Review of experimental studies of exposures to nitrogen dioxide

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1. Introduction

Nitrogen dioxide (NO_2) has been associated with adverse effects on hospital admissions, decrements in measures of lung function and lung function growth, increases in respiratory symptoms, asthma prevalence and incidence, cancer incidence, adverse birth outcomes and mortality (US EPA, 2015; WHO Europe, 2013).

In recent years, it has not been clear whether the effects are caused by NO_2 itself, or by some other air pollutant(s) with which it is correlated in the ambient air. However, as the associations between NO_2 and short-term health effects in many studies remain after adjustment for other air pollutants and that there is consistent short-term epidemiological evidence and some mechanistic support for causality, particularly for respiratory outcomes, the REVIHAPP project concluded that NO_2 also has direct effects (WHO Europe, 2013).

It is more difficult to assess the independent long-term effects of NO_2 as the correlations between concentrations of NO_2 and other pollutants are often high, so that NO_2 might represent the mixture of traffic-related air pollutants. However, some epidemiological studies do suggest that long-term exposures to NO_2 have independent effects on respiratory and cardiovascular mortality and on children's respiratory symptoms and lung function. The mechanistic evidence, particularly on respiratory effects, and the weight of evidence on short-term associations are suggestive of a causal relationship (Committee on the Medical Effects of Air Pollutants, 2015; WHO Europe, 2013).

The US EPA's current draft conclusion also is that there is a causal relationship between NO_2 exposure and respiratory effects (US EPA, 2015). The evidence of associations of ambient concentrations of NO_2 with a range of effects on health has strengthened in recent years and is robust to adjustment for other pollutants including some particle metrics. The combined evidence from epidemiologic and experimental studies is suggestive of, but not sufficient to infer, a causal relationship between short-term NO_2 exposure and cardiovascular effects (US EPA, 2015).

A recent review of the effects of traffic related air pollution (Health Effects Institute, 2010) reported that there was

- Sufficient evidence for increased asthma prevalence and increased asthma symptoms and exacerbations in children;
- Suggestive but not sufficient evidence for asthma causation, asthma symptom exacerbations in adults and reduced lung function;

- Suggestive evidence for mortality, especially cardiovascular mortality; and,
- Insufficient evidence for allergic sensitization, adult onset of asthma, increased health-care use both in children and adults, development of chronic obstructive pulmonary disease, birth outcomes, childhood cancers and lung cancer.

The current consensus is that there is no evidence that there is a threshold for NO_2 exposure (WHO Europe, 2013). However, the evidence base for assessing the existence of a threshold or the shape of the concentration–response curve is weaker than for fine particles (WHO Europe, 2013).

The investigation of the effects of inhaled air pollutants on human health has followed a multidisciplinary approach using animal toxicology, molecular and cellular biology, controlled human exposure studies, and epidemiology. Each approach has specific strengths and weaknesses in evaluating the effects of air pollution on human health.

Epidemiological studies examine the relationship between exposure and health effects in the community setting. Accurate estimation of exposure and effects of a particular air pollutant is difficult, as air pollution is a complex mixture. The extent of potential confounding, time considerations in air pollution effects (lags and latent periods), individual variation in air pollution exposure and exposure misclassification leads to uncertainty about the causal nature of any observed association with the health outcome under investigation.

Human experimental studies (or chamber studies) are directed at understanding mechanisms of injury and permit strict control of the exposures and the characteristics of the exposed persons. These studies typically involve small homogeneous groups of subjects and outcome measures can be carefully characterised. However, ethical and practical considerations often limit the use of such studies. Exposures must be at concentrations below the levels that are likely to produce unacceptable short-term or long-term effects. Feasibility usually limits exposure to a few hours, and chronic effects cannot be readily addressed. Despite these constraints, controlled human exposure studies have been used to investigation the acute effects of exposure to pollutants including NO_2, SO_2 and O_3 in both healthy people and in people with asthma. Although experimental studies generally only address a single pollutant, this can be helpful when it is unclear whether the health effects reported in epidemiological studies are related to the pollutant itself, or a mixture of pollutants for which the pollutant serves as a surrogate. The results from the experimental studies are therefore useful in determining whether NO_2 should be considered toxic to the

population in its own right or should be considered a marker for the complex mixture of air pollution.

2. Scope of work

The scope of work is to undertake a literature review and develop a summary report to NSW Health summarising the health evidence health effects from in-tunnel and short term exposure to NO_2. The literature review should include, but not be limited to, the following:

- evidence of health effects from 'short-term' exposure to NO_2 at 15 and 30 minutes exposure;
- whether the proposed NO_2 'in-tunnel' 15 minute rolling average of 0.5ppm is appropriately protective of health;
- health effects of traffic pollution with NO_2 as a marker;
- any evidence of independent effects of NO_2 when other traffic pollutants are controlled for; and
- whether NO_2 is considered a 'non-threshold' pollutant

This report will review:

- Experimental studies where the NO_2 exposure is ≤ 30 minutes;
- Experimental studies where the NO_2 exposure is ≥ 60 minutes; and
- Ambient studies in road tunnels, subways and busy roads.

3. Experimental studies where the NO_2 exposure is ≤ 30 minutes

The papers (N=18) reviewed in this section are presented in Table 1 and were published between 1985 and 2014 (Barck et al., 2005a; Barck et al., 2005b; Barck et al., 2002; Bauer et al., 1986; Bylin et al., 1988; Bylin et al., 1985; Ezratty et al., 2014; Helleday et al., 1994; Jorres and Magnussen, 1990; Jorres and Magnussen, 1991; Kim et al., 1991; Koenig et al., 1987; Rubinstein et al., 1990; Sandstrom et al., 1990; Sandstrom et al., 1991; Strand et al., 1997; Strand et al., 1996; Strand et al., 1998).

In three studies (Helleday et al., 1994; Sandstrom et al., 1990; Sandstrom et al., 1991), NO_2 concentrations were between 2.25ppm and 5.5ppm to mimic occupational exposures. These three studies are discussed separately at the end of this section.

In the remaining 15 studies, NO_2 exposure was less than 0.5ppm except in the study by Ezratty et al. (Ezratty et al., 2014) where in addition to 0.2ppm, an exposure of 0.6ppm was also used. These studies have only a small number of subjects (generally less than 20) who

are either healthy or have mild asthma. The duration of exposure ranged from 15 to 30 minutes. Exposure to NO_2 in the experimental chamber was either at rest or whilst engaging in mild to moderate intermittent exercise. Main outcome measures were lung function, airway responsiveness, and cellular and inflammatory markers in blood, bronchoalveolar lavage fluid (BALF) and nasal washings.

Thirteen of the 15 studies measured changes in lung function before and after NO_2 exposures and filtered air. The lung function indices that were commonly measured included forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), peak expiratory flow (PEF) and specific airway resistance (sRaw). In 12 of these studies, there were no differences in any of the lung function indicators after exposure to NO_2 compared to filtered air. In these 12 studies, the concentration of NO_2 ranged from 0.12ppm to 0.5ppm and the duration of exposure was from 15 to 30 minutes. In the remaining study (Bylin et al., 1985), healthy subjects demonstrated an increase in sRaw when exposed to 0.22ppm NO_2 for 20 minutes but a decrease in sRaw when exposed to 0.44ppm NO_2 for 20 minutes. Changes in sRaw were not seen in subjects with asthma. In the study by Koenig et al. (Koenig et al., 1987), there were no differences in lung function between healthy and asthmatic adolescents.

Eight of the fifteen studies measured airway responsiveness to non-specific bronchoconstricting agents (histamine, methacholine, sulphur dioxide, cold air) (Bauer et al., 1986; Bylin et al., 1988; Bylin et al., 1985; Jorres and Magnussen, 1990; Jorres and Magnussen, 1991; Rubinstein et al., 1990; Strand et al., 1997; Strand et al., 1996) or allergen challenge (Strand et al., 1997). Five studies (Bauer et al., 1986; Bylin et al., 1988; Bylin et al., 1985; Jorres and Magnussen, 1990; Strand et al., 1996) showed increased airway responsiveness after exposure to NO_2 compared to exposure to air whereas three studies (Jorres and Magnussen, 1991; Rubinstein et al., 1990; Strand et al., 1998) did not show an effect after exposure to NO_2. Seven of these studies recruited only subjects with asthma. Bylin et al. (Bylin et al., 1985) also studied healthy adults. In contrast to subjects with asthma, there were no changes in airway responsiveness after exposure to NO_2 in healthy subjects.

Six studies (Barck et al., 2005a; Barck et al., 2005b; Barck et al., 2002; Ezratty et al., 2014; Strand et al., 1997; Strand et al., 1996), also measured inflammatory markers in blood, sputum, nasal fluid and/or BALF. In these six studies, NO_2 exposure ranged from 0.2ppm to 0.6ppm and the duration of exposure ranged from 15 to 30 minutes. A range of inflammatory markers were measured and include eosinophil cationic protein (ECP; six studies),

myeloperoxidase (MPO; four studies), tryptase (one study) and interleukins (one study) as well as different types of white blood cells (for example, eosinophils and neutrophils; five studies). The results are mixed. There were increases in ECP after NO_2 exposure in three of six studies (Barck et al., 2005a; Barck et al., 2002; Ezratty et al., 2014) but no increases in MPO, tryptase or interleukins. Eosinophils and/or neutrophils were increased in two of the five studies.

