical role in protecting the epithelium against air pollutants and xenobiotics. Humans have high levels of uric acid and low levels of ascorbic acid in nasal lavage fluids (NALF), and levels of these antioxidants could affect the relative susceptibility of the nasal epithelium to oxidant pollutants (Hatch, 1992; Housley et al., 1995a,b; Peden et al., 1990; 1993; Rapheal et al., 1989). Maintenance of the mucus layer through various active transport processes, regulation of the secretory rate of mucin, and epithelial integrity are important factors contributing to the normal function of the mucociliary apparatus in the nose.

Secretory immunity is an important defence system of the nasal airway mucosa (Bachert and Moller, 1990). Mainly through secretory immunglobulin A (SIgA). Secretory IgM (SIgM) and serum derived and locally produced IgG also contribute to epithelial surface protection, but to a lesser extent (Brandtzaeg, 1995). Winther et al. (1987) estimate the lymphocyte to monocyte/macrophage ratio in nasal mucosa from healthy adults to be approximately 10:1, the T cell to B cell ratio to be 3:1, and the T helper/inducer cell to T supppressor/cytotoxic cell ratio is 2.5: 1. In addition, the authors observed regional differences with a relatively increased number of T suppressor/cytotoxic cells around submucosal glands, and a relatively large number of B cells in lymphocyte aggregates in the lamina propria. The HLA-DR antigen was expressed in epithelial cells, suggesting involvement of the surface epithelium in local immune responses. Diesel exposure causes enhanced expression of chemokines including RANTES (Regulated upon activation, normal t-cell expressed and secreted), MIP-I causing the immune system to produce more IgE and allergic inflammation (Nel et al., 1998).

Plasma extravasation from the profuse subepithelial microvessels and the subsequent exudation of plasma across the mucosa into the lumen of upper airway is not just an exaggeration of the normal baseline exchange of fluid and solutes between the capillary circulation and the mucosa (Erjafalt and Persson, 1989). Plasma exudation seems to be a specific inflammatory response in human airways. It is also a general response in the sense that it occurs regardless of which cellular or non-cellular mechanism drives the inflammation. Therefore, mucosal exudation of plasma reflects equally the inflammatory airway response in allergy and in infection. Plasma exudation is not only reflecting the intensity of inflammation but also contributing to the vicious circles and sequelae of airway inflammation (Persson et al., 1992).

1.2.2 Nasal sampling techniques

From the above it is clear that an intact nasal function is important for the respiratory system. A wide range of techniques is available to assess and quantify nasal function in human subjects. If pollutants are water-soluble, respiratory irritant capable of causing cellular damage, the effects should be detected in the nasal passages (Calderon-Garciduenas et al., 1994). Since many of the cell types found in the nasopharyngeal region are the same or similar to cells found in the trachea or bronchi, we can learn about inflammatory mechanisms by studying the readily accessible human nasal mucosa rather than the relatively inaccessible bronchial mucosa. Lymphocytes, eosinophils, mast cells, many other migrating cells and the epithelial lining cells in the airway have the capacity to procedure and release different mediators and enzymes as well as cytokines. Much of the current research in rhinitis and asthma is focused on cellular changes in biopsy material and lavage fluid. These materials are analysed by molecular biological techniques and have shown similarities between the nasal and bronchial mucosa.

There are several methods of obtaining nasal cells and different techniques sample different compartments of the nasal mucosa. A summary of these methods is given in the following paragraphs.

1.2.2.1 Blown secretions

Blowing the nose onto wax-paper or directly onto a glass slide, followed by air drying and staining of the cells is the first method to sample and investigate nasal epithelial cells. A modification of this technique involves instillation of 1 ml of normal saline solution into each nostril followed by blowing of the nostril being studied, while the other is gently blocked with a finger . The technique is specially suitable for a quick view of the cell population in nasal secretions in such clinical situations as allergic rhinitis (Tonnesen and Hindberg, 1988). The disadvantages include obtaining only those cells present in nasal secretions, dependence on the presence of secretions at the time of specimen acquisition, and restriction of sampling to the anterior regions of the nose.

1.2.2.2 Nasal smears

Nasal smears are obtained with cotton-wool swabs gently moved along the anterior to the posterior part of the inferior and/or the middle turbinates. The swab is then either smeared over a glass slide and the specimen is fixed and stained or it is immersed in a transport fluid for microbiology culture techniques. This method yields cells from nasal secretions and the superficial portion of the nasal mucosa. The results are not uniform and are difficult to evaluate from different subject populations. One application of this technique is to determine the presence or absence of a specific cell type (e.g.eosinophils) as a relative proportion of total cells. This technique has also proven useful for detection of upper respiratory tract carriage of Streptococcus pneumonae and Haemophilus influenzae in healthy children (Capeding et al., 1995).

1.2.2.3 Imprints

In this method, plastic strips covered with 1% albumin are introduced into the nasal cavity under direct vision and are gently pressed over a nasal mucosa surface. This strips are then fixed, stained and examined under the microscope. The material obtained contains nasal secretions and superficial epithelium. The disadvantages of this technique include the presence of mucus on the smears that obliterates the cell morphology owing to heavy mucopolysaccharide staining, and the small numbers of epithelial cells obtained (Pipkorn and Enerback, 1984).

1.2.2.4 Nasal brush

In this technique, epithelium is harvested with a small brush made of plastic-coated steel wire with nylon bristles. The brush is introduced into the nasal cavity under direct vision and is rotated while being introduced and removed. The brush is then submerged into a saline-buffered solution or transport media if cell culture is the final choice (Black et al., 1998). The brush is shaken so the harvested cells go into suspension. The advantages of the procedure are the quantification of the total number of cells per

volume by hemocytometer, staining of multiple slides for quantification of different cell types, and the use of cell pellets for biochemical analysis. The disadvantages include a mild and transient nasal discomfort and possible limitation of the number of cells obtained, depending on the dexterity of the operator and the brush surface (Jacobson et al., 1999)

1.2.2.5 Nasal scrapings

A specimen of nasal secretions and epithelium is obtained by using a plastic curette to scrape the nasal mucosa under direct vision. The inferior and middle turbinates are easily reached with good tolerance from the subject and minimal discomfort (sneezing and tearing in the ipsilateral eye). The scraping can then be smeared on a slide, submerged in neutral formaldehyde, or used for tissue culture in an appropriate culture media. The advantages of this technique are the specificity of sampling site, ease of repetition, good numbers of cells and minimal discomfort. The disadvantage is that the samples are fragmented and small, thus if the tissue is abnormal to begin with, it is difficult to keep is architecture intact when the histo-technician embeds the tissue in paraffin and cuts the material. Therefore, this technique requires a skilled person to handle the tiny specimens and orient the histology cuts (Lin et al., 2001).

1.2.2.6 Nasal biopsies

Both the inferior and the middle turbinates are accessible for biopsy procedures and control of possible complications. The procedure requires local anaesthesia by topical application of a decongestant such as oxymetazoline or phenylephrine, followed by a local anaesthetic (Lidocaine or tetracaine). Some subjects require injecting the biopsy site with a solution of 1% Lidocaine and 1:100 000 epinephrine. The biopsy specimen can be obtained with a punch, Geristma or Takahashi forceps, and post biopsy bleeding is controlled by a vasoconstrictor. The tissue can be frozen or fixed for molecular, immunhistochemical or microscopic studies. The major advantages of biopsy specimens is that all layers of the nasal mucosa are sampled from the epithelium to the basal

membrane to the submucosa, and one is able to sample macroscopically abnormal areas and include adjacent unremarkable areas for comparison. Any number of special techniques can be used with these tissues and it is possible to obtain several dozen slides from each biopsy. The disadvantages include discomfort for the patient, the need for anaesthesia, limitation of the procedure to adults, and the limited number of specimens that can be obtained from the same person. Possible complications include bleeding, pain and synechiae formation. A good clinical history should be taken first by a physician with special attention to previous bleeding disorders, consumption of drugs that alter bleeding time, previous family and personal history of local anaesthetic reactions, and use of prescribed or non-prescribed medications or drugs that may alter the nasal mucosa. This procedure should be done by a physician with previous experience in this technique (Calderon-Garciduenas et. al, 1992).

1.2.3 Nasal Lavage as a tool to detect inflammatory effects

Since 1870 nasal lavages have been used. At that time this technique was used for cleaning the nose, where the fluid was introduced through one nostril and was run out through the other nostril. Now nasal lavage is used extensively to assess many biomarkers including inflammatory cell influx, antioxidants, eicosanoid mediators, neuropeptide release, nasal glandular products, increased vascular permeability products, cytokines, and other products from cells such as neutrophils, eosinophils and mast cells (Graham et al., 1988; Graham and Koren, 1990; Koren et al., 1989; Noah et al., 1995; Peden, 1996; Tonnesen and Hindeberg, 1988).

Nasal lavage (NAL) is a simple and economical method to perform, relatively non-invasive, and allows multiple sequential sampling of both nasal secretion and cells from the same subject. However, most nasal lavage methods are limited by the fact that the lavage fluid does not remain in contact with the mucosa for long time . Most variations of the technique require the subject to bend the head back, which contaminates the nasal lavage with nasopharyngeal secretions. Housley et al. (1995a,b) developed a method that allows for prolonged nasal lavage and avoids contamination from the nasopharynx. Subjects are asked to gently blow their noses and then report which nasal cavity had the

greatest resistance to airflow. This cavity is recorded as open and the opposite one as closed. The balloon of a modified Foley catheter is then inflated inside one of the nostrils to occlude it. Subjects are asked to lean forward at 45^{o} and 5ml of 0.9% sterile saline at room temperature are passed into the cavity and left for periods of up to 10 min. The procedure is then repeated for the other cavity and samples are collected. A modification of the above method is the uses a uretral catheter provided with an inflatable balloon. The balloon is inserted into the nostril, and gently inflated by a syringe. While the subject is sitting in writing position, a syringe is filled with 10ml of pre-warmed Hanks`balanced salt solution and the fluid is recorved by retracting the syringe. This procedure is carried out in each nostril (Grunberg et al., 1996).

A more labour-intensive method was developed by Peden et al., in which the nasal lavage is performed using a disposable metered-dose nasal inhaler (nasal sprayer) filled with sterile, room –temperature normal saline solution. This device is actuated 5 times in one nostril while occluding the other nostril, then the subject is instructed to exhale through the lavaged side into a specimen cup. The subject then repeats the entire cycle six times in each nostril. Total input of saline solution is approximately 7 ml, and median return is 37% for normal subjects. One advantage is that the study subject is in control. The disadvantages are that the final volume depends on the force used in the insufflation, and the sampling is limited to the anterior part of the nose (Peden, 1996).

Greiff et al. (1990) used a compressible plastic container “nasal pool” (NP) is used. This device has a nasal adapter, or nozzle, attached to its upper ending. The nasal adapter is inserted into the nasal opening of the subject sitting in a forward –flexed neck position (60^{o}) from the upright position. The NP device, filled with 14 ml fluid is fully compressed by the subject. Approximately 12 ml fluid is thus introduced into one nostril. The instillate can be maintained in the nasal cavity as long as the container is compressed and the subject feels comfortable, which may be for 15 min or even longer. The advantage is the possibility of the controlling of comfortable feeling for the subject. The drawback is that he sampling is limited to the anterior part of the nose.

The nasal lavage technique which we used for our study was first described by Graham and Koren (1990). The volunteer in sitting position, lift his neck 45^{o} backwards and elevate the palate to close their nasopharynx. Five ml of sterile phosphate buffered saline without Ca^{2++} and Mg^{2++}, heated to $37^{o}C$, is instilled into each nostril with a 10ml sterile pipette, while the volunteers is not breath and swallow. After 10 seconds the

volunteers put his head forward and the nasal lavage fluid is pelled into a 15 ml centrifuge tube via a polyamide gauze (100 mesh) filtered funnel to separate mucous. Tubes are immediately placed on ice and the total volume (at 0.1ml confidence) is determined. It is important that NAL are stored on ice continuously from the moment of sampling until further processing. Cytospins should be stained within 24 hrs after preparation and fixed. The samples are centrifuged (600g/ 5 min at 4^{o}C) and the supernatant removed and transfer into glass tube in ice . Later this is used to take 8 time 250µl portions of NALF to be frozen in Micronics at –20^{o}C for measurement of soluble mediators analysis. The total cell count is determined using a cell-count chamber. The cytospins preparations are made of the remaining cells, which are later on stained with the May-Grünwald–Giemsa method for cell differentials. The counting of differential cells cover the neutrophils, eosinophils, lymphocytes, monocytes and epithelial cells.
This technique samples cells in nasal secretions and has the advantage of being easy to perform, reproducible, painless and repeatable. Measurements can be taken at frequent intervals to follow the kinetics of cellular and biochemical changes. Harvested cells can be used for ex vivo studies and there is usually a high cellular yield (McCaffrey, 1997).

1.2.4 Biomarkers measured in NAL

Parameters that are assayed in nasal lavage fluid include cells and a number of soluble immune mediators and exudate markers. It has been shown that in experimental studies in which volunteers were exposed to ozone and in epidemiological studies at elevated concentrations of photochemical air pollution the number of polymorhnuclear leukocytes (PMNs), like neutrophils, and to a lesser extent eosinophils has been increased in NAL (Frischer et al., 1993; Graham and Koren 1990; Seltzer et al., 1986), and in BAL (Graham and Koren, 1990). For this reason, an increase of the number of PMNs may be considered as a biomarker of effect for exposure to ozone. NAL has also been used to study antioxidant status of the upper respiratory tract (Kelly et al., 1996), mediators produced in human exposed to ozone (Graham and Koren, 1990; Frischer et al., 1993; Devlin et al., 1994; Steerenberg et al., 1996), cytopathology of the nasal mucosa of humans exposed to diesel engine exhaust (Diaz-Sanchez et al., 1996). Also cytokines have been measured in epidemiological studies of air pollution, and they

showed increased production of interleukins (e.g.IL-6 and IL-8) (Steerenberg et al., 1995; Keman et al., 1998; Hiltermann et al., 1997). Also clinical studies in patients with nasal disease have shown increased secretion of cytokines including IL-6, Il-8, IL-1 and TNFα (Bachert et al., 1995; Bachert and Van Cauwenberge, 1997).

