The Effects of Long-Term Exposure to Ambient Air Pollution on Cardiovascular Morbidity: Mechanistic Evidence

A report by the Committee on the Medical Effects of Air Pollutants

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Foreword

Air pollution is a significant public health problem, with increasing concerns over the adverse health effects on human health. In 2010 COMEAP published a report that estimated the size of the effect of long-term exposure to particulate air pollution on deaths (mortality) in the UK. This report, and others following it from several international sources, brought into sharp perspective the impact of ambient pollution on public health.

The COMEAP report of 2010 noted (largely underpinned by the American Cancer Society (ACS) study findings) that a large part of the effect of exposure to particulate air pollution on deaths in the UK was likely due to effects on the cardiovascular (CV) system. In the current work programme the Committee has extended this work to effects on CV illness or morbidity. The Committee previously considered this issue in its 2006 report 'Cardiovascular Disease and Air Pollution' (COMEAP, 2006). The Committee noted that air pollution has an impact on deaths from CV disease, with clear associations reported between both daily and long-term average concentrations of pollutants, but were unable to determine which components of the air pollution mixture were responsible for the effects. Since then, a vast literature on the health effects of ambient air pollutants has emerged suggesting a need to re-evaluate the topic.

The work has involved three avenues of activity. The evidence relating to mechanisms of effect has been reviewed and is presented in the present report. The epidemiological evidence is currently under review and on completion the third, and most important step: estimating the size of the effects of long-term exposure to air pollution on CV morbidity in the UK will be undertaken. Reports on this and on the epidemiological evidence will be published in the near future.

The present report was prepared by a Working Group of the COMEAP Sub-Group on the effects of exposure to long-term exposure to air pollution on CV morbidity. A large body of evidence was reviewed by Members of the Working Group between 2015 and early 2017. To complete this task, Members devoted a great deal of time and effort and for this. I am extremely grateful. I am also grateful to the COMEAP Secretariat, Sarah Robertson and Alison Gowers, who have worked with and alongside Members: without their help the work would not have been completed.

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Executive Summary

This report deals with the mechanisms of effect of air pollutants as they impact on cardiovascular (CV) morbidity. We have concluded that the mechanistic evidence suggests that long-term exposure to current levels of air pollution is likely to affect CV morbidity. The epidemiological evidence will also be important in coming to a view on these effects, and whether it is possible to quantify them. This evidence is being examined by other COMEAP Working Groups.

The work reported here forms part of COMEAP's work on the impact of long-term exposure to air pollutants on CV morbidity in the UK, undertaken by a COMEAP Sub-group on the effects of exposure to long-term exposure to air pollution on CV morbidity. Working Groups were established to consider three different aspects of the Sub-group's work: 1) Mechanisms of action; 2) Epidemiological evidence; and 3) Quantification of effects. The work reported here was undertaken by the Mechanisms Working Group. The terms of reference of the Working Groups are set out in Appendix 1.

It was decided that Members of the Mechanisms Working Group would focus on evidence published since publication of the review by Brook et al (2010): "Particulate matter air pollution and cardiovascular disease: An update to the Scientific Statement from the American Heart Association". It was noted that Brook et al (2010) had focused on particulate air pollution; Members of the Working Group decided to extend the scope of their work to include gaseous air pollutants.

Members of the Working Group found that the large body of evidence could be covered in a constructive manner by using the following themes:

a The effects of exposure to air pollutants on cardiac electrophysiology

b The effects of exposure to air pollutants on the vascular system

c The effects of exposure to air pollutants on haemostasis

d The role of inflammation and oxidative stress in CV outcomes following exposure to ambient air pollution

It was appreciated that though the focus of COMEAP's work was on the effects of long-term exposure to pollutants, a far greater volume of evidence dealt with the effects of short-term exposure. This was seen as inevitable in that studies of mechanisms often involve the study of effects on human volunteers and on animal models: long-term exposure studies of these kinds are difficult and, in some cases, impossible. It was agreed therefore to review the work on the effects of short-term exposure.

Members of the Working Group adopted a systematic approach to their reviews of the literature. Formal systematic review was beyond the resources of the Working Group, but an exhaustive search and detailed review of the literature was undertaken, as described in Appendix 2.

Members of the Working Group presented their findings as a series of reviews. These were discussed by the full COMEAP Committee. The reviews addressing the role of oxidative stress and haemostasis in CV disease outcomes following exposure to ambient air pollution has been published (Kelly and Fussell 2017; Robertson and Miller 2018).

The conclusions of the Working Group, reproduced below, are set out in Chapter 6 of this report. Chapter 6 also includes a short discussion of the implications of the findings.

a In each of the areas we reviewed we found clear evidence that exposure to air pollutants, primarily fine particulate matter ($PM_{2.5}$), affected a range of physiological and patho-physiological variables and effects. These included indices of inflammatory and oxidative status, reduced heart rate variability (HRV), arrhythmias, endothelial dysfunction, raised blood pressure, progression of atherosclerotic disease and promotion of thrombosis (blood clotting).

b Multiple biological mechanisms were identified for each of the themes described above. No single mechanism is predominant over others, and it is likely that many mechanisms work in concert to produce wide-ranging effects of air pollution throughout the CV system. Oxidative stress (OS) and inflammation remain the main mechanisms of investigation, however, a few novel mechanisms have been described.

c We find that the evidence supporting the assertion that exposure to fine particulate air pollution, namely $PM_{2.5}$ can have detrimental effects on the CV system has strengthened both in terms of volume of studies and range of effects since the review by Brook et al (2010). Overall, the studies we reviewed were carried out in a scientifically appropriate manner based on clearly defined hypotheses.

d There have been few studies examining the potential adverse effects of gaseous pollutants on CV morbidity. The studies published in this area have reported inconsistent findings and do not allow any firm conclusions to be reached.

e The effects observed were not always consistent across studies. Comparison between studies was often difficult, for example due to differences in species, population, exposure scenario, duration of exposure, endpoints, age and co-morbidity.

f Some of the effects recorded were not large and many were demonstrated using exposure to concentrations well above those in ambient air.

g Studies using validated human exposure chamber techniques and exposures that are considered to be representative of 1-2 hours of exposure to roads with very heavy traffic, provide strong evidence that exposure to air pollution may affect CV health.

h The problems of extrapolating from short-term studies to the effects of long-term exposure, from animals to human, and from healthy subjects to those suffering from CV disease were discussed in detail.

i We note that studies of long-term exposure are few in number and essentially limited to epidemiological approaches to the study of mechanisms of effect. We see this as inevitable given the nature of the problem. Toxicological studies, and to a more limited extent epidemiological studies, with longer-term exposure (for example 6 months) of relevant doses are emerging, and the findings from these are expected to be valuable.

j Current data are too preliminary to allow meaningful conclusions about effect modifications by, for example, sex, age and co-morbidities, on the associations between air pollution and CV morbidity to be drawn. Investigations that directly compare potentially susceptible populations with healthy counterparts in the same study are required.

k We have found nothing to persuade us that the CV health effect associations of particulate air pollution reported in relevant epidemiological studies are not causal in nature; indeed, that they are causal in nature seems, to us, likely.

Contents

Chapter 1
Introduction

1.1 Background

Cardiovascular disease (CVD), the diseases of the heart and blood vessels, is responsible for more than a quarter of all deaths in the UK – around 160,000 deaths each year. The British Heart Foundation estimates that deaths from myocardial infarction (heart attack) and stroke account for 70,000 and 40,000 deaths respectively. Furthermore, across the UK more than 500,000 people have been diagnosed as suffering from heart failure and 188,000 hospital episodes are attributed to heart attacks each year (BHF 2017). These figures make clear the impact that CVD has on public health and the burden it imposes on the National Health Service.

While there are some CVD risk factors that cannot be controlled, there are many risk factors that are modifiable. Lifestyle risk behaviours, such as poor diet, high alcohol consumption, smoking and physical inactivity have been linked to increased risk of CVD. In addition, a growing literature points to exposure to ambient air pollution as contributing to the risk of developing CVD. In 2010 COMEAP estimated (COMEAP 2010).

"The current (2008) burden[1] of anthropogenic particulate matter is, with some simplifying assumptions, an effect on mortality in 2008 equivalent to nearly 29,000 deaths in the UK at typical ages and an associated loss of total population life of 340,000 life-years. The burden can also be represented as a loss of life expectancy from birth of approximately six months."

Of these, the majority of deaths were attributed to CVD, and this was recently echoed in the 2015 Global burden of disease study (Cohen et al. 2017). In 1998 COMEAP explored the effects of short-term exposure to air pollutants on hospital admissions from respiratory diseases, based on estimates from time-series studies (COMEAP 1998).The effects of long-term exposure to air pollutants were, then, in the process of being established and less emphasis was placed on effects on the CV system than is the case today. Nonetheless, in 2001 COMEAP recommended an approach for quantifying CV hospital admissions based on estimates from time-series studies of particles (COMEAP 2001). The Committee also noted in its 2006 report 'Cardiovascular Disease and Air Pollution' (COMEAP 2006) that air pollution has an impact on deaths from CV disease and hospital admissions for treatment of CV diseases, with clear associations reported between both daily and long-term average concentrations of pollutants. The Committee was unable to determine which components of

[1] "burden" means the burden placed on Public Health by long-term exposure to anthropogenic particulate matter

the air pollution mixture were responsible for the effects (although it was considered likely that fine particles played an important part). The Committee focused on possible mechanisms of effects of particles: several hypotheses were agreed to be plausible and not mutually exclusive. The findings of this report suggested the need for further analysis of the quantitative effects of air pollutants on hospital admissions for treatment of CV diseases.

A number of reviews on the adverse effects of air pollution on CV health have been undertaken and published. One of the most comprehensive and widely recognised is the 2010 American Heart Association scientific statement on Particulate Matter Air Pollution and Cardiovascular Disease (Brook et al. 2010). It is now generally accepted that exposure to air pollutants plays a role in the development and severity of CVD. Additionally, the triggering of events, such as myocardial infarction and stroke, has been shown to occur within hours of exposure (Peters et al. 2001; Sullivan et al. 2005)

1.2 Purpose

It is accepted that short-term exposure, as reflected by the findings of time-series studies, triggers potentially life-threatening CV events. What is not so well understood is whether long-term exposure exacerbates the effects of disease and, perhaps, increases the likelihood of potentially life-threatening events being triggered by factors including, but not limited to, short-term exposure to air pollutants. Understanding and interpreting the evidence on the long-term effects of air pollution exposure on CV morbidity is likely to be important in terms of public health. Given that long-term exposure has an effect on CV mortality it seems an at least plausible inference that such exposure should affect CV morbidity. COMEAP was asked to examine the evidence for the effects of long-term exposure to ambient air pollution on CV morbidity, and to estimate the size of the effects in the UK today. A COMEAP Sub-group was set up for this purpose. A smaller Mechanisms Working Group was established to examine the biological plausibility of the assertion that long-term exposure to ambient air pollution has an effect on CV morbidity.

Evidence of biological plausibility is helpful in making the argument for the causality of associations reported in epidemiological studies of air pollutants and effects on health. A lack of such evidence does not negate the assertion that such findings are causal in nature: it is clearly possible that mechanistic evidence may not be available at the time when decisions need to be made; it is also possible that the mechanisms underlying effects may not have been proposed. Mechanistic evidence, when supportive, tends to suggest plausibility, rather than actuality, of mechanisms of effect. This is because mechanistic studies are often not undertaken in people and, importantly, not in people susceptible to effects of air pollutants, but in healthy subjects, in animals or in *in vitro* systems.

1.3 Approach

Working Groups were established to consider three different aspects of the Sub-group's work: 1) Mechanisms of action; 2) Epidemiological evidence; and 3) Quantification of effects. Findings of the Mechanisms Working Group are provided in this report.

An extensive body of literature exists on the effects of air pollution on CV health. It was recognised that a complete review of this literature was a formidable task and beyond COMEAP's resources. The Committee agreed that the 2010 American Heart Association

scientific statement on "Particulate matter air pollution and cardiovascular disease" (Brook et al. 2010) provided a good starting point for their work. A comprehensive search strategy was therefore developed to identify the relevant studies published since the literature searches conducted by Brook et al (2010). This decision limited the task but it remained a demanding one. Research on the effects of air pollutants on the CV system is expanding rapidly and what might seem a simple task, identifying studies as they appear, is actually very difficult. It was appreciated that no review can be completely up to date: the appearance of papers during and after initial searching of the literature always presents a problem. Publication cut-off dates for the literature reviews undertaken by the Working Group were agreed. COMEAP decided that a systematic approach to literature identification and retrieval should be taken. Initial thinking suggested that a formal systematic review of the literature should be undertaken. However, due to the wealth of research in this area and limited resources this proved to be impossible and a less demanding approach was adopted. This approach is outlined below.

1.3.1 Search strategy

The Working Group developed an initial list of search terms as well as pre-defined eligibility criteria for studies. Key search terms included air pollution or particulate matter or ozone or carbon monoxide or nitrogen dioxide or sulphur dioxide plus an endpoint of cardiovascular morbidity. Preliminary examination of the literature and Members' reading and individual familiarity with the field suggested four possible mechanisms linking air pollution and cardiovascular disease and it was agreed that these might usefully be reviewed:

a Effects of exposure to air pollutants on cardiac electrophysiology (Robert Maynard)

b Effects of exposure to air pollutants on the vascular system (Mark Miller)

c Effects of exposure to air pollutants on haemostasis (Sarah Robertson)

d The role of inflammation and oxidative stress (OS) in cardiovascular disease outcomes following exposure to ambient air pollution (Frank Kelly and Julia Fussell)

Members of the Working Group and Secretariat were allocated to each of these four areas (indicated in brackets) and then the search strategy was modified accordingly. For full details see Appendix 2. All searches were conducted in PubMed and the following limits were applied to all searches: publication dates from 1st January 2009 until the end of 2015/2016 (key earlier publications are discussed for contextual background); the criteria used in selecting or rejecting studies were similar. The following key exclusion criteria were used in the review:

a Duplicate references

b No original data included (for example, reviews, editorials, letters[2])

c Language: full text not available in English

[2] Reviews, editorials and letters were screened for potential additional references

d Exposure not relevant to ambient air pollution (for example, indoor air pollution, occupational exposure, biomass[3], cigarette smoke, manufactured nanoparticles)

e Did not provide mechanistic data beyond mortality or hospital admissions[4]

Whilst the focus was on the effects of long-term exposure, it was decided that observations from short-term exposure studies could potentially provide insights into the effects of long-term exposures. Studies investigating short-term associations were therefore retrieved and reviewed for relevance. After removing duplicates, a two-stage selection procedure was undertaken to identify relevant studies (title/abstract screening followed by full-text screening). Members of the Working Group then wrote their own appreciations of the literature focussing on key studies deemed to have produced results of specific interest and thereby advancing our understanding of how air pollution contributes towards CVD. This process of systematic searching and selective reviewing and reporting brought the task within the resources of the Working Group.

Epidemiological studies reporting single pollutant associations were taken at face value; many studies did not examine multi-pollutant models and these can, in any case, be complicated to interpret. It should be borne in mind that associations reported to be due to one pollutant can still be fully or partially representing effects of other pollutants. Combining our review of the epidemiological mechanistic evidence with the toxicological evidence was thus important.

1.4 Report Structure

Chapters 2, 3, 4 and 5 are the reports by the Members of the Mechanism Working Group and Secretariat, based around the 4 thematic areas: (1) Chapter 2: cardiac electrophysiology; (2) Chapter 3: vascular system; (3) Chapter 4: haemostasis; and (4) Chapter 5: inflammation and OS. In Chapter 6, we present our conclusions and recommendations for future work. Chapter 6 also discusses the issues and uncertainties in interpreting the mechanistic evidence

[3] Occupational exposure to manufactured nanoparticles, industrial accidents and environmental events (e.g. volcanic eruptions or wildfires) were not included. Workplace exposures that potentially spill into communities or were representatives of the main aspects of general urban air pollution (e.g. engine emissions from garages, bus depots.) were included

[4] Studies solely reporting mortality or morbidity will be reviewed by the Epidemiology working group

Chapter 2
Effects of exposure to air pollutants on cardiac electrophysiology

For the purposes of this review the term "cardiac electrophysiology" has been taken to include the control of heart rate (HR) and rhythm, depolarisation and repolarisation of myocardial tissue and myocardial contractility. Emphasis has been placed on measurement of Heart Rate Variability (HRV) parameters. HRV is a complex physiological phenomenon that reflects the inter-play of sympathetic and parasympathetic control of the heart. Reviews of HRV and its use in clinical cardiology have been provided by Billman et al (Billman 2011) and Malik et al (Malik et al. 1996), the latter being particularly helpful for details of the various parameters monitored. In brief: two sets of parameters are widely used. These are those based on time domain measurements (for example, the standard deviation of the intervals between normal electrocardiogram (ECG) complexes (SDNN) and the square root of the mean of the sum of squares of the differences between adjacent NN (Normal to Normal) intervals (RMSSD); and those based on frequency domain analysis of the ECG power spectrum: High frequency (HF) and Low Frequency (LF) power. Many other parameters, including the QT interval (or the QT interval corrected for heart rate, QTc) which reflects the depolarisation-repolarisation process, depression of the ST segment which reflects the effects of ischaemia and T wave characteristics (reflecting repolarisation) have also been monitored. It is not possible, here, to review these parameters in detail: the reviews mentioned above provide detailed explanations. An outstanding review of the principles of cardiac electrophysiology has been provided by Levick (Levick 2010).

Ninety two studies were reviewed; these have been sub-divided by the pollutants studied.

2.1 Animal studies

2.1.1 Studies of the effect of particulate matter

Studies of the effects of exposure to concentrated ambient particles (CAP) have produced varying results. Chen et al (Chen et al. 2010) and Lippmann et al (Lippmann et al. 2013) showed that exposure of apolipoprotein E$^{-/-}$(ApoE$^{-/-}$) mice to Manhattan CAP was strongly associated negatively with HR and positively with indices of HRV SDNN and RMSSD) but that Tuxedo CAP had the opposite effects. Composition of the particulate material appeared to be important in controlling the direction of the effects. Kamal et al (Kamal et al. 2011) also reported varying effects of CAP on heart rate and HRV depending on the prevailing source

category. Farraj et al (Farraj et al. 2015) reported that neither CAP nor ozone (O_3) had any effect on HRV but that the combination of CAP and O_3 reduced SDNN. De Brito et al (Brito et al. 2014) also found no effect of CAP on HRV. Carll et al (Carll et al. 2015) studied the effects of metal-rich CAP in mice with isoprenaline-induced cardiomyopathy: RMSSD was increased. Studies in animal models of human disease have produced varying results: Wagner et al (Wagner et al. 2014) found that HRV in rats with induced metabolic syndrome was unaffected by CAP, O_3 or CAP + O_3. Wang et al (Wang et al. 2013) on the other hand showed that Baltimore particulate matter (PM) decreased HRV in mice that had been genetically engineered to display cardiomyopathy. Some authors have shown that exposure to PM can increase the occurrence of arrhythmia in models of cardiac ischaemia: Gao et al (Gao et al. 2014), and Wellenius et al (Wellenius et al. 2011) reported an increased frequency of premature beats on exposure of rats with acute myocardial infarctions to stack emissions.

Studies of the effects of diesel PM have also produced mixed results. Lamb et al (Lamb et al. 2012) reported that filtered diesel exhaust (DE; without particulate material) produced immediate effects on the ECG of spontaneously hypertensive (SH) rats but that whole DE did not. The latter, however, produced post-exposure ST segment depression. Neither filtered nor whole DE had any effect on HRV. Several groups (Gordon et al. 2012; Huang et al. 2010; Kim et al. 2012) showed that exposure to DE was associated with impaired contractility. Carll and colleagues (Carll et al. 2012; Carll et al. 2013a; Carll et al. 2013b) reported a series of studies which showed that DE slowed atria-ventricular conduction and ventricular repolarisation, and had a bipolar effect on HRV. The authors suggested that early sympathetic dominance might be replaced by parasympathetic dominance and then returned to sympathetic dominance. Brito (Brito et al. 2010) reported that exposure to diesel particle increased RMSSD.

Reports of effects of metal-rich PM have come from several groups. Metal rich PM increased RMSSD and the incidence of AV block arrhythmia in a rat model of cardiomyopathy (Carll et al. 2015). Nickel sulphate was shown to have no effects on HRV in normal rats but to increase HRV (LnRMSSD) in hypertensive animals (Chuang et al. 2013). Ultrafine particles (UFPs) have been studied. Upadhyay et al (Upadhyay et al. 2014) showed that ultrafine carbon particles decreased HRV in a rat model of hypertension. Savi et al (Savi et al. 2014) reported adverse effects of ultrafine titanium dioxide (TiO_2) on cardio-myocytes. Kan et al (Kan et al. 2014) reported results that suggested that ultrafine TiO_2 had an effect on the nodose ganglion (vagus nerve, visceral afferent) in rats. Jin et al (Jia et al. 2012a) reported that ultrafine black carbon (BC) reduced HRV in mice.

2.1.1.1 Summary

Many studies of exposure to supra-ambient concentrations of PM have shown effects on cardiac-electrophysiology. The effects reported are inconsistent. The hypothesis that PM leads to decreased parasympathetic activity and thus to reduced HRV is not entirely sustained by these studies. It seems that a much more complex relationship between exposure to PM and cardiac -electrophysiology exists and that the relationship might depend on disease states and on particle composition.

2.1.2 Studies on the effects of gases

A significant number of studies of effects of gases have been published. Farraj et al (Farraj et al. 2012) showed that exposure to O_3, 0.2 ppm for 4 hours had no effect on HRV in rats but that exposure to 0.8ppm for 4 hours did: RMSSD, SDDN and HF HRV all increased. In

addition, ST segment depression, lengthening of the PR interval, and an increase in atrial premature beats were reported. The authors suggested an increase in parasympathetic activity. Gordon et al (Gordon et al. 2014) reported no effects of 1.0 ppm O_3 (6 hours per day, 2 days per week for 13 weeks) in aged rats. Wang et al (Wang et al. 2013) exposed rats to 0.81 ppm O_3 with and without PM. In the case of the combination exposure, HF HRV increased and despite a general reduction in HRV, it was suggested that vagal activity had increased. A number of studies have looked at effects of carbon monoxide (CO) but with the exception of work by André et al (Andre et al. 2011) which showed an increased sensitivity to isoprenaline in rats exposed to ambient concentrations of CO, none seems to shed light on the effects of ambient exposure to the gas. The same might be said of studies of sulphur dioxide (SO_2). No studies using nitrogen dioxide (NO_2) were found. The study comparing filtered and non-filtered diesel exhaust (Lamb et al. 2012) [see section 2.1.1] suggests a possible different effect of diesel exhaust gases but it is unknown whether NO_2, CO or other volatile components are contributing to this.

2.1.2.1 Summary

Only O_3 has been studied in detail: it is clear that exposure to high concentrations of O_3 can affect cardiac electrophysiology but this has not been consistently found. It seems likely that O_3 and PM have interactive effects. It is not clear whether O_3 is more likely to increase or reduce HRV, but the former seems more probable.

2.2 Volunteer studies

Twenty eight studies were reviewed. Studies of the effects of ambient and CAPs on volunteers have, in general, confirmed that such exposure reduces HRV as a result of reducing parasympathetic dominance of the autonomic control of the heart (Brook et al. 2014; Sivagangabalan et al. 2011; Tong et al. 2012). Studies of the effects of exposure to DE have produced conflicting results. Tong et al (Tong et al. 2014) reported work in which six subjects characterised by the GSTM-1 null mutation were exposed to whole DE (PM concentration: 100-300 $\mu g/m^3$). No effects on SDNN or indices of repolarisation were found but, 18 hours after exposure to the highest concentration, a reduction in LF and very LF (VLF) power was found. Complex studies by Langrish et al (Langrish et al. 2013) have revealed effects of DE on the control of blood vessel diameter. Mills et al (Mills et al. 2011) reported that exposure of healthy subjects, and some who had suffered a myocardial infarction, to diesel PM (300 $\mu g/m^3$), alone or in combination with NO_2 or O_3, had no effect on HRV. The general lack of effects in these studies is surprising: diesel particles make a significant contribution to ambient PM. Studies of the effects of exposure to UFPs have also produced mixed results (Samet et al. 2009; Vora et al. 2014). Samet et al (Samet et al. 2009) reported that exposure increased HRV in a group of young volunteers. The authors commented that reductions in HRV were more likely in the elderly and that there might be an age-dependent effect. Vora et al (Vora et al. 2014) reported that exposure of middle aged subjects with type 2 diabetes to elemental carbon produced non-significant decreases in SDNN, pNN50 and RMSSD, but that HF HRV decreased and HR increased. It was suggested that diabetes might blunt the cardiac response to particles: the point being that diabetes is associated with autonomic neuropathy. Devlin et al (Devlin et al. 2014) reported that exposure of subjects with metabolic syndrome to concentrated UFPs led to an increase in the QT interval (QTc). The authors pointed out that though the effect was small it approached that associated with pro-arrhythmic risk. Two studies (Huang et al. 2012; Scaife et al. 2012) reported that exposure to NO_2 (400 and 500 ppb for 1 or 2 hours) had no effect on HRV.