Strand et al. (Strand et al., 1996) investigated whether any adverse effects from exposure to NO_2 persisted over seven days. Exposure to 0.26ppm NO_2 or purified air for 30 minutes was followed by histamine challenge at 30 minutes, five hours, 27 hours and seven days after the end of exposure. There was a significant increase in airway responsiveness to histamine only at five hours after exposure. There were no changes in specific airway resistance, inflammatory markers (ECP, MPO, tryptase) or in self-reported symptoms in the 24 hours after exposure. This suggests that any change in airway responsiveness is transient.

Three studies have also exposed subjects to different concentrations of NO_2 in an attempt to establish a dose-response relationship (Bylin et al., 1988; Ezratty et al., 2014; Koenig et al., 1987). Koenig et al. (Koenig et al., 1987) did not demonstrate differences in lung function between exposures to 0.12ppm NO_2, 0.18ppm NO_2 or filtered air for 30 minutes. Bylin et al. (Bylin et al., 1988) exposed adults with mild asthma to 0.13ppm, 0.25ppm, 0.49ppm NO_2 or filtered air for 30 minutes and were only able to demonstrate increased airway responsiveness to histamine challenge in subjects exposed only to NO_2 concentration of 0.25ppm. These two studies did not demonstrate a dose-response relationship. Ezratty et al. (Ezratty et al., 2014) exposed adults with seasonal allergic rhinitis and mild asthma to 0.2ppm NO_2, 0.6ppm of NO_2 or filtered air and measured lung function as well as inflammatory markers in sputum. Exposure to NO_2 or filtered air occurred on two consecutive days - one exposure for 30 minutes on day one and twice for 30 minutes each on day two. There were no differences in lung function (FEV_1, PEF) after exposure to NO_2 (0.2ppm and 0.6ppm) or filtered air. However, the percentage of eosinophils and ECP in sputum increased after exposure to 0.6ppm NO_2 but not after 0.2ppm NO_2 or filtered air.

A small number of studies have also studied the impact of repeated exposures to NO_2 (Barck et al., 2005a; Ezratty et al., 2014; Strand et al., 1998). Strand et al. (Strand et al., 1998) exposed subjects with mild asthma to 0.26ppm of NO_2 for 30 minutes for four consecutive days and measured lung function (FEV_1, sRaw) after inhaling an allergen, as well as self-reported symptoms and medication use. There were small but significant changes in FEV_1 with both single and repeated exposures but no evidence of a cumulative

effect. There were no changes in airway responsiveness to histamine challenge, reported symptoms and beta-agonist use. Barck et al. (Barck et al., 2005a) exposed subjects with mild asthma to 0.26ppm NO_2 for 15 minutes on day one followed by two exposures of 0.26ppm NO_2 for 15 minutes each on day two. Lung function tests and sputum were collected after an allergen challenge. There were no changes in lung function and sRaw. However, ECP in sputum increased significantly after the second day of NO_2 exposure compared to the first NO_2 exposure suggesting that brief repeated exposures are necessary to promote an airway inflammatory response to allergen. Ezratty et al. (Ezratty et al., 2014) exposed adults with seasonal allergic rhinitis and mild asthma to 0.2ppm NO_2, 0.6ppm NO_2 or filtered air and measured lung function as well as inflammatory markers in sputum. Exposure to NO_2 or filtered air occurred on two consecutive days - one exposure for 30 minutes on day one and twice for 30 minutes each on day two. There were no changes in eosinophil inflammation after the first day of NO_2 exposure but there were significant increases in eosinophil inflammation after the second day of NO_2 exposure suggesting either a delayed response or a cumulative effect of NO_2.

Lastly, the three studies where NO_2 exposures were greater than 2.25ppm are presented here. Sandstrom et al. (Sandstrom et al., 1990) studied 32 healthy males and showed increased mast cells and lymphocytes in BALF at four to 24 hours and increased macrophages at 24 and 48 hours following exposure to 4ppm NO_2 and light exercise for 20 minutes. In a further study, Sandstrom et al. (Sandstrom et al., 1991) exposed healthy adults to 2.25, 4.0 and 5.5ppm NO_2 and light exercise for 20 minutes. There were no changes in FVC and FEV_1 or non-cellular markers after any of the NO_2 exposures. There was an increase in lymphocytes (at 4.0ppm and 5.5ppm NO_2) and mast cells at all three NO_2 exposures. Finally, Helleday et al. (Helleday et al., 1994) exposed smokers and non-smokers to 3.5ppm NO_2 and light exercise for 20 minutes. Smokers had increased alveolar macrophages and neutrophils in BALF at 24 hours after exposure while non-smokers had increased lymphocytes in BALF and increased neutrophils in bronchial fluid. There were no changes in white blood cell counts in blood in either of the groups.

In summary:

- There were no differences in lung function after exposure to NO_2 compared to filtered air. NO_2 concentrations ranged from 0.12ppm to 0.5ppm for 15 to 30 minutes.
- There is an effect of exposure to NO_2 on airway responsiveness. Five of eight studies showed increased airway responsiveness after exposure to NO_2

compared to exposure to filtered air. All five studies recruited subjects with asthma.

- The results for inflammatory markers are mixed. The most consistent results were for increases in ECP after NO_2 exposure (increases in three of six studies).
- The one study that measured airway responsiveness over a longer period (at 30 minutes, five hours, 27 hours and seven days after exposure) suggests that any change in airway responsiveness is transient.
- There is no clear evidence of a dose-response relationship for effects on lung function and airway responsiveness at NO_2 levels below 0.5ppm.
- There is no clear evidence that repeated exposure to NO_2 lead to cumulative effects. The two studies that demonstrated probable cumulative effects exposed subjects to a higher dose on the second day. Therefore it is not clear whether it was the cumulative doses or the increased dose that lead to greater effects after the second day of exposure.

4. Experimental studies where the NO_2 exposure is ≥ 60 minutes

Fourteen studies were reviewed in this section (Table 2). These studies were conducted between 1989 and 2012, with NO_2 exposures between 0.3 and 4ppm and duration of exposure between one to six hours (1 hour: 4 studies; 2 hours: 2 studies; 3 hours: 4 studies; ≥4 hours: 4 studies).

Of the nine studies that investigated changes in lung function (NO_2 exposures ranged from 0.3 to 4ppm and the duration of exposure ranged from one to four hours) (Blomberg et al., 1999; Frampton et al., 2002; Frampton et al., 1991; Frampton et al., 1989; Langrish et al., 2010; Rubinstein et al., 1991; Salome et al., 1996; Vagaggini et al., 1996; Witten et al., 2005), only one study demonstrated decreases in lung function after NO_2 exposure compared to exposure to air. In this study (Blomberg et al., 1999), 12 healthy subjects were exposed to 2ppm NO_2 or air for fours a day on four consecutive days. There were significant reductions in FEV_1 and FVC after the first NO_2 exposure. Lung function changes attenuated with repeated exposures.

Five studies investigated airway responsiveness to a number of agents (carbachol, histamine, house dust mite allergen, hypertonic saline). Airway responsiveness was increased in two studies (Frampton et al., 1991; Salome et al., 1996). In these two studies, NO_2 exposures were 0.6ppm for one hour and 1.5ppm for three hours. In the studies where airway responsiveness was not altered, the NO_2 exposures were 0.6ppm and 0.05ppm with

three 2ppm peaks for three hours (Frampton et al., 1989); 0.3ppm for one hour (Vagaggini et al., 1996) and 0.4ppm for three hours (Witten et al., 2005).

Seven of these experimental studies also measured various inflammatory markers in BAL, nasal lavage or blood (Blomberg et al., 1997; Blomberg et al., 1999; Frampton et al., 2002; Pathmanathan et al., 2003; Rubinstein et al., 1991; Wang et al., 1995; Witten et al., 2005). Three studies did not show changes to inflammatory markers (Rubinstein et al., 1991; Wang et al., 1995; Witten et al., 2005). Rubinstein et al. (Rubinstein et al., 1991) did not show any changes in lymphocytes in blood or BALF in healthy adults after 0.6ppm NO_2 exposure for two hours per day on four consecutive days; Wang et al. (Wang et al., 1995) did not show changes in nasal lavage after 0.4ppm NO_2 exposure for six hours except that in the subjects who were exposed to a nasal allergen challenge prior to NO_2 exposure, there was increased ECP in nasal fluid compared to those subjects who did not have the nasal allergen challenge; and, Witten et al. (Witten et al., 2005) showed no difference in inflammatory markers in sputum after exposure to 0.4ppm NO_2 for three hours and house dust mite allergen. The remaining four studies showed increases in various inflammatory markers in bronchial washings, bronchial biopsy and BALF. All four studies recruited healthy adults and NO_2 exposures ranged from 2ppm for four hours (Blomberg et al., 1997), 2ppm for four hours on four consecutive days (Blomberg et al., 1999; Pathmanathan et al., 2003) and 0.6 or 1.5ppm for three hours (Frampton et al., 2002).