NAL was also used to study inflammatory responses of humans occupationally exposed to wood dust (Borm et al., 1997) and cotton dust (Keman et al., 1998); Schins and colleagues also used the nasal lavage technique to study nasal inflammatory and respiratory parameter in human volunteers during and after repeated exposure to chlorine (Schins et al., 2000). These studies clearly showed the utility of this technique in various settings. There is a possibility that the non-invasive NAL procedure can be used as a surrogate for the more costly and invasive broncho-alveolar lavage (BAL) procedure in epidemiological studies. It should be considered that mucosal inflammatory responses in the tracheo-bronchial airways agree better with nasal mucosa responses than with responses occurring in the terminal airways and parenchymal region as shown by bronchoalveolar lavage material (Persson et al., 1992).

In **Table 1-2** normal values of cell numbers, PMN count and IL-8 are listed from various studies to show the consistency of our method.

Cells: After nasal lavage in a normal population the average proportion of neutrophils between 70 and 80% of all leucocytes. The average proportion of eosinophils is 11.1 ± 24% and for lymphocytes 0.2 ± 0.6 (Steerenberg et al., 1996). In asthmatic and allergic subjects eosinophilic and mast cells will be more prominent present (Bascom et al., 1996; Noah et al., 1995). In all studies PMNs correlated with increased ozone exposure (Graham and Koren, 1990; Koren et al., 1989; Frischer et al., 1993; Graham et al., 1988), and can therefore be considered as a measure for a non specific inflammatory reaction.

Myeloperoxidase (MPO): The granules of neutrophils contain a large number of different enzymes. One is MPO, which is present in the primary granules. The MPO-H_2O_2-halide system is toxic to many organisms including bacteria, fungi, viruses, mycoplasmata and parasites (Klebanoff and Rosen, 1978), as well as to many cells of the host (Klebanoff et al., 1976). About 95% of the cellular MPO in the circulation in present in the neutrophils whereas the remaining MPO is contributed by the less numerous monocytes (Bos et al., 1978). Activation of the neutrophil's degranulation causes increased MPO concentration in NAL. Moreover, the concentration of MPO correlated with the number of leukocytes in NAL (Steerenberg et al., 1996) and increased after experimental (Graham and Koren, 1990) and ambient exposure (Frischer et al., 1993) of ozone.

Eosinophilic cationic protein (ECP): Eosinophilic granulocytes may be activated locally at the site of the inflammatory reaction. After stimulation they are capable of releasing highly toxic proteins e.g. ECP that are able to distrupt the integrity of the epithelial lining of the airways (Svensson et al., 1990). It is not clear whether ECP is produced locally or whether it is due to the leakage of plasma levels of ECP (Klementsson et al., 1990). In general high levels of ECP are in accordance with high numbers of eosinophilic granulocytes in allergic patients (Svenson et al., 1990). Moreover, the ECP concentration is increased after elevated ambient ozone concentrations (Frischer et al., 1993; Kopp te al, 1999).

Tryptase: This tetrametic neutral protease is found in basophilic granulocytes and mast cell secretory granules (Juliusson et al., 1991; Castells and Schwartz, 1988) and together

with histamine it is released during degranulation (Schwartz et al., 1981). Tryptase is absent in NAL of healthy subjects (Juliusson et al., 1991; Castells and Schwartz, 1988), but , it seems that tryptase is detectable immediately after ozone exposure (Koren et al., 1989).

Interleukin 8 (IL-8): This interleukin belongs to the super family of chemokines characterised by potent neutrophil-activating properties (Van Damme, 1991). In animal studies intradermal injection of picomolar amounts of IL-8 induces neutrophil and neutrophil dependent plasma protein extravasation (Rampert et al., 1989). IL-8 can be produced in vitro by several cell types including macrophages, lymphocytes, neutrophils, fibroblasts and endothelial cells in response to a variety of stimuli such as endotoxin, viruses, other cytokines and ozone (Bazzoni et al., 1991). Nasal Challenge with IL-8 suspensions in atopic and non-atopic subjects induced a significant increase of neutrophils and eosinophils infiltration, indicating that IL-8 is a powerful polymorphnuclear leucocyte chemoattractant in nasal mucosa (Bachert and Ganzer, 1993; Douglass et al.,1994).
IL-8 in nasal lavage fluid is probably produced by the nasal mucosa epithelium, and its release is enhanced by organic types of dust, viruses, chemical noxes and also by TNF - α; increased concentrations of IL-8 were found in NAL of cotton workers (Keman et al., 1998) and in patients with chronically inflammatory illnesses of the nasal mucosa (Kremer et al., 2000) .

Interleukin 6 (IL-6): This is a multifunctional cytokine produced by a variety of cells, including T-lymphocytes, macrophages, neutrophils, eosinophils, fibroblasts, endothelial cells and mast cells (Snick, 1990; Juliusson et al., 1991). IL-6 has been found to play a central role in inflammatory. IL-6 mRNA was found in nasal mucosa of only 6 patients among 49 with polyposis or sinusitis (Davidsson et al., 1996). In a similar group of patients IL-6 protein was detected 14 out of 25 patients (Kremer et al., 2000). In healthy individuals, only 5 out 27 subjects showed detectable IL-6 protein in nasal lavage. Similar data were reported in a smaller set of individuals measured repeatedly over time (Steerenberg, 1996).

Exudate markers: In the secretions lining the human nasal cavity uric acid has been shown to be the only low molecular weight antioxidant present in abundance. Because uric acid originates from the plasma, it is possible that factors affecting the levels of plasma uric acid will also alter the levels recovered in lavage fluids (Housley et al., 1996). Small changes in permeability of membranes by ozone may increase the concentrations of low molecular compounds (uric acid (M= 168) and urea(M=60). Larger changes may increase the concentration of macromolecules such as albumin (M= 65.000) or clara-cell protein (Koren et al ., 1989). Moreover , it is suggested that uric acid is probably the most important scavenger of ozone in human blood plasma and that uric acid secretion by the airway mucosa may serve as an important protective response to respiratory ozone exposure (Peden et al., 1993). In a number of studies increased albumin concentration correlated with ozone exposure (Graham and Koren, 1990).

Nitric oxide (NO): Nitric oxide is an important intercellular messenger in blood vessels, airways and in the brain. It is also an important toxin produced by macrophages and neutrophils, along with high concentrations of reactive oxygen species such as superoxide and hydrogen peroxide, as a defence against infections. Endogenous NO may play an important signalling role in the physiological control of airway function and in the pathophysiological of airway diseases (Barnes and Kharitonov, 1996). The endogenous NO is generated from the amino acid L-arginine by the enzyme NO synthase, of which three distinct isoforms exist. The concentration exhaled NO is increased in patients with inflammatory diseases of the airways such as e.g. asthma, cystic fibrosis, and lower respiratory tract infection. Furthermore it has been hypothesised that NO regulates the T-helper 1 and T-helper 2 balance in favour of T helper 2 type IgE mediated allergic reactions. Besides in exhaled air, NO can also be measured in nasal lavages (Barnes and Lieuw, 1995).

Table 1-1: Summary of several human studies on inflammatory markers in nasal lavage fluid in response to airborne exposure in volunteer an field conditions

Exposure	Markers	Results	Reference
Ozone: 0.4 ppm 86-230µg/m^3	PMNs, Cells ,MPO ECP, Tryptase, IL-6 Albumine Uric acid Urea	Increased Increased Increased Increased Increased	Koren et al., 1989 Fischer et al , 1993 Devlin et al., 1994 Graham and Koren, 1990
Diesel exhaust particles 0.15 mg DEPs suspended in 200µl of saline	IL-2, IL-4 IL-5, IL-6 IL-10, IL-13 IFN-γ IgE	Increased Increased Increased Increased Increased	Diaz-Sanchez et al., 1996
Ozone: ambient and time-series studies, children	PMNs ECP	Increased Increased	Kopp et al., 1999 Calderon, 1999
Ozone	PMNs, ECP,MPO IL-8 Tryptase IL-6	Increased Increased No change No change	Steerenberg et al., 1995
Swine dust, 21 mg/m^3 (1.2 µg/m^3)	PMNs IL-1, IL-6, TNFα	Increased Increased	Wang et al., 1997
Wood dust (Meranti) Different exposure classes (up to 5 mg/m^3), n=980	Total cells	No difference between exposure classes	Borm et al., submitted
Cotton dust, 1.3 mg/m^3 (1500 – 3800 EU/m^3), n=11	IL-8 Total cells, LBP Albumin BPI, BPI/LBP-ratio	No change decreased Increased Increased	Keman et al., 1998 Borm et al., 2000
Chlorine (Cl_2) 0.1- 0.4 ppm, cross-over design, n=7	PMNs, Total cells IL-8, Albumin	No change No change	Schins et al., 2000

Abbreviations: see list on page V. For those not in this list: EU, Endotoxin Units; IL_x: Interleukin-x; PMN: Polymorphonuclear leucocytes; MPO: Myeloperoxidase; ECP:Eosinophilic cationic protein; IFN-γ: Interferon-gamma; INFα: Inter-feron alpha; BPI: Bactericidal-permeability protein; LPS: Lipopolysaccharide; LBP: LPS-binding protein

Table 1-2: Comparison of several biomarkers in nasal lavage among different studies using the same technique

	Log Total cells	% PMN	IL-8	References
Wood workers				
Male (456)	3,66 ± 0,85	ND	ND	Borm et al., in press.
Female (385)	3,69 ± 0.78			
Volunteers (n=12)	3,17 ± 0,56	88 ± 24	103 ± 74	Steerenberg et al., 1995
Volunteers exposed to chlorine (n=8), 32 measurements per subject	4,24 ± 0,49	82 ± 6	653 ± 171	Schins et al., 2000
General population				
Adults (n=501)	4,27 ± 0,64	71± 24	285 ± 392	This study
Children (n= 381)	4,57 ± 0,69	81± 14	1056 ± 942	

ND: Not Determined

1.3 Study aims and design

In the previous text we have documented that environmental air pollution, including oxidant gases and particles, can cause inflammatory effects in the respiratory tract that can be estimated by using the nose, and more specifically nasal lavage and brush as tools. Various biomarkers have been described as useful in this context, and have been validated for this purpose. Inflammatory alterations in the rat lung have also been shown to be associated to mutagenic (Driscoll et al., 1997), proliferative and malignant processes (Borm et al., 2000). Whether a similar connection exists in the human airways remains unknown.

In this study we investigated inflammatory, proliferative and genotoxic effects in the upper respiratory tract in subjects from 3 cities in a relatively small area. More specific study purposes were:

1) *to study the relation between the above effects and exposure,*
2) *to study the interaction between inflammation, proliferation and genotoxicity.*

Therefore a cross-sectional study among 1200 pre-school children and their mothers of 3 cities in Germany (NRW) including a smaller control site was performed from February until June 2000. We anticipated that in our subjects inflammation measured by total cell counts, IL-8 and number of neutrophils in nasal lavage is up regulated by (1) ambient exposure to PM and oxidant gases (2) the constituents of PM such as transition metals and endotoxins, and (3) individual risk factors such as allergy, smoking and the presence of disease. The impact of environmental exposure on genotoxicity and cell proliferation was considered secondary to inflammation, but nevertheless investigated directly as well. To do so we selected 80 non-allergic children from the upper (30), middle (20) and lower (30) quartiles of nasal inflammation based on their total cell count. In nasal epithelium from these subjects obtained by nasal brush both oxidative DNA damage and cell proliferation were estimated by immunohistochemistry and the outcomes related to personal characteristics, inflammation measured by nasal lavage and environmental exposure. In addition, all mothers of these children were included for similar determinations. Chapter 2 describes the outcome of the first study purpose, i.e.

to relate nasal inflammation to environmental exposure. Chapter 3 describes the second study purpose, i.e. to study the interaction between nasal inflammation and DNA damage and proliferation in nasal epithelium. All outcomes and their meanings are discussed in Chapter 4 as a General Discussion.

1.4 References

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CHAPTER 2

2 Ambient exposure and nasal inflammation in adults and children[1]

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2.1 Abstract

The epidemiological evidence that ambient exposure, including particulate matter (PM) is related to adverse health outcomes continues to mount. Inflammation and disease of the upper respiratory tract are commonly suggested as effects of ambient exposure. Therefore we studied both ambient exposure and nasal effects in a 4 month cross-sectional survey in Nordrhein-Westfalen (Germany). At 4 locations in 3 different cities (sites A, B1, B2, and, C) ambient exposure to TSP, O_3, NO_x and SO_2 was derived from compliance measurements by governmental offices and 1176 subjects (actual nasal lavage samples: 501 mothers and 381 children, 6-7-years) were screened using nasal lavage, with success rates of 90 and 75 %, respectively. No differences in total cell counts or percentage of neutrophils were found between mothers or children from the 4 different sites, despite small but significant differences in ambient exposure to TSP, SO_2, O_3, and NO_x between different cities during this period. A higher epithelial cell

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count in mothers and children from one city might be related to general higher ambient pollution in that location. Interestingly, total cells and interleukin-8 levels in children were higher than in mothers and possibly reflects their increased susceptibility to effects of air pollution. Future analysis will include individual risk factors such as allergy, smoking and the presence of disease.

2.2 Introduction

The epidemiological evidence that ambient exposure is related to adverse health outcomes continues to mount. The effects of ambient particular matter, including morbidity and mortality, are most evident in the elderly and those with pre-existing impairments in cardiopulmonary health (Samet et al., 2000; WHO, 1999) and impose considerable costs to current society (Künzli et al., 2000). These findings and the stronger adverse health associations with the fraction of ultra fine particles has been reason to promulgate an additional standard such as $PM_{2.5}$. However, major uncertainties are still present regarding the biological mechanisms involved in the dramatic responses to PM in susceptible groups to such small variations in particle mass and the effect of other co-exposures such as ozone, NO_x and SO_2.