2.2.1 Summary

No new and persuasive body of evidence suggesting that exposures to ambient concentrations of O_3 or NO_2 have significant effects on cardiac electro-physiology has appeared. The evidence for effects of diesel exhaust is conflicting; that for concentrated ambient particles is rather stronger. UFP have been shown to produce effects though the effects are not large and not entirely consistent across studies.

2.3 Epidemiological studies of the effects of long-term exposure to air pollutants on cardiac electrophysiology and cardiac function

Thirteen studies were reviewed. A number of cohort studies have revealed effects of long-term exposure to air pollutants on cardiac electrophysiology. In general there is more evidence for effects on repolarisation (indicated by lengthening of the QT interval) than on HRV. Very interestingly, Adam et al (Adam et al. 2012) showed that long-term exposure to traffic associated PM produced HRV effects only in those subjects taking ACE inhibitor therapy. Further work from this group (Adam et al. 2014) showed that long-term exposure to PM was associated with a reduction in HRV only in a sub-group characterised by a genotype likely to lead to an increased inflammatory response (two pro-inflammatory G-alleles of the functionally-relevant IL6-174G/C polymorphism). Mordukhovich and colleagues (Mordukhovich et al. 2015) reported an increase in QT interval associated with long-term exposure to fine PM ($PM_{2.5}$) in a cohort of elderly people; work from the same group (Mordukhovich et al. 2016) from the Normative Aging Study cohort showed a rather weak effect of long-term exposure to $PM_{2.5}$ on HRV. Leary et al (Leary et al. 2014), multi-ethnic study of atherosclerosis (MESA) cohort, reported that right ventricular function was negatively related to annual average concentrations of NO_2.

2.3.1 Summary

Evidence to show that long-term exposure to PM and NO_2 has a negative effect on cardiac function has accumulated. The evidence is, however, not entirely consistent.

2.4 Epidemiological studies of the effects of short-term exposure to air pollutants on cardiac electrophysiology and cardiac function

Ninety five studies were reviewed. This substantial group of studies provides a large data-set and one that is difficult to summarise briefly. The studies include some of healthy subjects, others of patients with a variety of diseases: diabetes, cardiovascular disease (CVD; recent myocardial infarction, heart failure), chronic obstructive pulmonary disease (COPD), and asthma. Studies with continuous, on-line monitoring of HRV and concentrations of pollutants have led to associations between a considerable number of individual pollutants and a similar or larger number of HRV parameters and other physiological and biochemical variables being reported. If, to these, are added findings at a number of time-points it will be appreciated that large tables of data can be generated. Authors have tested for the significance of individual associations by multiple pair-wise comparisons. The meaning or importance of the occasional p value of $< .05$ in such large tables of data is open to some doubt. Additionally, some studies

have looked at associations between end-points and concentrations of pollutants using lagged concentrations in the models developed to describe the associations. Few studies have used an *a priori* approach and authors have focussed on the lags that have generated significant associations. The picture is further complicated by our lack of knowledge of what changes, usually small and perhaps transient changes, in parameters of HRV actually mean both in healthy subjects and in patients suffering from a variety of diseases. It is not true to say that only reductions in HRV are important; several authors have suggested that increases in HRV are also related to an increased probability of adverse cardiovascular (CV) events. The effects of disease upon the responses of the body to pollutants further complicate the problem. For example, diabetics suffering from autonomic neuropathy may be "unable" to respond with a change in HRV in the same way as healthy subjects.

2.5 Healthy subjects

Forty seven studies involved healthy subjects. In the great majority of those that monitored effects of air pollutants on HRV, and most did, there was clear evidence, not discussed in detail here, that exposure to air pollutants reduced HRV. A few studies (Davoodi et al. 2010; Jia et al. 2012b; Shields et al. 2013; Weichenthal et al. 2014) reported that exposure to air pollution was associated with an increase in HRV. Most studies involved ambulatory monitoring of HRV whilst subjects walked, cycled or commuted in motor vehicles through areas affected by urban air pollution. In many cases personal exposure to air pollutants was measured. One group of studies (Wu et al. 2010, 2011a, b; Wu et al. 2013) is especially interesting. These studies were focused on a small group of Beijing taxi drivers (N = 14) who were monitored before, during and after the Olympic Games of 2008. Levels of pollutants were sharply reduced during the period of the Games. Several important findings should be noted. Firstly, traffic related exposures to high concentrations of $PM_{2.5}$ are associated with, in general, a reduction in HRV; secondly, ambient temperature and $PM_{2.5}$ are associated with HRV in a non-linear fashion; thirdly, the effects of temperature and $PM_{2.5}$ (and CO) on HRV interact with one another. Additionally, it seems that RMSSD is a better index of effects of $PM_{2.5}$ on cardiac autonomic control than SDNN. These are important findings and need to be taken into account when interpreting and designing studies in this field. The Inhalation Toxicology paper of 2011 (Wu et al. 2011a) is unusual in that modelling revealed the complexity of the exposure-response relationship between $PM_{2.5}$ and HRV. The relationship between $PM_{2.5}$ and indices of HRV was found to be far from linear: SDNN, for example, rose as $PM_{2.5}$ increased, then fell back only to increase again at high PM concentrations.

Wu et al (Wu et al. 2013) also reported marked alinearities in the relationship between temperature and HRV. Hampel et al (Hampel et al. 2014) showed that changes in HRV followed rapidly on changes in exposure to PM.

Both $PM_{2.5}$ and particle number concentration (PNC) were related to reductions in HRV. Weichenthal et al (Weichenthal et al. 2014) reported an interaction between the effects of $PM_{2.5}$ and O_3 on HRV: on days when $PM_{2.5}$ concentrations were low, O_3 was associated with a decrease in HRV; when $PM_{2.5}$ was high: O_3 increased HRV. Langrish et al (Langrish et al. 2009) reported an interesting study of the effects of reducing exposure to particles by the use of a face mask. In comparison with not wearing a mask, wearing a mask increased HRV. Lee et al (Lee et al. 2016) reported a study of 21 subjects which showed a clear relationship between HRV and nocturnal $PM_{2.5}$. A rapid onset of effect was seen. Effects on repolarisation have been studied: Liao et al (Liao et al. 2010) showed that $PM_{2.5}$ was associated with a reduction in

repolarisation parameters, effects occurred 3-4 hours after exposure. Effects on the ST segment of the ECG and on the T wave have been reported by several groups, for example Zhang et al (Zhang et al. 2009). The interaction between temperature and effects on $PM_{2.5}$ on HRV was studied by Ren et al (Ren et al. 2011): warm season effects (HRV decreased as temperature increased) were greater when O_3 concentrations were raised but unaffected by changes in $PM_{2.5}$. But not all studies showed effects: Lux and Pope (Lux and Pope 2009) showed no association between $PM_{2.5}$ and repolarisation parameters in a group of elderly subjects. In one study, by Feng et al (Feng et al. 2015), the effect of what might have been expected to be an index of increased susceptibility was studied. However, it was shown that HRV was more markedly reduced in subjects with a low Framingham Score (estimates the risk of developing CVD within a 10 year period; those with a score <10% are classified as low risk, 10-20% as moderately high risk and > 20% as high risk) than in those with a high score.

2.5.1 Summary

Exposure to ambient air pollutants has been repeatedly shown to affect cardiac electrophysiology. In general, HRV has been shown to be reduced and indices of repolarisation to be changed in a direction suggestive of deleterious effects, by exposure to air pollutants. Alinearities in the response have been reported. Most studies have focused on the effects of PM, very few have addressed the possible effects of gases.

2.6 Patients

Forty eight studies were concerned with effects in patients suffering from a variety of diseases: diabetes, metabolic syndrome, heart failure, myocardial infarction, COPD and asthma. Not all monitored effects on cardiac electrophysiology. Most studies focused on a group of patients and did not include a control group of healthy subjects. Several authors have pointed out the possible effects of drugs on the autonomic response to air pollutants: beta-blocking agents have been shown to reduce the response (de Hartog et al. 2009); there is a suggestion that ACE inhibitors increase the response (Bartell et al. 2013). These findings are illustrated by the following two figures: the first (Figure 2.1) from de Hartog et al (de Hartog et al. 2009); the second (Figure 2.2) from Bartell et al (Bartell et al. 2013).

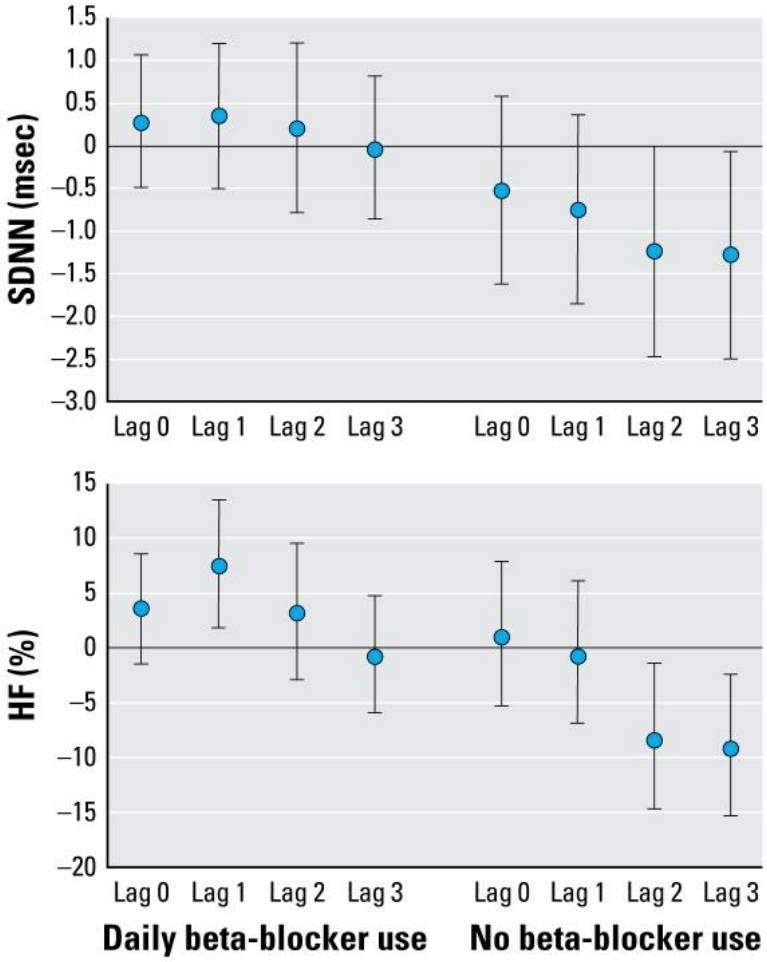

Figure 2.1 Pooled effect estimates (95% CIs) for three study panels for the association of outdoor $PM_{2.5}$ with HRV (SDNN and HF) stratified by beta-blocker use. Effect estimates are calculated for an increase of 10 μg/m³ $PM_{2.5}$. (Reproduced with permission from Environmental Health Perspectives, Hartog et al., 2009)

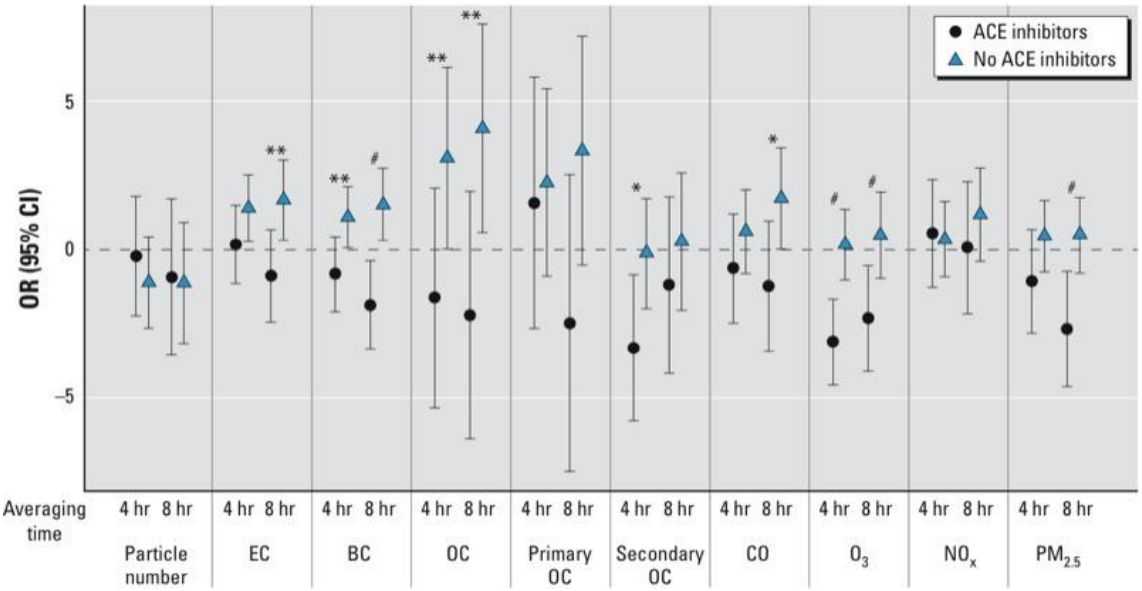

Figure 2.2 Associations of SDNN with outdoor air pollutants: effect modification by ACE inhibitor use. (Reproduced with permission from Environmental Health Perspectives, Bartell et al., 2013)*p<0.1, **p<0.05, #p<0.01, compared with no effect modification by ACE inhibitor use

Folino et al (Folino et al. 2009) also reported a protective effect of beta blocking drugs. Effects on patients with diabetes have been shown by Sun et al (Sun et al. 2015), Laumbach et al (Laumbach et al. 2010), Whitsel et al (Whitsel et al. 2009), Peters et al (Peters et al. 2015) and Hampel et al (Hampel et al. 2012) who showed that effects were increased in subjects with an abnormality of a gene controlling the choline transporter: this suggests a mechanistic link with effects on autonomic function. Sarnat et al (Sarnat et al. 2014) reported a study comparing effects of commuting on healthy subjects and subjects with asthma. Indices of lung function, airway inflammation and HRV were monitored at a number of time points. Subjects with asthma continued their usual medication during the study. More marked effects on RMSSD in the asthmatic subjects were noted. The authors were cautious in their interpretation of these findings: they did speculate that patients with asthma may be more sensitive than healthy subjects as regards the effects of traffic-associated air pollutants on cardiac electrophysiological function. Effects on patients who had had a myocardial infarction have been reported by several groups. Rich et al (Rich et al. 2012b) reported effects in a group of patients undergoing cardiac rehabilitation therapy. This well-designed and detailed study involved measurements of UFPs, accumulation mode particles and $PM_{2.5}$. The findings are shown in Table 2.1.

It will be seen that of the several HRV parameters monitored (the original paper should be seen for details of the measurements), particle sub-groups and time points, few statistically significant effects were found and that these were related to a variety of time points with effects on RMSSD occurring early and, perhaps, suggesting different mechanism from that affecting repolarisation (TpTe). The authors concluded that sub-clinical reductions in parasympathetic modulation and prolonged late repolarisation were associated with exposure to particles. Langrish et al (Langrish et al. 2012) reported that use of a face mask to reduce exposure to particles reduced the effects of Beijing air pollution on HRV in a group of subjects with coronary heart disease. Huang et al (Huang et al. 2012) also reported an association.

Table 3.1 Change in each outcome, measured in the pre-exercise resting period, associated with each IQR increase in UFP, accumulation mode particle (AMP), and $PM_{2.5}$, by lag hour and time when outcome measurement was made. Reproduced with permission from Environmental Health Perspectives, Rich et al., 2012)

	UFP[a]	AMP[b]	$PM_{2.5}$[c]
Outcome and lag hr	Change in outcome (95% CI)	Change in outcome (95% CI)	Change in outcome (95%CI)
MeanNN (msec)			
0–5	−3.49 (−9.20, 2.23)	−1.21 (−7.61, 5.19)	−2.78 (−9.27, 3.70)
0–23	−2.53 (−8.76, 3.69)	−1.56 (−8.44, 5.33)	−0.21 (−7.41, 6.99)
24–47	−3.29 (−9.17, 2.59)	1.92 (−5.16, 9.00)	−0.08 (−7.05, 6.89)
48–71	3.40 (−2.89, 9.68)	3.37 (−3.47, 10.22)	0.52 (−6.19, 7.23)
72–95	3.71 (−2.14, 9.55)	1.75 (−4.64, 8.13)	−0.54 (−6.78, 5.70)
96–119	−3.45 (−9.56, 2.66)	−3.26 (−9.46, 2.95)	0.79 (−5.19, 6.77)
SDNN (msec)			
0–5	−1.07 (−2.72, 0.58)	−0.66 (−2.50, 1.19)	−1.37 (−3.25, 0.51)
0–23	−0.68 (−2.47, 1.12)	−0.68 (−2.66, 1.30)	0.12 (−1.96, 2.19)
24–47	0.05 (−1.65, 1.74)	0.58 (−1.46, 2.61)	1.61 (−0.39, 3.60)
48–71	−0.47 (−2.29, 1.35)	0.50 (−1.47, 2.47)	1.83# (−0.09, 3.75)
72–95	−1.31 (−3.00, 0.38)	−0.44 (−2.29, 1.40)	2.67** (0.88, 4.46)
96–119	−0.05 (−1.80, 1.71)	−0.01 (−1.79, 1.77)	0.68 (−1.03, 2.40)
rMSSD (msec)			
0–5	−2.31# (−4.78, 0.16)	−3.65** (−6.39, −0.91)	−2.81# (−5.67, 0.06)
0–23	−2.45# (−5.13, 0.24)	−4.33** (−7.27, −1.38)	−1.53 (−4.67, 1.61)
24–47	−2.01 (−4.53, 0.52)	0.40 (−2.63, 3.43)	1.69 (−1.34, 4.73)
48–71	−0.91 (−3.64, 1.82)	−0.45 (−3.39, 2.49)	0.73 (−2.19, 3.66)
72–95	−1.74 (−4.29, 0.81)	−2.19 (−4.96, 0.57)	−0.22 (−2.95, 2.51)
96–119	−0.59 (−3.26, 2.09)	−1.87 (−4.58, 0.83)	−0.05 (−2.65, 2.55)
QTc (msec)			
0–5	0.43 (−1.03, 1.88)	0.17 (−1.44, 1.78)	−0.27 (−1.97, 1.42)
0–23	1.14 (−0.43, 2.71)	0.83 (−0.91, 2.57)	−0.13 (−1.98, 1.72)
24–47	0.17 (−1.34, 1.67)	−0.05 (−1.84, 1.74)	−0.93 (−2.72, 0.86)
48–71	0.19 (−1.40, 1.77)	0.85 (−0.87, 2.58)	0.23 (−1.49, 1.95)
72–95	−1.09 (−2.59, 0.42)	−0.68 (−2.32, 0.97)	−0.04 (−1.64, 1.57)
96–119	0.53 (−1.05, 2.10)	1.02 (−0.58, 2.62)	0.56 (−0.97, 2.09)
TpTe (msec)			
0–5	0.21 (−0.41, 0.84)	0.21 (−0.49, 0.91)	0.07 (−0.63, 0.76)
0–23	0.34 (−0.33, 1.02)	0.78* (0.02, 1.53)	0.24 (−0.52, 0.99)
24–47	0.33 (−0.32, 0.98)	1.05** (0.28, 1.82)	−0.10 (−0.83, 0.63)
48–71	0.60# (−0.09, 1.29)	0.53 (−0.22, 1.28)	−0.50 (−1.20, 0.20)
72–95	−0.24 (−0.89, 0.41)	−0.64# (−1.35, 0.07)	−0.53 (−1.19, 0.14)
96–119	−0.25 (−0.92, 0.43)	0.04 (−0.65, 0.72)	−0.06 (−0.69, 0.57)

[a]IQR increases of 2,885 particles/cm^3 (6-hr mean) and 2,680 particles/cm^3 (24-hr mean). [b]IQR increases of 897 particles/cm^3 (6-hr mean) and 838 particles/cm^3 (24-hr mean). [c]IQR increases of 7.2 μg/m^3 (6-hr mean) and 6.5 μg/m^3 (24-hr mean).
*$p < 0.05$. **$p < 0.01$. #$p < 0.10$.

between ambient concentrations of air pollutants and reduction of HRV in a group of patients with heart disease. Zanobetti et al (Zanobetti et al. 2010) reported an interesting study of 46 patients with heart disease. The effects of different sub-fractions of PM on HRV are shown in Figure 2.3 taken from Zanobetti et al (Zanobetti et al. 2010). Independent responses of HRV to $PM_{2.5}$, O_3 and NO_2 were found by Zanobetti et al (Zanobetti et al. 2010).

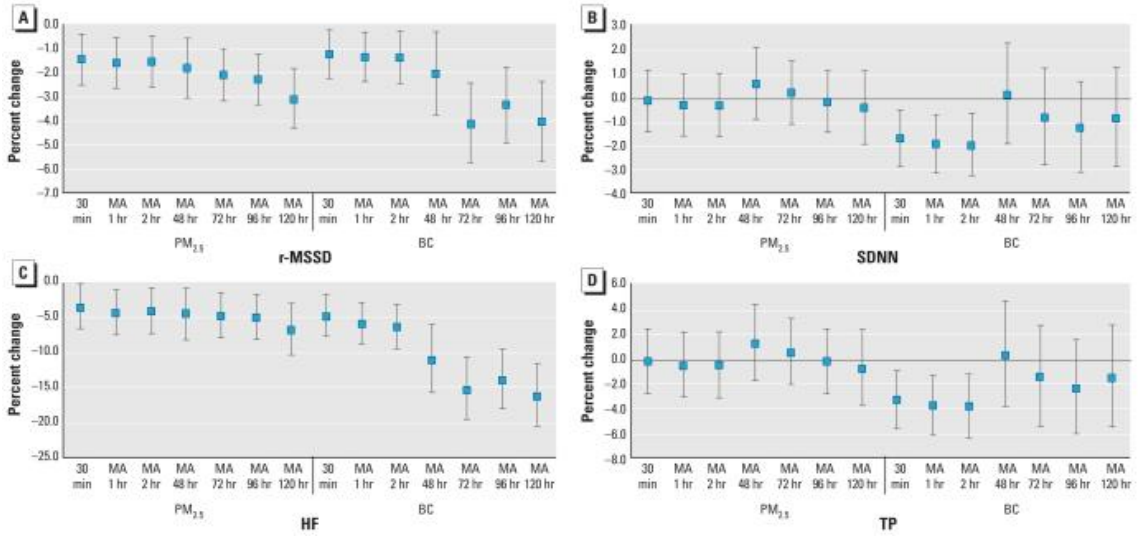

Figure 2.3 Percent change (95% CI) in r-MSSD (msec; A), SDNN (msec; B), HF (msec^2, C), and TP (msec^2, D) associated with different averaging $PM_{2.5}$ and BC exposure. MA, moving average. $PM_{2.5}$ and BC effects are scaled to their IQR. (Reproduced with permission from Environmental Health Perspectives, Zanobetti et al., 2010)

2.6.1 Summary

A number of authors have shown that exposure of patients suffering from heart disease to air pollutants can affect HRV. It is clear that these effects can themselves be affected by medication, especially by that which affects the ANS. In general, small and not always statistically significant reductions in HRV were found but the interpretation of the findings is complicated by the range of indices of cardiac electrophysiology that have been studied. In some studies a large number of endpoints were studied and, of these, only some showed significant effects. There is some evidence to suggest that air pollutant gases, O_3 and NO_2 have effects though more emphasis has been placed on particulate air pollutants.