Three studies also examined cardiovascular disease markers following NO_2 exposures (Channell et al., 2012; Langrish et al., 2010; Scaife et al., 2012). Langrish et al. (Langrish et al., 2010) did not show any differences in blood flow after vasodilators or in fibrinolytic markers in 10 healthy adults exposed to 4ppm NO_2 compared to filtered air for one hour. There were no differences in heart rate, blood pressure and heart rate variability in 18 older adults with a history of heart disease exposed to 4ppm NO_2 compared to filtered air for one hour (Scaife et al., 2012). In an interesting study, Channell et al. (Channell et al., 2012) exposed 14 healthy adults to diesel exhaust (PM=100ug/m^3, NO_2=0.8ppm), 0.5ppm or filtered air for two hours and then probed human coronary artery cells for intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). VCAM-1 was increased after both NO_2 and diesel exhaust exposure and ICAM-1 was increased only after NO_2 exposure.

In summary:

- There were no differences in lung function after exposure to NO_2 compared to filtered air. NO_2 concentrations ranged from 0.3 to 4ppm and the duration of exposure ranged from one to four hours.
- Two of five studies showed increased airway responsiveness after exposure to NO_2 compared to exposure to filtered air.
- The results for inflammatory markers are mixed. Overall, inflammatory markers increased after exposure to NO_2.
- There are only a few experimental studies investigating cardiovascular endpoints after exposure to NO_2. Currently, it is not possible to make a judgment on the cardiovascular effects of NO_2.
- In the one study where subjects were exposed to NO_2 or air for four consecutive days, there were significant reductions in FEV_1 and FVC after the first NO_2 exposure which then attenuated with repeated exposures.

5. Ambient air studies in road tunnels, subways and busy roads

Eight studies were reviewed which investigated health effects after exposure to ambient air in road tunnels (n=4), along busy roads (n=1) or in subways (n=3) (Table 3). All eight studies also studied subjects in a control environment.

Seven of the eight studies measured lung function in a number of exposure scenarios where NO_2 levels ranged from 0.01 to 0.5ppm and the duration of exposure from five minutes to two hours (Huang et al., 1991; Klepczynska Nystrom et al., 2012; Klepczynska Nystrom et al., 2010; Larsson et al., 2010; Larsson et al., 2007; McCreanor et al., 2007; Svartengren et al., 2000). Only one study showed a decrease in lung function (McCreanor et al., 2007) where there were significant decreases in FVC and FEV_1 after walking along a busy road for two hours (NO_2=0.07ppm, $PM_{2.5}$=28ug/m^3) compared to spending two hours in a park (NO_2=0.01ppm, $PM_{2.5}$=12ug/m^3). McCreanor et al. (McCreanor et al., 2007) however did not show changes in FEF_{25-75} or in exhaled nitric oxide. Six of the studies did not show any changes in lung function although Larsson et al. (Larsson et al., 2010) showed a decrease in PEF but not in VC, FVC and FEV_1.

Six studies (Klepczynska Nystrom et al., 2012; Klepczynska Nystrom et al., 2010; Larsson et al., 2010; Larsson et al., 2007; McCreanor et al., 2007; Svartengren et al., 2000) also collected information about respiratory symptoms and in only one study was there no difference in symptoms (McCreanor et al., 2007). In the five remaining studies, NO_2

exposures ranged from 0.01 to 0.15ppm and $PM_{2.5}$ from 28 to 95ug/m^3. Upper respiratory symptoms were reported in two studies (Larsson et al., 2010; Larsson et al., 2007) and lower respiratory symptoms were reported in four studies (Klepczynska Nystrom et al., 2012; Klepczynska Nystrom et al., 2010; Larsson et al., 2010; Larsson et al., 2007). Svartengren et al. (Svartengren et al., 2000) reported that exposure to road tunnel air resulted in significantly more asthma symptoms in the late phase of the allergen challenge.

Three studies measured airway responsiveness (Huang et al., 1991; Larsson et al., 2010; Svartengren et al., 2000). Twelve children with moderate asthma inhaled concentrated road tunnel air for 5, 15, 35, 65 and 105 breaths where NO_2 levels were 0.45 to 0.5ppm (Huang et al., 1991). Methacholine and dust mite allergen challenges after 105 breath exposure did not show increased airway responsiveness. Larsson et al. (Larsson et al., 2010) also did not demonstrate increased airway responsiveness in adults with mild asthma exposed to road tunnel air (NO_2=0.17ppm, PM_{10}=170ug/m^3, $PM_{2.5}$=95ug/m^3) compared to exposure to a hospital laboratory. On the other hand, Svartengren et al. (Svartengren et al., 2000) demonstrated increased airway responsiveness to allergen challenge after exposure a 30 minute to road tunnel air (NO_2=0.13ppm, $PM_{2.5}$=80ug/m^3) compared to exposure to a low air pollution suburban environment (median NO_2=0.005ppm).

Six studies which investigated inflammatory markers. Inflammatory markers were measured in BALF, nasal fluid, sputum and blood. Three studies (Klepczynska Nystrom et al., 2012; Klepczynska Nystrom et al., 2010; Larsson et al., 2010) did not demonstrate increases in BALF inflammatory markers (interleukins, tumour necrosis factor) or in blood counts in mild asthmatics after exposure to road tunnel air for two hours (median NO_2=0.13ppm; median $PM_{2.5}$=80ug/m^3) (Larsson et al., 2010) or to a subway for two hours (mean NO_2=0.01ppm, $PM_{2.5}$=71-77ug/m^3) (Klepczynska Nystrom et al., 2012; Klepczynska Nystrom et al., 2010). On the other hand, McCreanor et al. (McCreanor et al., 2007) demonstrated increased neutrophils and MPO in subjects exposed to a busy road but not in IL-8 and ECP; Larsson et al. (Larsson et al., 2007) showed that subjects exposed to road tunnel air had increased lymphocytes in BALF but no changes to peripheral blood (blood count, plasminogen activator inhibitor-1, fibrinogen), fibronectin and total metallo-proteinease-9; Klepczynska Nystrom et al. (Klepczynska Nystrom et al., 2010) showed increases in blood fibrinogen and regulatory T-cells in blood after subway exposure but not in plasminogen activator inhibitor-1; and, Steenhof et al. (Steenhof et al., 2014) showed that exposure to NO_2 (geometric mean 0.02ppm) in a number of environments was associated with decreased lymphocytes and eosinophils and that exposure to PM was associated with increased neutrophils and monocytes in blood.

In summary:

- In these eight studies of exposure to road tunnel air and subway air, NO_2 concentrations were relatively low (less than 0.2ppm in seven studies and 0.5ppm in one study). NO_2 concentrations were similar to published mean concentration in other road tunnels (0.015-0.15ppm) (National Health and Medical Research Council, 2008). $PM_{2.5}$ concentrations in these eight studies were at the lower end of reported concentrations from other road tunnels (100-300ug/m^3) (National Health and Medical Research Council, 2008).
- There were generally no differences in lung function after exposure to road tunnels or subways compared to control environments.
- Both upper and lower respiratory symptoms were more commonly reported after exposure to road tunnel and subway environments.
- The effect of exposure to road tunnels on airway responsiveness is unclear. Two of three studies did not show increased airway responsiveness. All three studies recruited subjects with asthma.
- There are mixed results for inflammatory markers following exposure to road tunnels or subways.

6. Discussion

Folinsbee (Folinsbee, 1992) reviewed experimental studies of exposures to NO_2 (20 studies of people with asthma and five studies of healthy people) to test the hypothesis that exposure to NO_2 increased airway responsiveness. In the 20 studies of people with asthma, the NO_2 exposures ranged from 0.1ppm to 0.6ppm (17 studies ≤0.5ppm) and the duration of exposure ranged from 20 to 120 minutes (14 studies ≤60 minutes and nine studies ≤30 minutes). In the 5 studies of healthy people, the NO_2 exposures ranged from 0.1ppm to 2ppm (three studies ≤0.5ppm) and the duration of exposure ranged from 20 to 180 minutes (four studies ≤60 minutes and one study ≤30 minutes). Overall, a significant proportion (59%) of subjects demonstrated increased airway responsiveness on exposure to NO_2 compared to exposure to filtered air. In a subgroup analysis, increased airway responsiveness was only seen in subjects resting when exposed to NO_2 and not when exercising during exposure. There was no dose response relationship for subjects with asthma ($0.05<NO_2<0.2$ppm; $0.2<NO_2<0.3$ppm; $0.3<NO_2$). In healthy subjects, increased airway responsiveness was only demonstrated in those exposed to more than 1ppm of NO_2.