Despite this lack of understanding of the exact mechanisms, most studies indicate the involvement of oxidative stress, inflammation and immune-modulating effects in airways and lungs (MacNee & Donaldson, 1999; Nel et al., 2001). Since there is a need for sensitive markers that can identify early responses to ambient exposure and in particular PM inhalation (Schlesinger, 2000), the above processes form the basis of current and future biomarkers to ambient PM exposure.

We designed a study among 1200 inhabitants of 2 major cities in Germany and a smaller control site to compare in vivo biological response in the upper respiratory tract with effects that can be induced in vitro by particulates sampled during the health effect screening period. The study is also linked to the ongoing East-West comparison since subgroups of 6-7 year old children were included for both purposes. By application of nasal lavage and nasal brush we investigated inflammation and will relate the outcomes to in vitro activity of sampled particulates using similar endpoints. We anticipated that in our subjects inflammation in nasal lavage is upregulated by (i) total ambient exposure

to TSP and oxidant gases, (ii) the constituents of PM such as transition metals and endotoxins, and (iii) individual risk factors such as allergy, smoking and the presence of disease. We now report on the first theme and have studied total cell counts, cell differentials and interleukin-8 (IL-8) in nasal lavage as biomarkers of inflammation in the nose and describe differences between mothers and children as well as between different measurement sites.

2.3 Methods

Both exposure and human adverse effects were studied in a 4 month cross-sectional survey in Nordrhein-Westfalen (Germany) between February and June 2000. At 4 locations in 3 different cities (sites A, B1, B2 and, C) ambient exposure data are related to inflammation measured in the upper respiratory tract using nasal lavage. At each location 150 children and their mothers were investigated in vivo using nasal lavage and nasal brush measuring the in-vivo inflammation (PMN and IL-8 in nasal lavage fluid). Nasal lavage in adults was done and neutrophilic granulocytes (PMN) and IL-8 were evaluated as described previously (Borm et al., 2000). Nasal lavage in children has been done by instillation of 4.0 ml PBS in one nostril, to have a chance for a second effort. For all subjects personal characteristics were scored through a parent-completed questionnaire. Ambient exposure data on total suspended matter (TSP), SO_2, O_3, NO, NO_2, CO and weather data were retrieved from the local Landesumwelt-website as non-verified daily averages from the various measurement stations in these areas. These data were used to calculate the mean ambient exposure during the exact interval when subjects were screened. Statistical analysis was done by log-transformed data of both PMN cell counts and IL-8, since both are not normally distributed. Differences were tested using analysis of variances (ANOVA) between groups (cities), using post-hoc LSD testing with significance at 0.05 (two-sided). All statistical analyses were done using SPSS 9.0 for Windows NT.

2.4 Results

2.4.1 Exposure

Comparing the annual concentrations of pollutants over the period 1997-1999 shows that site B1 has higher daily means of annual TSP levels compared to the rural site A (**Figure 2-1**). The effect is even stronger looking at the peak-exposures that determine the 98 % percentile. The ambient exposure during the screening interval **(Figure 2-2)** in the 4 sites shows a TSP profile similar to the annual mean with highest levels in B1 (mean: 78μg/m^3) that are significantly different (ANOVA, Post-hoc testing, $P<0.05$) from the three other sites (means: 45-47 μg/m^3). Own measurements of PM_{10} during the entire interval using weekly means did not reveal significant differences in PM_{10} levels between the 4 different sites (Data not shown; ANOVA, post-hoc testing, $P <0.05$). Also the levels of SO_2, NO, NO_2 and CO were significantly higher in B1 compared to the other sites (ANOVA, post-hoc Bonferroni, $P<0.05$). On the other hand ozone concentrations were highest in A (52 μg/m^3) and B1 (34 μg/m^3) compared to the other sites. Since screening was not done randomly in the total interval, we also calculated the mean pollutant levels in each site during the same period when screening the subjects with nasal lavage **(Table 2-1)** but found no difference between the total interval and the screening weeks per site.

2.4.2 Nasal Lavage

A total of 1176 subjects (mothers and their 6-or 7-old child) were investigated and nasal lavage and total cell counts were obtained from 501 mothers and 383 children, with success rates of 90 and 75 %, respectively. Cell differentials were obtained from 460 mothers (92%) and 353 children (93 %), while interleukin-8 was measured in 96% and 84 % of samples. Total number of cells as well as number of neutrophils levels were significantly higher in children (Table 3-1), despite the instillation of less (i.e. 4 ml) of saline for nasal lavage. Also IL-8 levels were significantly higher in children (1056 ± 53

pg/ml) than in mothers (285 ± 18 pg/ml); these values are more than proportional to the lower NALF-volume and suggest higher IL-8 in the juvenile nasal epithelium. There has been an increase in the efficacy of nasal lavage in children since the success rate and recovered volume increased from the start (site C, 46 %) to the end in B1 (87%). This difference is taken into account when comparing parameters that appeared to be dependent on recovery volume in a rank correlation; the number of total cells was positively correlated with recovery-volume ($r=0.138$, $P< 0.01$) in children only, while IL-8 concentration was negatively correlated to recovery in both children ($r= -0.34$) and mothers ($r= -0.26$).

No significant differences between nasal lavage parameters of children or mothers living in different cities (**Table 2-1**, **Figure 2-3**) were observed. However, it needs to be emphasised that such analysis did not include differences in (passive) smoking, atopy, respiratory disease and specific allergies. Among cell differentials a significant difference was found between neutrophilic granulocytes, monocytes, lymphocytes (both $P<0.01$, ANOVA) and epithelial cells ($P<0.04$, ANOVA) in both children and mothers from different cities. Differences in children were mainly due to statistical differences (LSD post-hoc testing) between the control site A and B, with higher percentual epithelial counts (and NAL-volume) in B1. Differences in mothers were more scattered, but the higher epithelial cell count between B (B1 and B2) and rural site A (LSD, post hoc testing) was confirmed in this group as well.

IL-8 levels were not significantly different between children or mothers from different sites but IL-8 levels and total cells were quite different between mothers and children (ANOVA, Table 1). In both groups a significant correlation was present between log-transformed values of IL-8 and total PMN cell number ($r=0.46$, $P< 0.001$) with a highly similar fit as shown in **Figure 2-4**. This suggests that IL-8 is the cytokine primarily responsible for cell-influx in the nasal cavity. Current and future analysis concentrate on the temporal relation between nasal parameters and environmental parameters such as PM_{10} and ozone.

2.5 Discussion

Previous epidemiological studies have shown that infants and young children are a group at risk for the effects of air pollution including PM_{10} (Dockery et al., 1996; Krämer et al., 1999; Heinrich, 2000; Boezen et al., 2000). Studies using nasal lavage have demonstrated small but significant changes in PMN count and eosinophilic cationic protein , related to ozone exposure in children (Frischer et al., 1993). However, later work of the same research group also suggested a possible adaptation of nasal mucosa in spite of constant high ozone levels during summer season (Kopp et al., 1999). Although the nose is also a primary deposition organ for coarse as well as ultra fine particulates not much work has been reported to relate nasal inflammation to ambient PM exposure. A body of work has been published by Calderon and co-workers relating inflammatory and genotoxic endpoints in nasal epithelium to duration and extent of exposure to O_3 in Mexico-City (Calderon-Garciduenas et al., 1995, 1999). Saxton and co-workers (review: Nel et al., 1998) exposed volunteers to diesel exhaust particles (DEP) and found that a nasal instillation of 150 μg diesel (less than the daily inhaled dose) induced cell-numbers and cytokines such as RANTES in NALF dramatically within 24 hrs of challenge (Nel et al., 2001). Also several occupational studies have shown that inflammatory markers such as IL-8 or ECP in nasal lavage respond very sensitive to particles and/or co-exposures in cotton workers (Borm et al., 2000) or boiler-makers (Woodin et al., 1998).

Our data on cell counts, differentials and IL-8 levels in 381 children and 501 mothers did not reveal differences between the different locations, despite differences in all ambient pollutants including TSP and SO_2 that are expected to act on the upper respiratory tract. More statistical analysis is however required to digest the interference with time, air pollution, and differences in personal characteristics (such as allergy, pre-existing disease, passive smoking). An interesting finding is the increased epithelial cell count in both children and mothers living in B1 compared to the rural site. Nel et al. (2001), also reported an increased recovery of epithelial cells 24 hrs after challenge of subjects with 150 μg DEP, but did not show the data. Previously, we also noted increased epithelial cell counts in subjects after controlled exposure to low levels of chlorine (< 0.5 ppm) but found that the effect was not statistically consistent among the different dose-groups (Schins et al., 2000). Therefore, we suggest that the epithelial

shedding is a non-specific result of the combined exposure to pollutants, which is generally higher in site B1 **(Figures 2-1 and 2-2)**. However, also here a correction for (passive) smoking and other cofounders needs to be done.

In addition, the data show that the total cell count and IL-8 concentration (as well as amount) are much higher in children than adults, despite the use of a smaller lavage volume and one-nostril only. Although data needs filtering out of the allergic and asthmatic subjects, which is usually up to 15 %, it suggests that the juvenile epithelium is producing more IL-8 in normal conditions than adults, which might be linked to the increased susceptibility of this group to environmental pollutants. Current work concentrates (i) on the link between nasal inflammation and genotoxicity and proliferation in nasal epithelium obtained by nasal brush, and (ii) link the in vivo data to in vitro effects obtained with PM sampled at the 4 different sites.

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Table 2-1: Ambient exposure values (mean and SD) and nasal lavage parameters in children and mothers sampled at the different sites

City	A		B1		B2		C	
Parameter	C	M	C	M	C	M	C	M
Nr of subjects	91	121	104	105	132	146	56	129
Total cells (x 10^5)	12.7	4.87	14.2	3.77	14.5	7.69	7.38	3.77
NAL-volume (ml)	2.01	5.98	2.27	6.17	2.28	6.35	1.94	6.09
Recovery (%)	50.3	59.8	56.8	61.7	57.0	63.5	48.5	60.9
IL-8 (pg/ml)	1165	302	1001	297	1104	275	846	271
Sampling interval	20/03-07/04		10/04-29/05		10/04 – 29/05		21/02- 19/03	
Temperature (^{o}C)	8.4 (2.0)		15.7 (5.0)		17.8 (6.3)		9.5 (2.5)	
TSP ($\mu g/m^3$)	49.1 (23.9)		78.5 (29.8)		46.7 (19.3)		34.9 (12.6)	
Ozone ($\mu g/m^3$)	42.5 (18.4)		43.3 (16.9)		ND		ND	
SO_2 ($\mu g/m^3$)	5.3 (1.2)		22.5 (13.2)		9.0 (3.7)		6.1 (1.7)	
NO ($\mu g/m^3$)	6.8 (4.5)		16.5 (12.2)		6.9 (5.5)		13.7 (16.1)	
NO_2 ($\mu g/m^3$)	16.9 (8.6)		39.4 (12.0)		27.7 (10.0)		32.6 (11.1)	

C: Children; M: Mothers; NAL: Nasal lavage; TSP, Total suspended matter

Figure 2-1: Annual mean TSP concentrations and 98 % percentiles (1997-1998) in several LUA measurement stations in and around our screening areas A, B1, B2 and C

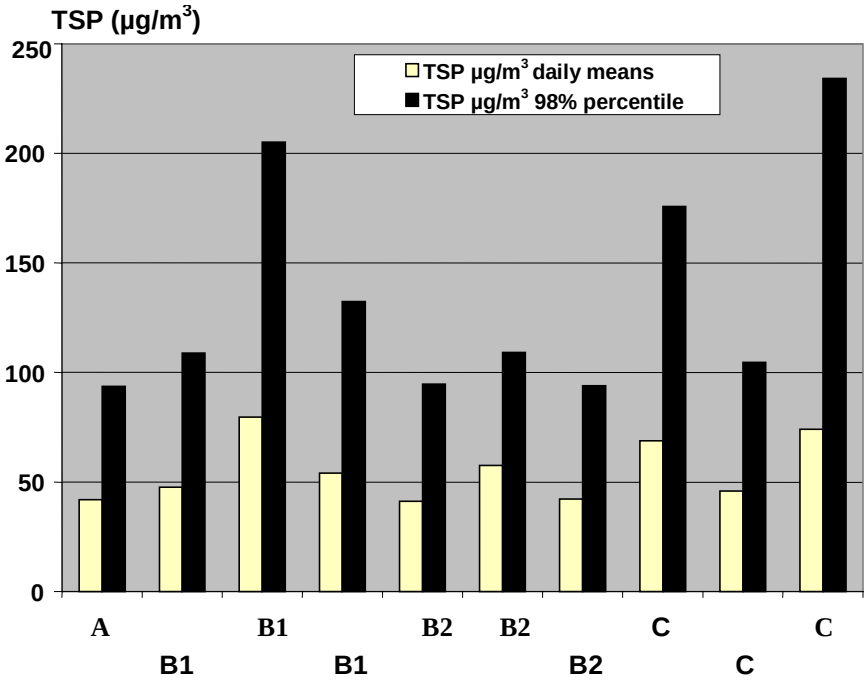


Figure 2-2: Mean exposure to SO_2, TSP, NO, NO_2 and O_3 (all in ug/m^3) measured in 4 LUA-stations that co-locate best with our sampling sites

B1 is used for north-site of B, and B2 for South-site of B. Values are the mean of all available daily exposures in each station during the entire screening period (21-02-01 through 29-05-01). Exposure values in the weeks that screening took place in each site are listed in **Table 2-1**.