2.7 Overall summary

It is clear that exposure to ambient concentrations of air pollutants can affect cardiac electrophysiology: this effect is reflected by a reduction in HRV. On the whole reductions in HRV have been small and some inconsistency in responses is evident. In general these studies support the contention that exposure to air pollutants might increase the likelihood of episodes of cardiac morbidity. This supports the conclusions of the Brook et al report of 2010.

Chapter 3
Effects of exposure to air pollutants on the vascular system

3.1 Introduction

Over the last few decades it has become apparent that air pollution has effects that are not restricted to the lung, but occur throughout the body. The cardiovascular system (CVS) in particular appears to be a prominent target for the actions of air pollution, whereby inhalation of pollutants has multiple detrimental actions on different facets of the CVS. This review provides a summary of recent evidence on the biological mechanisms by which exposure to air pollution can affect the vasculature. It considers mechanisms and markers of vascular dysfunction in otherwise healthy arteries, as well as for the progression of vascular disease, in particular the formation of atherosclerotic plaques and the clinical consequences that arise from them. Appendix 2 presents details of the search process, including reasons for exclusion. A detailed document was written providing a brief description of all references deemed relevant to the topic. Given the large number of references, the review is extensive (>80 pages). The current document is a more concise, descriptive summary of the key points. A list of references found is available upon request.

3.2 Air pollution and blood pressure

Blood pressure (BP) is one of the easiest parameters to measure non-invasively in man, yet one that is invaluable in terms of diagnosing cardiovascular (CV) risk. While BP is required to direct blood to the organs of the body based on demand, elevated blood pressure (or "hypertension"; > 140/90 millimetres of mercury (mmHg) systolic blood pressure (SBP)/diastolic blood pressure (DBP)) is a recognised risk factor for cardiovascular disease (CVD), as well as many non-CVDs. The strength of associations between hypertension and CVD, as well as the prevalence of CVDs in many demographics across the world, has led to the belief that very small increases in blood pressure (for example,< 5 mmHg) across a population long-term would be accompanied by significant levels of mortality (Rahimi et al. 2015).

3.2.1 Epidemiological studies

Given the relative ease of measuring BP non-invasively, it is perhaps unsurprising that many researchers have looked at this important indicator of health in regards to air pollution in human populations. The number of epidemiological studies using this parameter has increased

dramatically over the past decade in line with increasing recognition of the CV effects of air pollution. These include large cohort investigations (40 original research articles) and smaller panel investigations (37 articles). A full-text description of these studies is unwieldy; instead the details and findings of these studies have been tabulated in the detailed document.

Since 2009, numerous epidemiological studies have been published examining potential relationships between exposure to environmental pollutants and BP. The heterogeneity of study design is daunting and, unsurprisingly, there is wide variation in the outcomes observed, not only between different investigations but also within studies (the same publication may report significant associations for some pollutants but not for closely related constituents). Nevertheless, despite this and the possibility of publication bias against negative findings, it is difficult to deny the strong evidence that exposure to air pollution is associated with elevated BP. Most studies have focused on particulate matter (PM,) but there are also data that support associations between exposure to gaseous pollutants, such as nitrogen dioxide (NO_2) or sulphur dioxide (SO_2), and increased BP.

In contrast to results from animal studies that show differences in direction of the BP responses (see below), epidemiological studies consistently report increases in BP in response to pollutants. Most epidemiological studies suggest the size of the effect is relatively small, for example 1-4 mmHg for a 10 $\mu g/m^3$ increase in, or interquartile range of, fine PM ($PM_{2.5}$). Based on the current evidence this appears to be the case irrespective of large differences in monitoring time or the method used.

Efforts have also been directed to ascertaining whether certain populations are more susceptible to the hypertensive effects of air pollution. The groups that have been most frequently studied include the elderly, young and patients with CVD or metabolic disease. Results suggest that air pollution can raise BP in all these groups. However, at present, the lack of direct comparisons and differences in study design make it difficult to maintain that these individuals are significantly more susceptible to the hypertensive effects of air pollution than healthy adults.

Several recent studies have sought to determine the active agents responsible for the hypertensive effects of PM. Many of the usual candidate constituents are positively associated with increased BP, including both organic and elemental carbon, and a long-list of other elemental constituents. Some studies have reported stronger associations for specific metals as compared with total PM mass, but there is no consensus as to which metal or other component of PM is the key driver of the hypertensive effects.

While epidemiological studies have clearly added to the understanding of hypertension and air pollution in man, gaps in mechanistic understanding still remain. Some panel studies have looked at lung function in addition to BP. While the majority show detrimental effects of pollution on both sets of endpoints, biological pathways linking the two have yet to be defined. Studies measuring heart rate variability (HRV) or vasodilator responses in peripheral organs suggest that an increase in sympathetic activity is a plausible pathway, although these parameters have their own strengths and weaknesses (see Chapter 2 and miscellaneous vascular section below). A detailed appraisal of potentially confounding variables (for example, noise, socioeconomic status, medication) is warranted; at present, the contribution of these factors has been addressed to varying extents with little clarity emerging as to how these factors interact with the biological effects of pollutant exposure.

In summary, the body of recent epidemiological evidence supports a link between air pollution and raised BP, and provides a foundation for future mechanistic investigations in controlled exposure studies in man and *in vivo* animal models.

3.2.2 Controlled human exposures

Controlled human exposure studies offer a unique means to regulate exposure to address specific pollutants and individuals with techniques that can probe mechanistic aspects in more detail. Concentrations of pollutants can be set at defined levels, although the acute nature of exposure usually means that exposure levels are in the upper end of those found in heavily polluted environments (usually 1- or 2-h exposure to PM in the region of 100-300 $\mu g/m^3$ PM). A limited number (8 articles reviewed) of controlled exposure studies measuring BP during/after controlled exposure in man have largely been restricted to concentrated ambient particles (CAPs) or dilute diesel exhaust (DE). Investigations have largely been in healthy individuals, with low subject numbers (usually less than 20 volunteers). Nevertheless, evidence exists for an increase in BP in response to inhalation of CAPs or DE. Typically, alterations in BP (usually 2-5 mmHg) bear close relation to the magnitude of effects observed in epidemiological studies. Exposure to CAPs has been shown to produce small increases (~2 mmHg) in some BP parameters (Bellavia et al. 2013). BP responses to DE were larger (4-12 mmHg) but more variable (Mills et al. 2011), (Tong et al. 2014), and were still evident 24-h after exposure (Cosselman et al. 2012). Effects are frequently observed for SBP, but not DBP, the underlying mechanisms for which are not clear, but may have implications for specific CV conditions. While a few controlled exposure studies have suggested greater BP effects in susceptible individuals, this has not been satisfactorily tested in a sufficient number of studies that include a direct comparison to healthy volunteers. Controlled exposures have been useful in determining the time course; clearly demonstrating the rapidity of hypertensive effects of pollutants and, concerningly, showing indications of long-lasting (>6-h) actions following cessation of acute exposure.

3.2.3 Animal studies: *in vivo* exposures

While BP is readily measurable in man, animal studies offer additional options for mechanistic insight. However, measurement of BP is more challenging practically. Three broad methods exist: surgical implantation of telemetry devices, insertion of a monitoring catheter into the arteries of anaesthetised animals, or non-invasively using a tail-cuff; a technique similar to that in man. Each technique has its own advantages and disadvantages.

Since 2009, approximately 25 studies have measured BP in animals following pulmonary exposure (inhalation or instillation) to air pollutants with periods of exposure that range from a few hours to several months. The pollutants investigated have largely been CAPs, DE or diesel exhaust particulate (DEP), with an array of different concentrations and periods of exposure. In relation to CAPs, five studies were associated with increases in some BP parameters (Bartoli et al. 2009), one with a decrease in BP (Wagner et al. 2014) and two with no effect on any BP parameter (Brito et al. 2014; Ying et al. 2009b). For DE inhalation or DEP instillation studies, three studies were associated with increases in some BP parameters, four with decreases and one study with no effect on BP (Labranche et al. 2012). Ozone (O_3) in the absence of particles did not alter BP (Wang et al. 2013), but did in another study by the same group (Wagner et al. 2014). NO_2 and SO_2 have only been investigated in combination with PM (Zhang et al. 2015).

Several studies have been able to prevent/reverse the effect of pollutants on BP by using agents to block inflammation/oxidative stress (OS) pathways (Ying et al. 2009a), inhibitors of autonomic function (Bartoli et al. 2009) or blockage of pulmonary sensory receptors (Robertson et al. 2014). Hypertensive rodents (Labranche et al. 2012) and rat models of metabolic syndrome (Wagner et al. 2014) have been used to investigate susceptibility to air pollutants, with mixed results.

Other studies worthy of specific mention include an investigation showing that the autonomic imbalance caused by long-term inhalation of CAPs was associated with structural changes in the hypothalamus of the brain (Ying et al. 2014), a potential link between DEP-induced hypertension and renal damage (Nemmar et al. 2014), and alterations in BP control of the offspring of mice following in utero exposure to DE (Weldy et al. 2014).

In summary, animal studies demonstrated extremely variable BP responses to pollutants. While increases in BP are the most common response to different types of PM, decreases in BP are also frequently reported. Similarly, there is inconsistency as to which BP parameters (SBP, DBP, mean arterial blood pressure (MAP), pulse pressure (PP)) are affected. Responses vary from those that are similar to studies in humans, to those of a far greater magnitude, although it should be noted that acute periods of high-level exposure are common. While there is almost no BP data from animal exposures to gaseous pollutants alone, mixtures of gases and particles or DE (ie after removal of particles by filtration) appear to have the capacity to alter BP. Inflammation and OS remain the mainstay of mechanistic investigations, with good evidence for a role of both (see Chapter 5). Alterations in autonomic balance and resetting of baroreceptor sensitivity also appear to play a role, although the underlying mechanisms are complex. Animal models of potentially susceptible groups have largely focused on pre-existing hypertension. There are certainly indications that these animals are susceptible to an additional increase in BP from air pollutants, however, evidence for a greater susceptibility in these models required further investigation.

3.3 Air pollution and vascular contractility

This section describes methods to assess vascular health other than BP, many of which can provide further insight into the biological mechanisms involved.

3.3.1 Epidemiological – large cohort studies

Pulse propagation through arteries (within individual arteries, or by comparison between different vascular beds) can provide a surrogate of vascular health, based on the loss of vascular compliance due to structural and functional changes of blood vessels. The methods used are non-invasive and can be performed with relative ease by a trained individual. Several cohort studies of varying size have demonstrated impaired pulse wave characteristics in individuals living in close proximity to roads (Hoffmann et al. 2009b), or with higher PM exposure (Chen et al. 2012). Others have not found a statistically significant association with particulate air pollution (Lenters et al. 2010). However, overall there is reasonable evidence that short-term exposure to particulate air pollution, or from living close to a major road, is associated with both a loss of arterial compliance and decreased responsiveness to stimuli. The evidence for effects of long-term exposure is also emerging. Existing cohort studies are of sufficient power to demonstrate trends over broad populations, but at present there is little consensus in relation to higher risk individuals (for example, gender, age, disease status). The

role of gaseous co-pollutants, and identification of PM harmful constituents, in these vascular actions has not been adequately addressed by large cohort studies. Ultrasound techniques have been used to provide an indication of mechanisms underlying alterations in the diameter of peripheral arteries (usually the forearm) in response to pollutants. Flow-mediated dilatation (FMD) in particular appears to be sensitive to $PM_{2.5}$ exposure (Krishnan et al. 2012), suggesting impaired responsiveness of the vascular endothelium. Retinal photography has also been used as non-invasive means to demonstrate that PM can impair microvascular function (Adar et al. 2010).

3.3.2 Epidemiological – smaller panel studies

A large number of small panel studies (23 articles reviewed) have taken advantage of the increasing options to measure the vascular effects in man to address the subject of air pollution. These include those described above for the large cohort studies, such as arterial stiffness (Weng et al. 2015). Panel studies have made use of a variety of methods to induce vasodilation of arteries through different mechanisms, for example, nitroglycerin-mediated (endothelium-independent) dilatation (NMD), in addition to FMD and baseline brachial artery diameter. A general lack of effect of pollutants on NMD provides further confirmation that impairment is based at the level of the endothelium (Lanzinger et al. 2014).

Microvascular function (MVF) is an important predictor of more widespread endothelial dysfunction, as well as an important contributor to disease pathology, especially in diseases such as diabetes. Use of an EndoPAT to measure MVF has generated mixed results in regards to various pollutants (Karottki et al. 2014). A number of blood biomarkers give insight into vascular function. These include measurement of the oxidation products of the endothelium-dependent mediator nitric oxide (NO) (Gandhi et al. 2014; Pettit et al. 2015) and endothelin-1, a potent endogenous vasoconstrictor (Calderon-Garciduenas et al. 2015; Poursafa et al. 2016).

Overall, the weight of evidence suggests that PM in air pollution is associated with a loss of compliance and decreased responsiveness of both conductance and resistance arteries. There is not enough evidence to draw meaningful conclusions as to whether gaseous co-pollutants exert similar effects. Personal monitoring of air pollution has added strength to these observations in small panel studies, demonstrating significant vascular changes that were not necessarily apparent from stationary monitoring data. Heterogeneity of study designs currently limits the certainty of findings relating to the time course of effects, interactions with other environmental conditions (for example, climate) and identification of susceptible groups. Mechanistically, changes in autonomic function, reduced production of endothelium-derived vasodilator substances and increases in circulating vasoconstrictors are the dominant hypotheses.

3.3.3 Controlled human exposures

23 studies were reviewed in detail. Controlled exposure studies have provided convincing evidence that transient inhalation of particulates can impair vascular function in different arterial beds. Studies using CAPs exposure (Brook et al. 2009) have used a variety of endpoints, with the majority demonstrating an impaired vascular responsiveness of some form. A greater consensus has been found following inhalation of vehicle exhaust (Lundback et al. 2009). Gaseous co-pollutants from vehicle exhaust appear to have limited actions on vascular responsiveness (Langrish et al. 2010). The constituents within PM that drive the vascular

effects are the subject of on-going research. Free radical generation by surface constituents such as reactive transition metals or specific organic species is an attractive possibility. The inconsistent response of blood biomarkers and rapidity of the effects suggest that inflammation alone is not the primary cause of the impaired vascular function. A wide range of other biological mechanisms have been proposed, and it is likely that multiple pathways contribute to the overall action of inhaled PM. There is a distinct lack of controlled exposure data that directly compares healthy volunteers with potentially susceptible individuals.

3.3.4 Animal studies: *in vivo* exposures

A wealth of animal data (52 studies reviewed in-depth) exists using *in vivo* exposures to look at vascular function *in situ* (see also the section on blood pressure) and *ex vivo,* using a wide array of endpoints. A variety of biological pathways have been argued persuasively. However, the study protocols are so varied (for example, type of pollutant, length of exposure, dose, method of administration, animal model and endpoints explored) that it is difficult to identify patterns within the data. Nevertheless, the findings from these studies broadly agree with those from panel studies on humans; in that a number of sources of PM can impair vascular function, whereas the evidence for gaseous co-pollutants alone is less certain. Synergism in the toxicological effect of gaseous and particulate pollutants remains a possibility. The possibility that acute vascular effects of air pollution could build-up over time to produce the more-widespread structural and functional changes seen in disease has not been definitively shown, but remains likely.

3.4 Air pollution and atherosclerosis

Atherosclerosis is the formation of lipid- and inflammatory cell-rich plaques that build up on the inner surface of large arteries. 'Unstable' plaques can become eroded and/or rupture, exposing the highly thrombogenic constituents of the inside of the plaque to the blood, potentially leading to thrombotic occlusion of the blood vessel. Depending on the location of this event, this can trigger a heart attack (coronary arteries of the heart), a stroke (carotid artery or artery in the brain) or limb ischaemia that can ultimately lead to loss of the limb (for example, the leg). Atherosclerosis is a chronic disease (developing over decades) bringing further challenges in terms of the monitoring of pollutants over relevant periods, or establishing long-term exposure protocols.

3.4.1 Epidemiological – large cohort studies

Measuring atherosclerosis in humans centres on non-invasive imaging techniques to look at the size of atherosclerotic plaques (thickness of the vascular wall or loss of lumen diameter) or certain plaque constituents. Since 2009, a surprisingly large number of cohort studies have looked for associations with various parameters of air pollution exposure and the development of vascular plaques (most commonly carotid intimal medial thickness (CIMT) or coronary artery calcium (CAC)). 21 original research articles were reviewed, in addition to 3 meta-analyses. There is strong evidence from the measurement of CIMT that particulate air pollution (Sun et al. 2013), or proximity to traffic (Kunzli et al. 2010), is associated with atherosclerosis in humans. More consistent associations are seen for $PM_{2.5}$ compared to PM_{10} (L Perez et al. 2015). Meta-analyses support these findings (Provost et al. 2015), whereas several smaller cohort studies do not. Findings appear to be remarkably consistent across different countries and continents. While the magnitude of the effect is small, it is usually within the range that

could be associated with increased numbers of CV events. Other measures of atherosclerosis, such as coronary artery calcium, generally do not show statistically significant associations. This could be related to the technical aspects of the methods employed or the stage of disease that the procedure is optimal for. There remains a shortage of large cohort studies looking at the effect of gases on atherosclerosis in man. Identification of harmful constituents of PM and susceptible populations also require additional investigation.

3.4.2 Epidemiological – smaller panel studies

There has been a distinct lack of small panel studies (6 reviewed) looking at air pollution and atherosclerosis since 2009. This may, in part, be due to the relatively large numbers of larger epidemiological studies making use of cohorts that measure CIMT, however, it perhaps represents a missed opportunity to look at mechanisms of atherosclerosis. Nevertheless, there is a growing number of panel studies, while not measuring atherosclerosis, that do provide evidence for exposure to air pollution altering the blood lipids profile in a manner that is linked to atherogenesis (Calderon-Garciduenas et al. 2015; Fan et al. 2014).

3.4.3 Animal studies: *in vivo* exposures

The chronic developmental process of atherosclerosis lends itself to the study of the effects of long-term exposures, and provides the opportunity to test more realistic dose rates in genetically/diet-accelerated models. Since the first animal study in 2002 carrying out instillation to PM in hypercholesterolaemic rabbits (Suwa et al. 2002), there has been a proliferation of studies in rodent models (usually Apolipoprotein-E (ApoE) knockout mice or low density lipoprotein (LDL) receptor knockout mice) investigating the atherosclerotic effects of air pollutants (comprehensively reviewed in (Moller et al. 2011) & (Miller et al. 2012)). The use of murine models to address this topic has only gained momentum since 2009.

Overall, the studies have produced a rather mixed set of findings that encompasses a wide range of exposures in terms of pollutant of interest, length of the exposure period and means to deliver pollutants to the lung. In terms of environmental sources of air pollution, the focus has remained on PM, through inhalation of CAPs, ultrafine particle-rich vehicle exhaust or instillation techniques to deliver particulates from these sources in the absence of gases. A noticeable trend in more recent studies is the longer duration of exposures, usually 4 weeks, but not infrequently many months. Both urban PM and vehicle exhaust increase the size of atherosclerotic plaques and often markers of plaque vulnerability. The role of gaseous pollutants remains understudied, although there is stronger evidence here for a pro-atherosclerotic effect of gases in comparison with other aspects of vascular function. Fittingly for this complex and progressive disease, multiple pathways have the potential to drive the pro-atherosclerotic actions of pollutants, and a number of mechanistic studies have successfully used pharmacological antagonists of candidate pathways to prevent these actions. Inflammation and OS are prominent mechanisms and could contribute to the atherosclerotic actions of air pollution at several stages of the pathway. Overall, there is compelling evidence from animal models that various air pollutants promote atherosclerotic disease.

3.5 Overall conclusions

Since 2009, over 400 studies have been published which address the vascular effects of air pollution. Together they provide extensive insight into the different means by which both

short- and long-term exposure to air pollution could lead to vascular dysfunction and disease. Varied study designs, and a diverse array of techniques and endpoints, have been employed. While this contributes to a comprehensive assessment of the area, it is perhaps not surprising that inconsistent or contradictory findings are commonplace. Nevertheless, given the weight of positive evidence, it is the author's opinion that there is a strong case for air pollution causing detrimental effects on the vasculature that would contribute to CV morbidity in the long-term.

BP is one of the most significant risk factors for CVD. Measurement of BP in humans is straightforward and inexpensive, and consequently there is now a large volume of investigations looking at how air pollution affects BP. The epidemiological evidence for air pollution being associated with elevated BP is one of the most consistent areas examined by the current review. Modest increases in PM can be associated with small increases in BP; an observation that seems relatively consistent across different study types, populations and periods of monitoring. There are limited data from human controlled exposure investigations; however, the magnitude of BP alteration in these studies does provide some support for epidemiological observations. In contrast, findings from animal exposure studies are more inconsistent, both in terms of direction and size of the alteration in BP. Heterogeneity in study design (with marked differences in dose, period of the exposure and timing of measurements) will contribute to the inconsistencies. Nevertheless, animal studies have provided useful mechanistic insight that supports observations in man. While the alterations in BP appear small, changes of this magnitude across populations are likely to be accompanied by significant levels of CV morbidity and mortality.

A diverse range of techniques has been used to provide other measures of vascular health in peripheral vascular beds. Here, discrepancies are more noticeable, but overall the evidence still suggests that exposure to air pollution is associated with a shift in the balance towards vasoconstriction and impaired vasodilation. The observations suggest a widespread vascular dysfunction following exposure to air pollution, of both conduit and resistance arteries. A variety of mechanistic pathways have been proposed including an imbalance in the autonomic nervous system, loss of endothelial function (in particular, reduced synthesis, and increased inactivation, of NO), increases in circulating vasoconstrictors, and a combined insult of inflammation and OS. Whilst relatively few novel biological pathways have emerged since 2009 (especially those linking the pulmonary system and vascular effects), the detail of existing pathways has been advanced greatly.

The vascular impairment outlined above provides the foundations by which air pollution contributes to vascular disease and atherosclerosis. Findings of several large epidemiological cohort studies also support such an association. A rapidly expanding number of animal investigations using prolonged exposure to pollutants has strengthened the feasibility of a range of biological pathways. It is plausible that air pollution could contribute to the initial pathological processes (for example, oxidation of circulating lipoprotein, endothelial dysfunction), promote the progression of existing disease (for example, OS, increasing lipid and inflammatory cell content of plaques) and also potentially trigger atherosclerotic plaque rupture that underlies acute CV events (for example, structural remodelling, breakdown of the plaque matrix).

This review focused on the post-2009 literature to ascertain how the field has advanced since the 2010 American Heart Association review. Arguably, in many ways the conclusions drawn parallel those reached seven years previously. This in part reflects the practical limitations of investigations that continue to challenge researchers. These include;

a) the limited spatial variation provided by stationary monitoring of pollutants;

b) the need for long-term monitoring of pollutants, especially over the course of disease;

c) the large number of subjects required to identify differences across populations;

d) confounding variables (for example, noise, socioeconomic differences);

e) separating the effects of closely correlated pollutants;

f) obtaining suitable differentials in pollutants within a defined population and;

g) the use of surrogate measurements to assess CVD in man

Identification of groups who may be especially susceptible to air pollution remains a pressing issue, and one that is confounded by practical difficulties, for example baseline functional differences in control groups prior to pollutant exposure, and use of medication masking the actions of pollutants. The role of gaseous pollutants remains woefully understudied. There is a temptation to suggest gaseous co-pollutants can impair vascular function; however, given the paucity of data it is difficult to speculate further as to comparisons to, and interaction with, particulate components. Animal models have been used to overcome many of these obstacles. The requirement for high doses of exposure to observe measurable effects is a recurring problem, as is identifying mechanisms that directly contribute to the pathophysiological effects of pollutants (rather than being downstream consequences of the disease burden) and the consequent uncertainty in translating results to humans in real-world scenarios. Nevertheless, our understanding of the mechanistic pathways has grown considerably. Imbalance in autonomic nervous activity, neuroendocrine activation, endothelial dysfunction, and generation of systemic inflammation and OS remain the key mechanisms. There is substantial interplay between all these pathways, and no single pathway alone can satisfactorily explain the multifaceted nature of CV impairment. While in some way recent evidence has served to complicate rather than simplify the network of mechanistic knowledge, overall it provides a solid foundation for a causal association between air pollution and CV morbidity.