Hesterberg et al. (Hesterberg et al., 2009) reviewed more than 50 experimental studies that focussed on inhaled NO_2 concentrations between 0.1 to 3ppm during short-term exposures (30 minutes to six hours) both at rest or combined with exercise. Their findings were:

- Airway responsiveness in asthmatic individuals is not affected by NO_2 up to about 0.6ppm, although some sensitive asthmatics may be affected by NO_2 levels as low as 0.2ppm;
- Healthy subjects exposed to NO_2 below 1ppm do not show pulmonary inflammation;
- At 2ppm for four hours, neutrophils and cytokines in lung-lavage fluid can increase, but these changes do not necessarily correlate with significant or sustained changes in lung function;
- There is no consistent evidence that NO_2 concentrations below 2ppm increase susceptibility to viral infection;
- For asthmatics and individuals with chronic obstructive pulmonary disease, NO_2 induced lung inflammation is not expected below 0.6ppm;
- Changes in blood chemistry generally required NO_2 concentrations above 1–2ppm.

Goodman et al. (Goodman et al., 2009) conducted a meta-analysis and meta-regression of controlled human NO_2 exposure and airway responsiveness studies in asthmatic subjects. The three endpoints (compared to filtered air) were: change in provocative dose of a challenge agent necessary to cause a specified change in lung function; the change in FEV_1 after an airway challenge; and, the proportion of subjects with increased airway responsiveness. The authors examined 41 exposure scenarios from 38 studies published between 1976 and 2002. Concentrations of NO_2 were between 0.2ppm and 0.6ppm and the duration of exposure between 30 minutes and six hours. The overall meta-analysis results were statistically significant for all three endpoints. Dose-response relationships assessed by meta-regression for all the three endpoints were not significant. The conclusion was that although there were NO_2 effects on airway responsiveness, the effects were too small to be considered significant at NO_2 levels below 0.6ppm. Similar conclusions were expressed by Hesterberg et al. (Hesterberg et al., 2009).

Brown (Brown, 2015), in a recent analysis, examined the effects of exposure to NO_2 on airway responsiveness in asthmatics using data from human experimental studies. Analysis was stratified by whether exposure occurred while subjects were resting (16 exposure scenarios, 12 studies, 0.1-0.53ppm, duration 20-60 minutes) or by whether exposure was combined with exercise (17 exposure scenarios, 12 studies, 0.15-0.60ppm, duration 30-360 minutes). There were significant increases in airway responsiveness in individuals with

asthma exposed to NO_2 (at rest) to between 0.2ppm and 0.3ppm for 30 minutes and at 0.1ppm for 60 minutes. There was also a median decrease of 25% in the provocative dose with a clinically relevant halving of the provocative dose occurring in 25% of the asthmatic subjects (three studies with exposures ≤30 minutes and two studies with exposures of 60 minutes). This is in contrast to the conclusions drawn by Hesterberg et al. and Goodman et al. (Goodman et al., 2009; Hesterberg et al., 2009) who suggested that the effects on airway responsiveness are sufficiently small so as not to be clinically significant. The meta-analyses showed no effect for exposures during exercise. Linear regression models did not show an association between provocative dose and NO_2 exposure.

7. Summary

From the review of the experimental studies and meta-analytical studies of short-term (≤30 minutes) exposures to low levels of NO_2 (≤0.5ppm), the strongest evidence is for effects on airway responsiveness. These effects are generally seen in asthmatics, and the effects are small and transient. However, clinically relevant halving of the provocative dose occurred in 25% of asthmatic subjects.

In New South Wales, the proposal is to set the in-tunnel 15 minute rolling average NO_2 guideline at 0.5ppm. The NO_2 concentration will be a marker for air pollution concentration within the road tunnels. However a number of salient points should be borne in mind when setting in-tunnel NO_2 guidelines:

- The above studies examined small numbers of healthy or mildly asthmatic subjects, whereas the general population will include people who are more sensitive to NO_2 and may therefore experience more pronounced effects at low concentrations.
- Clinically relevant halving of the provocative dose occurred in 25% of asthmatic subjects in experimental studies where NO_2 concentrations were generally <0.5ppm and the duration of exposure between 20-60 minutes.
- Subjects exposed to road tunnel, subway or a busy road were more likely to report upper and lower respiratory symptoms. The NO_2 concentration ranged from 0.01 to 0.15ppm and the exposure duration from 30 minutes to two hours.
- In-tunnel air pollution is a complex mixture and NO_2 is a marker for this complex mixture. Therefore, even if there are no health effects at a certain level of NO_2 in experimental studies (for example, 0.5ppm in this instance), this does not mean that there will not be any health effects if in-tunnel NO_2 levels are <0.5ppm. Other components of in-tunnel air pollution, such as fine and ultra fine particles and air toxics, may yet be at high enough levels to lead to adverse health effects.

- Although the respiratory effects of NO_2 may be small in any given individual, the fact that large numbers of people will be unavoidably exposed means that the overall burden of illness could be large.

Whether the proposed NO_2 in-tunnel 15 minute rolling average of 0.5ppm is appropriately protective of health is an important question. At the moment, experimental studies suggest that brief exposures to NO_2 (≤30 minutes) at concentrations ≤0.5ppm increases airway responsiveness and that in some people with asthma such adverse effects may be clinically relevant.

Therefore, it is recommended that further consultations take place prior to finalising the NO_2 in-tunnel guideline and that consideration be given to setting a lower NO_2 in-tunnel guideline.

8. References

Barck, C., Lundahl, J., Hallden, G. and Bylin, G., 2005a. Brief exposures to NO_2 augment the allergic inflammation in asthmatics. Environmental Research, 97: 58-66.

Barck, C., Lundahl, J., Holmstrom, M. and Bylin, G., 2005b. Does nitrogen dioxide affectinflammatory markers after nasal allergen challenge? American Journal of Rhinology, 19: 560-566.

Barck, C. et al., 2002. Ambient level of NO2 augments the inflammatory response to inhaled allergen in asthmatics. Respiratory Medicine, 96(11): 907-917.

Bauer, M.A., Utell, M.J., Morrow, P.E., Speers, D.M. and Gibb, F.R., 1986. Inhalation of 0.30 ppm nitrogen dioxide potentiates exercise-induced bronchospasm in asthmatics. American Review of Respiratory Disease, 134(6): 1203-1208.

Blomberg, A. et al., 1997. The inflammatory effects of 2 ppm NO2 on the airways of healthy subjects [published erratum appears in Am J Respir Crit Care Med 1997 Dec;156(6):2028]. American Journal of Respiratory and Critical Care Medicine, 156(2): 418-424.

Blomberg, A. et al., 1999. Persistent airway inflammation but accommodated antioxidant and lung function responses after repeated daily exposure to nitrogen dioxide. American Journal of Respiratory and Critical Care Medicine, 159(2): 536-543.

Brown, J.S., 2015. Nitrogen dioxide exposure and airway responsiveness in individuals with asthma. Inhal. Toxicol., 27(1): 1-14.

Bylin, G., Hedenstierna, G., Lindvall, T. and Sundin, B., 1988. Ambient nitrogen dioxide concentrations increase bronchial responsiveness in subjects with mild asthma. European Respiratory Journal, 1(7): 606-612.

Bylin, G., Lindvall, T., Rehn, T. and Sundin, B., 1985. Effects of short-term exposure to ambient nitrogen dioxide concentrations on human bronchial reactivity and lung function. European Journal of Respiratory Diseases, 66(3): 205-217.

Channell, M.M., Paffett, M.L., Devlin, R.B., Madden, M.C. and Campen, M.J., 2012. Circulating factors induce coronary endothelial cell activation following exposure to inhaled diesel exhaust and nitrogen dioxide in humans: evidence from a novel translational *in vitro* model. Toxicological Sciences, 127(1): 179-186.

Committee on the Medical Effects of Air Pollutants, 2015. Statement on the evidence for the effects of nitrogen dioxide on health UK Ministry of Health, London.

Ezratty, V. et al., 2014. Repeated nitrogen dioxide exposures abd eosinophilic airway inflammation in asthmatics: a randomised crossover study. Environmental Health Perspectives, 122: 850-855.

Folinsbee, L.J., 1992. Does nitrogen dioxide exposure increase airways responsiveness? Toxicology & Industrial Health, 8(5): 273-283.

Frampton, M.W. et al., 2002. Nitrogen dioxide exposure: effects on airway and blood cells. American Journal of Physiology: Lung Cellular and Molecular Physiology, 282: L155-L165.

Frampton, M.W. et al., 1991. Effects of nitrogen dioxide exposure on pulmonary function and airway reactivity in normal humans. American Review of Respiratory Disease, 143(3): 522-527.

Frampton, M.W. et al., 1989. Nitrogen dioxide exposure in vivo and human alveolar macrophage inactivation of influenza virus in vitro. Environmental Research, 48(2): 179-192.