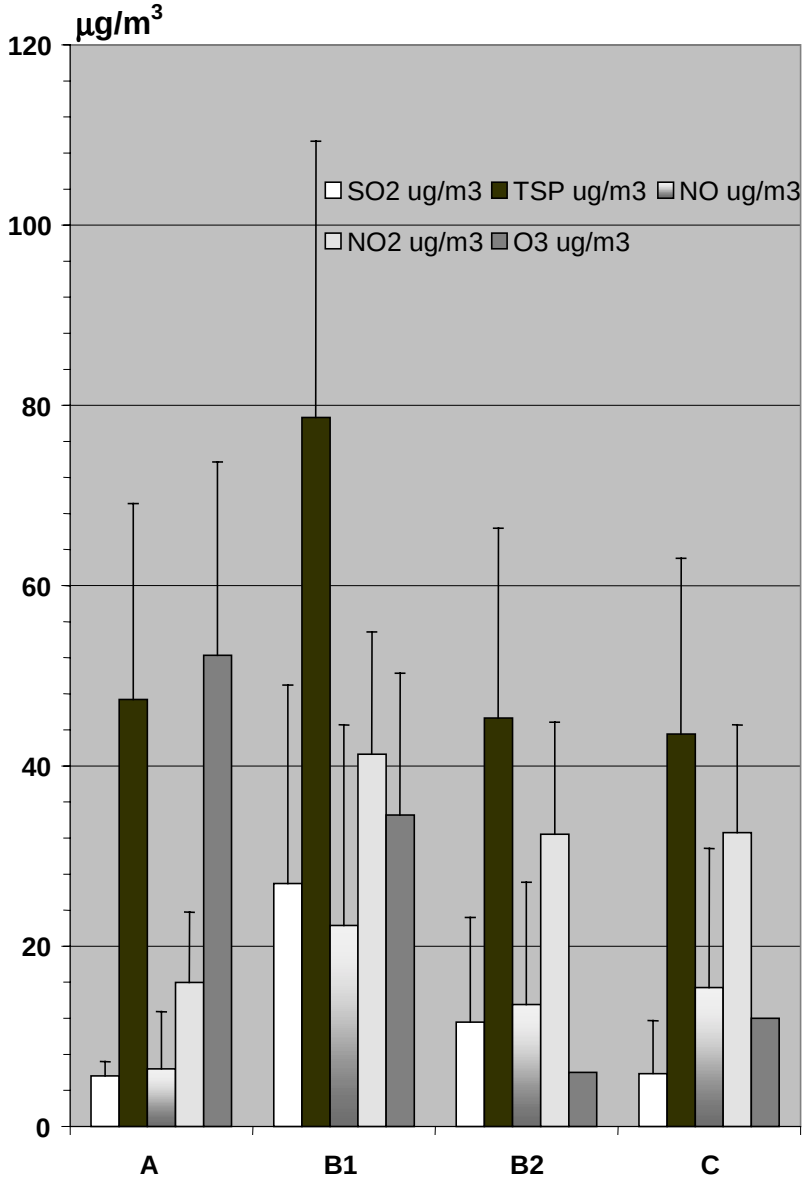


Figure 2-3: Cell differentials in nasal lavage of Children and Mothers from the 3 different cities in this study (left white bar: A, then B1, B2 and C as the right black-bar). Means plus one standard deviation are shown

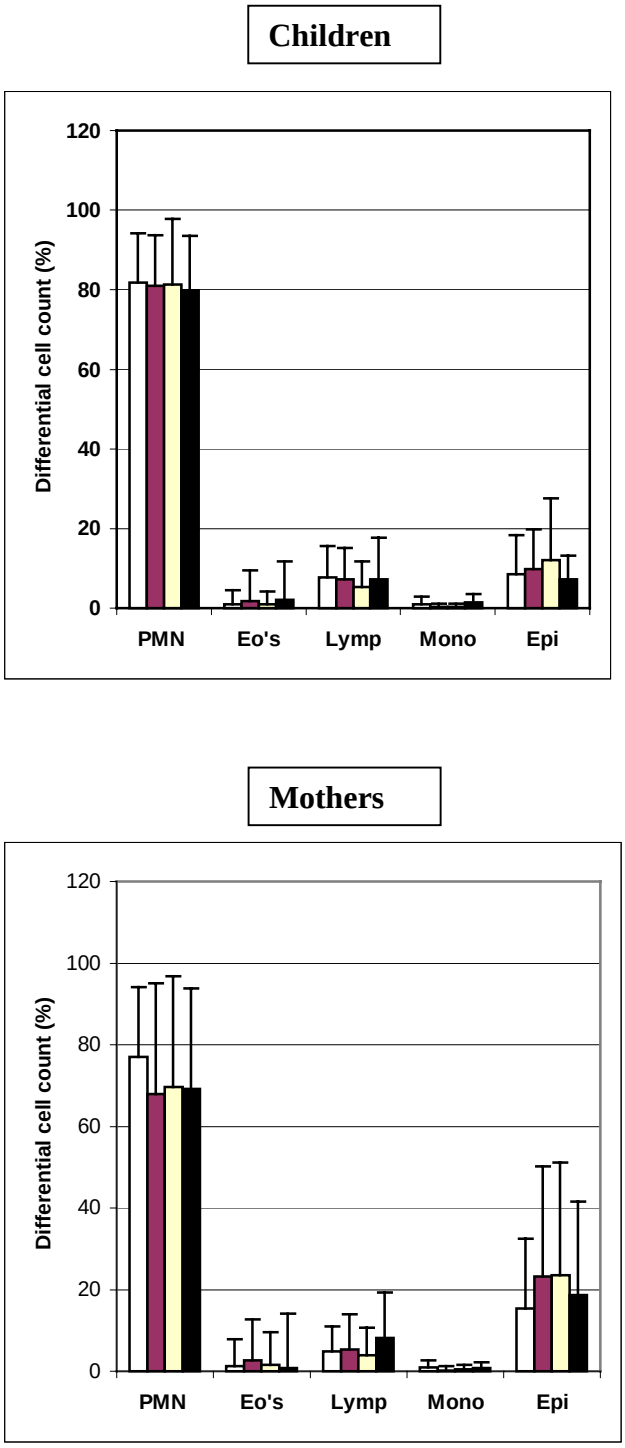
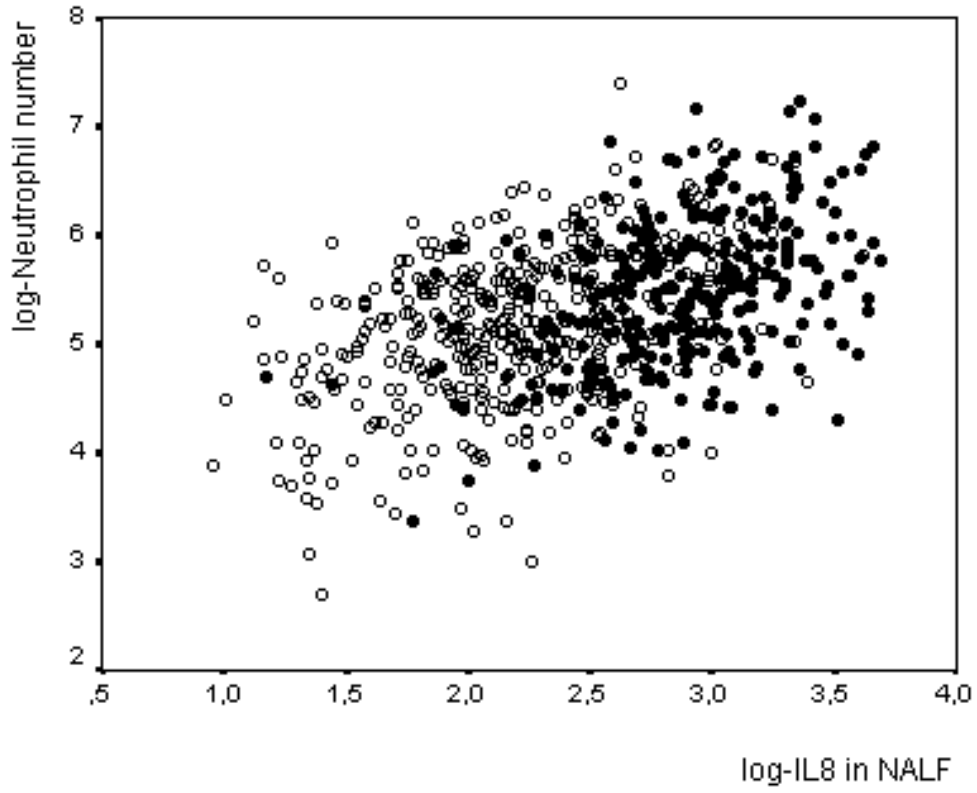


Figure 2-4: Figureal relation between log-transformed values of IL-8 and neutrophil-counts in nasal lavage of children (•) and their mothers (o)

Pearson's correlation is 0.458 (N=724, P < 0.001).



CHAPTER 3

Oxidative DNA damage and proliferation in nasal epithelium in relation to nasal inflammation[2]

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3.1 Abstract

In animal models inflammation in the respiratory tract has been shown to cause DNA damage as well as cell proliferation. It was the purpose of this study to evaluate oxidative DNA damage and cell proliferation in a human model of inflammation. The human nose was used as a target organ, comparing neutrophilic inflammation and the oxidative DNA lesion 8-Hydroxydeoxyguanosine (8-OHdG) and Ki-67 induction in epithelial cells obtained by nasal brush. Non-atopic children (n=80) and their mothers (n=80) were selected from a larger cohort (n= 1176) based on their inflammatory status (total cell count) in the nasal lavage. No association between inflammation and 8-OHdG was observed, but a clear correlation between inflammation and proliferation in epithelium was seen. In children, but not in adults, a weak link between proliferation and oxidative DNA damage was seen. Our data in the human nose forward the importance of cell proliferation as an alternative mechanism by which inflammatory cells may cause neoplastic changes in the respiratory tract.

Key words: neutrophils, oxidative DNA damage, 8-OHdG, nasal epithelial cells, inflammation, proliferation, Ki-67

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[2] Part of this chapter have been submitted (1) as an extended abstract to Annals of Occupational Hygiene and (2) Free Radical Biology & Medicine.

3.2 Introduction

Inflammation has been recognised as an important factor in cancer development. In the lung the mechanism underlying this response is still unclear, although it is postulated that reactive oxygen species (ROS), derived from inflammatory neutrophils are thought to play a key role (Dungworth et al., 1994; Driscoll et al., 1997). Upon activation, neutrophils and other phagocytes generate superoxide ($O_2^{\bullet-}$) by the reduction of oxygen, a reaction catalysed by the enzyme NADPH-oxidase. During this 'respiratory burst' basically four oxidants are generated: superoxide, hydrogen peroxide (H_2O_2), nitric oxide (NO•) and hypochlorous acid (HOCl) (Babior, 2000), which are originally manufactured for killing invading micro-organisms. Earlier experiments performed in our lab, have demonstrated that neutrophils or H_2O_2 caused the induction of the hydroxyl radicals (•OH) specific DNA base lesion 8-hydroxydeoxyguanosine (8-OHdG) in alveolar type II cells in vitro (Knaapen et al., 1999). These in vitro data are in line with observations in rat lungs, where 8-OHdG was induced in epithelial cells during particle-elicited inflammation (Nehls et al., 1997). Recent studies by Johnston et al. (2000) showed that inflammation alone is not responsible for the development of mutagenic effects in rat lungs after high doses of particle exposures. The observation of mutagenic effects in the study from Johnston only after crystalline silica exposures suggest additional factors, including biopersistence of particles and direct or indirect cytotoxicity to target cells, to be important determinants of secondary genotoxic events (Johnston et al., 2000). Apart from DNA damage, cell proliferation is a major mechanism in chemical carcinogenesis by both genotoxic and non-genotoxic agents (Swenberg et al., 1993). Increased cell proliferation decreases the time that is available for DNA repair of adducts and DNA-reactive chemicals are far more effective when there is also increased cell turnover. DNA synthesis is involved in chromosomal aberrations, insertions, deletions and gene amplification, which in turn are important mechanisms in chemical carcinogenesis. Formaldehyde- induced nasal cancer in rats illustrates the importance of cell turnover as a driving force in cancer. Although formaldehyde is a weak mutagen, formaldehyde induced necrosis and regenerative cell proliferation determine the non-linearity in concentration response to tumor induction and the site specificity of formaldehyde-induced squamos cell carcinomas in the nasal passages of rats (Morgan et al., 1997). The increase in cell proliferation correlates with

the tumor response curve, supporting the concept that sustained increases in cell proliferation are crucial in formaldehyde carcinogenesis (Monticello et al., 1994). Increased cell proliferation was also demonstrated in the nasal respiratory epithelium of lifelong adults residents of Mexico City and in newly arrived adult individuals in a period as short as 1 week (Calderon-Garciduenas et al., 1999a). Since inflammatory alterations of the lower airways have been associated to proliferative and malignant processes (Driscoll et al., 1997; Johnston et al., 2000) it was our objective to clarify whether a similar connection exists in the upper airways. Therefore we investigated inflammation in the nasal cavity in relation to oxidative DNA damage and cell proliferation in nasal epithelium.

3.3 Methods

3.3.1 Chemicals

Diaminobenzidine-tetrahydrochloride (DAB), Hank‘s balanced salt solution (HBSS), Ham’s RPMI medium, Phosphate buffered saline (PBS) were obtained from Sigma (St. Louis, MO). Hydrogen peroxide (H_2O_2) was purchased from Fluka (Germany) and RNAse was obtained from Boehringer (Mannheim, Germany).

3.3.2 Study population

The study group consisted of 80 children **(Table 3-1)** and 80 mothers **(Table 3-2)** selected from a cohort of 558 children and 557 mothers screened during a large health effect survey in Nordrhein-Westfhalen (Germany). Only children without hayfever or specific allergy, based on questionnaire and skin-prick test to 5 common allergens were included for this study. A questionnaire was obtained from each child and parent including items on place and length of residence, passive smoking, average daily outdoor time; parents occupational history; family history of respiratory diseases; personal history of allergies and respiratory and otolaryngological symptoms.