Chapter 4
Effects of exposure to air pollutants on haemostasis

5.1 Introduction

Haemostasis is a complex, orchestrated series of events to maintain circulating blood in the liquid state and to prevent blood loss following injury through the formation of a blood clot. Excessive clotting, especially in patients with pre-existing CVD, can block major arteries leading to a loss of downstream blood flow, and potentially leading to clinical events such as heart attack, ischaemic stroke or death. In contrast, a reduction in the ability of blood to clot can lead to uncontrolled bleeding with severe blood loss from injury or escape of blood following aneurysm, e.g. haemorrhagic stroke. Thus the body maintains an intricate balance to preserve haemostasis; a process that involves the interplay of circulating blood cells, a variety of coagulation factors, platelets and fibrinolytic factors, as well as interactions with the vascular wall and endothelial cell-derived mediators (Figure 4.1). Clotting may be initiated by either the intrinsic (contact activation) or extrinsic pathway (tissue factor; TF). These two pathways converge into a final common pathway of thrombin production and fibrin clot formation (Figure 4.2). We regard the term "haemostasis" to include the whole process: platelet activation, coagulation and fibrinolysis. A seminal review by the American Heart Association (AHA) (Brook et al. 2010) concluded that there was evidence, albeit somewhat inconsistent, suggesting that particulate matter (PM) may adversely affect haemostasis shifting the balance to a pro-coagulant and anti-fibrinolytic state and that this may, in part, contribute to the effects of air pollution on cardiovascular disease (CVD). The mechanisms underlying such an effect were poorly defined but systemic inflammatory activation and alterations in platelet function are proposed as key processes involved in the alterations in haemostasis (Brook et al. 2010).

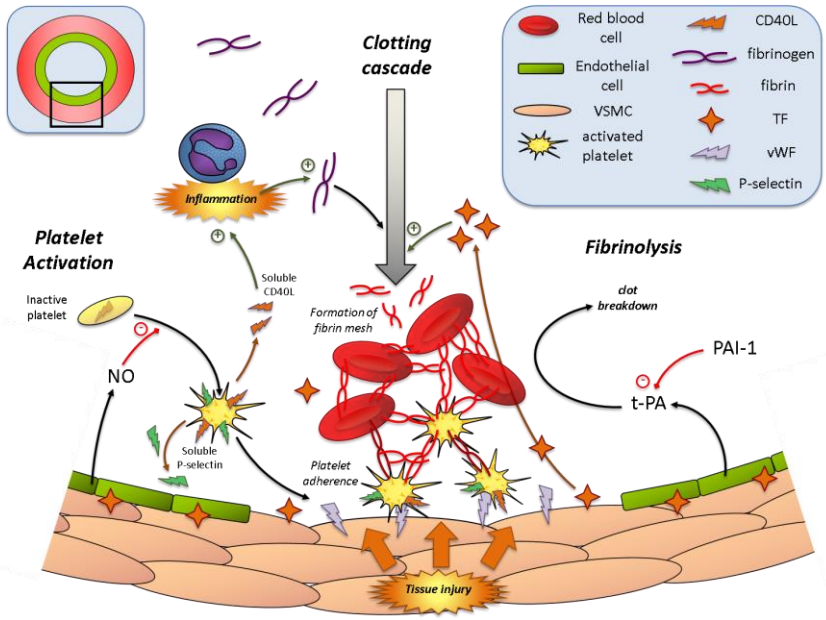

Figure 4.1: Processes and components involved in normal haemostasis (these are discussed in more detail in the text).

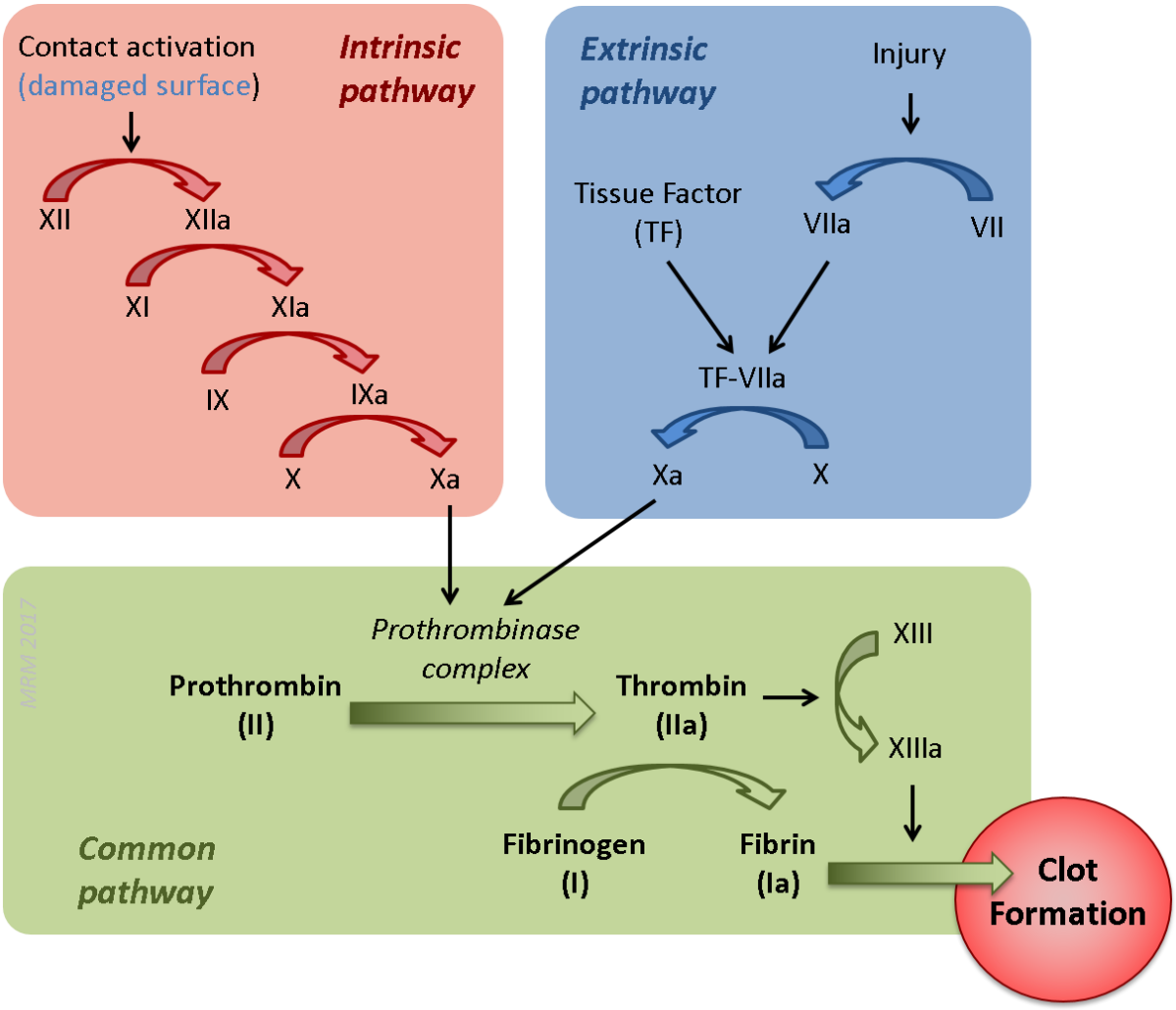

Figure 4.2: The intrinsic, extrinsic and common pathways of the coagulation (clotting) cascade. Air pollution has been shown to affect several steps in blood coagulation (these are discussed in more detail in the text).

This review appraises the newly accumulated scientific evidence (2009-2016) on contribution of haemostasis and thrombosis towards CVD induced by exposure to both particulate and gaseous pollutants. The review provides a narrative summary of the seventy four publications that fulfilled all inclusion criteria (see Appendix 2.4 for details). Emphasis has been given as to whether the conclusions of the AHA statement have been strengthened during the intervening 8-year period and where new insight has been made as to possible underlying mechanisms. Of note, we expand the remit from the AHA statement by inclusion of gaseous pollutants (for example. nitrogen dioxide (NO_2), ozone (O_3), carbon monoxide (CO) and sulphur dioxide (SO_2)).

5.2 Epidemiological studies

5.2.1 Short-term exposure to air pollutants

The vast majority of epidemiological studies addressing the haemostatic effects of air pollution have used time-series approaches to investigate the effects of short-term (acute) exposures (<7 days), with most evaluating alterations in plasma biomarkers of systemic coagulation and fibrinolysis. Fibrinogen, an essential coagulation protein associated with an increased risk of coronary events (Danesh et al. 2005; van de Loo 1995), has been one of the most studied. Hildebrandt and colleagues reported PM to be associated with a 2.4% increase in fibrinogen from 3.1 g/L (baseline) in 38 male patients with chronic obstructive pulmonary disease (COPD) (Hildebrandt et al. 2009). However, no clear pattern emerged for the other plasma haemostatic biomarkers evaluated. Thus, the increase in fibrinogen in response to exposure may be somewhat artificial, perhaps arising from small sample bias. With a larger sample size (N=242), another study noted no increase plasma fibrinogen levels in patients with COPD per interquartile range (IQR) increase in fine PM ($PM_{2.5}$;particles less than 2.5 μm in diameter) at all lag days (0-10) (Dadvand et al. 2014). Similarly, there were no significant changes in fibrinogen with PM exposure among young and older adults (Gong et al. 2014; Thompson et al. 2010; Wu et al. 2012). Besides fibrinogen, other biomarkers having pro-coagulant properties or reflecting a pro-thrombotic state have been investigated (including (plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA)) (Delfino et al. 2009; Emmerechts et al. 2012; Gong et al. 2014). Earlier studies typically analysed single biomarkers of haemostasis, whereas more recent studies have used a multiple biomarker approach. While most studies have observed pollution-related changes in at least one biomarker, the direction and statistical significance of the pollutant-biomarker association has not been consistent, making it difficult to generalise conclusions. More convincing evidence for associations between exposure to ambient particles and biomarkers of haemostasis comes from studies conducted during the 2008 Beijing Olympics (Gong et al. 2014; Rich et al. 2012a). The air pollution control measures that were put in place in Beijing during the 2008 Olympic Games provided an unprecedented opportunity for quasi-experimental studies to assess the effect on systemic biomarkers of haemostasis and inflammation, among others. The short-term reduction in PM during the Olympic Games was associated with decreases in biomarkers of haemostasis (Gong et al. 2014; Rich et al. 2012a). During the 2008 Beijing Olympic Games, Rich et al. (2012) observed statistically significant improvements in soluble P-selectin (sP-selectin; -34%) and von Willebrand factor (vWF; -13.1%) among the 125 healthy young adults studied (Rich et al. 2012a). An interesting study by Delfino et al. (2009) examined whether medication use modifies the association between particulate air pollution and biomarkers of haemostasis. Here, an association between $PM_{2.5}$ exposure and blood levels of sP-selectin in patients with coronary artery disease (CAD) was only observed in those not taking clopidogrel, providing further evidence for a role of platelets (Delfino et al. 2009). Further studies are warranted to disentangle the modifying effects of health status and use of different medications, in addition to whether such preventative action of anti-platelet agents are more marked in those with greatest exposure to air pollutants.

Differences in particle composition across study locations could be a reason for the inconsistent findings, as physical and chemical characteristics of PM are known to vary widely both with space and time (Son et al. 2012; Steenhof et al. 2011). Very few studies have analysed associations between individual PM constituents and changes in biomarkers of haemostasis. There is limited/suggestive epidemiological evidence that the haemostatic responses are more

intrinsically related to chemical composition rather than just particle mass. For example, Wu et al (2012) demonstrated that certain transition metals (for example, iron, titanium, cobalt and cadmium) within $PM_{2.5}$, but not PM mass, was associated with increased plasma fibrinogen levels among healthy adults in Beijing. Besides fibrinogen, biomarkers of the fibrinolytic pathway (PAI-1 and t-PA) were also significantly and positively associated with transition metals in $PM_{2.5}$. These epidemiological observations are consistent with animal and *in vitro* studies showing a role for transition metals in PM-induced toxicity (Gray et al. 2015). Additionally, studies have shown the administration of chelating agents (chemical compounds that react with metal ions, to decrease their availability for other reactions) can block the effects of air pollution on blood coagulation (Sangani et al. 2010). Several transition metals have been shown to participate in Fenton and Fenton-like reactions and thus induce oxidative stress. Oxidative stress is a recurring mechanism in actions of air pollution (Miller et al. 2012), and associations between exposure and biomarkers of oxidative stress have been observed in both *in vivo* and *in vitro* studies (Kelly and Fussell 2017). To the best of our knowledge, only one study has investigated the effects of genetic polymorphisms related to oxidative stress and particulate pollution exposure on markers of haemostasis (Frampton et al. 2012). Stronger associations between PM and haemostatic biomarkers were observed among individuals who were genetically predisposed to oxidative damage (Frampton et al. 2012). However, unexpectedly, exposure was associated with a decrease in the levels of circulating biomarkers of haemostasis, counter intuitively suggesting a potentially protective effect of PM against thrombosis (Frampton et al. 2012). Besides oxidative-stress related polymorphisms, other epidemiological studies have investigated the effect of polymorphisms in the fibrinogen genes on responses to PM exposure (Peters et al. 2009). For example, carriers of the FGB rs1800790 minor allele elicited greater fibrinogen responses to PM than those with a major allele (Peters et al. 2009). Such 'unforeseen' functional consequences of genetic polymorphisms may, in part, account for discrepant findings between studies investigating different populations. Further work in this area is clearly warranted.

The size distribution of PM in ambient air may also be important. An increasing body of experimental data (see below) has suggested that the ultrafine fraction within PM may be more toxic than the fine ($PM_{2.5}$) or coarse (PM_{10}; particles < 10 μm in diameter) fractions, attributed to the larger surface area per unit mass of ultrafine particles (UFPs). The large majority of these studies have focussed on pulmonary responses and as yet, there are too few population studies to conclude whether different particle size fractions have different magnitudes of association with haemostatic outcomes. Only a few studies have specifically investigated the effect of particle size on the haemostatic effects of PM and results have been mixed (Chen et al. 2015; Delfino et al. 2009; Frampton et al. 2012; Gong et al. 2014; Ruckerl et al. 2014). This is likely the consequence of limited statistical power of studies together with methodological heterogeneity. Of particular interest is the study by Ruckerl et al. (2014) which used a particle counter with a lower particle cut-off size than the more conventional methods. Nevertheless, analysis of the different size fractions of UFP (3-10 nm, 10-30 nm, 30-50 nm, 50-100 nm) did not reveal certain size range to be more influential than others. Also, associations were stronger for $PM_{2.5}$ than for UFPs; inconsistent with the notion that UFPs have greater biological activity, although it is noteworthy that this study found a reduced thrombus formation in association with PM.

There is some limited evidence that reactions to particles of different sizes are characterised by different intervals of time (lags) between exposure and biomarker changes, suggesting that the mode of action may be different for the various particle sizes (Gong et al. 2014). There is some limited evidence to suggest that inhaled UFPs may translocate into the circulation (Miller et al.

2017; Oberdörster et al. 2002; Shimada et al. 2006) with the potential for direct pro-aggregatory effects on platelets. However, particle translocation remains controversial and a recent study showed that the translocated fraction is less than 1% (mass) of the delivered dose to the lung (Buckley et al. 2017). The exact pathway for the translocation into the circulation also remains unclear. While some studies (Gong et al. 2014), report associations between haemostatic biomarkers and concentrations of ambient UFPs for time lags representing direct effects (ie. Lag 0), the time course of others (Rudez et al. 2009) is more likely to represent indirect effects, with findings very rarely being consistent across studies for the same biomarker-pollutant pair. It is also not clear whether inflammatory processes mediate the exposure-response relationship. It has been shown that exposure to particulate air pollution can induce platelet aggregation and increased thrombin generation without inducing significant levels of inflammation (Rudez et al. 2009). Others have suggested that an inflammatory response is an essential component in the haematological changes associated with short-term exposure to air pollution (Emmerechts et al. 2012; Gong et al. 2014; Hajat et al. 2015). Of note, most studies have looked at a limited number of inflammatory biomarkers and these may not necessarily encompass all aspects of the inflammatory response.

There has been limited progress in the use of global coagulation tests. Measurement of individual biomarkers provides only 'snapshots' of the overall coagulation process, and global coagulation tests can provide a more complete picture of haemostatic status. The prothrombin time (PT) test (which measures activity of the extrinsic and common pathways) and the activated partial thromboplastin time (APTT) test (which measures activity of the intrinsic and common pathways) are widely used global screening tests for blood coagulability. To date, attention has primarily focused on PM. Associations between daily ambient PM_{10} and shortened PT (ie. greater coagulability of the blood) has been demonstrated in 1218 normal subjects from the Lombardia Region, Italy (Baccarelli et al. 2007). No changes in APTT were noted, perhaps instead pointing towards TF-dependent changes in thrombin generation. This result is contrary to a more recent study showing prolonged clotting times with increased levels of PM_{10} in a group of 233 patients with diabetes (Emmerechts et al. 2012). However, reduced clotting times were associated with increasing PM_{10} concentrations of the sub-acute exposure time windows (day 0 to day 3) investigated in this study (Emmerechts et al. 2012).

One of the most exciting areas to have developed in recent years, has been increasing interest in uncovering whether environmental exposures can impact on health through epigenetic mechanisms (alterations in gene expression and function without changing the underlying DNA sequence), such as DNA methylation, histone modifications and non-coding RNA expression (Marczylo et al. 2016). Alterations of DNA methylation have been linked to various human diseases, and epidemiological studies have shown distinct DNA methylation abnormalities with exposure to air pollutants (Baccarelli et al. 2009; Tarantini et al. 2009). Evidence for associations between (i) air pollution and epigenetic alterations and (ii) between disease states (including CVD) and epigenetic changes, lends biological plausibility for epigenetic change underpinning the effects of air pollution on haemostasis. The most compelling evidence comes from a recent study demonstrating that exposure to black carbon (BC) particles was associated with a decrease in DNA methylation (hypomethylation) of the tissue factor (F3) gene and subsequent increases in fibrinogen protein expression (Bind et al. 2014b). The specific mechanism of how air pollution exposure may alter DNA methylation has not been elucidated at present.

Unlike PM, there have been relatively few studies evaluating the relationship between gaseous pollutants and biomarkers of haemostasis, and the evidence has been mixed. Some studies have

reported positive associations, some no association and a few inverse associations (Dadvand et al. 2014; Delfino et al. 2009; Hildebrandt et al. 2009; Rich et al. 2012a; Ruckerl et al. 2014; Rudez et al. 2009; Wu et al. 2012). The heterogeneity of these studies makes the data difficult to interpret as studies have tended to use different exposures (NO_2, CO), target populations or outcome measures.

5.2.2 Long-term exposure to air pollutants

As with the studies of short-term exposure to PM discussed above, fibrinogen has been the most widely studied biomarker in long-term exposure (>1 week) epidemiological studies. Using data from the Heinz Nixdorf Recall Study (a prospective population-based cohort of 4,814 German adults) plasma fibrinogen levels were found to be elevated by 3.9% among men, but not women, for each 3.91 $\mu g/m^3$ increase in annual average $PM_{2.5}$ (Hoffmann et al. 2009a). This was found to be independent of short-term changes in air pollution. However, a comparative study using a higher spatial resolution (1 km as compared to 5 km) did not confirm this finding (Viehmann et al. 2015). Large population-based cohort studies in the US and UK have also reported no association between fibrinogen and PM (Dabass et al. 2015; Forbes et al. 2009; Hajat et al. 2015; Lanki et al. 2015). Studies also varied in their ability to adjust for potential confounding factors (medication use, co-morbidities, socio-economic status (SES)). Additionally, different exposure patterns and sources are likely to contribute to the inconsistent results among studies. Studies have generally relied on data collected from the nearest fixed-ambient monitoring stations as a surrogate for personal exposure. Unless complex modelling has been performed a key weakness in using fixed-site monitoring data is that it ignores spatial variability. Differences in particle composition across study locations could also be a reason for the inconsistent findings, as physical and chemical characteristics of PM are known to vary widely both with space and time (Son et al. 2012; Steenhof et al. 2011). Evidence from a multi-centre meta-analysis found that long-term exposure to zinc within $PM_{2.5}$, but not other constituents (for example, iron and nickel) increased fibrinogen concentrations (Hampel et al. 2015). These findings add to the growing evidence that mass alone does not fully capture the toxicity potential of PM.

A few studies have looked at other markers of clotting and fibrinolysis, with considerable differences in the magnitude and direction of responses obtained (Emmerechts and Hoylaerts 2012; Green et al. 2015; Hajat et al. 2015; Viehmann et al. 2015). Factor VII (FVII) plays a central role in initiating the process of coagulation. A cohort study of 2,086 mid-life women found an inverse relationship with FVII (-3.6%; 95% Cl, -7.8 to 0.8%) for each additional 10 $\mu g/m^3$ of annual $PM_{2.5}$ pollution (Green et al. 2015), pointing towards a more hypocoagulable state. However, the measurement and analysis of one or more coagulation biomarkers may not provide a complete picture of the balance between thrombosis and lysis. For example, long-term exposure to PM led to enhanced thrombin generation in patients with diabetes, in the absence of pro-coagulant changes in coagulation parameters (FVII, FVIII, FXII) (Emmerechts et al. 2012). The evidence has, for the most part, been directed at particulate air pollutants. Differences in the focus and design of investigations addressing gaseous air pollutants make it difficult to draw meaningful conclusions as to the long-term relationship with coagulation markers (Dadvand et al. 2014; Green et al. 2015; Hajat et al. 2015; Lanki et al. 2015; Mostafavi et al. 2015; Panasevich et al. 2009).

A small number of studies have made use of global coagulation tests in long-term epidemiological studies. Reduced clotting times were observed in association with PM_{10} average

over one year among healthy controls and patients with deep vein thrombosis (Baccarelli et al. 2008). Patients with diabetes showed hypercoagulability, using endogenous thrombin potential (ETP) with PM_{10} for time windows up to 6 months (Emmerechts et al. 2012). Interestingly, associations between for time windows up to one month, but not longer, and thrombin generation were dependent on TF. The authors proposed microvesicles (small membrane-bound structures secreted from different cell types) to be the main source of the TF (Emmerechts et al. 2012). Increased levels of TF-positive microvesicles in plasma from diabetic subjects were also found to be correlated with annual average concentrations of PM monitored as PM_{10} (Emmerechts et al. 2012).

Induction of systemic inflammation by air pollution could contribute to a pro-thrombotic state. Consistent with this notion, Viehmann et al. (2015), using data from the Heinz Nixdorf Recall Study, observed a positive correlation between C-reactive protein (CRP; a marker of systemic inflammation) and platelet count in relation to air pollution (Viehmann et al. 2015). However, other studies have found no clear patterns of association between markers of haemostasis and markers of inflammation (Emmerechts et al. 2012; Lanki et al. 2015; Panasevich et al. 2009). In the study by Emmerechts et al. (2012), a systemic inflammatory state could not explain the pro-coagulant state during the longer time windows (1 month to 1 year) (Emmerechts et al. 2012). Only a limited number of studies have examined whether age, gender, and pre-existing co-morbidities influence the haemostatic effects of long-term air pollution exposure and results have been inconsistent. For example, Forbes et al. (2009) found no effect modification by gender for associations for PM and fibrinogen, whereas PM exposure was associated with increasing fibrinogen levels in men, but not women, in the population-based Heinz Nixdorf Recall cohort study (Hoffmann et al. 2009a). The latter study is of particular interest as it controlled for a large number of confounding variables. Nevertheless, the study produced some unexpected results, showing no effect modification by medication use or co-morbidities on the association between PM and fibrinogen.

5.2.3 Summary

In summary, much of the recent epidemiological research has strengthened the evidence of associations between short- and long-term particulate air pollution exposure and changes in haemostasis. Increased use of assays of global coagulation, reflecting events from beginning of clot formation to fibrinolysis, has contributed to strengthening the evidence that particulate pollutants could cause thrombotic events. However, reported changes have been modest and, at times, inconsistent. Furthermore, it is not clear whether differences in susceptibility exist within populations (for example, age, sex, pre-existing disease). The magnitude of these changes is in general small, and their clinical relevance has yet to be ascertained. It is, however, possible that, if untreated, these haemostatic changes over the long-term could ultimately lead to exacerbation of myocardial ischaemia and other clinical outcomes in response to a triggering factor. The current evidence is too sparse to draw conclusions about the effects of exposure of gaseous pollutants on haemostasis.