Goodman, J.E., Chandalia, J.K., Thakali, S. and Seeley, M., 2009. Meta-analysis of nitrogen dioxide exposure and airway hyper-responsiveness in asthmatics. Critical Reviews in Toxicology, 39(9): 719-742.

Health Effects Institute, 2010. Traffic-Related air pollution: a critical review of the literature on emissions, exposure, and health effects, Health Effects Institute, Boston, Massachusetts.

Helleday, R., Sandstrom, T. and Stjernberg, N., 1994. Differences in bronchoalveolar cell response to nitrogen dioxide exposure between smokers and nonsmokers. European Respiratory Journal, 7(7): 1213-1220.

Hesterberg, T.W. et al., 2009. Critical review of the human data on short-term nitrogen dioxide (NO_2) exposures: evidence for NO_2 no-effect levels. Critical Reviews in Toxicology, 39(9): 743-781.

Huang, J.L., Wang, S.Y. and Hsieh, K.H., 1991. Effect of short-term exposure to low levels of SO_2 and NO_x on pulmonary function and methacholine and allergen bronchial sensitivities in asthmatic children. Archives of Environmental Health, 46(5): 296-299.

Jorres, R. and Magnussen, H., 1990. Airways response of asthmatics after a 30 min exposure, at resting ventilation, to 0.25 ppm NO2 or 0.5 ppm SO2. European Respiratory Journal, 3(2): 132-137.

Jorres, R. and Magnussen, H., 1991. Effect of 0.25 ppm nitrogen dioxide on the airway response to methacholine in asymptomatic asthmatic patients. Lung, 169(2): 77-85.

Kim, S.U., Koenig, J.Q., Pierson, W.E. and Hanley, Q.S., 1991. Acute pulmonary effects of nitrogen dioxide exposure during exercise in competitive athletes. Chest, 99(4): 815-819.

Klepczynska Nystrom, A. et al., 2012. Health effects of a subway environment in mild asthmatic volunteers. Respiratory Medicine, 106: 25-33.

Klepczynska Nystrom, A. et al., 2010. Health effects of a subway environment in healthy volunteers. European Respiratory Journal, 36: 240-248.

Koenig, J.Q., Covert, D.S., Marshall, S.G., van Belle, G. and Pierson, W.E., 1987. The effects of ozone and nitrogen dioxide on pulmonary function in healthy and in asthmatic adolescents. American Review of Respiratory Disease, 136(5): 1152-1157.

Langrish, J.P. et al., 2010. Exposure to nitrogen dioxide is not associated with vascular dysfunction in man. Inhal. Toxicol., 22(3): 192-198.

Larsson, B.-M. et al., 2010. Limited airway effects in mild asthmatics after exposure to air pollution in a road tunnel. Respiratory Medicine, 104: 1912-1918.

Larsson, B.-M. et al., 2007. Road tunnel air pollution induces bronchoalveolar inflammation in healthy subjects. European Respiratory Journal, 29: 699-705.

McCreanor, J. et al., 2007. Respiratory effects of diesel traffic in persons with asthma. The New England Journal of Medicine, 357: 2348-58.

National Health and Medical Research Council, 2008. Air quality in and around traffic tunnels, Canberra, Australia.

Pathmanathan, S. et al., 2003. Repeated daily exposure to 2 ppm nitrogen dioxide upregulates the expression of IL-5, IL-10, IL-13 and ICAM-1 in the bronchial epithelium of healthy human airways. Occup Environ Med, 60: 892-896.

Rubinstein, I., Bigby, B.G., Reiss, T.F. and Boushey, H.A., Jr., 1990. Short-term exposure to 0.3 ppm nitrogen dioxide does not potentiate airway responsiveness to sulfur dioxide in asthmatic subjects. American Review of Respiratory Disease, 141(2): 381-385.

Rubinstein, I., Reiss, T.F., Bigby, B.G., Stites, D.P. and Boushey, H.A., Jr., 1991. Effects of 0.60 PPM nitrogen dioxide on circulating and bronchoalveolar lavage lymphocyte phenotypes in healthy subjects. Environmental Research, 55(1): 18-30.

Salome, C.M. et al., 1996. Effect of nitrogen dioxide and other combustion products on asthmatic subjects in a home-like environment. European Respiratory Journal, 9(5): 910-918.

Sandstrom, T., Andersson, M.C., Kolmodin-Hedman, B., Stjernberg, N. and Angstrom, T., 1990. Bronchoalveolar mastocytosis and lymphocytosis after nitrogen dioxide exposure in man: a time-kinetic study. European Respiratory Journal, 3(2): 138-143.

Sandstrom, T. et al., 1991. Inflammatory cell response in bronchoalveolar lavage fluid after nitrogen dioxide exposure of healthy subjects: a dose-response study. European Respiratory Journal, 4(3): 332-339.

Scaife, A. et al., 2012. Lack of effect of nitrogen dioxide exposure on heart rate variability in patients with stable coronary heart disease and impaired left ventricular systolic function. Occup Environ Med, 69: 587-591.

Steenhof, M. et al., 2014. Air pollution exposure affects circulating white blood cell counts in healthy subjects: the role of particle composition, oxidative potential and gaseous pollutants - the RAPTES project. Inhal. Toxicol., 26(3): 141-165.

Strand, V., Rak, S., Svartengren, M. and Bylin, G., 1997. Nitrogen dioxide exposure enhances asthmatic reaction to inhaled allergen in subjects with asthma. American Journal of Respiratory and Critical Care Medicine, 155(3): 881-887.

Strand, V., Salomonsson, P., Lundahl, J. and Bylin, G., 1996. Immediate and delayed effects of nitrogen dioxide exposure at an ambient level on bronchial responsiveness to histamine in subjects with asthma. European Respiratory Journal, 9(4): 733-740.

Strand, V., Svartengren, M., Rak, S., Barck, C. and Bylin, G., 1998. Repeated exposure to an ambient level of NO2 enhances asthmatic response to a nonsymptomatic allergen dose. European Respiratory Journal, 12(1): 6-12.

Svartengren, M., Strand, V., Bylin, G., Jarup, L. and Pershagen, G., 2000. Short-term exposure to air pollution in a road tunnel enhances the asthmatic response to allergen. European Respiratory Journal, 15(4): 716-724.

US EPA, 2015. Integrated science assessment for oxides of nitrogen-health criteria (second external review draft), EPA/600/R-14/006 National Center for Environmental Assessment-RTP Division, U.S. Environmental Protection Agency.

Vagaggini, B. et al., 1996. Effect of short-term NO2 exposure on induced sputum. European Respiratory Journal, 9(9): 1852-1857.

Wang, J.H., Devalia, J.L., Duddle, J.M., Hamilton, S.A. and Davies, R.J., 1995. Effect of six-hour exposure to nitrogen dioxide on early-phase nasal response to allergen challenge in patients with a history of seasonal allergic rhinitis. Journal of Allergy & Clinical Immunology, 96(5): 669-676.

WHO Europe, 2013. Review of evidence on health aspects of air pollution - REVIHAPP Project, WHO Regional Office for Europe, Copenhagen, Denmark.

Witten, A. et al., 2005. Effects of nitrogen dioxide on allergic airway responses in subjects with asthma. Journal of Occupational & Environmental Medicine, 47: 1250-1259.

Table 1 Experimental studies where the NO_2 exposure is ≤ 30 minutes

Study	Experimental group	Level and duration of exposure to NO_2	Health outcomes measured	Results and comments
Bylin 1985 (Bylin et al., 1985)	N=8, 20-36 years, healthy. N=8, 17-45 years, asthma.	230ug/m^3 (0.11ppm), 460ug/m^3 (0.22ppm) or 910ug/m^3 (0.44ppm) NO_2 or filtered air at rest for 20 minutes.	Airway responsiveness to histamine (only for NO_2 at 0.44ppm and filtered air), sRaw.	In healthy adults, sRaw increased with 0.22ppm NO_2 and decreased with 0.44ppm NO_2. No changes in airway responsiveness. In the asthmatic group, increase in airway responsiveness with exposure to 0.44ppm NO_2. No differences sRaw following NO_2 exposure compared to filtered air between the two groups.
Bauer 1986 (Bauer et al., 1986)	N=15, 20-45 years, asthma, non-smokers.	0.3ppm NO_2 or air for 30 minutes. Exercise for last 10 minutes of the exposure.	Lung function (FEV_1, PEF) and airway reactivity to isocapnic cold air hyperventilation.	No difference in FEV_1 and PEF between NO_2 and air exposures during resting exposures. No fall in FEV_1 and PEF between NO_2 and air exposures during exercise. Exposure to NO_2 potentiated airway reactivity to isocapnic cold air hyperventilation.
Koenig 1987 (Koenig et al., 1987)	N=10, 14-19 years, healthy adolescents. N=10, 11-19 years, adolescents with asthma.	0.12ppm, 0.18ppm NO_2 or filtered air for 30 minutes. Moderate exercise for 10 minutes following exposures.	Lung function (PEF, FEV_1, maximal flow at 50% and 75% of VC).	No differences in any of the endpoints following NO_2 exposure compared to filtered air both within and between the two groups of subjects.
Bylin 1988 (Bylin et al., 1988)	N=20, 17-56 years, mild asthma, non-smokers.	260ug/m^3 (0.13ppm), 510ug/m^3 (0.25ppm) or 1000ug/m^3 (0.49ppm) NO_2 or purified air at rest for 30 minutes.	Airway responsiveness to histamine (about 25 minutes after exposure), sRaw, sGaw.	Increased airway responsiveness to 0.25ppm NO_2 only. No differences in sRaw and sGaw with the different NO_2 exposures.
Rubinstein 1990	N=9, 23-34 years, stable asthma, non-	0.3ppm NO_2 or filtered	Airway responsiveness to sulphur dioxide (0.25-0.4ppm following	No differences in any of the endpoints following NO_2 exposure compared to filtered