3.3.3 Nasal lavage and nasal brush

Nasal lavage and processing of lavage fluid was done as described previously (Schins et al., 2000) except that 4 ml of fluid was instilled in one nostril for children. Inflammatory effects were assessed by counting total number of cells and the proportion of neutrophils, eosinophils, lymphocytes and monocytes. To obtain nasal epithelial cells, the ventral surface of the inferior nasal turbinates was brushed using an interdental brush (Deflogrip). The brush containing the epithelial cells was immediately immersed in RPMI medium (4 ºC), and cells were removed from the brush by shaking followed by vortexing. Cells were centrifuged (600 g, 5 min.), resuspended in 300 µl HBSS, and 200 µl was further diluted in 600 µl Carbowax-buffer (0.8% Polyethyleneglycol in 70% ethanol). From this suspension cytospin preparations were made (Shandon, 800 rpm, 5 min). Slides were dried on air (30 min), fixed in 100% acetone (-20ºC, 5 min), and stored at -80ºC until analysis for 8-OHdG or Ki-67. For final analysis, 80 non-allergic children were selected based on total cell numbers present in the nasal lavage and grouped into 4 quartiles. Levels of 8-OHdG and Ki-67 in subjects from upper (n = 30), middle (each n = 10), and lower (n = 30) quartiles were then analysed as described below.

3.3.4 8-Hydroxydeoxyguanosine analysis

A semi-quantitative immunostaining described by Knaapen et al (2000), was used to detect 8-OHdG using a monoclonal antibody (N45.1, Toyokuni et al., 1997). Staining was developed using an ABC kit (Vectastain, Vector Laboratories, USA). Slides were mounted with Depex (Boehringer, Germany), and staining of 8-OHdG was then visualised and quantified using a microscope-coupled software analysis system (Soft Imaging System). Staining contrast distribution of 100 epithelial cells per slide **(Figures 3-1 A, B)** was measured and used to calculate a median score (8-OHdG/MEDIAN) as well as a score weighed for the intensity of the staining (8-OHdG/SCORE). This score was based on the number of cells in each quartile, multiplied by a certain weighing factor, to obtain larger contrast between individuals.

The following three score indices are used:

SCORE1 = K1 + 2 K2 + 3 K3 + 4 K4,

SCORE2 = K2 + 2 K3 + 4 K4,

SCORE 3= K2 + 3 K3 + 9 K4.

3.3.5 Immunnohistochemical staining and analysis of Ki-67

Cell proliferation was evaluated using immunohistochemical staining for Ki-67 by using a monoclonal antibody .The monoclonal antibody Ki-67 detects a nuclear antigen that is present only in proliferating cells, but absent in resting cells (Gerdes et al., 1983). Slides with nasal epithelial cells were washed with PBS twice. Endogenous peroxidase was blocked by treating the cells with 3% H_2O_2 in PBS-solution for 20 minutes at room temperature. After washing in PBS slides were treated with normal serum (horse) in 10ml 3% bovine serum albumin solution at room temperature for 1hour to block non-specific binding sites, then incubated with the primary antibody Ki-67 in a dilution of 1:20 (Boehringer Mannheim, Germany) at 4^oC overnight. After washing with PBS an ABC reagent (avidin and biotinylated horseradish peroxidase complex, Vectastain, Vector Laboratories, USA) was added, and the slides were incubated for 45 min at room temperature. Incubation was followed with washing the slides again. For the localisation of the peroxidase, slides were treated with diaminobenzidine for 15 min at room temperature. The next step was washing the slides in distilled water, followed by dehydration by a series of increasing alcohol of 70, 96 and 100% ethanol and also washing in xylol. Finally the slides were mounted with cover glasses using Depex (Serva). Analysis of this immune-histochemical staining was done by the light microscopy. We investigated 100 cells per slide to determine Ki-positive (weak,strong) and negative cells.

3.3.6 Statistical analysis

Statistical analysis was done using log-transformed data of both PMN cell counts and IL-8, since both are not normally distributed. Differences were tested using analysis of variances (ANOVA) between groups , using post-hoc LSD testing with significance at

0.05 (two-sided). Multiple linear regression was used to investigate the effect of personal characteristics on immunohistochemistry data and to investigate relation between proliferation and DNA damage. All statistical analyses were done using SPSS 9.0 for Windows NT.

3.4 Results

3.4.1 Characteristics of selected subjects

Both in children and mothers total nasal cells were distributed log-normally with a significantly higher cell count in children. We considered total cells (> 70 % PMN) as the endpoint of inflammation, and selected 80 children from different quartiles of cell number distribution **(Table 3-1)** for further study. Mothers **(Table 3-2)** were included through their children. The data in Table 1 show that IL-8 concentrations in lavage are different between the quartiles, but no differences in age, PMN (%), epithelial cells (%) or passive smoking were detected. A homogenous distribution of mothers over all quartiles was obtained **(Table 3-2)** which shows that no link is present between nasal inflammation in children and mothers. Interestingly, an increasing trend ($P < 0,05$) for PMN (%) with increasing inflammation was found, although the number of smokers in the highest quartile (n=2) was lower than in the other groups. Total cells counts in mothers and children were also paralleled by IL-8 levels in lavage. Among mothers each quartile contained about 20 % atopics.

3.4.2 Quantification of 8-OHdG immunnohistochemical staining

Quantification of 8-OHdG was first developed using rat lung epithelial cells (RLE) and pre-treatment with H_2O_2 (50 and 100 μM) as described by Knaapen et al (1999). Using nasal brushes of 10 subjects, first the influence of cell number was evaluated and showed that intensity of 100 (epithelial) cells was as accurate as counting up to 500 cells. Although nasal brushes predominantly contained epithelial cells, inflammatory cells such as granulocytes were commonly seen in fixed and stained cytospin preparations. Since the density of staining is highest in fragmented granulocytes, we systematically compared intensity measurements in 16 subjects scoring (i) 100 cells without looking at the cell-type, and (ii) 100 structurally intact epithelial cells. From these 16,000 epithelial cells (160 participants x 100 cells) the grey-tone distribution was determined leading to 4 classes of grey-tone, defined by the quartiles of the distribution. For average distribution of grey-tones for 16 subjects is shown in **Figure 3-2A**, and demonstrates that scoring all cells leads to a higher variation, skewed towards the left by

more cells with a lower grey-tone (= highly intense (PMN) cells compared to evaluating epithelial cells only. This causes our score type 3 (with a strong weighing of highly intense cells) to be significantly increased compared to evaluation of epithelial cells only (Paired Student-t test, P< 0.01, **Figure 3-2B**).

3.4.3 8-Hydroxydeoxyguanosine analysis in relation to inflammation

The analysis of children and their mothers showed no significant differences in 8-OHdG score between children with different inflammatory status **(Figure 3-3A)**. No significant difference was found in the intensity weighed scores between children with low nasal inflammation (n=30) and high inflammation (n=30). Although only SCORE 3 is shown in **Figures 3-3A and B**, also no difference was found using different weighing factors (SCO1, SCO2). Children without symptoms at the day of study (Table 1) did not reveal any differences compared to asymptomatic children (ANOVA, P>0.1). Mothers with different inflammatory status **(Figure 3-3B)** also showed no difference in 8-oxo-dG score. Additional analysis showed no effects of smoking, passive smoking (children) atopy or respiratory symptoms at the day of study. No effect of these variables on oxidative DNA damage in nasal epithelial cells was observed in a multiple linear regession.

In staining procedures, each day a randomly selected set of 18 subjects (9 children, 9 mothers) and two frozen samples of an epithelial cell were treated for immunohistochemistry and microscopic analysis was done at the end but in the same order. To check possible drift of staining and scoring we studied the intensity score of both epithelial cells and subjects versus time and found no trend between sample intensity and time of treatment or analysis (data not shown).

3.4.4 Ki-67 in relation to inflammation

In contrast to oxidative DNA damage, a significant increasing trend in Ki-67 positive cells was observed in quartiles of inflammation in both children **(Figure 3-4A)** and mothers **(Figure 3-4B)**. Also on a individual level **(Figures 3-5A, B)** the number of neutrophils was closely related to the percentage of Ki-67 positive cells. Among

personal characteristics that could affect cell proliferation, the incidence of (previous) respiratory or allergic disease did not play role. No effect of passive smoking (in hrs/wk) on Ki-67 in children was seen, but Ki-67 positive cells were slightly more presented in non-smoking (37.7%) women than in smoking (31%) women.

However, this 'smoking' effect appeared to be caused the low percentage of smokers among women in the upper quartile of inflammation **(Table 3-2)**.

3.4.5 DNA damage in relation to cell proliferation

The association between cell proliferation (expressed as % Ki-67 positiv cells) and oxidative DNA damage (8-OHdG) in nasal epithelial cells is illustrated in **Figure 3-6A** for children and in **Figure 3-6B** for mothers. A multiple linear regression using Ki-67 positive cells as a dependent variable and 8-OHdG, smoking, age and inflammation as independent variables showed a significant fit (R=0.62), due only to inflammation (P<0.00). No relation with the DNA damage (P=0.23) was present. A similar analysis in children showed a significant fit (R=0.75) with inflammation again as a main descriptor (β=0.73, P<0.00) as a descriptor of Ki-67. However, also oxidative DNA damage showed borderline significance (β=0.14, P=0.08). This significance was maintained when 8-OHdG was taken as the dependent variable (β=0.31, P=0.08), whereas inflammation was lost as a significant descriptor. These data indicate a weak relation between DNA damage and proliferation in nasal epithelium of children but not in their mothers.

3.5 Discussion

Previous and current in vitro experiments showed that oxidants released by activated neutrophils can cause oxidative DNA damage in epithelial lung cells in a co-incubation model. However, only few data describe the link between neutrophils and oxidative DNA damage in an in vivo target tissue. In rats, levels of 8-OHdG or mutations in the HPRT gene, both observed in lung epithelial cells after particle exposure, were positively correlated with the percentage of neutrophils present in the BAL fluid (Nehls et al., 1997; Driscoll et al., 1997). For humans it is even more complicated to study whether neutrophils directly cause oxidative DNA damage in respiratory tract cells, mainly by the fact that invasive techniques have to be used. In earlier studies we and others showed that the nose can be used as a model to investigate both inflammatory and genotoxic effects caused by exposure to environmental air pollutants (Schins et al., 2000; Calderon et al., 1999). In an attempt to find a model to elucidate the possible DNA damaging effects of the neutrophil on respiratory epithelial cells in a human situation, we analysed both neutrophil numbers and levels of oxidative DNA damage in nasal epithelial cells in 80 non-allergic children and their mothers. Nasal lavage was used to obtain information about the inflammatory status of the nose (total and differential cell count, IL-8 levels). Furthermore, from the same subjects, nasal epithelial cells were obtained by a nasal brush. By analysis of the chemical structure of modified DNA bases, it was shown that neutrophil-induced oxidative DNA base damage in target cells can largely be attributed to a hydroxyl attack of the DNA (Dizdaroglu et al., 1993). Therefore, we decided to analyse 8-OHdG as a marker of the possible neutrophil-induced oxidative DNA damage, as it is a base modification highly specific for the hydroxyl radical (Floyd et al., 1988). Subjects were selected based on total cell number in nasal lavage **(Table 3-1)**. Total cell number ranged between 5800 cells/ml in lowest quartile to about 500,000 cells/ml in the upper quartile, and closely correlated to IL-8 levels in nasal lavage fluid (Table 3-1). As shown in Table 3-1, 79 % of the total cells are neutrophils, and no statistical difference in percentage was observed between the 4 categories. Therefore this approach mimics a dose response effect for neutrophil-induced damage, and it was hypothesised that a higher neutrophil numbers would cause by an increased level of oxidative DNA damage. However, as illustrated in **Figure 3-7A** no relation was found between total neutrophil numbers

present in the lavage and the level of 8-OHdG in the epithelial cells. This finding seems to be conflicting with the general consensus on the link between neutrophils and DNA damage in target cells. However, there are several possible explanations for this discrepancy. In the described model, the sampling site of the neutrophils is slightly different from that of the target cells. This means that the sampled neutrophils might not have been in close contact with the sampled epithelial target cells. This adhesion is thought to be a crucial factor not only in neutrophil-induced cytotoxicity (Simon et al., 1986), but also in genotoxicity since no DNA damage was found in epithelial cells exposed to neutrophil-conditioned medium (data not shown). Furthermore, apart from possible neutrophil derived oxidants, epithelial cells in the nose are directly exposed to oxidant gases (e.g. ozone) and other pollutants present in ambient air, such as particulate matter. These factors are suggested to have a direct impact on respiratory tract epithelial genotoxicity by inducing oxidative DNA damage (Calderón et al., 1999b; Knaapen et al., 2000).

The outcomes of cell proliferation forward careful interpretation since it is an important step during the development of neoplastic lesions. Calderon and colleagues demonstrated increased and sustained cell proliferation in lifelong adults residents of Mexico City and in newly arrived adult individuals in a period as short as 1 week (Calderon-Garciduenas et al., 1999a). Nasal cell proliferation was evaluated as the percentage of cells in replicative DNA synthesis (S phase) using DNA staining with propidium iodide in a flow cytometer on RNase and Tween 20 treated cell suspensions. Although the methods section mentions ‘biopsies’, nasal scrapings were obtained for DNA cell cycle analysis from 109 adults and 43 children from Mexico City and 16 adults and 27 children from a Caribbean island with low pollution. Control children, control adult and exposed Mexico City children all had a similar percentages of cells in the S phase (12-15 %) A significant increase (20-25 % S-phase cells) was seen in adult residents from Mexico City at different time-points. In addition, newly arrived adults (N=61) showed a level of cell turnover at day 2 after coming to the city equal to controls (8-10 %). However at days 7, 14 and 25 after arrival a significant increase in S-phase cells up to 20 % was seen. These data demonstrate an increased and sustained nasal cell turnover in the adults within a week of residence in Mexico City.

In our study cell proliferation was evaluated using immunohistochemical staining for Ki-67 by using a monoclonal antibody (Gerdes et al., 1983). Previous studies have revealed immunostaining withKi-67 in many different cell types but theKi-67 antibody does not react with cells known to be in resting stage, such as lymphocytes, monocytes, hepatocytes and brain cells (Gerdes et al., 1983). The Ki- 67 antibody detects a nuclear antigen that is present in all proliferating cells (S, G2, and M phase of the cell cycle), but is absent in G0 (Gerdes et al., 1984). This explains why the percentage of proliferative cells in adult and children residents (20- 40%) in our study is higher than the control values in residents (10-12 %) found for S-phase cells only (Calderon et al., 1999a). Although the method is only semi-quantitative it allows to study cell proliferation in larger cohorts in epidemiological settings, since the cells can be spun, frozen and kept for later staining.