5.3 Controlled human exposure studies

Controlled human exposure studies provide a means to investigate biological mechanisms of pollutants in isolation with better control of exposure and fewer confounding factors than epidemiological studies. Despite the heterogeneity in study design and the possibility of publication bias against negative findings, studies have presented compelling evidence that

particulate air pollution promotes a pro-thrombotic state. In recent years, much of the research has focussed on DE and CAPs. A series of controlled human exposure studies have demonstrated impaired fibrinolysis, by measurement of t-PA (the activator of fibrinolysis) in healthy volunteers after exposure to DE generated under either transient engine speed and load (300 μg/m^3; 1 h) (Mills et al. 2005) or idling (250 μg/m^3; 1 h) conditions (Barath et al. 2010). Similar observations have been observed in patients with coronary heart disease (CHD) following DE exposure (Mills et al. 2007) and in healthy volunteers after exposure to coarse CAPs (89.0 μg/m^3; 2 h) (Graff et al. 2009).

In recent years, major advances have been made in demonstrating increased thrombus formation following DE exposure. *Ex vivo* thrombus formation has been assessed, using a Badimon chamber (which mimics flow conditions within the coronary circulation of man), after DE exposures (320-350 μg/m^3) lasting 1 and 2 hours in healthy volunteers (Lucking et al. 2008; Lucking et al. 2011). Interestingly, DE exposure was associated with increased platelet-leukocyte aggregates, as well as increased circulating levels of soluble forms of CD40L, suggesting that the enhanced thrombus formation was mediated through platelet activation (Lucking et al. 2008; Lucking et al. 2011). DE exposures did not have any significant effect on cellular and soluble markers of inflammation (Lucking et al. 2008; Lucking et al. 2011). These results suggest that short-term exposure to short-term exposure to DE may lead to a pro-thrombotic state independent of systematic inflammation. However, these studies have been typically limited to measuring a small number of blood markers of inflammation, and cannot exclude the possibility that other markers of inflammation may have been increased. The question as to whether particulate air pollutants affect platelets through direct and/or indirect effects thus remains unresolved.

Significant advances have been made in recent years regarding whether the anti-fibrinolytic and pro-thrombotic responses are due to diesel PM or the associated DE gaseous components (or both). Acute exposures of healthy volunteers to DE with a particle trap abolished the effects on endogenous fibrinolysis and *ex vivo* thrombus formation (Lucking et al. 2011). While the filters were effective in terms of particle removal (reducing particle concentration by 98%), the catalytic oxide coatings led to measurable increases in concentrations of NO_2. Nevertheless, a study by the same investigators found that bradykinin-induced release of tPA into plasma did not change significantly after exposure to NO_2 at 4 ppm for 1 h (Langrish et al. 2010), suggesting that NO_2 does not appear to be a major factor in the anti-fibrinolytic effects of dilute DE inhalation. Strengthening the role of particles, thrombotic effects appear to be relatively consistent in inhalation studies performed using diesel engine emissions at equivalent particle mass concentrations but different gaseous components (Lucking et al. 2008). Controlled exposure to O_3 (0.3 ppm for 2 h) reduced plasma PAI-1 within 24 h in healthy volunteers, also suggesting a pro-thrombotic effect of O_3 (Devlin et al. 2012).

The source and chemical nature of the particles appears to be important. For example, exposure to CAPs from non-exhaust traffic related sources, did not significantly affect the fibrinolytic balance in patients with CHD (Mills et al. 2008). Furthermore, a pure carbon nanoparticulate exposure alone had no discernible effect on blood coagulation and fibrinolysis in healthy volunteers (Mills et al. 2011). However, to date these effects have not been studied extensively and therefore only limited information is available on these issues. Although diesel motor emissions constitute a significant proportion of UFPs in the urban environment, it is as yet unclear which fraction of particulate from DE plays the largest role in the pollution-induced pro-thrombolytic and anti-fibrinolytic effects. Studies using inhaled laboratory-generated ultrafine carbon particles (50 μg/m^3; count medium diameter, 32 nm; 2 h) – as surrogates for

ambient UFP- have reported platelet activation and increased vWF (which mediates platelet-platelet or platelet-vessel interaction) levels in people with type 2 diabetes (Stewart et al. 2010)

Gene expression profiling analysis using microarray techniques has demonstrated changes in expression levels of genes involved in the clotting cascade (Pettit et al. 2012). Expression of the F2R gene (located at chromosome 5q13.3), which encodes the thrombin receptor, were elevated 30-fold in 14 healthy participants after a 1 h exposure to diluted DE (300 μg/m^3) compared to clean air (Pettit et al. 2012). In addition, DE exposure decreased expression of the gene encoding the urokinase-type plasminogen activator (PLAU), a secreted serine protease involved in the breakdown of clots (Pettit et al. 2012). Genes related to oxidative stress pathways have been shown to be differentially regulated in response to exposure to air pollution. In particular, inducible nitric oxide synthase 2A (NOS2A), a biomarker of oxidative stress, was upregulated in healthy volunteers 24 h after DE exposure (300 μg/m^3, 60 min on 2 separate days) (Pettit et al. 2012). Further evidence is required to confirm a role for oxidative stress in causing PM-induced pro-coagulant/thrombotic effects. It is also worth noting that the changes in the gene expression level of coagulation markers have not frequently been reflected by changes in concentrations of coagulation markers in blood after DE exposure.

5.3.1 Summary

Taken as a whole, controlled exposure studies in man provide support for the notion that particulate air pollution exposure favours a pro-thrombotic state. The most common human controlled inhalation studies are of the effects of DE. Exposures have been short (1-2 h) and concentrations used have been high, but within the range that could occur in the urban environment (for example, during high air pollution episodes or in areas of dense traffic). It has been reported that O_3 exposure, but not NO_2, promotes thrombosis, but these are isolated studies. A number of different mechanisms have been postulated to mediate thrombogenic actions of particulate air pollution. Arguably, one of the strongest hypotheses for the enhanced thrombotic profile in response to exposure suggests an increase in platelet activation. Inflammation and oxidative stress continue to be implicated as a mechanism underlying pollution-induced pro-thrombotic and anti-fibrinolytic effects, although findings are inconsistent. Most investigations used young, healthy subjects and there is insufficient data to ascertain whether pre-existing conditions further promote the thrombogenic effects of air pollution.

5.4 *In vivo* studies in animals

5.4.1 Effects of air pollution on TF-and factor X-dependent activation

Animal models provide greater flexibility to explore the biological mechanisms of air pollutant exposure. In relation to the haemostatic system in particular, *in viv*o models allow induction of thrombosis to be directly studied, as opposed to relying on surrogate measures. Together with biomarkers, a detailed assessment of effect and mechanism is possible. In recent years, persuasive evidence for a number of biologically plausible pathways has been put forward. The focus has remained on PM, through inhalation of urban ambient particles or instillation techniques to deliver particulates in the absence of gases. There is increasing evidence that the association between exposure to PM and alterations in haemostasis is mediated, at least in part, by interleukin-6 (IL-6). Mutlu et al. (2007) first drew attention to a role of IL-6 in mediating

PM-related pro-thrombotic effects (Mutlu et al. 2007). In C57 mice, intratracheal administration of ambient particles (PM_{10}; 10 μg), collected from an urban background site in Düsseldorf, Germany, increased lung production of IL-6, reduced clotting times and enhanced thrombin generated within 24 h compared with saline instillation (Mutlu et al. 2007). Additionally, the role of alveolar macrophages as the critical source of IL-6 was confirmed by depleting alveolar macrophages using intracheally instilled liposomal clodronate (Mutlu et al. 2007). IL-6 dependent activation of coagulation has also been suggested in studies using different particle size fractions and other exposure methods (Budinger et al. 2011). Work by Budinger et al. (2011) demonstrated an IL-6-dependent increase in coagulation activation markers (thrombin-anti-thrombin (TAT)) following inhalation exposure (8 h per day, for 3 days) to fine CAPs ($PM_{2.5}$; 88.5 ± 13.4 μg/m^3) and after the instillation of $PM_{2.5}$ (200 μg) (Budinger et al. 2011). Unfortunately, because studies have used different methods in assessing the haemostatic effects, making comparisons between studies is difficult.

Budinger et al. (2011) also showed a role of IL-6 in the increased levels of TF antigen following PM exposure (Budinger et al. 2011). IL-6 knockout mice did not display the increased levels of TF following inhalation exposure (8 h per day, for 3 days) to fine CAPs ($PM_{2.5}$; 88.5 ± 13.4 μg/m^3) and after the instillation of $PM_{2.5}$ (200 μg) (Budinger et al. 2011). However, measurements of TF antigen do not necessarily reflect the functional capacity and integrity of TF (Kilinc et al. 2011). Perhaps the most convincing evidence for a role of TF in mediating thrombus formation following exposure to PM comes from the study by Kilinç and colleagues (Kilinc et al. 2011). In mice intratracheal administration of UFPs (approx. 0.36 μg) collected near a Dutch roadside tunnel (mainly used by heavy diesel trucks) increased plasma thrombin generation at 4 and 24 h post-exposure (Kilinc et al. 2011). The extrinsic pathway is initiated when TF comes in contact with and activates factor VII (Figure 5.2). Kilinç et al (2011) showed increased thrombin generation 4 h after exposure to UFP to be blocked by administration of TF/FVIIa inhibitor (Kilinc et al. 2011). TF-driven thrombin generation was supported by observations that thrombin generation parameters were similar in wildtype and mice deficient in FXII (an important protein involved in the initiation of the intrinsic pathway; Figure 5.2) 4 h after UFP exposure (Kilinc et al. 2011). Of note, this study did not provide evidence for a causal link between IL-6 and TF. The use of FXII knockout mice to study the role of the intrinsic coagulation pathway provided support for the intrinsic pathway in the later phases of thrombin generation (20 h after UFP exposure) (Kilinc et al. 2011). While intratracheal instillation of UFP increased thrombin generation in wildtype mouse plasma at 20 h post-exposure, no effect was seen in FXII knockout mice. Similar results were obtained by pharmacological inhibition of FXII by the inhibitor corn trypsin inhibitor (Kilinc et al. 2011). Overall, these results suggest that distinct mechanisms regulate pollution-related haemostatic effects over different timescales. However, more studies are needed with particulate pollution from different sources and different particle sizes. Inhalation studies are also required. Intratracheal administration is an artificial route of delivering pollutants to the lungs and does not simulate normal animal inhalation exposure conditions (Driscoll et al. 2000). Nevertheless, intratracheal instillation of particle suspensions has been shown to be a reliable way of producing excellent dispersion of particles throughout the lobes of rodent lungs and across the surface of the alveoli, leading to pulmonary effects that are directly comparable to that of inhalation studies (Henderson et al. 1995; Miyabara et al. 1998).

The study by Kilinç et al (2011) did not assess whether the initiation of the intrinsic coagulation pathway following exposure to UFP was a consequence of an inflammatory response and/or particle translocation processes (Kilinc et al. 2011). Interestingly, Budinger et al (2011) suggested that IL-6 is significantly associated with PM-induced thrombogenic effects

independent of other inflammatory markers (Budinger et al. 2011). Two studies have been published examining the effect of anti-inflammatory agents on thrombogenic factors in mice following exposure to DEP (15-30 μg) (Ali et al. 2015; Nemmar et al. 2012). Both studies demonstrated a critical role for inflammation in mediating DEP-induced thrombotic effects (Ali et al. 2015; Nemmar et al. 2012). Interestingly, the Nemmar et al. (2003) found that inflammation and thrombosis were associated events at 18 h, but not at 4 h (Nemmar et al. 2003). Particle translocation could play a role in the early pro-thrombotic effects of DEP, with inflammation playing a greater role at later stages. Indirect evidence for this concept is provided by the finding that intravenous administration of DEP to the blood has the capacity to increase *in vivo* thrombosis formation at 2 h, without inducing inflammation (Tabor et al. 2016). The link between inflammation and thrombosis at later time points after pulmonary instillation is possible (6-24 h), but complex (Tabor et al. 2016). Smyth et al. (2017) showed intratracheal instillation of DEP (25 μg) in mice to induce platelet aggregation independent of lung inflammation (Smyth et al. 2017). The study also showed that platelet aggregation persisted in endothelial nitric oxide synthase (eNOS) knockout mice (Smyth et al. 2017), suggesting a lesser influence of vascular-derived mediators in actions of DEP on platelets. There is some discrepancy regarding the role of platelets in mediating the pro-thrombotic effects of particulate air pollution (Budinger et al. 2011; Emmerechts et al. 2012; Kilinc et al. 2011; Lucking et al. 2008; Lucking et al. 2011; Mutlu et al. 2007; Smyth et al. 2017; Tabor et al. 2016). This is likely due to differences in study designs, including species, particle types, doses, exposure methods and different measurable indicators of platelet function. An especially noteworthy study is that by Emmerects et al. (2012) suggesting that continuous exposure of mice to traffic-related air pollution, in a real-life setting (mice were placed in a highway tunnel for 25 or 26 days; mean 24.9 $\mu g/m^3$ $PM_{2.5}$) may affect platelet function with an increased release of platelet derived pro-coagulant microvesicles (Emmerechts et al. 2012). This study also looked at how age modifies the pollution-induced changes in platelet counts and activation but no clear patterns emerged (Emmerechts et al. 2012). More studies assessing the potential effect modification by age, as well effects of co-morbidities are clearly needed. One study has examined hypertension and demonstrated enhanced platelet aggregation and thrombin generation 24 h after intratracheal instillation of DEP (15μg) in mice induced with experimental hypertension compared with wildtype controls (Nemmar et al. 2011). There is limited evidence to support the assertion that exposure to air pollution may have a priming effect that leads to an augmented response to subsequent stimuli (Wilson et al. 2010). Platelets from CAPs-exposed mice ($PM_{2.5}$; 88.5 $\mu g/m^3$; 6 h/day; 5 days/week for 2 weeks) showed a 54% increase in fibrinogen binding in response to the agonist adenosine diphosphate (ADP), compared to saline exposed mice (Wilson et al. 2010).

5.4.2 Effects of air pollution on fibrinolytic function

As discussed above, impairment of endogenous fibrinolysis has been suggested from studies exposing humans to DE by inhalation under controlled experimental conditions (Mills et al. 2005; Mills et al. 2007). Subsequent studies showed that this effect was directly attributable to the exhaust particles (Kodavanti et al. 2011). Increased aorta PAI-1mRNA levels, suggesting the presence of vascular injury and/or fibrinolytic activation were observed in rats exposed to O_3 (0.38 ppm) or diesel exhaust particles (DEP; 2.2 mg/m^3) alone for 16 weeks (5 h/day, 1 day/week) (Kodavanti et al. 2011). Interestingly, aortic thrombodulin (TM) and t-PA mRNA levels were also increased, an effect opposite to the expected direction of change (Kodavanti et al. 2011). Further studies are needed to determine whether these exposures would induce similar changes in corresponding protein levels or activity. A great advantage of the study

design employed by Kodavanti et al. (2011) was that they analysed the effects of exposure to both acute (2 days) and sub-chronic (16 weeks) pollution in the same study. The analysis found that the associations between exposure and mRNA markers of haemostasis to be strongest after a longer series of exposures (Kodavanti et al. 2011). This study also showed a synergistic decrease in effects following DE and O_3 (0.5 ppm + 2 mg/m^3 5 h/day; 1 day/week for 16 weeks) co-exposure (Kodavanti et al. 2011). Concentrations of PAI-1 mRNA in mouse fat tissue has also been shown to increase following inhalation exposure (8 h per day, for 3 days) to fine CAPs ($PM_{2.5}$; 88.5 ± 13.4 μg/m^3) and after the instillation $PM_{2.5}$ (200 μg) (Budinger et al. 2011). Notably, studies in IL-6 null mice revealed no significant effect of the loss of IL-6 on the induction of PAI-1 expressions following exposure (Budinger et al. 2011). Treating wildtype mice with the TNF-α receptor antagonist etanercept (10 mg/kg i.p.) prevented upregulation of PAI- 1 expression following exposure (Budinger et al. 2011). In contrast etanercept had no effect on PM-induced thrombus formation (Budinger et al. 2011). Another study showed curcumin (45 mg/kg), a compound with anti-inflammatory and anti-oxidant properties, to block the DEP-induced up-regulation of TNF-α and PAI-1 in mice (15 μg DEP via intratracheal instillation, 4 times/week, for 1 week), but to only partially inhibit PM-induced thrombosis (Nemmar et al. 2012). Collectively, these data suggests that PM exposure is associated with both the activation of coagulation and impairment of fibrinolysis, but that these facets are regulated via distinct mechanisms.

Lastly, there was limited evidence linking oxidative stress to the effects of pollution exposure on thrombosis and fibrinolysis. For example, the pulmonary and systematic inflammation induced by ultrafine carbon particles (180 μg/m^3, 24 h) in aged spontaneously hypertensive (SH) rats was associated with increased hemeoxygenase-1 levels (HO-1; a marker of oxidative stress), alongside changes in biomarkers of thrombosis (Upadhyay et al. 2014). Additionally, oxidation of the low density lipoprotein (LDL) receptor has an influence on the thrombotic effects of inhaled vehicular emissions (Kodavanti et al. 2011).

5.4.3 Summary

In summary, the majority of animal studies support the notion that particulate air pollution exposure leads to an enhanced thrombogenicity. The observation of *in vivo* of thrombus formation in blood vessels in situ is a strength of animal studies given that clot formation will be heavily influenced by the vascular wall and flow conditions of the blood prior to clot formation. Whilst *in vivo* studies generally use high air pollution exposures, the similarity of many of the mechanistic pathways with those shown in epidemiological and controlled exposure studies in man suggests that these exposures are relevant. Multiple mechanisms have been postulated, including inflammation, oxidative stress, interplay between TF and IL-6 (potentially independently of other inflammation pathways), increases in coagulation factors, and impaired fibrinolysis. A role of platelet activation in the enhanced thrombosis is one of the most consistent observations. The thrombotic effects of gaseous pollutants and the use of models of susceptible populations is an avenue for future research, and one that would provide a useful foundation addressing these matters in human studies, particularly if dose and time responses can be defined.

5.5 Overall summary

In 2010, a seminal review of literature suggested a role of the haemostatic system in the overall cardiovascular (CV) effects of air pollution, although a great deal of uncertainty remained

(Brook et al. 2010). The present chapter discusses the growing body of evidence from 2009 to 2016, expanding the remit to include gaseous co-pollutants as well as airborne particulates of different size fractions.

A large body of work (2326 references screened, 74 assessed) has been published on the topic in between 2009 and 2016. Overall, examination of this literature supports the contention that exposure to air pollution promotes coagulation and impairs fibrinolysis, leading to an unfavourable imbalance in haemostatic factors that would be expected to increase the risk of thrombotic events in susceptible individuals. As noted in the AHA review, inconsistent findings between studies are commonplace. However, the volume of publications supporting the pro-thrombotic effect of air pollution far outweigh those showing no effect or the opposite effect, even given the assumption that there may be a publication bias towards studies suggesting a health risk of pollutants. Potential reasons for the inconsistencies are many. In general, the investigations assessed were, in our opinion, of high scientific quality – both in their design, implementation and analysis. Instead, a major source of discrepancies most likely reflects the differences in study design and increasing complexity of the endpoints investigated. Additionally, the nature of exposure is also likely to play a significant role in the differences between studies, in a manner that will depend on the study type under investigation. For example, distinguishing the effects of individual pollutants remains a challenge for epidemiological studies, especially where there is high correlation between pollutants (for example, $PM_{2.5}$ and NO_2 from traffic-derived sources). Controlled exposure studies in man and animals are frequently criticised for the high concentrations used, a criticism that still applies to many of the studies performed in recent years. Indeed, little consensus has been reached in striking a balance between using a realistic dose that models long-term exposure with obtaining suitably high exposure to explore pathogenic mechanisms in short-term studies. However, the number of studies, both epidemiological and experimental, assessing the effects of longer periods of exposure are increasing. Overall, the direction of effect seen in these studies matches those of short-term studies.

While the remit of this literature review was to include consideration of whether gaseous co-pollutants have haemostatic effects, there have been too few studies draw this conclusion with any certainty. Epidemiological studies suggest that it is likely that ambient levels of gases such as NOx, O_3, and possibly SO_2, are associated with biomarkers of thrombotic pathways, however, a paucity of experimental studies means it is not possible to draw any conclusions as to the wider functional consequences of such observations, or address more taxing issues such as causality. Of those few studies available, it does appear that gases can alter thrombotic pathways, however, initial indications suggest that inconsistences between studies could be even greater than for PM; an observation that might not be especially surprising given that our focus is on a physiological system (the blood) some distance from the initial organ of exposure (the lung). Obtaining evidence of a dose-dependency of the effects of gases will again be important for advancing this area.

While the understanding of the pathophysiologic mechanisms by which air pollution promotes thrombosis remains incomplete, significant progress has been made. Similarities in the pattern of biological actions on the haemostatic system suggest that the findings of short-terms studies provide relevant, and valuable, information. Multiple mechanistic pathways are plausible, including platelet activation, oxidative stress, interactions between inflammatory mediators and impaired fibrinolysis. Emerging pathways of interest include the role of circulating microvesicles, epigenetic modifications and alterations in sensitivity to the above caused by genetic polymorphisms. A schematic overview of these pathways is shown in Figure 4.3. There

is great complexity in the coagulation cascade and its interaction with other pathways leading to thrombosis (see Figures 4.1 and 4.2), with many levels of feedback regulation (positive and negative). Consequently, it has been difficult to disentangle which points in the pathway are pivotal in driving the haemostatic effects of PM. An increase in circulating fibrinogen is one of the more consistent observations across studies, thus the availability or activation of this mediator could be important. Platelets (and platelet-derived mediators) also appear to play an important role in driving the pro-thrombotic effects of air pollution, and there has been increasing evidence suggesting the importance of inflammation and oxidative stress. It is likely that these mechanisms act in concert, and potentially act synergistically to amplify the overall effect on the blood. Experimental studies using interventions (for example, pharmacological inhibition, genetic modifications) to inhibit specific pathways will be useful in dissecting out the key pathways of interest. Ultimately, reducing the health effects of air pollution will rely on removal of pollutants and a better understanding of these mechanisms will help ascertain which pollutants are most harmful and which populations and individuals are most at risk.

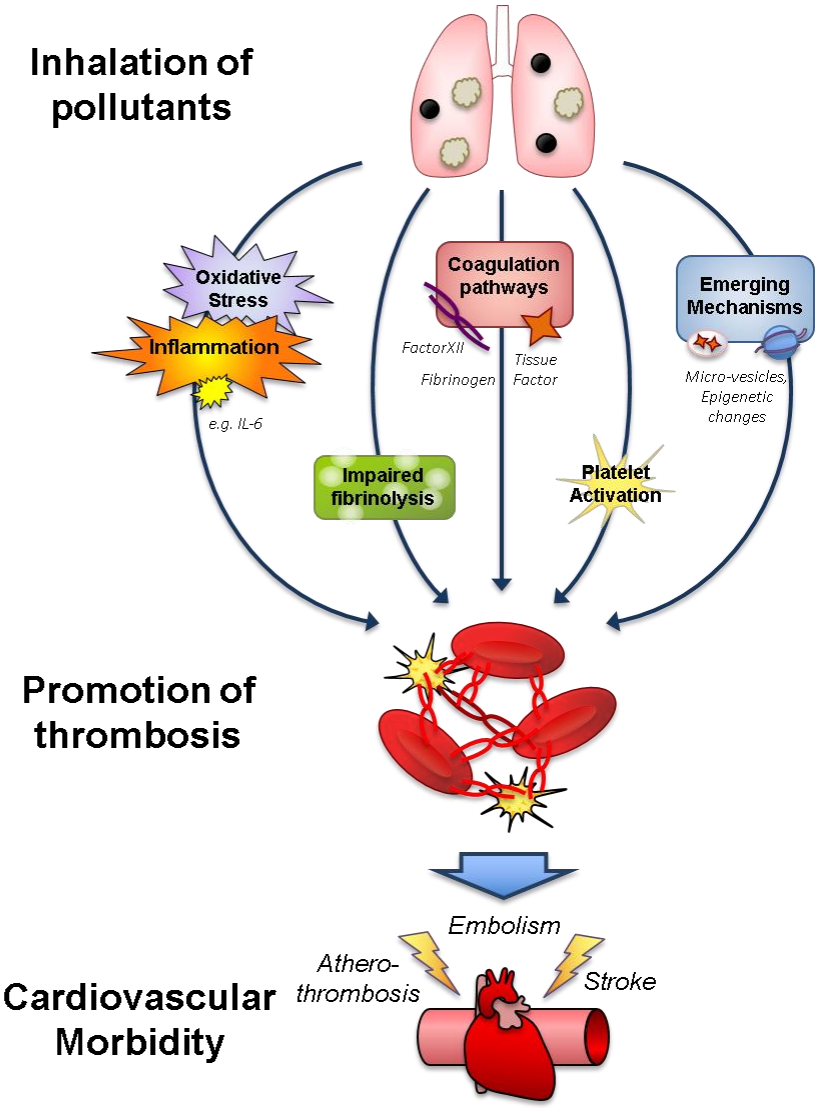

Figure 4.3: Summary of the main and emerging mechanisms described in the review.