(Rubinstein et al., 1990)	smokers.	air for 30 minutes. Light to moderate exercise.	NO_2 exposure), lung function (FVC, FEV_1), sRaw, and respiratory symptoms.	air.
Sandstrom 1990 (Sandstrom et al., 1990)	N=32, 21-37 years, healthy, non-smokers, males.	4ppm NO_2 for 20 minutes. Light exercise.	BAL prior to and 4, 8, 24 and 48 hours after exposure for cell count and differential, mast cells, macrophages and albumin.	Increased mast cells and lymphocytes in BALF 4-24 hours after exposure and increased lysozyme positive macrophages at 24 and 48 hours after exposure.
Jorres 1990 (Jorres and Magnussen, 1990)	N=14, 21-55 years, mild asthma non-smokers.	0.25ppm NO_2, 0.5ppm SO_2 or filtered air for 30 minutes at rest.	Lung function (sRaw) and airway responsiveness to isocapnic hyperventilation with 0.75ppm SO_2.	Increased airway responsiveness after NO_2 exposure compared to SO_2 or air exposures.
Jorres 1991 (Jorres and Magnussen, 1991)	N=11, 17-55 years, mild stable asthma.	0.25ppm NO_2 or filtered air for 30 minutes. Exercising for the last 10 minutes of exposure.	Airway responsiveness to methacholine one hour after exercise.	No differences in airway responsiveness after NO_2 exposure compared to filtered air.
Kim 1991 (Kim et al., 1991)	N=9, 18-23 years, healthy competitive athletes.	0.18ppm, 0.3ppm NO_2 or filtered air for 30 minutes. Exercise on treadmill during exposure.	Lung function (FEV_1, PEF, R_T, Vmax50%) just prior to and immediately after exposure.	No differences in FEV_1, PEF, R_T and Vmax50% after NO_2 exposures compared to filtered air.
Sandstrom 1991 (Sandstrom et al., 1991)	N=18, 22-32 years, healthy, non-smokers, males.	2.25ppm, 4.0ppm or 5.5ppm NO_2 for 20 minutes. Eight subjects in each exposure category. Light exercise.	BAL prior to and 24 hours after exposure for cell count and differential, mast cells, macrophages and albumin and for non-cellular components (albumin, fibronectin, angiotensin-converting enzyme, hyaluronan, β_2-microglobulin).	No changes in FVC and FEV_1or non-cellular markers after NO_2 exposures. Increase in lymphocytes (at 4.0ppm and 5.5ppm NO_2) and mast cells at all three NO_2 exposures.

			Lung function (FVC, FEV_1) at baseline and prior to bronchoscopy.	
Helleday 1994 (Helleday et al., 1994)	N=8, 24-35 years, non-smokers. N=8, 28-32 years, smokers, median 20 cigarettes/day.	3.5ppm NO_2 for 20 minutes. Light exercise.	BAL and peripheral blood sampling for cell count and differential prior to and 24 hours after exposure.	After NO_2 exposure, smokers had increased alveolar macrophages and neutrophils in BALF. After NO_2 exposure, non-smokers had increased lymphocytes in BALF and increased neutrophils in bronchial fluid. No differences in blood cell count and differential.
Strand 1996 (Strand et al., 1996)	N=19, 20-48 years, mild asthma, atopic, life-time non-smokers and ex-smokers.	488ug/m^3 (0.26 ppm) NO_2 or purified air for 30 minutes. Intermittent exercise.	Airway responsiveness to histamine and sRaw measured 30 minutes, 5 hours, 27 hours and 7 days after exposure. Peripheral blood inflammatory markers (ECP, MPO or tryptase) and expression of an adhesion molecule (MAC-1) on granulocytes measured at baseline and at 30 minutes and 27 hours after the end of exposure. Self-reported respiratory symptoms during exposure and for the week after the end of exposure.	Significant increase in airway responsiveness to histamine (at 5 hours after exposure). No change in aRaw. Increased expression of MAC-1 30 mins after exposure. No change in ECP, MPO or tryptase. No differences in self-report symptoms in the 24 hours after exposure.
Strand 1997 (Strand et al., 1997)	N=18, 18-50 years, mild asthma, pollen allergy (birch, timothy), life-time non-smokers and ex-smokers.	500ug/m^3 (0.26 ppm) NO_2 or purified air for 30 minutes followed by allergen challenge at 4 hours after exposure. .	Airway responsiveness to allergen and sRaw to histamine challenge 24 hours after NO_2 exposure. Lung function (FEV_1, PEF, FVC, $FEF_{50\%}$, $FEF_{75\%}$). Peripheral blood inflammatory	Pre-exposure to NO_2 made no differences to airway responsiveness to allergen or to histamine challenge, sRaw, inflammatory markers (ECP, eosinophils) and respiratory symptoms. No difference in lung function immediately after allergen challenge but a significant decrease in

			markers (ECP, eosinophils). Self-reported respiratory symptoms.	PEF and FEV_1 with NO_2 3-9 hours after allergen challenge.
Strand 1998 (Strand et al., 1998)	N=16, 21-52 years, mild asthma, atopic, life-time non-smokers.	500ug/m^3 (0.26 ppm) NO_2 or purified air for 30 minutes on 4 consecutive days. Allergen challenge 4 hours after end of exposure. At rest.	Lung function (FEV_1), sRaw, respiratory symptoms, medication use. Self-reported respiratory symptoms. Histamine challenge on day 5.	No changes in FEV_1 after NO_2 exposure. Small but significant changes in lung function (FEV_1) with single and repeated NO_2 exposure and allergen challenge. No change in histamine responsiveness, respiratory symptoms or medication (inhaled beta-agonist) use.
Barck 2002 (Barck et al., 2002)	N=13, 23-39 years, atopy, mild-asthma, non-smokers.	500ug/m^3 (0.26ppm) NO_2 for 30 minutes. Allergen inhalational challenge 4 hours after exposure.	Lung function (FEV_1 3-10 hours after allergen challenge) and sRaw Bronchial wash and BAL performed 19 hours after allergen challenge for inflammatory mediators. Respiratory symptoms.	No effect on lung function, airway resistance or symptoms. Increased neutrophils in bronchial wash and BAL and increased ECP in bronchial wash after NO_2 and allergen challenge. No differences in other inflammatory markers (eosinophils, IL-5, IL-8, sICAM-1, eotaxin and MPO) between the two groups. No change in respiratory symptoms or medication use.
Barck 2005 (Barck et al., 2005a)	N=18, 23-48 years, atopy, mild-asthma, non-smokers.	Day 1: 500 ug/m^3 (0.26ppm) NO_2 or filtered air for 15 minutes. Day 2: 500ug/m^3 (0.26ppm) NO_2 or purified air for 15 minutes, after 1 hour a further 500ug/m^3 NO_2 or purified air for 15 minutes On both days, exposure	Lung function (FEV_1), sRaw and symptoms following NO_2 exposure and allergen challenges. Inflammatory mediators and cells in sputum and blood at baseline (day 1) and days 2 and 3.	No effect of NO_2 exposure on FEV_1 and sRaw. Significant increase in ECP between days 1 and 3 in both sputum and blood after NO_2 exposure plus allergen challenge. No changes in neutrophils. Decreases in the levels of MPO in blood but not in sputum after NO_2 exposure. No change in respiratory symptoms or medication use.

		followed by allergen challenge 4 hours.		
Barck 2005 (Barck et al., 2005b)	N=16, 22-48 years, seasonal allergic rhinitis, mild asthma.	500ug/m^3 (0.26ppm) NO_2 or filtered air for 30 minutes at rest.	Nasal challenge (cells, ECP, MPO) with birch or timothy pollen 4 hours after exposure. Blood sampling at baseline and 24 hours later (ECP, MPO).	No enhancing effect of NO_2 exposure to nasal challenge compared to purified air. No differences in ECP, MPO, eosinophils and neutrophils in blood and nasal fluid.
Ezratty 2014 (Ezratty et al., 2014)	N=19, 20-69 years, mild intermittent asthma, non-smokers.	0.2ppm or 0.6ppm NO_2 or filtered air. One exposure for 30 minutes on day 1 and twice for 30 minutes each on day 2.	Inflammatory response (cell count and differential, ECP) in sputum 6, 32 and 48 hours after first exposure. Lung function (FEV_1, PEF) at baseline and daily for 3 days.	Percentage of eosinophils and ECP in sputum increased after 0.6ppm NO_2 but not after 0.2ppm NO_2 or filtered air. Dose related trend for percentage eosinophil increase on days 2 and 3 and for ECP on day 3. No differences in FEV_1 and PEF.