Interestingly, cell proliferation in nasal epithelium assessed by Ki-67 positive staining was clearly associated to the total number of inflammatory cells in the nasal lavage. This relation was consistent among both children (all non-atopic) and adults and was not confounded by smoking, allergy or atopy in the adults. This suggests that nasal inflammatory cells release persistent soluble mediators able to drive epithelial cell proliferation. In previous studies where we measured EGF and soluble EGF-R in nasal lavage of patients with rhinitis or polyposis (Kremer et al., 2000) we could not detect differences compared to controls, despite an increased total cell number and IL-8 in NAL of patients. This could mean that either these growth factors are not involved or the proliferative process in these patients had already ceased. In agreement with this, addition of EGF to SV40-transformed epithelial cell line A549 did not stimulate the high baseline proliferation as measured by Ki-67 (data not shown). Alternative explanations include the involvement of other growth factors (such as TGF-β) or that cell contact between inflammatory and epithelial cells is necessary for stimulation of growth.

A clear association between proliferation and oxidative DNA damage was not present in this study. However, a weak but statistically robust association was found in children also after correction for differences in inflammation and passive smoking. Although Calderon showed both increased 8-OHdG and proliferation in nasal cells from children

in Mexico City, no report was made on their potential association (Calderon-Garciduenas et al., 1999a). Although intuitively one expects more oxidative DNA damage in a proliferating cell (due to DNA vulnerability and dimished repair), not much in vivo data is available on the link between cell proliferation and 8-OHdG adducts. In human colon adenocarcinomas the fraction of PCNA-positive cells was proportionally associated with the staining intensity for 8-OHdG (Kondo et al., 1999). In vitro studies have shown enhanced oxidative DNA damage after hydrogen peroxide treatment in various actively dividing cells (Duthie & Collins, 1997; Villani et al., 2000). In addition, 8-OH-dG adducts were shown to increase ^{3}H-Thymidine in mouse splenocytes and B16F10 melanoma cells (Kwee et al., 1998), but not in 3T3 fibroblasts. These data suggest a mutual interaction between oxidative DNA damage and cell proliferation. To further investigate this relation in the nasal model, it is recommended to use a double-staining procedure that allows determination on a single-cell level. Since our data show a strong link between inflammation in the nasal cavity and nasal respiratory epithelial cell proliferation but not with oxidative DNA damage, we suggest that proliferation is mainly driven by inflammatory derived factors, among whom oxidants play a minor role.

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3.7 Tables and Figures

Table 3-1: Characteristics of children included in the sub-study relating inflammation (quartiles) to genotoxicity and proliferation

	Lower-Quartil (N= 30)	SecondQuartil (N=10)	Third-Quartil (N=10)	Upper-Quartil (N=30)
Age (yrs)	6,4 ± 0,4	6,4 ± 0,3	6,4 ± 0,5	6,4 ± 0,3
Total cell count (GM)	5,769 ± 2,920	21,865 ± 7,766	62,845 ± 25,668	496,922 ± 532,435
% of PMN in NALF	79 ± 17	89 ± 5	76 ± 28	77 ± 9
% of epithelial cell in NALF	15 ± 16	6 ± 3	18 ± 29	10 ± 10
IL-8 pg/ml in NALF (GM)	685 ± 786	838 ± 561	1408 ± 769	1641 ± 1122
Allergy				
- atopic	0	0	0	0
- nonatopic	30	10	10	30
Passive Smoking (y/n)	10	3	4	7
Cold [a]				
+ Cough	3	3	1	3
+ Runny nose	4	3	4	7
Fever [b]	0	0	0	1

[a]) on the day of investigation, [b]) within 8 days before study

Abbreviations: GM= geometric mean

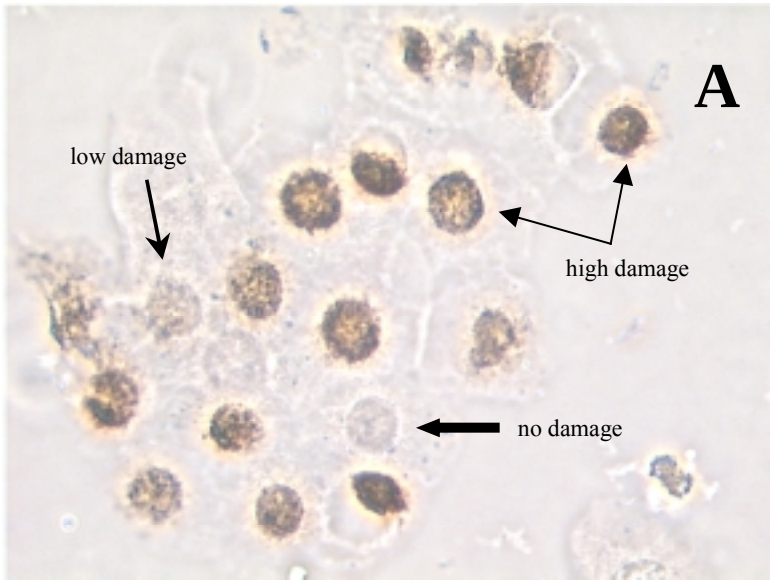
Table 3-2: Characteristics of mothers included in the sub-study relating inflammation (quartiles) to genotoxicity and proliferation

	Lower-Quartil (N= 18)	Second-Quartil. (N= 22)	Third-Quartil (N= 17)	Upper-Quartil (N= 16)
Age	34,8 ± 5,4	33,7 ± 6,2	36,2 ± 4,8	35,1 ± 5,7
Total cell count	3,546 ± 1,769	12,283 ± 3,283	31,794 ± 11,981	139,997 ± 127,131
% of PMN in NALF	44 ± 30	67 ± 23	78 ± 17	82 ± 8
% of epithelial cell in NALF	48 ± 32	22 ± 21	13 ± 17	10 ± 6
IL-8 pg/ml in NALF	162 ± 170	219 ± 335	200 ± 206	262 ± 177
Allergy				
atopic	5	5	3	6
nonatopic	13	13	14	8
Active Smoker	9	6	6	2
Passive Smoker	15	22	15	17
Cold [a]				
+ Cough	1	1	2	1
+ Runny nose	4	4	3	2
Fever [b]	0	0	0	0
Smoking [a]	9	4	5	2

[a]) on the day of investigation, [b]) within 8 days before study

Abbreviations: GM= geometric mean

Figure 3-1: 8-OHdG-Staining (A)



Representative pictures of 8-OHdG and Ki-67 staining in nasal epithelial cells. Both were detected using a specific antibody as described in the methods section. Staining contrast distribution of 100 epithelial cells per slide was measured and used to calculate a score weighed for the intensity of the staining. Only cells exhibiting a clear epithelial morphology were used for quantification. Pictures was taken at 1000x magnification.

Figure 3-1: Ki-67-Staining (B)

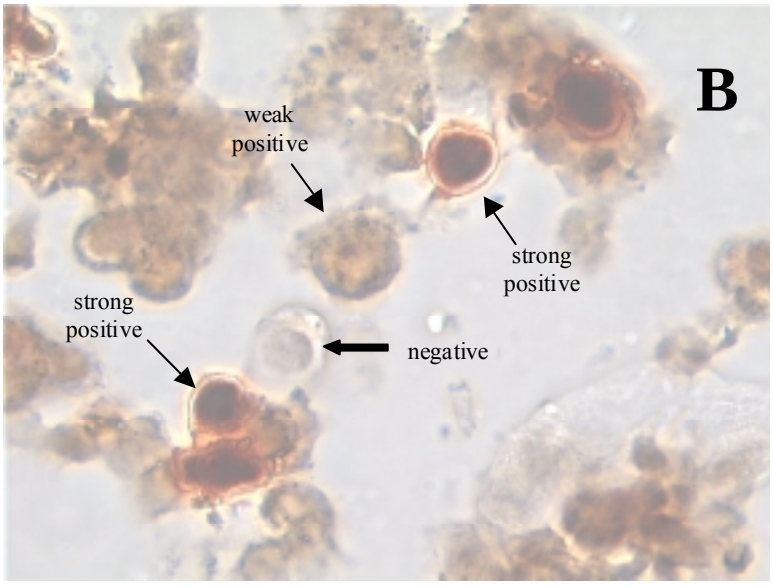
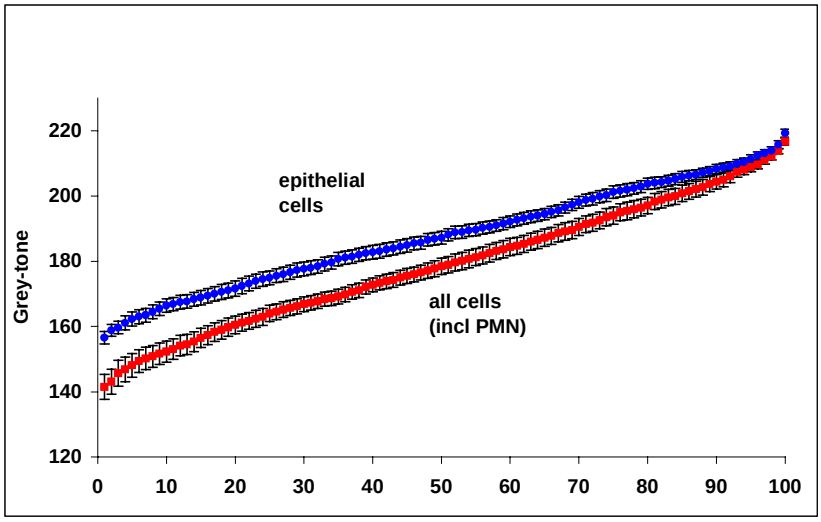


Figure 3-2: 8-OHdG Staining intensity in nasal epithelial cells and PMNs

Comparison of immunhistochemical staining in 100 epithelial cells without PMNs (only epithelial cell) and 100 epithelial cells including PMNs (all cells). Results are mean of 100 cells in 16 different subjects.



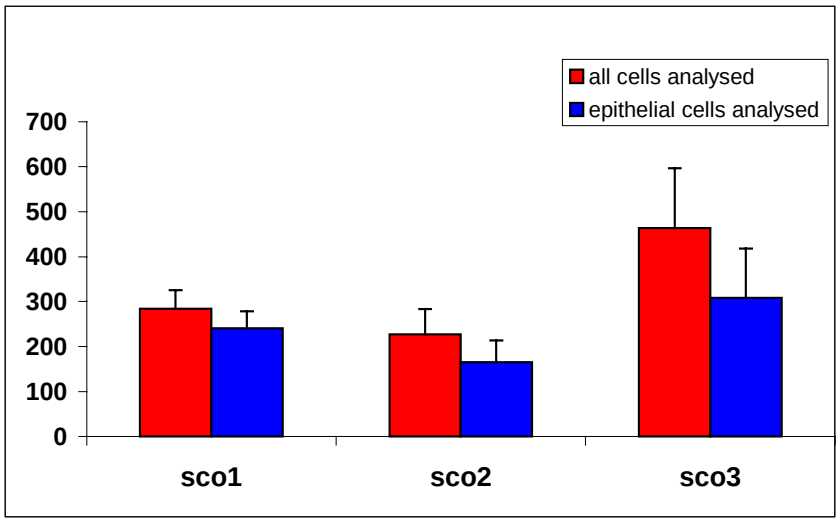
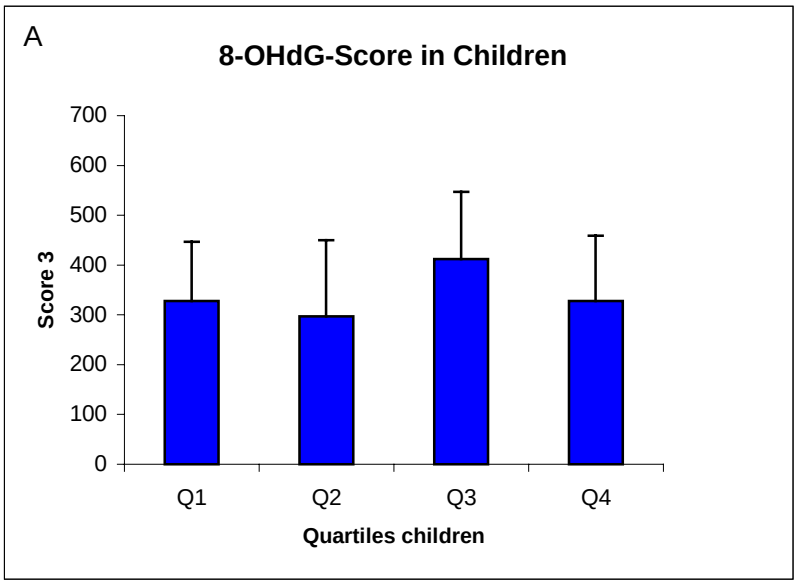


Figure 3-3: Comparision of 8-OHdG-Score in Children/Mothers

Oxidative DNA damage in nasal epithelial cells of non-allergic children (A) and their mothers (B). Levels of 8-hydroxydeoxyguanosine (8-OHdG) were analysed using a specific immunostaining and expressed as a score weighed for the intensity of the staining (8-OHdG score). A) Mean 8-OHdG score and standard deviation in nasal epithelial cells from children divided into 4 quartiles based on nasal inflammation (total cell number in nasal lavage). B) Mean 8-OHdG score and standard deviation in nasal epithelial cells from mothers divided into 4 quartiles based on nasal inflammation.