In summary, the recent evidence supports a role for a pro-thrombotic effect of air pollution, through the activation of multiple pathophysiological processes. It is likely that these effects will contribute to the overall CV morbidity associated with air pollution and increase the risk of thrombotic effects in those with pre-existing CVD. A clearer understanding of dose-dependency of effects, and of the effects of longer-term exposures, would greatly add to the case for causality of the associations between long-term average ambient concentrations of air pollutants and indices of CV morbidity. Key areas of future research will be to assess the role

of gaseous pollutants and studies that directly compare potentially susceptible individuals/models with healthy counterparts.

Chapter 5
Role of oxidative stress and associated inflammation in cardiovascular disease outcomes following exposure to ambient air pollution

5.1 Introduction

Exposure to ambient air pollution is associated with adverse cardiovascular (CV) outcomes (Brook et al. 2010; Franchini and Mannucci 2007; Mustafic et al. 2012; Pope and Dockery 2006). These are manifested through several, likely overlapping, pathways including at the functional level, endothelial dysfunction, atherosclerosis, pro-coagulant changes, alterations in autonomic nervous system (ANS) balance and changes in blood pressure (BP) (Brook et al. 2010). At the molecular level, principal pathways involved in eliciting such effects will depend upon the nature of the air pollutants (i.e. particles plus their components versus gases). These include

a) inflammatory responses within the alveoli, secondary systemic inflammation through pulmonary and systemic oxidative injury which, via pro-oxidative and/or pro-inflammatory mediators, initiates or exacerbates CV responses (Miller et al. 2009);

b) translocation of ultrafine and nanosize particles and/or particle constituents across the alveolar membrane into the systemic circulation possibly giving rise to direct interaction and localised toxicity within the vascular endothelium and/or cardiac tissue (Miller 2014; Moller et al. 2011);

c) activation of airway sensitive receptors or nerves leading to ANS imbalance (CM Perez et al. 2015).

At numerous points along each of these functional and molecular pathways, there is potential for cellular oxidative imbalances to occur (Miller et al. 2012). In 2010 Brook and colleagues (Brook et al. 2010) presented persuasive evidence that oxidative stress (OS) is a critically important cause and consequence of particulate matter (PM)-mediated CV effects. This review examines subsequent evidence, stemming from epidemiological, occupational and controlled exposure studies and research employing healthy and diseased animal models, isolated organs

and cell cultures in assessing the importance primarily of the pro-oxidant potential of particulate and gaseous air pollution, as well as associated inflammatory processes, in the development of cardiovascular disease (CVD). Two hundred and sixty-seven articles were appraised in detail. Of these, the studies that have been incorporated in this review are limited to those deemed to be have produced results of special interest, thereby adding to our understanding of how OS contributes towards cardiovascular disease induced by exposure to ambient air pollution. Of note, care was taken to include publications from the current literature search that reported negative findings.

5.2 Endothelial dysfunction

5.2.1 Human studies

On examining an impact of antioxidant gene polymorphisms on the relationship between black carbon (BC) exposure and serum concentrations of soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1) in 809 participants of the Normative Aging Study, Madrigano et al (Madrigano et al. 2010) reported magnified associations in subjects with a glutathione S-transferase M1 (GSTM1) deletion. A genetic score approach, investigating interactions between relevant pathways and the environment, also suggests that OS plays a role in the association of ambient particles derived from oil combustion and endothelial dysfunction (Bind et al. 2014a; Dai et al. 2016). Research examining the association between PM oxidative potential (OP; the capacity of particles to cause damaging oxidative reactions) and adverse health outcomes includes a cohort panel study in 93 elderly non-smoking adults. Results suggest that short-term exposures to traffic-related air pollutants with a high OP contribute to microvascular endothelial dysfunction, represented by reactive hyperaemia index (RHI) (Zhang et al. 2016). In 12 healthy subjects, acute (2 h) exposure to diesel exhaust (DE; 300 $\mu g/m^3$ $PM_{2.5}$ [particulate matter less than 2.5 μm in diameter]), impaired nitric oxide (NO)–mediated endothelial vasomotor function and promoted superoxide anion radical (O_2^{*-}) production in human umbilical vein endothelial cells (HUVEC) pre-incubated with serum from 5 of the subjects (Wauters et al. 2013).

5.2.2 Animal studies

Acute DE (300 $\mu g/m^3$ PM, 5 h) inhalation impaired acetylcholine (ACh)-mediated relaxation and increases O_2^{*-} production in coronary arteries of rats, signals that were blocked with O_2^{*-} scavenging, nitric oxide synthase (NOS) inhibition or tetrahydrobiopterin supplementation (Cherng et al. 2011). A murine 3-month nickel (Ni) plus concentrated ambient particle (CAP) exposure (Ying et al. 2013), induced endothelial dysfunction, systemic OS and a redox-dependent decrease in endothelial (e)NOS dimers in the aorta. Researchers have also demonstrated that a chronic CAP exposure (92.4 $\mu g/m^3$, 6 h/d, 5 d/wk for 20 weeks) in mice impairs aortic tonal responses and induces the generation of O_2^{*-} in monocytes, aortic tissue and perivascular fat through Toll like receptor 4/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–dependent mechanisms (Kampfrath et al. 2011). OS following exposure to vehicular source air pollutants has been implicated in the imbalance of vasoactive factors produced by the endothelium. A 7-day inhalation exposure to gasoline engine exhaust (GEE; 60 $\mu g/m^3$, 6 h/d) or mixed vehicle exhaust (250 $\mu g/m^3$ PM diesel plus 50 $\mu g/m^3$ PM gasoline exhaust) was associated with increased lipid peroxidation and O_2^{*-} in the aorta of apolipoprotein $E^{-/-}$ ($ApoE^{-/-}$) mice along with an upregulation of circulating and vascular

factors (matrix metalloproteinase [MMP]-2, MMP-9, tissue inhibitor of metalloproteinases [TIMP]-2, endothelin-1 [ET-1]) mediated in part through activation of ET-1-endothelial receptor A (ET_A) and lectin-like oxidised low-density lipoprotein (LOX-1) receptor pathways (Lund et al. 2009; Lund et al. 2011). When normotensive and spontaneously hypertensive (SH) rats were exposed via intratracheal (IT) instillation to diesel exhaust particles (DEP) for 4 weeks (0.8 mg, 3 times a week for 4 weeks), impaired ACh-induced relaxations, accompanied by a significant upregulation of the p22phox NADPH oxidative component in aortas, were only observed in the SH rat group, possibly suggesting a synergism between DEP-induced OS and classical risk factors (Labranche et al. 2012).

High concentrations of nitrogen dioxide (NO_2; 5-20 mg/m^3, 6 h/d for 7 days) produced mild pathology in the heart of rats, marked OS and an upregulation of the vasoconstrictor ET-1 (Li et al. 2011). Sub-chronic (0.5 ppm for 1 or 5 days, 8 h/day) ozone (O_3) exposure has been shown to decrease superoxide dismutase (SOD) activity, eNOS protein and indices of NO production in the mouse aorta (Chuang et al. 2009). Following weekly episodic (5 hr/day, 1 day/week for 16 weeks) exposure of rats to either O_3 (0.4 ppm) or DEP (2.1 mg/m^3), mRNA biomarkers of OS (haemoxygenase-1[HO-1]), vasoconstriction (ET-1, ET_A, ETB, eNOS) and proteolysis (MMP-2, MMP-3, and TIMP-2) were increased in the aorta, but not in the heart (Kodavanti et al. 2011). Twenty-four hours following a single inhalation of 1 ppm O_3, the coronary vascular bed of rats exhibited a markedly diminished dilatory response to ACh, which was restored to different degrees by SOD, catalase (CAT) and NADPH oxidase inhibition (Paffett et al. 2015).

Evidence exists that PM-mediated suppression of the number and function of bone marrow-derived endothelial progenitor cells (EPCs) (Haberzettl et al. 2012; O'Toole et al. 2010) is related to an increased level of oxidants (Cui et al. 2015a; Cui et al. 2015b). PM treated (as low as 10 µg, 3 times per week for 1 month via intranasal instillation) mice exhibit a reduced bone marrow stem cell (BMSC) population in association with increased dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation, decreased phosphorylated protein kinase B and inhibited proliferation of BMSCs (Cui et al. 2015a). The exposure also decreased the circulating EPC population and promoted apoptosis of EPCs in association with increased oxidant production (Cui et al. 2015b). These effects were effectively prevented with N-acetylcysteine (NAC) treatment or use of a transgenic model that overexpresses the antioxidant enzyme.

5.2.3 *In vitro* studies

Cell culture work with end points encompassing cell activation, altered expression and production of messenger molecules and NO are shedding further light on the mechanism by which various sources of particulate air pollution induce OS and subsequent dysfunction in vascular endothelial cells (Buchner et al. 2013; Cao et al. 2014; Du et al. 2013; Forchhammer et al. 2012; Li et al. 2009; Mo et al. 2009; Montiel-Davalos et al. 2012; Rui et al. 2016; Wei et al. 2011). Ultrafine particles (UFPs) from diesel engines dose-dependently induce OS via c-Jun NH_2-terminal kinases (JNK) activation in human aortic endothelial cells (Li et al. 2009), whilst in mouse pulmonary microvascular endothelial cells, NADPH has been identified as the major source of oxidants generated following ambient UFP exposure, involving the translocation of p47phox, p67phox and rac 1 to the plasma membrane (Mo et al. 2009). On investigating the subsequent activation of endothelial cells by UFP-induced oxidants, these investigators demonstrated that this was mediated through phosphorylation of p38 mitogen activated kinase (MAPK) and extracellular signal-regulated kinase (ERK1/2). Wood smoke particles (1 µg/ml)

increased VCAM- 1 expression on HUVECs and at 50 or 100 μg/ml caused adhesion of THP-1 monocytes without having any effect on DCFH-DA oxidation (Forchhammer et al. 2012). Although increased oxidants were observed in HUVECs after exposure to DEP and carbon black (CB) nanoparticles, results from inhibitor studies indicate that this is not a prerequisite for increased adhesion factor expression and foam cell formation, (Cao et al. 2014; Frikke-Schmidt et al. 2011). Using NAC and selective kinase inhibitors, Rui et al (Rui et al. 2016) however demonstrated that $PM_{2.5}$-induced increase in intracellular oxidants generation triggers cell surface expression of ICAM-1 and VCAM-1 and adhesion of THP-1 cells to HUVECs through JNK, ERK1/2, p38 MAPK and AKT phosphorylation, and nuclear translocation of nuclear factor kappa B (NF-κB) in human EA.hy926 cells in a dose- and time-dependent manner. OS has also been implicated in the regulation of eNOS activity by ambient UFP, involving an increase in glutathione oxidation, protein S-glutathionylation and eNOS S-glutathionylation, leading to a decrease in NO production (Du et al. 2013). Investigations into the mechanisms behind changes in vascular permeability exerted by DEP, using an endothelial tube model, has also identified the dependence on OS. This is accompanied by the release of pro-inflammatory tumour necrosis factor (TNF)-α and interleukin (IL)-6, stimulating the secretion of vascular endothelial growth factor-A (Tseng et al. 2015). Exposure of isolated rat aortic rings (thereby ruling out prior interaction with the lung or vascular tissue) to DEP (10–100 μg/ml) generates O_2^{*-} and inhibits endothelium-dependent NO-mediated relaxation – effects that are reversed in the presence of SOD (Labranche et al. 2012; Miller et al. 2009).

5.3 Atherosclerosis

5.3.1 Human studies

A panel of 40 university students relocating between two campuses with contrasting air pollution scenarios in Beijing, China indicates that certain $PM_{2.5}$ chemical constituents/pollution sources are more closely linked with changes in biomarkers of OS associated with atherosclerosis (Wu et al. 2015). Whilst $PM_{2.5}$ iron (Fe) and Ni and $PM_{2.5}$ from traffic emissions and coal combustion were positively associated with oxidized low density lipoprotein (ox-LDL), calcium was associated with an increase in soluble CD36. Another study reported increased urinary 1-hydroxypyrene levels in taxi drivers that were positively correlated with ox-LDL and homocysteine but negatively correlated with antioxidants (CAT and GST) (Brucker et al. 2013). In 2348 participants living in London, the strength of association between intima-media thickness, a measure of subclinical atherosclerosis, and particulate matter less than 10μm in diameter (PM_{10}) mass concentration was stronger than that for the OP of PM_{10}, possibly however owing to the OP assay underestimating the total oxidative burden of PM (Tonne et al. 2012). In 22 randomly selected participants in the Normative Aging Study cohort, a significant association was found between long-term ambient $PM_{2.5}$ exposures and levels of multiple extracellular vesicle-encapsulated microRNAs (evmiRNAs) circulating in serum (Rodosthenous et al. 2016). *In silico* pathway analysis on $PM_{2.5}$-associated evmiRNAs identified several key related pathways, including OS, inflammation and atherosclerosis.

5.3.2 Animal studies

Evidence stemming from earlier studies using transgenic mouse models (deficient in ApoE [$ApoE^{-/-}$] or LDL [$LDLR^{-/-}$] receptors) in demonstrating the role of a localised oxidative insult in the development and/or enhancement of atherosclerosis following PM exposure has been

strengthened by research investigating the molecular pathways through which OS operates following experimental exposure to different types of PM. For DE, Bai et al (Bai et al. 2011) showed that exposure (200 μg/m^3; 6h/day, 5 days/week for 7 weeks) in ApoE$^{-/-}$ mice augmented plaque lipid content, cellularity, foam cell formation and smooth muscle as well as increasing expression of plaque OS markers, inducible NOS, CD36, and 3-nitrotyrosine, and enhancing systemic lipid and DNA oxidation. Miller et al (Miller et al. 2013) employed a DEP instillation study at a dose (35 μg twice a week for 4 weeks) representing the upper range a person may be exposed to in a heavily polluted city over 24 hours. This procedure increased plaque size, number, lipid rich area and frequency of buried fibrous caps in ApoE$^{-/-}$ mice, which despite the lack of systemic inflammation, correlated with lung inflammation and liver antioxidant gene expression (HO-1, NADPH-quinone oxidoreductase 1 and NF-E2-related factor-2), indicative of a counter-regulatory response to systemic pro-oxidative effects. Lipid peroxidation in plasma and liver following inhalational exposure of ApoE$^{-/-}$ mice to DE (250 μg/m^3, 2 weeks) was associated with impaired HDL anti-oxidant capacity (Yin et al. 2013). UFP exposure has also been shown to trigger reduced HDL antioxidant capacity, pro-atherogenic lipid metabolism and a greater atherosclerotic lesion in LDLR$^{-/-}$ mice, whilst D-4F (an apolipoprotein A-1 mimetic peptide) attenuated these effects, suggesting a role for lipid oxidation in UFP-mediated atherosclerosis (Li et al. 2013). Inhalational exposure of ApoE$^{-/-}$ mice to environmental air pollutants from vehicular sources (250 μg/m^3 diesel PM and 50 μg PM/m^3 gasoline exhausts; 6 h/d for 7 days) resulted in LOX-1-mediated vascular OS, expression of MMP-9 and ET-1 and monocyte/macrophage infiltration (Lund et al. 2011). In a Manhattan CAP exposure (6 h/day, 5 days/week, 4 months) study using ApoE$^{-/-}$ mice, Ying et al (Ying et al. 2009a) reported that ambient PM enhances atherosclerosis through the NADPH oxidase–dependent induction of O_2^{*-} and reactive nitrogen species in the aorta, causing decreased guanine cyclase–dependent arterial constriction in response to adrenaline.

5.3.3 *In vitro* studies

The pro-oxidative effects of PM have been observed in a number of cell types that are key players in the development of atherosclerotic lesions including endothelial cells (Cao et al. 2014; Li et al. 2009; Montiel-Davalos et al. 2010), macrophages/monocytes (Cao et al. 2014) and possibly smooth muscle cells (Sun et al. 2008). Increased OS may not however completely explain particle-induced lipid accumulation (Cao et al. 2014; Cao et al. 2015). Whilst exposure of THP-1 derived human macrophages to CB nanoparticles (2.5 μg/ml 24 h) increased cellular lipid load suggesting that the monocytes were transforming into foam cells, this occurred at concentrations lower (0.25 ng/ml) than those required to trigger increased intracellular oxidant production (Cao et al. 2014). Furthermore, whilst the presence of the antioxidant buthionine sulphoximine increased the CB-induced DCFH-DA oxidation, it showed no effect on particle-induced lipid accumulation. Automobile DEP (10 μg/ml, 24 h) has also been demonstrated to induce lipid droplet formation in macrophages but again, at concentrations that were not associated with increased generation of oxidants (Cao et al. 2015).

5.4 Pro-coagulant changes

5.4.1 Human studies

In a panel of 60 elderly subjects with coronary artery disease, traffic-related air pollutants were associated with increased systemic inflammation and soluble platelet selectin (sP-selectin; a

biomarker of thrombosis) as well as decreased erythrocyte antioxidant enzyme activity (glutathione peroxidase (GPx), Cu,ZnZn-SOD) (Delfino et al. 2009). Within subject, inverse associations of sP-selectin with Cu,ZnZn-SOD were detected in mixed models. Changes in gene expression connected with key OS and coagulation pathways have also been identified in 14 young healthy subjects exposed to DE (300 μg/m^3, 60 min on 2 separate days) (Pettit et al. 2012). A decrease in proteins involved in the fibrinolytic pathway (plasminogen, thrombomodulin) was detected in 34 subjects with metabolic syndrome (MeS) post a 2-hour controlled exposure of concentrated ambient ultrafine particles however, GSTM1-individuals were not more responsive than the entire study population (Devlin et al. 2014). Coagulation pathways have also been investigated in a panel of 31 healthy, young volunteers semi-experimentally exposed for 5 h to ambient air pollution at 5 locations with contrasting air pollution characteristics (Strak et al. 2013). Whilst organic carbon, nitrate and sulfate were most consistently associated with coagulative markers, PM_{10} OP did not display a strong or consistent association with the examined endpoints.

5.4.2 Animal studies

Gaseous and particulate air pollution animal exposures are associated with both OS and disturbed coagulatory haemostasis. These include an effect of an ultrafine carbon particle (180 μg/m^3, 24h) inhalation on pulmonary and systemic inflammation in aged SH rats that was associated with increased pulmonary expression of HO-1 as well as systemic changes in biomarkers of thrombosis (fibrinogen, tissue factor (TF)) (Upadhyay et al. 2014). In rats exposed to O_3 (0.4 ppm) or DEP (2.1 mg/m^3) for 16 weeks, biomarkers of OS (HO-1) and thrombosis (TF, plasminogen activator inhibitor-1 [PAI-1], tissue plasminogen activator [tPA], and von Willebrand factor [vWF]) were increased in the aorta, but not in the heart, possibly triggered by oxidatively modified lipids and proteins through LOX-1 and/or RAGE signalling (Kodavanti et al. 2011). Pre-treatment of mice with cysteine (the amino acid that is limiting for glutathione [GSH] synthesis) prodrug l-2-oxothiazolidine-4-carboxylic acid, before IT instillation of DEP (30 μg), suggests that OS is at least partly responsible for the pulmonary and systemic inflammation and thrombotic events in the pial cerebral microvessels (Nemmar et al. 2009). Similarly, emodin (1,3,8-trihydroxy-6-methylanthraquinone; an anthraquinone that is extracted from the rhubarb plant root), which has strong antioxidant and anti-inflammatory effects, ameliorated the following effects of DEP (1 mg/kg): increased heart tissue levels of IL-1β and TNF, decreased SOD and glutathione reductase activities and the prothrombotic effect of DEP in pial arterioles and venules (Nemmar et al. 2015). Furthermore, emodin prevented platelet aggregation *in vitro* in whole blood, and the shortening of activated partial thromboplastin time and prothrombin time caused by DEP.

5.4.3 *In vitro* studies

A causal link between the oxidative effects of PM exposure and pro-coagulant responses has also been implicated in human endothelial cells following exposure to soluble UFPs (Snow et al. 2014). The exposure induced thrombin generation and fibrin clot formation via TF upregulation, involving intracellular hydrogen peroxide production and the NOX-4 isoform of NADPH oxidase. Exemplifying the likely overlapping effects of cellular oxidative imbalances on CV events, mitochondrial OS is believed to play a role in a recently uncovered mechanism by which activation of the sympathetic nervous system following particulate air pollution exposure increases the risk of thrombotic CV events (Chiarella et al. 2014). In an extensive series of animal and *in vitro* experiments utilising non-selective and mitochondrial antioxidants

as well as electron transport chain inhibitors, Chiarella et al (Chiarella et al. 2014) describes a systemic increase of catecholamine induced by CAPs ($PM_{2.5}$), which augments the release of IL-6 from lung macrophages via a pathway that requires mitochondrial oxidants and adenylyl cyclase, and cAMP response element-binding protein, which in turn contributes to a hypercoagulable state.

5.5 Autonomic nervous system dysfunction

5.5.1 Human studies

Baja et al (Baja et al. 2010) reported a stronger association between the heart-rate–corrected QT interval and elevated short-term exposure to BC among 580 Normative Aging Study participants who had a high number of unfavourable genotypes related to OS, as well those who were obese or diabetic. Another study conducted among a panel of elderly Asian residents using genetic risk scores again suggests that the association of air pollution with heart rate variability (HRV), as well BP, are mediated by OS pathways (Kim et al. 2016). In a post-infarction population, total antioxidant capacity (TAC) however did not appear to modify the association between short-term ambient $PM_{2.5}$ concentrations and biomarkers of HRV and repolarisation (Wang et al. 2016). That oxidative pathways exacerbate the adverse effects of ambient levels of air pollution on the cardiac autonomic function has also been investigated by looking at associations between air pollution exposure, biomarkers of OS and HRV (Hemmingsen et al. 2015; Lee et al. 2014; Lin et al. 2013; Sarnat et al. 2014; Zhang et al. 2013). Whilst Lee et al (Lee et al. 2014) reported that OS modified the association between continuous personal exposure to $PM_{2.5}$ and HRV, the markedly improved air quality brought about by the 2008 Olympic Games in Beijing was not mirrored by a change in HRV measurements despite decreases observed in urinary 8-oxodGuo (Zhang et al. 2013). Exhaled malondialdehyde (MDA) levels were slightly, but insignificantly, elevated in a group of asthmatic and non-asthmatic subjects who experienced decreases in HRV following a 2-hour highway commute during rush hour (Sarnat et al. 2014). Another study observed that a 5-hour exposure to real-life levels of $PM_{2.5}$ from an urban street (24 $\mu g/m^3$) reduced HRV as well as causing vasomotor dysfunction in overweight middle-aged and elderly adults. However these effects were not associated with the altered biomarkers chosen to measure OS (dihydrobiopterin, biopterin, uric acid, ascorbic acid and dehydroascorbate) (Hemmingsen et al. 2015). Studies examining the impact of antioxidant supplementation on the CV effects of pollution exposure include that of Romieu et al (Romieu et al. 2005) who reported that omega-3 polyunsaturated fatty acid supplementation attenuated the effect of same-day indoor $PM_{2.5}$ on HRV among elderly residents of a nursing home in Mexico City. More recently, in a controlled human exposure study (CAP mean mass concentration 278 ± 19 $\mu g/m^3$ for 2 h) omega-3 fatty acid supplementation (3 g/day for 4 weeks), attenuated the CAP-induced changes in HRV and cardiac repolarisation in healthy, middle-aged healthy adults (Tong et al. 2012).