BAL=bronchoalveolar lavage; ECP=eosinophil cationic protein; $FEF_{50\%}$=forced expiratory flow at 50% volume of forced vital capacity; $FEF_{75\%}$= forced expiratory flow at 75% volume of forced vital capacity; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity; MPO=myeloperoxidase; PEF=peak expiratory flow; R_T=total respiratory resistance; sGaw= specific airway conductance; sRaw=specific airway resistance; Vmax50%=maximal expiratory flow at 50% volume of forced vital capacity

Table 2 Experimental studies where the NO_2 exposure is ≥60 minutes

Study	Experimental group	Level and duration of exposure to NO_2.	Health outcomes measured	Results and comments
Frampton 1989 (Frampton et al., 1989)	N=19, 19-37 years, healthy non-smokers.	Continuous 0.6ppm NO_2 for 3 hours (n=9). Continuous 0.05ppm NO_2 for 3 hours which included 3 2ppm peaks each of 15 minutes (n=15). Five subjects participated in both protocols.	Lung function (FVC, FEV_1) and carbachol challenge Macrophages from BALF (taken 3.5 hours after exposure) examined for inactivation of influenza virus and IL-1 production *in vitro.*	No changes in lung function (FVC, FEV_1) and carbachol challenge when exposed to NO_2 compared to air. No significant changes in inactivation of influenza virus and IL-1 production *in vitro* between NO_2 and air exposure.
Frampton 1991 (Frampton et al., 1991)	N=39, 19-37 years, healthy, never smoked.	9 subjects exposed to 0.6ppm NO_2 or purified air for 3 hours. 15 subjects exposed to 0.05ppm NO_2 with 3 peaks of 2ppm for 15 minutes each. 15 subjects exposed to 1.5ppm NO_2 or purified air for 3 hours. Exercise.	Lung function (sGaw, PEFR, MEFR, FVC, FEV_1) and airway reactivity to carbachol 30 minutes after exposure.	No effect on lung function for any of the three NO_2 exposure protocols. Only exposure to 1.5ppm NO_2 for 3 hours increased airway reactivity.
Rubinstein 1991 (Rubinstein et al., 1991)	N=5, 18-45 years, healthy, non-smokers.	0.6ppm NO_2 for 2 hours/day on 4 separate days within a six day period.	Respiratory symptoms, lung function (FVC, FEV_1, sRaw), circulating and BALF lymphocyte subtypes.	No effect on symptoms and lung function, or changes in circulating and BALF lymphocyte subtypes

		Light to moderate intermittent exercise.		
Wang 1995 (Wang et al., 1995)	N=16, 18-55 years, seasonal allergic rhinitis, non-smokers.	0.4ppm NO_2 or air for 6 hours.	8 subjects also had a nasal allergen provocation test prior to measurement of nasal airway resistance and nasal lavage (ECP, MPO, IL-8, MCT).	No change in nasal airway resistance, ECP, MPO, IL-8 and MCT after NO_2 or air exposure. After allergen challenge, those exposed to NO_2 compared to air had higher ECP in nasal fluid and there were no differences in increases in MCT. No changes in MPO and IL-8 after allergen challenge.
Salome 1996 (Salome et al., 1996)	N=9, 19-65 years, current asthma. N=11, 7-15 years, severe asthma.	1 hour exposures to 5 different scenarios: Ambient air 0.3ppm NO_2 in ambient air 0.6ppm NO_2 in ambient air Ambient air+gas heater combustion by-products+0.3ppm NO_2 Ambient air+gas heater combustion by-products+0.3ppm NO_2	Airway responsiveness to histamine 1 hour and 1 week after exposure. Lung function and symptoms during, 1 hour and 1 week after exposures.	No significant effect on symptoms or lung function (FEV_1, PEFR). Small significant increase in airway responsiveness at 0.6ppm NO_2 in ambient air but not in the mixture with combustion by-products.
Vagaggini 1996 (Vagaggini et al., 1996)	N=7, mean age=34 years healthy, non-	0.3ppm NO_2 for 1 hour. Moderate	Lung function (FEV_1) and symptoms before and 2 hours after exposure. Nasal lavage and induced sputum 2	No significant changes in lung function (except for a small significant decrease in COPD group), airway responsiveness to hypertonic saline

	smokers. N=8, mean age=29 years, mild asthma. N=7, mean age=58 years, COPD.	intermittent exercise.	hours after exposures.	challenge and nasal lavage eosinophils after exposure to NO_2 compared to air in all three groups. In the normal and COPD groups, an increase in symptom score after NO_2 exposure but not after air exposure.
Blomberg 1997 (Blomberg et al., 1997)	N=30, 20-30 years, healthy, non-asthmatics, non-smokers.	2ppm NO_2 or air for 4 hours. Light exercise.	Endobronchial biopsies and bronchial lavage at 1.5 (n=15) or 6 hours (n=15) after exposures.	Significant increases in IL-8, neutrophils in bronchial washings and increases in lymphocytes in BALF after exposure to NO_2. No up-regulation of adhesion molecules or increase in inflammatory cells in bronchial biopsies.
Blomberg 1999 (Blomberg et al., 1999)	N=12, 21-32 years, healthy, non-asthmatic non-smokers.	2ppm NO_2 or air for 4 hours/day on 4 consecutive days. Light exercise.	Lung function and inflammatory mediators in the lungs. Bronchial biopsies, BAL and bronchial washes 1.5 hours after last exposures.	Significant reductions in FEV_1 and FVC after first NO_2 exposure which attenuated with repeated NO_2 exposures. Significant increases in neutrophils and MPO in bronchial washings but not in BALF. No change in anti-oxidants.
Frampton 2002 (Frampton et al., 2002)	N=21, 18-40 years, healthy, lifetime non-smokers.	0.6ppm NO_2, 1.5ppm NO_2 or air for 3 hours. Intermittent exercise.	Spirometry (FEV_1, FVC) before and after exposure. Blood sampling and BAL 3.5 hours after exposure.	No differences in FEV_1 and FVC after NO_2 exposure compared to air. Decrease in haematocrit, haemoglobin and lymphocytes in blood with NO_2 exposure. Increase neutrophils and lymphocytes in bronchial lavage and increased CD4 lymphocytes in alveolar lavage with NO_2 exposure. Increased susceptibility of airway

				epithelial cells to NO_2 exposure.
Pathmanathan 2003 (Pathmanathan et al., 2003)	N=12, 21-32 years, healthy, non-asthmatics, non-smokers.	2ppm NO_2 or air for 4 hours/day on 4 consecutive days. Light exercise.	Inflammatory mediators in the lungs (bronchial biopsies at 1.5 or 6 hours after exposures).	Significant increases in IL-5, IL-10, IL-13, ICAM-1 following NO_2 exposure compared to air.
Witten (2005) (Witten et al., 2005)	N=15, 21-48 years, mild-moderate atopic asthmatics, non-smokers and past smokers, house dust mite allergy.	0.4ppm NO_2 or filtered air for 3 hours. Intermittent exercise.	Lung function (FEV_1) before and after exposure. House dust mite allergen challenge immediately after exposure. Sputum collected at 6 and 26 hours after allergen challenge.	No differences in FEV_1, allergen provocative dose and respiratory symptoms between NO_2 and filtered air. No differences in inflammatory markers (IL-5, IL-8, ECP, GM-CSF) between NO_2 and filtered air after allergen challenge.
Langrish (2010) (Langrish et al., 2010)	N=10, median age=24 years, healthy males, non-smokers.	4ppm NO_2 or filtered air for 1 hour. Intermittent exercise.	Lung function (FVC, FEV_1, VC) before and after exposure. Blood flow after vasodilators. Fibrinolytic markers (plasminogen activator, plasminogen-activator inhibitor type 1) measured at 4 hours after exposure. Exhaled nitric oxide measured at baseline, and 1 and 4 hours after exposure.	No differences in lung function, blood flow after vasodilators, fibrinolytic markers and exhaled nitric oxide between the two groups.
Scaife 2012 (Scaife et al., 2012)	N=18, 56-76 years, undergone CABG or had a previous history of a heart attack.	0.4ppm NO_2 or air for 1 hour.	Holter monitoring during and for 24 hours after exposure.	No significant changes in heart rate, blood pressure and indices of heart rate variability for exposure to NO_2 compared to air.
Channell 2012 (Channell et al., 2012)	N=14, healthy adults. Mean age diesel	Diesel exhaust ($PM=100ug/m^3$, NO_2=0.8ppm) or	Blood sampling before, immediately after and 24 hours after exposures. Human coronary artery endothelial	ICAM-1 expression increased at both time points after NO_2 exposure and VCAM-1 expression increased at both time points for NO_2 exposure and at 24

	exhaust exposure=24.9 years. Mean age NO_2 exposure=25.3 years.	filtered air. NO_2 (0.5ppm) or filtered air. All exposures for 2 hours with intermittent moderate exercise.	cells probed for ICAM-1 and VCAM-1 expression.	hours for diesel exhaust exposure. IL-8 increased in human coronary artery endothelial cells incubated with plasma after NO_2 exposure.