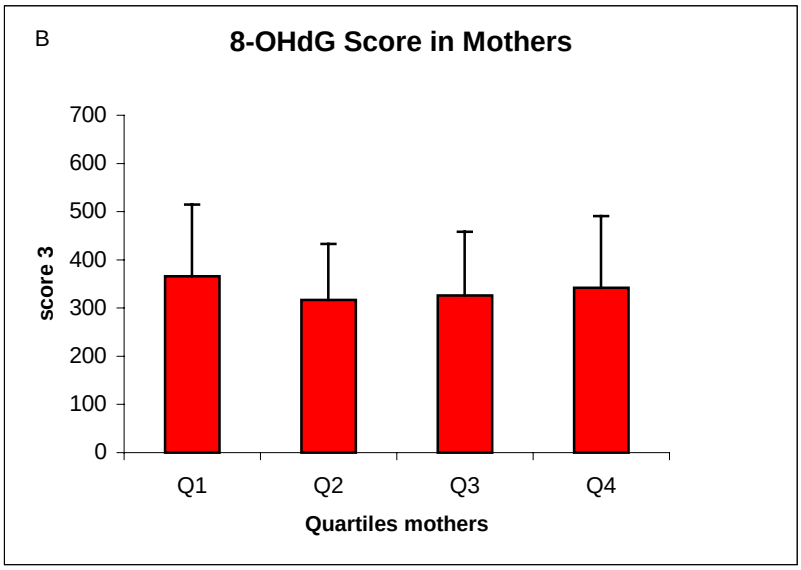
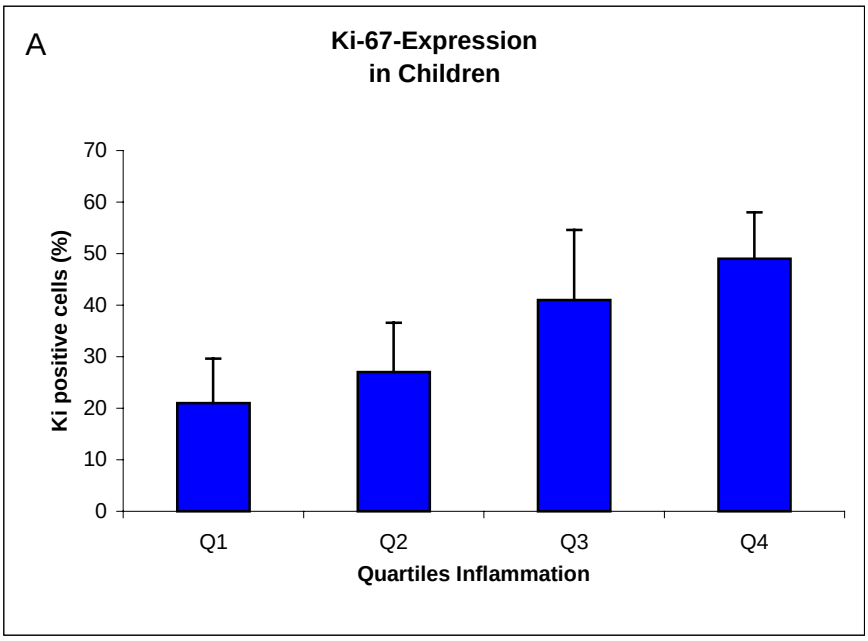


Figure 3-4: Ki-67-Expression in Children/Mothers

Epithelial cell proliferation based on percentage Ki-67 positive nasal epithelial cells of non-allergic children and their mothers. Positive cells were analysed using a specific immunostaining forKi-67 and were analysed without knowing the inflammatory status of the subject. A) Mean number of Ki-67 positive cells (%) score and SD in nasal epithelial cells from children divided into 4 quartiles based on nasal inflammation (total cell number in nasal lavage). B) Mean number of Ki-67 positive epithelial cells and SD in mothers divided into 4 quartiles based on nasal inflammation.



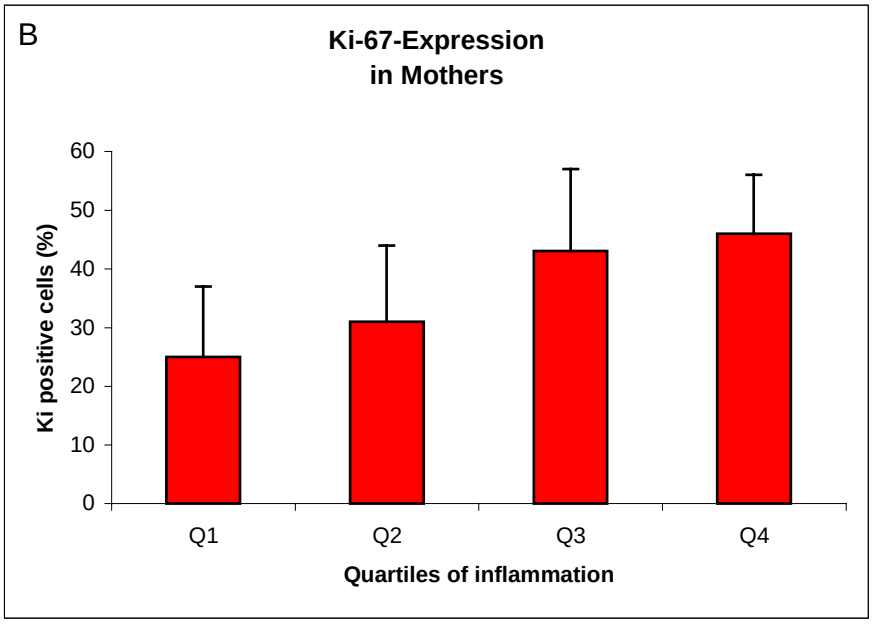
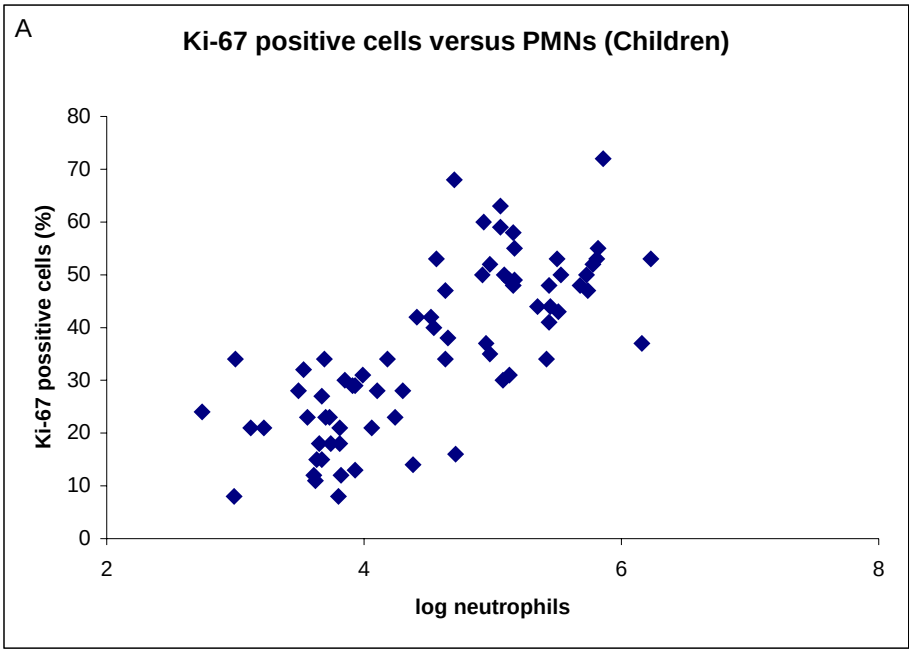


Figure 3-5: Ki-67 positive cells versus PMNs (Children/Mothers)

Association between cell proliferation (as % of Ki-67 positive cells) and neutrophil number on a group basis



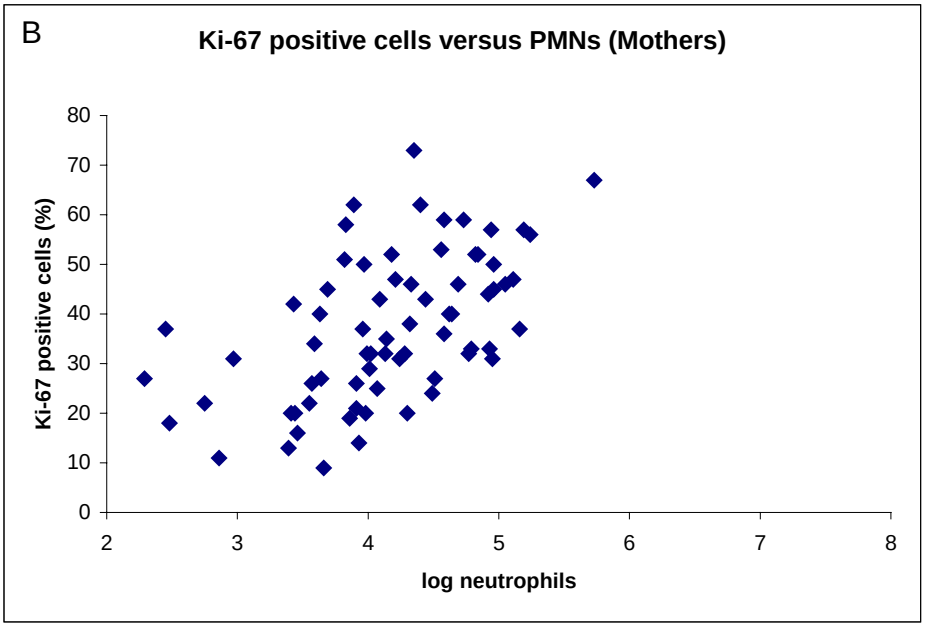
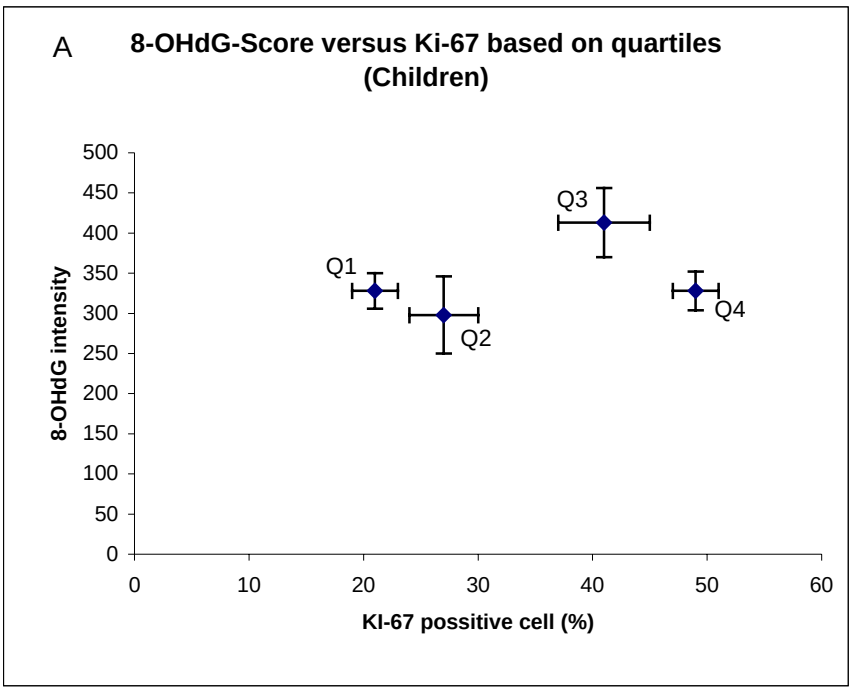


Figure 3-6: 8-OHdG-Score versus Ki-67 based on quartiles (Children/Mothers)
Association between cell proliferation (as % of Ki-67 positive cells) and intensity of 8-hydroxydeoxyguanosine (8-OHdG).



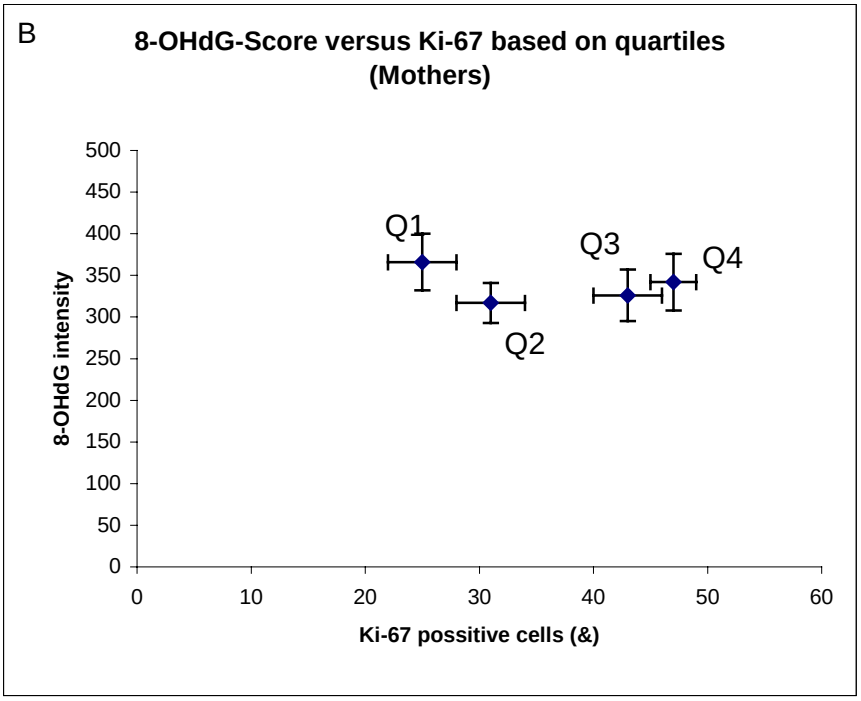
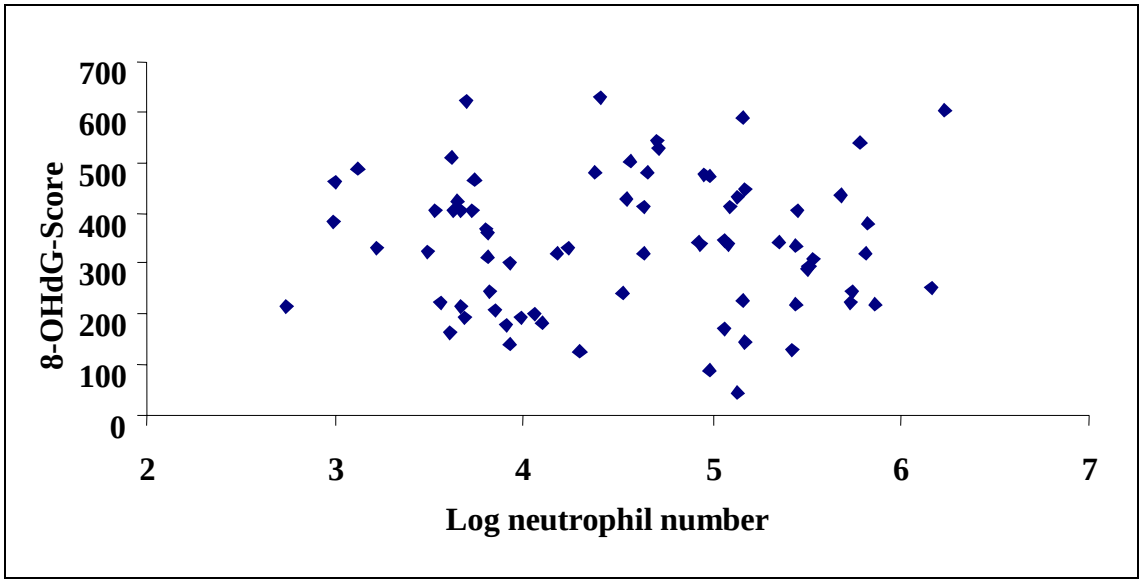


Figure 3-7A: Relation between neutrophils in nasal lavage and level of 8-OHdG in nasal epithial cells



CHAPTER 4

4.1 Summary and general discussion

This study was initiated to investigate the health of children and mothers of 3 different cities in Nordrhein-Westfalen (Germany) in relation to environmental exposure and personal characteristics. As a sub-study we have measured several biomarkers in nasal lavage and nasal brushes to evaluate our specific study aims.