5.5.2 Animal studies

Findings from animal studies investigating the arrhythmogenic mechanism of particulate pollution are also consistent with OS mediated pathways of injury (Ghelfi et al. 2010; Kim et al. 2012; Robertson et al. 2014). Following endotracheal DEP exposure to rats (200 µg/ml for 30 min) and perfused rat hearts (12.5 µg/ml for 20 min), Kim et al (Kim et al. 2012) observed

action potential duration prolongation, early afterdepolarisation and ventricular arrhythmia that were prevented by pretreatment with NAC as well as active Ca^{2+}/calmodulin-dependent protein kinase II blockade. Oxidant stress in the absence of recruited inflammatory cells, along with increased vulnerability to ischaemia associated arrhythmia has also been observed in rats following a large IT DEP instillation (0.5 mg) (Robertson et al. 2014). Whilst Wang et al (Wang et al. 2013) reported that O_3 alone (0.81 ppm) exposures did not significantly alter indicators (increased heart MDA, decreased HRV) adversely affected by $PM_{2.5}$ (0.2, 0.8, 3.2 mg/rat), the gaseous pollutant potentiated the effects induced by $PM_{2.5}$. The only other gaseous pollutant that has been investigated in this regard is carbon monoxide (CO) in a chronic exposure (30 ppm for 12 hours, including five 1 hour peaks at 100 ppm for 4 weeks) to rats, which promoted OS, altered Ca^{2+} homeostasis and increased the occurrence of ventricular arrhythmia (Andre et al. 2011).

5.6 Hypertension

5.6.1 Human studies

Traffic-related BC particles were associated with increases in systolic (SBP) and diastolic (DBP) blood pressure among 461 elderly men in the Normative Aging Study, but the effect was not modified by gene variants related to OS defence (GSTM1, GSTP1, GSTT1, GSTCD, NQO1, CAT, HMOX-1) (Mordukhovich et al. 2009). Furthermore, in a Swedish study (n=1429), associations between long-term exposure to vehicle NO_2 and hypertension were not stronger within individuals with potentially impaired antioxidant defence (gene variants in GSTP1, GSTT1, GSTCD) (Levinsson et al. 2014). In a panel study of post-infarction patients (n=76), no evidence of effect modification by TAC was found on the associations between ambient $PM_{2.5}$, accumulation mode particle and UFP concentrations in the previous 6 to 120 hours and SBP (Wang et al. 2016). A study conducted among elderly Asian participants, employing genetic risk scores did however suggest that associations of air pollution with BP were mediated, at least partly by OS pathways (Kim et al. 2016). Exposure to short-to moderate-term ambient BC concentrations was associated with increased BP and blood mitochondrial abundance, an adaptive mechanism to compensate for cellular-redox-imbalance following environmental challenges (Zhong et al. 2016). Furthermore, individuals with higher blood mitochondrial abundance appeared less susceptible to the impact of ambient BC on BP, possibly indicative of a compensatory response to the increases in mitochondrial abundance that attenuates the cardiac effects of traffic-related pollution. In a panel of 50 healthy people, short-term PM_{10} exposure was associated with retinal arteriolar narrowing and venular widening, both independent risk factors for cardiovascular disease. Furthermore, analysis of miRNA expression hinted to a possible role for OS and inflammatory pathways (Louwies et al. 2016).

5.6.2 Animal studies

Exposure to particulate pollution has been reported to increase BP in rodents along with increased oxygen free radicals in coronary perfusate (0.5 mg DEP by IT in adult rats) and an induction of HO-1 within the lung (180 $\mu g/m^3$ ultrafine carbon particle by inhalation in aged SH rats) (Robertson et al. 2014; Upadhyay et al. 2014). High dose IT $PM_{2.5}$ (3.2 mg per rat) alone or in combination with O_3 (0.81 ppm), but not O_3 alone, increases SBP and heart MDA (Wang et al. 2013). In healthy and SH rats, a 4-week DE inhalation (0.5 or 2 mg/m^3, 4 h/day,

5 days/week for 4 weeks) enhanced cardiac mitochondrial OS in both species and produced a hypertensive-like gene expression pattern in the ventricles of healthy rats, characterised by a generalised suppression of genes (including those related to mitochondrial function and compensatory response to OS) that are already suppressed in SH rats at baseline without DE (Gottipolu et al. 2009). Of interest, hearts of already hypertensive rats were spared from DE-induced further impairment in gene expression. On investigating the effect of different size PM fractions on renin-angiotensin-aldosterone and kallikrein-kinin elements in rats, Aztatzi-Aguilar et al (Aztatzi-Aguilar et al. 2015), observed an increased expression of the angiotensin II receptor type 1 in the heart that was accompanied by a decrease in HO-1 together with the induction of Acta1 and Col3a1 (markers of myocardial adaptive response to damage) and increased coronary wall thickness following an UFP sub-chronic exposure (107 $\mu g/m^3$, 8 weeks (5 h/day, 4 days/week).

5.7 Concluding remarks

The large and diverse evidence base indicates that cellular oxidative imbalances and associated inflammatory events can occur at each of the potential junctures (endothelial dysfunction, atherosclerosis, pro-coagulant changes, autonomic nervous system dysfunction, hypertension, cardiac dysfunction and increased susceptibility) at which inhaled air pollutants can exert adverse effects on the CV system (Figure 5.1).

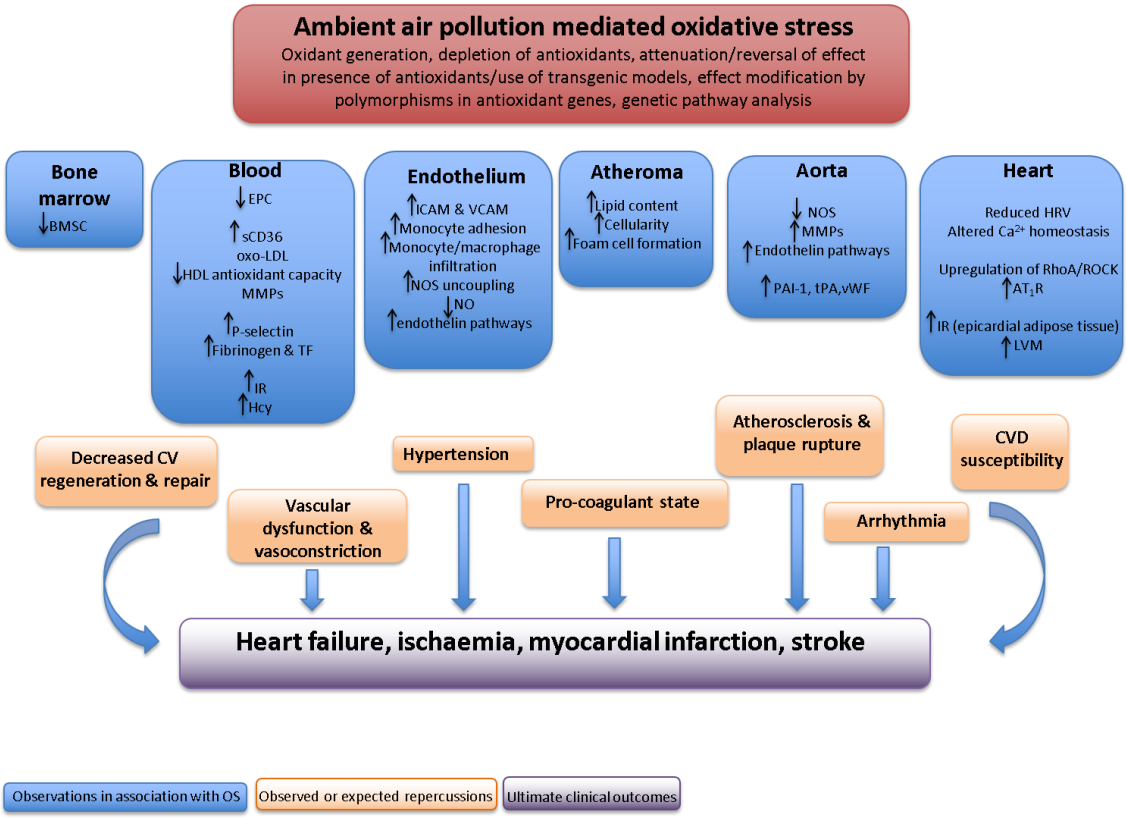

Figure 5.1 Illustration of potential oxidative stress-mediated mechanisms behind effect of ambient air pollution on CVDs. Observations of oxidative stress (for example, increased oxidant production; attenuation/reversal of effect in the presence of antioxidants/use of transgenic models; effect modification by polymorphisms in antioxidant genes; genetic pathway analysis) originate from epidemiological and experimental findings summarised in this review.

Evidence is particularly robust that vascular OS following particulate and gaseous pollutant exposure is central to the pathogenesis of endothelial dysfunction. Many studies pinpoint the production of oxidants and/or prevention to varying degrees by antioxidants in such circumstances, in association with decreased NO concentrations and impaired NO activity, endothelial cell activation and increased vascular permeability. *In vitro* studies investigating the role of particulate-generated-ROS in augmenting expression of adhesion molecules and adhesion of monocytes onto endothelial cells have however yielded conflicting results.

The use of transgenic rodent exposure models in demonstrating the role of an oxidative insult behind the pro-atherosclerotic properties of particulate air pollution from vehicular sources is also well documented. Numerous studies, using well controlled whole-body inhalation systems that mimic real world exposure in polluted cities, are consistent in showing that PM exposure enhances the pro-atherogenic properties of LDLs and plaque vulnerability and diminishes the anti-atherogenic function of protective HDLs and that, importantly, this is accompanied by increased markers of OS both systemically and in the atherosclerotic tissue. Although the pro-oxidative effects of various particle exposures has been observed *in vitro* in a number of cell types that are key players in the development of atherosclerotic lesions, studies using CB

nanoparticles and DEPs suggest that increased oxidant generation may not explain particle-induced lipid accumulation and transformation of monocytes into foam cells.

Much of the epidemiological support for a role of OS in influencing CV outcomes stems from evidence suggesting that an altered oxidant defence may impact on health effects associated with ambient pollutants. Studies, primarily limited to the Normative Aging Study cohort of elderly Caucasian men, have revealed an effect modification of the association between traffic pollutants and markers of endothelial function, changes in HRV and risk of acute CVD by gene variants related to antioxidant defences (Baja et al. 2010; Chahine et al. 2007; Levinsson et al. 2014; Madrigano et al. 2010; Park et al. 2006; Schwartz et al. 2005). Results from a smaller number of investigations into the interactions between such genetic polymorphisms and urban air pollution for hypertension and markers of thrombosis and coagulation have been more inconsistent (Levinsson et al. 2014; Mordukhovich et al. 2009). Further evaluation is therefore warranted, particularly for chronic CV outcomes and in more generalised populations to broaden our understanding of susceptibilities to pollutant-induced health effects.

The few studies exploring potential effect modification by either fish oil supplementation (Romieu et al. 2005; Tong et al. 2012) or statins (Schwartz et al. 2005) support the role of OS in an ambient PM-induced decline in HRV. These studies are supported by animal data, showing that pretreatment with antioxidants or functional foods represents a valuable approach for the prevention of DEP-induced thrombotic complications (Nemmar et al. 2009; Nemmar et al. 2015) - a caveat here however is that laboratory animals appear to be more responsive to dietary antioxidants compared with humans (Halliwell 2011). More epidemiological studies, ideally incorporating mechanistic investigations, into such beneficial effects are therefore needed.

Research focusing on the associations between the potential of PM to generate ROS and adverse CV health outcomes is in its infancy. Recent evidence however suggests that short-term exposures to traffic-related air pollutants with high OP play a major role in contributing to microvascular endothelial dysfunction (Zhang et al. 2016), whilst regional PM OP may modify the impact of $PM_{2.5}$ on the risk of myocardial infarction, with a possible magnified effect when taking into account the combined oxidative capacity of NO_2 and O_3 (Weichenthal et al. 2016).

Even in the absence of a polluted ambient atmosphere, the heart is inherently susceptible to oxidative injury (Zaky et al. 2015). It is an extremely active organ and as a consequence, has a high metabolic rate to satisfy the high-energy demand. This in turn leads to an increased rate of production of oxidising species but at the same time, compared with other tissues, the heart is characterised by lower concentrations of SOD, CAT and GPx (Costa et al. 2013; Damiani et al. 2012). It is therefore not surprising that an increased oxidative burden elicited by air pollution, in addition to being central to eliciting specific cardiac endpoints, is also implicated in the onset and progression of metabolic conditions and their associated complications. Studies have suggested that $PM_{2.5}$-induced OS increases obesity and IR later in life following early-life exposure (Xu et al. 2010), and influences adult CV health following *in utero* exposure (Weldy et al. 2014)

Although both gaseous and particulate emissions have been linked to detrimental outcomes, more evidence implicates the PM components for a large portion of the outcomes investigated. Much of the experimental data supporting an oxidative pathway originates from studies investigating the toxic effects of DEPs that occur at high concentrations in many of the world's

largest cities. Little is known however about OS pathways and CV effects caused by individual pollutants within air pollution mixtures (apart from documented effects in animal elicited following exposure to vehicle exhaust), or as a consequence of inter-pollutant interactions. In characterising free radical pathways and the endothelinergic system in rats after inhalation of urban PM, O_3 and a PM/O_3 combination, Kumarathasan et al (Kumarathasan et al. 2015) reported that such pollutant-specific changes can be amplified or abrogated following multi-pollutant exposures and called upon further studies, adopting a systems biology approach to validate and give greater insight into these air pollutant exposure-specific mechanistic pathways.

Uncertainties exist as to whether, for each of the CV endpoints discussed in this review, enhanced OS triggers the effect and/or is merely as end product of a given disease outcome. The contribution of OS pathways to acute versus chronic CVD following exposure to air pollutants is also currently unclear. However it is unlikely to be an either/or scenario. An acute oxidative insult could escalate into a cumulative effect following repeated environmental insults and/or provoke end stage adverse outcomes in already chronic cardiac states. It is highly likely that such questions are related to the substantial degree of interconnectivity between the pro-oxidant and pro-inflammatory effects that occur following exposure and inhalation of ambient pollutants and as such the difficulty in determining whether the induction of vascular OS precedes or follows that of inflammation.

In summary, an extensive and multi-disciplinary research effort, adopting multiple experimental approaches, points to OS as one of the most consistently proposed links between exposure to ambient air pollution and CVD outcomes. Whether this link involves a pathway that initiates or worsens a given endpoint, or is representative of the consequences of disease progression may well depend upon the nature of the ambient pollutant as well as the specific adverse CV event (for example, endothelial dysfunction, atherosclerosis, pro-coagulant changes, ANS imbalance, hypertension) or susceptibility to associated conditions (for example, obesity, insulin resistance (IR), diabetes mellitus (DM)) under question.

Chapter 6
Conclusions

6.1 Reflections on the evidence presented in the report

Studies of the mechanisms of effect of air pollutants are undertaken for a number of reasons. These include:

a to understand how air pollution exerts adverse health effects,

b to identify those at potentially higher than average risk of adverse outcomes and

c to develop strategies, including both pharmacological and non-pharmacological interventions, to reduce the impact of air pollutants on health.

In addition, mechanistic studies have added to confidence in, and biological plausibility of, associations reported in epidemiological studies. They may help in judgements of the relative importance of different pollutants, which can be difficult to distinguish when using epidemiological studies. It is recognised that not all associations revealed by epidemiological and, indeed, other techniques are causal in nature: some may reflect the effects of other, confounding, factors or may occur by chance. Distinguishing between casual and non-causal associations is thus of importance in the design of effective intervention policies and much effort has been devoted to this problem.

COMEAP has discussed this problem in the past, for example in the COMEAP report on Cardiovascular Disease and Air Pollution (2006). The report discussed at some length the nine features of associations that should be considered in judging whether a reported association is causal, often referred to as the Bradford Hill criteria. One of these features is biological plausibility. It is accepted that the absence of evidence of biological plausibility is not a reason for discarding the assertion that an association might be causal in nature: it is possible that the mechanisms underlying a causal association might be unknown. It is also accepted that whilst evidence of plausible mechanisms of effect does not prove causality it does add weight to the assertion that an association is causal in nature.

Before listing our conclusions we think it useful to make a few points that apply to the types of studies we have reviewed. These studies can be divided into two groups:

a human volunteer studies (which are conducted under controlled conditions and which, usually, involve small numbers of subjects receiving short-term exposures to often higher-than-ambient concentrations of pollutants) or animal studies, and

b epidemiological (observational) studies that include measurement of physiological variables of mechanistic interest

Epidemiological studies typically involve a large number of people and involve exposure to ambient concentrations of pollutants. Epidemiological studies of this type have been conducted in many countries, including those where ambient concentrations of air pollutants are higher than those in the UK. Studies of the effects of exposure to higher than UK ambient concentrations can shed light on possible mechanisms of effect but cannot show that such effects would, or do, occur in the UK. This raises the problem of thresholds of effect, again a subject on which COMEAP has deliberated in the past. The form of dose-response curves provides some guidance as to the likelihood of adverse effects occurring at concentrations lower than those studied. Very few of the studies identified in this report have examined dose-response relationships. Most have looked at the effects of relatively few concentrations, and in some cases studies have reported the effects of exposure to only one concentration.

We also recognise that mechanistic studies cannot, or are at least are very unlikely to replicate the effects of long-term exposures of humans. Studies of long-term exposure studies tend to be expensive and difficult, and, to date, only a handful of human and animal studies have assessed the long-term effects of air pollution exposure. Inferring the effects of long-term exposure from studies of the effects of short-term exposures involves a number of assumptions and uncertainties.

A further problem that applies to human volunteer studies, and to epidemiological studies of mechanisms, is the age-range of the subjects studied. Cardiac electrophysiology provides a good example of this: age can have a marked effect on heart rate variability (HRV), with older subjects tending to have a lower level of HRV at rest than young subjects (Stratton et al. 2003). Allowing for this effect would reduce the difficulty of predicting effects on older people from the results of studies of young people. Similarly the inferring of effects in persons suffering from cardiovascular diseases (CVDs) and in those taking pharmacological therapies is a problem. There is some evidence to show that effects, for example on HRV, can be reduced by some medications but increased by others. Not many studies of the effects of medication on responses to air pollutants have been undertaken and none has examined the dose-response relationship of the effects of medication on the effects of exposure to air pollutants on the endpoints we have reviewed.

Another point that has concerned us relates to transient changes in physiological variables. By physiological variables we mean for example, pulse rate or blood pressure or HRV. Physiological variables are regulated by systems that have evolved to ensure that the needs of the body under different conditions are adequately met and that when changes in demand are ended the system returns to a normal level. For example, exercise increases heart rate (HR), but once exercise is over the HR returns to normal. The rate at which it returns to normal is a measure of physical fitness. If, for argument's sake, it was discovered that the HR was increased as a result of short-term exposure to, say, fine particles ($PM_{2.5}$) would we be justified in calling this a patho-physiological as opposed to a physiological response? That it would be an effect that reflected a deviation from the normal HR is true, but whether such a deviation has significant consequences is much less clear. This is a problem that is not unique to the effects of air pollution on cardiovascular (CV) morbidity; it remains to be solved.

We have also considered the problem of inter-species variations in response to air pollutants. Toxicological studies often involve the exposure of experimental animals to concentrations of

toxicants well above those to which humans are likely to be exposed. The purpose of such studies is to discover whether, at high concentrations, effects occur. Whether this means that effects would occur in people exposed to much lower concentrations is a question that has exercised toxicologists for many years. Toxicologists deal with this problem by assuming that, in the absence of evidence to the contrary, effects shown to occur in animal models might occur in humans. Regulatory toxicologists use data provided by animal experiments to establish acceptably safe levels of exposure in humans. Uncertainty factors are used to allow for possible inter-species and inter-subject variations in sensitivity to test compounds; application of such factors provides a margin of safety. In the air pollution field things are a little different: people are exposed to ambient concentrations of air pollutants and their responses can be studied. In this report we have reviewed a number of such studies. Having established that effects can and do occur at ambient concentrations, animal and volunteer studies can be undertaken to explore the mechanisms underlying such effects. Of course there is a residual difficulty: the mechanisms that control responses to low concentrations might not be identical with those that control effects at high concentrations. Our work has led us to be cautious about interpreting the effects of animal and human volunteer studies.

What, then, can we hope to learn from studies of mechanisms? In our view we can expect to define mechanisms that might explain effects reported in epidemiological studies. We cannot hope to prove that long-term exposure to ambient concentrations of air pollutants has effects on human health. Nonetheless, mechanistic studies can help provide evidence of biological plausibility of associations reported in epidemiological studies.

Together, the four reviews presented cover a range of biological mechanisms. Ideally, we would wish to develop a mode of action pathway that includes all the mechanisms that have been outlined in the individual sections. However, the complexity and interplay between these pathways makes this challenging. Mechanisms can be categorised at different 'levels' (Figure 6.1):

a. the initial response of the pulmonary system to pollutant exposure,
b. the 'signal' that transmits the pulmonary response to the cardiovascular system (CVS),
c. the change in CV function and
d. the combined response to these changes in CV function over time that leads to morbidity and mortality

While largely discussed only tangentially, all the reviews above include consideration of the second 'level' in this sequence: the 'signal' from lung to CVS. Three main hypotheses have been proposed for this step in the pathway:

a. the passage of inflammatory mediators from the lung into the blood leading to systemic inflammation which impairs CV function (Seaton et al. 1995)
b. activation of pulmonary sensory afferents leading to effects on the functioning of neural systems (particularly the autonomic nervous system), which alters the regulation of the cardiac function, and potentially affects the rest of the CVS through release of neuroendocrine mediators (Pope et al. 1999),

c translocation of pollutants into the circulation to directly alter vascular function (Oberdörster et al. 2002)

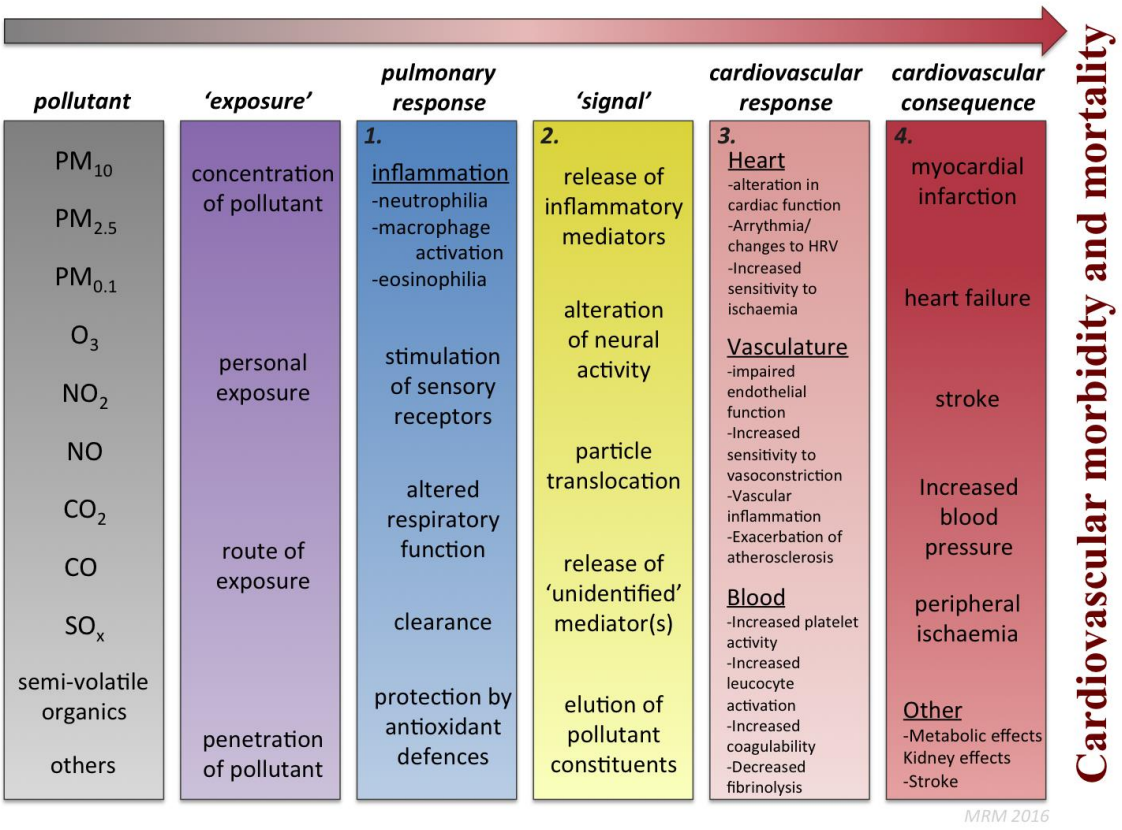

Figure 6.1: A sequential approach to the different 'levels' of mechanism through which air pollution could cause cardiovascular mortality

The evidence for and against these pathways is discussed elsewhere (Miller 2014). We acknowledge that the nature of the search processes we undertook led to focusing on specific pathways and to a lack of emphasis on others. In particular, the translocation pathway is not discussed in any depth in the present report because very few of the studies identified addressed this hypothesis directly. Studies that have been conducted have focussed on particulate air pollution. Part of the reason for this is that the reactivity of gaseous pollutants, such as ozone (O_3), prevent the direct translocation of these pollutants. For particulate air pollution, measuring the translocation of particles is challenging, requiring the use of model nanoparticles (for example, radiolabelled carbon nanoparticles, gold nanoparticles etc) rather than environmental particles *per se*, and thus were not targeted by our literature searches. It is worth noting, however, that the evidence for particle translocation has advanced in some respects, with greater understanding of some of the processes which influence translocation. However, these studies are largely confined to animal models (Buckley et al. 2017; Ganguly et al. 2017; Geiser and Kreyling 2010; Kreyling et al. 2014; Kreyling et al. 2017), although recent evidence has strengthened the case in man (Miller et al. 2017). Nitrative/nitrosative stress is another pathway that has not been discussed in detail (Peluffo and Radi 2007; Radi 2004; Sandhiya et al. 2014) but could, at least in theory, be influenced by air pollutants either directly by nitrogen dioxide (NO_2) (WHO 2013) or indirectly via the interplay with oxidative stress (OS) (Upmacis 2008).