BAL= bronchoalveolar lavage; BALF=bronchoalveolar fluid; CABG=coronary artery bypass graft; COPD=chronic obstructive pulmonary disease; ECP=eosinophil cationic protein; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity; GM-CSF=granulocyte/macrophage colony stimulating factor; ICAM=intracellular adhesion molecule; IL=interleukin; MCT=mast cell tryptase; MEFR=mid-expiratory flow rate; PEF=peak expiratory flow; sGAW=specific airway conductance; sRaw=specific airway resistance; VC=vital capacity; VCAM=vascular cell adhesion molecule

Table 3 Studies of exposures to ambient air studies in road tunnels, subways and near busy roads

Study	Experimental group	Level and duration of exposure to NO_2.	Health outcomes measured	Results and comments
Huang (1991) (Huang et al., 1991)	N=6, mean age=12 years, dust mite sensitive, moderate asthma.	Concentrated road tunnel air inhaled for 5, 15, 35, 65 and 105 breaths. Inhaling 105 breaths took about 5 minutes. SO_2=70-120ppb and NO_2=0.45-0.50ppm was which were 6 and 20 fold above ambient air respectively.	Lung function tests before and after each breath category. Methacholine challenge and mite allergen challenge before and after taking 105 breaths.	Exposure to concentrated road tunnel air did not affect lung function and did not increase airway sensitivity to methacholine challenge and mite allergen challenges.
Svartengren (2000) (Svartengren et al., 2000)	N=20, 19-55 years, mild atopic (birch, timothy grass) asthma. Exposed to pollutants inside a car within a road tunnel.	30 minute median exposure to: NO_2=313ug/m^3 (0.15ppm) (range 203–462ug/m^3) PM_{10}=170ug/m^3 (range 103–613ug/m^3) $PM_{2.5}$=95ug/m^3 (range 61–218ug/m^3). As a control, subjects also exposed to lower pollution levels in a suburban area – median NO_2=11ug/m^3 (0.005ppm) (range 0-51ug/m^3).	Following allergen challenge 4 hours after exposure, lung function (FEV_1), airway resistance, symptoms and medication used measured.	Concentrations of NO_2 in the car and in the tunnel were similar. After allergen exposure, a significant increase in airway resistance but no difference in fall in FEV_1 between tunnel air exposure and the control exposure. Exposure to road tunnel air had a significantly more asthma symptoms during the late phase after the allergen challenge.
McCreanor (2007) (McCreanor et al., 2007)	N=60, 19-55 years, mild to moderate asthma.	2 hours walking along a busy road (median NO_2=142ug/m^3 (0.07ppm); range 10.7-289ug/m^3; median $PM_{2.5}$=28.3ug/m^3, range 62-161ug/m^3). 2 hours in a park (NO_2=21.7ug/m^3 (0.01ppm);	Lung function (FEV_1, FVC, FEF_{25-75}), PEF, symptoms, exhaled nitric oxide. Sputum for inflammatory markers (IL-8, MPO, ECP).	Significantly greater decrements in FEV_1 and FVC in the street group. Changes were greater in moderate asthma group compared to the mild asthma group. No differences in FEF_{25-75}, asthma symptoms and medication use, and exhaled nitric oxide between the two exposures.

		range 2.4-146ug/m^3; median $PM_{2.5}$=11.9ug/m^3, range 3-55.9ug/m^3).		Higher neutrophils and MPO in the road exposure group. No differences in IL-8 and ECP.
Larsson (2007) (Larsson et al., 2007)	N=16, 19-59 years, healthy, non-smokers.	2 hour exposure to a road tunnel (median NO_2=230ug/m^3; range 180-269ug/m^3; median $PM_{2.5}$=64ug/m^3, range 46-81ug/m^3) 2 hour exposure to an urban area (24-hr mean NO_2=46ug/m3, $PM_{2.5}$=13ug/m^3). Intermittent light exercise.	Lung function (VC, FEV_1, FVC), symptoms, peripheral blood (fibrinogen) and BALF (lymphocytes, fibronectin, total metallo-proteinease-9).	No changes in lung function, markers in peripheral blood (blood count, plasminogen activator inhibitor-1, fibrinogen), fibronectin and total metallo-proteinease-9. Increase reporting of eye, lower airways and upper airways symptoms with road tunnel exposure. Increased alveolar macrophages and lymphocytes in BALF with road tunnel exposure.
Klepczynska-Nystrom (2010) (Klepczynska Nystrom et al., 2010)	N=20, 18-46 years, healthy, non-smokers.	2 hour exposure to a subway environment (mean NO_2=24ug/m^3 (0.01ppm), $PM_{2.5}$=77ug/m^3) 2 hour exposure to an office environment (NO_2 and $PM_{2.5}$ concentrations not provided). Intermittent light exercise.	Lung function (VC, FEV_1, FVC, PEF, exhaled nitric oxide), symptoms, peripheral blood (blood count, plasminogen activator inhibitor-1, fibrinogen), BALF (inflammatory markers – IL-1B, IL-6, IL-8, IL-10, IL-12p70, tumour necrosis factor).	Increased reporting of lower airways symptoms with subway exposure. No differences in upper airway, eye and nose symptoms. Increase in blood fibrinogen and regulatory T-cells in blood after subway exposure. No differences in lung function, plasminogen activator inhibitor-1and BALF inflammatory markers.
Larsson (2010) (Larsson et al., 2010)	N=14, 18-55 years, mild asthma, non-smokers.	2 hour exposure to a road tunnel (median NO_2=265ug/m^3 (0.13ppm); range 112-579ug/m^3; median $PM_{2.5}$=80ug/m^3, range 41-93ug/m^3) 2 hour exposure to hospital laboratory (NO_2 and $PM_{2.5}$ concentrations not provided).	Lung function (VC, FEV_1, FVC, PEF, exhaled nitric oxide), blood count, methacholine challenge for airway responsiveness, nasal lavage and induced sputum 7 hours after exposure.	Significantly increased reporting of upper and lower airways symptoms and decreased PEF with road tunnel exposure. No differences in VC, FEV_1, FVC, airway responsiveness after methacholine challenge, inflammatory markers (IL-1B, IL-6, IL-8, IL-10, IL-12p70, tumour necrosis factor, interferon-gamma) and blood count.

		Intermittent light exercise.		
Klepczynska-Nystrom (2012) (Klepczynska Nystrom et al., 2012)	N=16, 18-52 years, mild asthma, non-smokers	2 hours to a subway environment (mean NO_2=20ug/m^3 (0.01ppm), $PM_{2.5}$=71ug/m^3) 2 hours to an office environment (NO_2 and $PM_{2.5}$ concentrations not provided). Moderate light exercise.	Lung function (VC, FEV_1, FVC, PEF, exhaled nitric oxide), symptoms, peripheral blood (blood count, plasminogen activator inhibitor-1, fibrinogen), BALF (inflammatory markers – IL-1B, IL-6, IL-8, IL-10, IL-12p70, tumour necrosis factor).	Increased reporting of eye and nose symptoms with subway exposure. No differences in lower airway symptoms. Increase in CD4 cells expressing activation marker CD25 in BALF after subway exposure. No differences in lung function, plasminogen activator inhibitor-1, fibrinogen, blood count and BALF inflammatory markers.
Steenhof (2014) (Steenhof et al., 2014)	N=31, 19-26 years, healthy, non-smokers.	5 hour exposure to 5 different environments – 2 traffic sites, an underground train station, a farm and an urban background site. All outdoor sites combined: geometric mean NO_2=0.02ppm, range=0.009-0.034ppm; geometric mean $PM_{2.5}$=23ug/m^3, range=8-95ug/m^3. Underground train station: geometric mean NO_2=0.02ppm, range=0.014-0.026ppm; geometric mean $PM_{2.5}$=140ug/m^3, range=123-167ug/m^3. Intermittent exercise.	White blood count at baseline at 2 and 18 hours after exposure.	In regression models, decreased lymphocytes and eosinophils counts associated with NO_2. In regression models, increased white blood cell count, neutrophils and monocytes counts associated with PM at the underground train station.

BALF=bronchoalveolar fluid; ECP=eosinophil cationic protein; FEF_{25-}75=forced expiratory flow 25 to 75% volume of forced vital capacity; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity; IL=interleukin; MPO=myeloperoxidase; PEF=peak expiratory flow; VC=vital capacity