The first aim was to see if biomarkers in the nose were related to environmental exposure. This evaluation is partly presented in Chapter 3. Both in children and mothers biomarkers for nasal inflammation were used to assess potential effects of environmental pollutants (PM, O_3) on the upper respiratory tract. Several biomarkers, including IL-8, total and specific cell counts were used to study inflammation. No differences in these markers were found between children or mothers from different cities. Evaluation of annual mean exposure to several primary pollutants including ozone and TSP showed differences between these places, but apparently our measurement strategy and markers did not allow to detect these differences. On the other hand parallel studies in our lab, using coarse and fine PM sampled in these cities during the period of health effect screening did indeed exhibit statistically different pro-inflammatory activity (Schins et al., 2001). Several explanations can be given for the disconcordance between in vivo and in vitro findings. First, we suggest that nasal lavage assesses acute response to environmental pollutants as also shown in a time-series analysis of nasal inflammation in relation to O_3 exposure (Frischer et al., 1993). Although the inflammatory effects are expected to be regulated with a few days half-life they do not integrate the response to long-term exposure. Secondly, the intra- and interindividual variability of most markers in nasal lavage is rather large. For instance, previous repetitive measurements in a group of 10 subjects (Borm et al., 2000) revealed variation coefficients for albumin and IL-8 of 41% and 67%, respectively. Variation coefficients for cell counts were even considerably higher up to 150 % (Schins et al., 2000). This means that relatively large increases need to be present when using a cross-sectional design as applied here. In addition to this differences between cities were

based on annual or weekly means of ambient exposures averaged over the available stations in the specific area. How well this ambient exposure reflects personal exposure is highly dependent on confounding factors such as local immissions (living near roads, smoker in the house). Measurements in occupational hygiene have demonstrated that personal exposure can easily deflect two to 3-fold from stationary monitoring.

However, even though a relation with exposure seemed inaccessible, several interesting outcomes have been generated. First, the data show that nasal lavage after good instruction can have success rates up to 85 % in 6-year old children and that nasal brush seems to be better tolerated by children than by adults. In addition, children have much higher baseline levels of IL8 and cells in nasal lavage, suggesting a higher inflammatory status upon similar external exposure as their mothers.

When studying effects on nasal epithelium previous studies in Mexico City school children did show an increased proliferation nasal cells from school children with higher ambient exposure (Calderon-Garciduenas et al., 1999a). In another study by the same research group also oxidative DNA damage in nasal brushes (Calderon-Garciduenas et al., 1999b) was reported to be 2.3 to 3-fold higher in nasal scrapings of 98 children using immunohistochemical staining. In our hands however, we could not find a difference among 80 children or their mothers (also 80) from 3 different cities. In addition, no differences in cell proliferation using Ki-67 staining as a marker were found between different cities. However, we observed a significant relationship between the proliferation of nasal epithelium and the inflammatory status of the nose, measured by nasal lavage cell number and confirmed by IL-8 levels in nasal lavage fluid. Our data suggest that local inflammation in the nose does not augment oxidative DNA damage but is related to increased cell turnover. Findings on oxidative DNA damage are in agreement with ongoing in vitro studies in our laboratory (Knaapen et al., 1999) that have shown only increased oxidative DNA damage in co-cultures of PMN and epithelial cells if PMN are adherent and activated. We therefore hypothesise that growth and proliferation factors that are secreted by inflammatory cells do not need this intimate cell-cell contact and therefore proliferation is increased. On the other hand persistent ROS, such as hydrogen peroxide, can diffuse through the nasal passages and

has been forward to cause cell proliferation based on is activation of EGF-receptor (Goldkorn et al., 1998)

Differences between this work and studies among Mexico City school-children may also be due to methodological differences. We used a very homogenous group of school-children at school entry (age 6), and from those we randomly selected 80 healthy, non-atopic children from different cities and inflammatory status. In addition, our procedure for quantification of oxidative DNA-damage was carefully standardised and performed to eliminate bias by highly-stained mucosal neutrophils. This bias was demonstrated in a sub-study of 11 individuals where nasal brushes were evaluated by (i) epithelial cells only, or (ii) all cells.

Immission by environmental pollutants did neither affect DNA damage nor cell proliferation in nasal epithelium. A number of pollutants including ozone and (ultrafine) particles is well known to generate reactive oxygen species (ROS) as detected by several methods (Gilmour et al., 1996; Shi et al., 2001). We did not have particle counts as a measure for ultrafine particle, but ozone concentrations were significantly higher in the control site (A), but no increased oxidative damage was seen. Since many transition metals (present in PM) are able to generate ROS in the presence of hydrogen peroxide (Lloyd et al., 1998; van Maanen et al., 1999), we also assessed metals in the nasal lavage of a set of school children. Preliminary analysis showed no relation between Fenton active metals as Vanadium (V) or Chromium (Cr) with oxidative DNA damage. Neither of these metals were associated to proliferation as well, but Platinum (Pt) content nasal lavage was significantly related to inflammation (Borm et al., 2002). Pt in NALF could be considered as a marker for nasal deposition of (ultra) fines generated by autocatalyst-derived -Pt but needs further study (Jarvis et al., 2001). In summary, both particle-derived metals and oxidant gases such as ozone are suggested to generate ROS that could cause oxidative DNA-damage. We suggest however that the exposure contrast between the sample sites and the low sensitivity of our immuno-assay do not allow to pick-up a potential difference. On the other hand here the same argument as previously mentioned holds, i.e. that ambient exposure does not necessarily reflect personal exposure especially when local traffic emissions are concerned.

Our findings on the relation between proliferation and DNA-damage in the human nose forward further interpretation with regard to implications for human risk assessment. In rat models generally mutagenicity (described in the HPRT-gene) and neoplasm's are considered secondary to persistent inflammation and depletion of repair and anti-oxidant mechanisms (Driscoll et al., 1997; Greim et al., 2001). However, our data in this human model suggest that oxidative damage is not related to nasal inflammation, although methodological issues can also explain the absence of a relation. On the other hand nasal proliferation, assessed by Ki-67 staining in nasal brushes, seems to be associated with inflammation. This increased proliferation is not associated however with increased oxidative DNA damage. Previous findings in nasal passages of human subjects exposed to Mexico city air have shown increased squamous cells, increased inflammation and also increased strand breaks as a marker for DNA damage (Calderon-Garciduenas et al., 1999). However, recent studies with rats exposed to Mexico city air did not demonstrate significant adverse effects in nasal passages (Moss et al., 2001). Together these findings suggest that the human airways respond differently to the effects of ambient air pollutants than the airways of rats. Although 8-OHdG, strand breaks and mutations in the HPRT gene are all different endpoints of DNA damage, our findings do not support to use the concept of inflammation driven genotoxicity as developed in particle-induced lung carcinogenicity (Greim et al., 2001) for humans. Of course one should realise that pollutant deposition and defense mechanisms such as immune- and anti-oxidant factors in upper and lower respiratory tract are tremendously different and that nasal tumours are a rare event in man. On the other hand diseases characterised by proliferative responses of fibroblast or epithelium (Polyposis) are well known in the nose and might be caused by influx of inflammatory cells. However, previous studies in our lab among patients with rhinitis and polyposis could neither demonstrate increased levels of soluble EGF and soluble EGF-R in nasal lavage nor a relation between these proliferative markers and markers of inflammation such as IL-8 and total cells (Kremer et al., 2000). The crucial difference with those studies is that at present we investigated the target tissue itself using immunohistochemistry for a cell-cycle protein. Initial studies with EGF-staining were done but showed less contrast compared to Ki-67 in proliferating epithelial cell lines. Interestingly, the association between IL-8 levels in nasal lavage and proliferation in epithelium was much lower than when using total cell count. Part of this might be explained by the fact that a soluble

mediator as IL-8 recruits inflammatory cells but might have come from other sources, such as the blood during extravasation in the nose. Similarly, EGF-R and EGF could have been secreted in the nasal lining by plasma exudation.

In conclusion, we were unable to show differences of several parameters in nasal lavage and brush between inhabitants of different cities in NRW. This means that also no effect of environmental exposure could be detected. However, this health effect screening has enabled us to study inflammation, DNA damage and cell proliferation in the nose under normal conditions in healthy subjects. Under these background conditions we showed that cell proliferation in nasal brushes is associated with the total number of cells in the nasal lavage. These data forward application of these biomarkers to study this relation in patients with proliferative nasal diseases and warrant further interpretations with regard to human risk assessment of particle induced inflammatory and malignant effects.

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6 Curriculum vitae

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Grunschule	1973 - 1977	Hinis/ Türkei
Hauptschule	1977 - 1984	Hauptschule Dortmund
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BERUFLICHE AUSBILDUNG

MTA-Schule	06/1987 - 04/1990	Hygiene-Institut, Dortmund
Tätigkeit als MTLA	04/1990 - 10/1990	St. Johannes-Hospital, Dortmund
Studium der Humanmedizin	10/1990 - 03/1998	Ruhr-Universität Bochum
Praktisches Jahr	04/1997 - 03/1998	Augusta-Kranken-Anstalt Akademisches Lehrkrankenhaus, Bochum
Ärztin im Praktikum	06/1998 – 12/98	Innere Medizin, Elisabeth-Krankenhaus, Gelsenkirchen
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Wissenschafliche Mitarbeiterin	2/2000 - 12/2001	Medizinisches Institut für Umwelthygiene, Düsseldorf

7 List of publications

Abstracts

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Polat D, Becker A, Knaapen AM, Schins RPF , Borm PJA.
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Papers:

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Ambient exposure and nasal inflammation in adults and children- a preliminary analysis-Int. J.Hygiene Env. Health. Submitted for publication.

Borm PJA, Polat D, Knaapen AM, Becker A, Schins RPF.
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Knaapen AM, Schins RPF, **Polat D**, Becker A, and Borm PJA.
Mechanisms of neutrophil-induced DNA damage in respiratory tract epithelial cells.Free Radical Biology & Medicine. Submitted for publication.

Entzündung, Gentoxizität und Zellproliferation in der Nasalen Lavage und der Nasenschleimhaut bei Personen unter städtischer Umweltbelastung

Studienziele: Um die Beziehung zwischen der Umweltbelastung und den daraus resultierenden gesundheitsschädigenden Effekten zu erforschen wurden in 3 Städten in Nordrhein-Westfalen von Februar bis Juni 2000, 1176 Probanden (Mütter und deren 6 oder 7-jährige Kinder) mittels nasaler Lavage und nasalem Bürstenabstrich untersucht. Es sollten folgende Fragen beantworten werden: 1. Gibt es Unterschiede in den Entzündungsparametern der Nasal Lavage, in Zellproliferation und DNA-Schädigung zwischen Probanden in Abhängigkeit von Wohnsitz und persönlichen Eigenschaften? 2.Gibt es einen Zusammenhang zwischen Entzündung, DNA-Schaden und Zellproliferation in der Nase? **Ergebnisse:** Weder Gesamtzellzahl, Zelldifferenzierung, Interleukin-8 (IL-8), Albumin und Harnstoff als Biomarker in der Nasenspülflüssigkeit noch die oxidative DNA-Schädigung des Nasenepithels zeigten Unterschiede zwischen den verschiedenen Standorten. Die Untersuchung von Kindern und deren Müttern ergab einen deutlichen Zusammenhang zwischen der Entzündungsreaktion und der Zellproliferation in der Nase. Zusätzliche in-vitro- Studien mit vor Ort gesammelten groben und feinen Staubpartikeln (engl. particulate matter, PM) wiesen eine signifikante Toxizität der groben, nicht aber der feinen PM in Makrophagen (Zelllinie NR8383) sowie in humanen Lungenepithelzellen (Zelllinie A549) auf. Grobe PM von allen Standorten verursachten eine erhöhte Freisetzung von IL-8 in A549 Zellen, während nur die feinen PM des Industriestandortes eine Freisetzung von IL-8 induzierten. **Bewertung:** Mit der nasalen Lavage und dem Bürstenabstrich wurden keine Unterschiede in den Entzündungsreaktionen, der DNA-Schädigung und der Zellproliferation zwischen Kindern und Erwachsenen mit unterschiedlichem Wohnort ermittelt. Andererseits zeigten PM Proben in vitro Unterschiede in ihrem Potential, Entzündungsmediatoren freizusetzen. Es besteht ein Zusammenhang zwischen Entzündung (Gesamtzellzahl) in der nasalen Lavage und Zellproliferation in der Naseschleimhaut. Um die Wirkungen der Umweltbelastung auf die proliferativen Prozesse in der Nasenschleimhaut aufzudecken sind weitere Analysen erforderlich.

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