Overall, though, the three pathways remain central to our understanding of the effects of exposure to air pollutants on the CV system, as they were at the time of the American Heart Association Scientific Statement (Brook et al. 2010). Recent evidence has not refuted the suggestion that these pathways are important. At least for particulate air pollution it has defined these pathways in greater detail. The role of gaseous pollutants remains understudied. However, this review focussed on the post-2009 literature. An additional point to note is that there are associations between infectious respiratory diseases and CVD events (Rae et al. 2016; Violi et al. 2014; Vlachopoulos et al. 2015) and thus there is clearly the potential for pulmonary inflammation to contribute to the CV effects of inhaled pollutants. However, due to inconsistencies in measuring biomarkers of inflammation in the blood, the extent to which this particular pathway drives these effects, as opposed to the other potential mechanisms, has yet to be established and is a topic for future research.

Three of the reviews (cardiac electrophysiology, vascular function, haemostasis) centre on the evidence for a given change in 'endpoints' relating to CV function (Box 3. on Figure 6.1). The review of OS and inflammation represents a broader range of mechanisms that could occur across all four levels of the sequential pathway. We highlight this distinction because it emphasises the care that needs to be taken when formulating a chain of events. The evidence for a role of pathways involving OS and inflammation in contributing to the CV effects of air pollution is well established: these pathways are reoccurring processes following exposure to most air pollutants of interest, and their induction is likely to exacerbate the disease process at whichever level it occurs (Miller et al. 2012). However, this does not mean that these pathways are the key drivers of the CV dysfunction induced by air pollution. An important uncertainty is whether the generation of OS occurs prior to the change in CV function, or arises from the patho-physiology of CVD itself. OS and inflammation are hallmarks of disease in general, but are not necessarily the means by which air pollution causes disease in the first place. Studies with pharmacological inhibitors or genetic knockouts of OS/inflammation have been used to address this issue. However, while many animal studies have suggested that inhibition of these pathways can prevent many of the CV effects of exposure, these have not been replicated in human studies (limited as these are), and ultimately this uncertainty still persists.

An independent observation of the authors of all of the reviews was the degree of interaction, and occasionally overlap, between pathways. For each major pathway identified, a number of other pathways arose to add complexity and further mechanistic questions, for example do these mechanisms run in parallel or series? Do they have the capacity to amplify the effects of others? Are they up-regulated or down-regulated when another becomes redundant? This presents a challenge when trying to ascertain the relative importance of one biological mechanism over another and it has not been possible, based on the evidence reviewed, to draw such conclusions for effects on CV morbidity as a whole. What is clear, though, is that no single mechanism is likely to account for the wide-ranging effects of air pollution on the CV system. At present, we do not feel that any one pathway is predominant over others. Instead, we think it likely that many mechanisms work in concert and alter CV function. These mechanisms, over time and as exposure is prolonged, contribute to CV morbidity.

Having set out our reflections we now turn to our conclusions.

6.2 Conclusions

a In each of the areas we reviewed we found clear evidence that exposure to air pollutants, primarily fine particulate matter ($PM_{2.5}$), affected a range of physiological and patho-physiological variables and effects. These included indices of inflammatory and oxidative status, reduced heart rate variability (HRV), arrhythmias, endothelial dysfunction, raised blood pressure, progression of atherosclerotic disease and promotion of thrombosis (blood clotting).

b Multiple biological mechanisms were identified for each of the themes described above. No single mechanism is predominant over others, and it is likely that many mechanisms work in concert to produce wide-ranging effects of air pollution throughout the CV system. OS and inflammation remain the main mechanisms investigated, however, a few novel mechanisms have been described.

c We find that the evidence supporting the assertion that exposure to fine particulate air pollution, namely $PM_{2.5}$ can have detrimental effects on the CV system has strengthened both in terms of volume of studies and range of effects since the review by Brook et al (2010). Overall, the studies we reviewed were carried out in a scientifically appropriate manner based on clearly defined hypotheses.

d There have been few studies examining the potential adverse effects of gaseous pollutants on CV morbidity. The studies published in this area have reported inconsistent findings and do not allow any firm conclusions to be reached

e The effects observed were not always consistent across studies. Comparison between studies was often difficult, for example due to differences in species, population, exposure scenario, duration of exposure, endpoints, age and co-morbidity.

f Some of the effects recorded were not large and many were demonstrated on exposure to concentrations well above those in ambient air.

g Studies using validated human exposure chamber techniques and exposures that are considered to be representative of 1-2 hours of exposure to roads with very heavy traffic, provide strong evidence that exposure to air pollution may affect CV health.

h The problems of extrapolating from short-term studies to the effects of long-term exposure, from animals to human, and from healthy subjects to those suffering from CV disease were discussed in detail.

i We note that studies of long-term exposure are few in number and essentially limited to epidemiological approaches to the study of mechanisms of effect. We see this as inevitable given the nature of the problem. Toxicological studies, and to a more limited extent epidemiological studies, with longer-term exposure (for example 6 months) of relevant doses are emerging, and the findings from these are expected to be valuable.

j Current data are too preliminary to allow meaningful conclusions about effect modifications by, for example, sex, age and co-morbidities, on the associations between air pollution and CV morbidity to be drawn. Investigations that

directly compare potentially susceptible populations with healthy counterparts in the same study are required.

k We have found nothing to persuade us that the CV health effect associations of particulate air pollution reported in relevant epidemiological studies are not causal in nature; indeed, that they are causal in nature seems, to us, likely

6.3 Research recommendations

In the course of this work, the Working Group identified several areas for future research. Examples of some of these research recommendations are outlined below. A more detailed discussion of these and others can be found in the individual chapters.

a. Need for more refined exposure estimates at urban, local and personal levels

b. Better understanding of factors that modify the effects of air pollution

c. More mechanistic studies of the effects of long-term exposure to air pollution

d. Most of the studies have focussed on particulate air pollution, more studies are need assessing the effects of ambient gaseous pollution on CVD

e. Better characterisation of the effects of multiple pollutants

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Appendix 1
Terms of reference of the CV Working Groups

The following terms of reference were proposed and accepted.

Mechanisms Working Group

a Based on current knowledge, to propose plausible mechanisms by which long-term exposure to air pollutants could influence cardiovascular (CV) outcomes. To include consideration of mechanisms affecting initiation, progression and outcome of CV diseases

b To examine the evidence that air pollutants act in these ways

c Based on the weight of mechanistic evidence, to comment on the likelihood of causality for selected CV morbidity endpoints

d To comment on any insights that the mechanistic evidence provides on cessation lag, transience, permanence, and health implications

e To identify gaps in knowledge and to recommend research to close these gaps

Quantification Working Group

a To calculate, using the risk coefficients and approaches defined by the epidemiology working group (as above), the effect of long-term exposure to air pollutants on public health in the UK as represented by effects of CV morbidity. The working group will consider how these effects might best be expressed:

- as burden of disease and
- in terms of the benefit likely to be delivered by reductions in long-term average concentrations of air pollutants.

b Describe and explain the methods used to make these calculations;

c Make transparent and understandable the assumptions that underlie the calculations, and to discuss their importance;

d Clarify the relationships between different ways of expressing morbidity impacts and to comment on the appropriateness of their use.

Appendix 2
Search strategies adopted by Members of the CV Mechanisms Working Group

As the work developed it became apparent that a formal systematic review of the literature was beyond the resources of the Mechanisms Working Group and a less demanding approach was adopted. The Committee agreed that the 2010 American Heart Association scientific statement on "Particulate matter air pollution and cardiovascular disease" (Brook et al. 2010) provided a good starting point for building understanding and to review the evidence published since. Preliminary examination of the literature and Members' reading and individual familiarity with the field suggested four possible mechanisms linking air pollution and cardiovascular disease (CVD) which might usefully be reviewed. Members of the Working Group and Secretariat were allocated to one of the four mechanisms. Although there was variation in the search methods used between Members, all undertook a systematic and thorough PubMed search of the literature using the 2010 American Heart Association scientific statement (Brook et al. 2010), an expert review of the literature between January 2004 to March 2009, as a foundation for reviewing the research in this area in the subsequent years. The approaches taken by Members are outlined below in A2.1 to A2.4.

A2.1 Professor Frank Kelly: The role of inflammation and oxidative stress in CVD outcomes following exposure to ambient air pollution

The studies selected for inclusion were identified by searching the PubMed database with the following search string: : oxidative stress OR oxidative potential AND air pollution OR particulate matter (PM) OR ozone (O3) OR carbon monoxide (CO) OR nitrogen dioxide (NO2) AND cardiovascular OR cardiovascular disease OR myocardial OR heart OR cardiac OR stroke OR heart rate OR arrhythmia OR heart rate variability OR autonomic OR sympathetic OR atherosclerosis OR vascular OR blood pressure OR hypertension OR diabetes OR metabolic OR thrombosis OR coagulation. The search was limited to studies published in the English language from January 2009 to October 2016. Studies investigating the effects of particulate air pollution encompass PM in urban air, concentrated ambient particles (CAPs), diesel exhaust (DE) and PM from DE, i.e. diesel exhaust particles (DEPs). Manufactured nanoparticles are not included in this review. The titles and abstracts of over 1300 identified original research and review articles were examined (including studies that were identified from the reference lists of relevant review articles from the search results). Those that were not relevant to the focus of this review were discarded, leaving 265 articles that were then appraised in detail. Of these, the studies that have been included in this review are limited to those deemed to have produced results of special interest, thereby adding to our understanding of how oxidative stress (OS) contributes towards cardiovascular disease induced by exposure to ambient air pollution.

A2.2 Professor Robert Maynard: Effects of exposure to air pollutants on cardiac electrophysiology

The main structure of the search strategy comprised 2 concepts: 1) ambient air pollution; 2) cardiac electrophysiological effects, combined by using the Boolean operator "AND". A list of search terms was compiled for each of the concepts. In total, 72 PubMed searches were run using variations of these search terms, covering the period between January 2009 and the end of 2015. Searches were restricted to English language articles only. After exclusion of duplicates, 495 articles underwent title and abstract screening, of which 299 underwent full-text review. Main reasons for exclusion were publication type (for example, a review article) and measure (for example, no relevant outcome measures). After the full-text review, 228 studies remained and these were categorised by subject area: epidemiological studies (108), volunteer studies (28) and animal (*in vivo*) studies (92). The epidemiological studies were further sub-divided into long-term exposures (13), short - term exposure of healthy subjects (47) and short - term exposures of subjects suffering from cardiovascular diseases (CVDs) (48). Notes were prepared for each of these papers and used to construct a summary of the evidence. That summary is given in this report.

A2.3 Dr Mark Miller: Effects of exposure to air pollutants on the vascular system

PubMed was searching using the following search terms: air pollution *OR* particulate matter *AND* vasculature *OR* blood vessel *OR* artery *OR* vein *OR* vasodilation *OR* vasodilatation *OR* atherosclerosis *OR* atheroma *OR* endothelium *OR* endothelial *OR* blood pressure. The search was limited to studies published in the English language from January 2009 to March 2016.

A parallel search using terms specifically related gaseous pollutant terms yielded a prohibitively large number of references, the vast majority unrelated to air pollution, and thus these search results were not considered further. However, an initial screening of the references from the main search found that relevant publications on gaseous pollutants alone were identified by the main search. Only pollutants that were key components of outdoor urban air pollution were included (vehicle exhausts, urban particulate matter (PM), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), ozone (O_3), black carbon (BC), carbon monoxide (CO). Indoor air pollution, wood smoke, occupational sources, manufactured nanoparticles and cigarette smoke were excluded.

3172 publications were identified. After removal of inappropriate references and duplication, 1136 publications remained. The abstracts of these articles were reviewed as follows. Methodological details of potentially relevant references were tabulated under the following headings:

a Study Type (for example, primary data, meta-analysis or review),

b Study Design (for example, epidemiology controlled exposure in man, in animal),

c Study Population (for example, healthy/patient),

d Exposure Assessment (for example, stationary monitoring, personal monitoring, inhalation),

e Pollutants Investigated,

f Exposure Duration and

g Health Endpoints

Potential themes of interest were also noted, including mechanistic pathways, novel mechanisms, constituents of pollution investigated, methods of intervention/prevention (for example, facemasks, pharmacological agents) and susceptible populations.

Studies looking at solely mortality or hospital admissions, without further mechanistic insight were excluded. Meta-analyses were accepted as 'original research findings', but few extended far beyond gross endpoints such as mortality. A large quantity of *in vitro* studies prohibited review in the present manuscript and were excluded in favour of whole organism investigations with more immediate relevance to the physiological and pathophysiological processes being addressed here.

Arbitrarily, 440 references were merited as worthy of reading the full text in detail; these were grouped into the following categories:

a Blood pressure - considering blood pressure responses to both acute and chronic exposure to air pollution.

b Miscellaneous vascular - largely relating to vessel contractility using endpoints other than blood pressure.

c Atherosclerosis - studies looking at vascular disease, usually considering long-term exposure to air pollutants and chronic disease.

Each category has been broken down by study type to optimize comparisons of similar study designs and endpoints. Epidemiological studies were further divided into large cohort studies and smaller panel studies using an arbitrary cut-off of 200 individuals, which broadly separated the studies by their degree of their mechanistic exploration.

A2.4 Dr Sarah Robertson: Effects of exposure to air pollutants on haemostasis

Literature searches were performed in PubMed from the dates of 1st January 2009 to 28th February 2016, using the following search terms: "air pollution" or "particulate matter" *and* "blood" or "thrombosis" or "clot" or "fibrinolysis" or "coagulation" or "embolism" or "platelet". Terms for gaseous were not included due to the large number of irrelevant references these terms produced. Preliminary checks were performed to ensure that relevant references with gaseous pollutants were captured by the term "air pollution". Other papers were identified from prior knowledge, contact with experts in the field and hand searching of the bibliographies of the papers identified in the electronic search. References were downloaded into the referencing software program Endnote (version X8).

A total of 2,326 publications were identified following removal of duplicates. These were screened first at the abstract level, and then at the full article level. To be included in the final analysis studies had to meet the following inclusion criteria:

a) Peer-reviewed articles or published by a recognised institution between 1st January 2009 and 28th February 2016*

b) Study type: epidemiological studies, human controlled exposures and intervention studies, *in vivo* (animal) studies
c) Exposure: ambient (outdoor) air pollution – particulate air pollutants**, diesel exhaust (DE), ozone (O_3), nitrogen dioxide (NO_2), carbon monoxide (CO), sulphur dioxide (SO_2)
d) Health outcome: reported on coagulation, fibrinolysis, thrombophilia and platelet profile
e) Population: general population (all ages, those with pre-existing health conditions)

* Key earlier publications are discussed for contextual background
** Included PM size fractions, black carbon (BC), concentrated ambient particles (CAPs) and PM from DE (i.e. diesel exhaust particles (DEP))

Key exclusion criteria included:

a) No original data included (e.g. reviews, editorials and commentaries were excluded). However, reference lists from the identified original articles and reviews were screened to identify any other potentially relevant studies.
b) Language: full text not available in the English Language
c) Study type: *in vitro*
d) Exposure not relevant to ambient air pollution (e.g. indoor air pollution, occupational exposure***, biomass, cigarette smoke, manufactured nanoparticles)
e) Did not provide any mechanistic data beyond mortality or hospital admission

*** Occupational exposure to manufactured nanoparticles, industrial accidents and environmental events (e.g. volcanic eruptions or wildfires) were not included. Workplace exposures that potentially spill into communities or were representatives of the main aspects of general urban air pollution (e.g. engine emissions from garages, bus depts.) were included.

This review provides a narrative summary of the seventy four publications that fulfilled all inclusion criteria. The piece has been structured by study type: epidemiological studies (62%) controlled exposure studies in man (15%) and *in vivo* animal studies (23%). Epidemiological studies were further subdivided into short-term (7 days of exposure or less) versus long-term (>1 week of exposure).

Appendix 3
Glossary of terms and abbreviations

Terms are defined here in the context of their use in this paper

Acute phase protein	Protein released into the blood by the liver as a response to inflammation or injury.
Acetylcholine (ACh)	A neurotransmitter
Afferent and efferent nerves	A neural reflex involves nerves running towards and away from integrating centres in the spinal cord or brain: the former are described as afferent, the latter as efferent.
Ambient air	Outdoor air
Angiotensin converting enzyme (ACE)	The enzyme that converts angiotensin into angiotensin II located mainly in the lining of blood vessels.
Apolipoprotein E-/- (ApoE-/-)	Apolipoprotein E is a type of lipoprotein (a protein connected to fat). Knockout mice that lack ApoE-/- develop hypercholesterolemia (presence of high levels of cholesterol in the blood) when fed a high-fat diet. Have become a standard model for studies of atherosclerosis (see atherosclerosis)
Atheromatous disease	Disease of arteries involving the accumulation of fatty material of porridge-like consistency in the inner layer of the artery wall resulting in narrowing of the artery. These fatty deposits are known as plaques.
Atheromatous plaques	The discrete lesions of the arterial wall in atheromatous disease.

Atherosclerosis	Synonymous with atheromatous disease
Autonomic nervous system (ANS)	The involuntary nervous system (sympathetic and parasympathetic) that controls a wide range of physiological functions including activity of the gut, the heart and that of many glands.
Black carbon (BC)	Form of particulate air pollution produced from incomplete combustion. Measured by techniques based on colour, for example, by reflectance.
Blood pressure (BP)	The pressure of the blood against the walls of the blood vessels as measured in large arteries. Blood pressure readings consist of two figures: systolic blood pressure (SBP; the blood pressure when the heart is contracting) and diastolic blood pressure (DBP; the time when the heart is in a period of relaxation and dilation (expansion)).
Bronchoalveolar lavage fluid (BALF)	A diagnostic procedure where fluid is introduced into the lungs and then recollected and then recollected for analysis.
Carbon monoxide (CO)	A poisonous gas produced by incomplete oxidation of fossil fuels.
Cardiovascular disease (CVD)	Disorders of the heart and circulatory system
Carotid arteries	Major blood vessels in the neck that supply blood to the brain, neck and face.
Case-cross over studies	An epidemiological technique involving comparing ambient conditions during a period when an individual suffered from some effect on health with a period when no such effect occurred.
Cell adhesion molecules (CAMs)	Proteins located on the cell surface, involved in binding with other cells or with the extracellular matrix (ECM; collection of extracellular molecules

	secreted by cells that provides structural support to the surrounding cells) in the process called cell adhesion.
Chronic obstructive pulmonary disease (COPD)	Group of lung conditions in which there is limited airflow in the lungs because airways have narrowed
CAPs	Abbreviation for concentrated ambient particles
Carotid intimal medial thickness (CIMT)	Measurement of the thickness of the tunica intima and tunica media, the innermost two layers of the wall of the carotid artery. It is a non-invasive measure that uses ultrasound to detect the presence and extent of carotid atherosclerotic vascular disease.
Catalase (CAT)	Catalyses the reaction by which hydrogen peroxide is decomposed to water and oxygen.
Conduit artery	Elastic artery; conducting blood from the heart to other arteries.
Coronary arteries	The arteries that supply blood to the heart itself. The right and left coronary arteries arise from the root of the aorta as it leaves the heart
Coronary artery calcium (CAC)	Non-invasive way of obtaining information about the presence, location and extent of calcified plaque in the coronary arteries.
Coronary thrombosis	Partial or complete obstruction of blood vessel supplying the heart by a blood clot or ruptured atheromatous plaque
C-reactive protein (CRP)	A protein produced by the liver and released in the blood in response to injury and physiological stress and which is regarded as a marker of stress
Cytokine	A name given to a large group of molecules that are important in controlling cellular activities in tissues. Some dozens of such factors are known. They play an important part

	in controlling the inflammatory reaction to tissue damage. The interleukins, IL1-IL-23 are examples of cytokines
DCFH-DA	Dichlorodihydrofluorescein diacetate: cell permeable, sensitive indicator of peroxynitrite formation
DE	Abbreviation for diesel exhaust
DEP	Abbreviation for diesel exhaust particles
Diabetes mellitus (DM)	Chronic condition that occurs when the body cannot produce sufficient insulin.
Electrocardiogram (ECG)	A recording from electrodes places on the chest and limbs, of electrical changes originating in the muscle of the heart.
Endothelial progenitor cells (EPC)	Circulating, bone marrow-derived cell population that have a role in the repair of endothelial surfaces and injury.
Endothelin (ET-1)	A potent endogenous vasoconstrictor
Endothelial dysfunction	A pathological state of the endothelium (the inner lining of blood vessels) and is defined as the impaired ability of the vessel to dilate when stimulated.
Epidemiological studies	Investigations of diseases conducted at population level
GEE	Abbreviation for gasoline engine exhaust
Glutathione peroxidase (GPx)	Is an anti-oxidant enzyme family. GPx functions in protecting the body against oxidative stress (see oxidative stress).
Glutathione transferase (GST)	Part a superfamily of ubiquitous, multifunctional enzymes that play a crucial role in cellular detoxification.
Glutathione S-transferase M1 (GSTM1)	Member of GST superfamily. Has been extensively studied in the

	context of a lung cancer risk factor.
Granulocyte-macrophage colony stimulating Factor (GM-CSF)	See cytokine
h	Abbreviation for hour
Heart rate (HR)	The number of heart beats per minute
Haemeoxygenase-1 (HO-1)	Enzyme that catalyses the degradation of haeme
Heart rate variability (HRV)	The variability in the beat-to-beat interval of the heart
HF (LF and VLF)	High frequency, low frequency and very low frequency components of the Fourier transform of the wave pattern of heart rate variability. Complex wave forms may be analysed into a number of components using Fourier analysis.
High density lipoprotein (HDL)	Combinations of fats (lipids) and proteins. HDL transports cholesterol from the tissues of the body to the liver, so the cholesterol can be eliminated in the bile.
HUVEC	Abbreviation for human umbilical vein endothelial cells
Insulin resistance (IR)	The diminished ability of cells to respond to the action of insulin in transporting glucose (sugar) from the bloodstream into muscle and other tissues.
Interleukin-6 (IL-6)	See cytokine
Intratracheal (IT) instillation	Introduction of a substance directly into the trachea
Ischaemic heart disease (IHD)	Heart disease due to a reduction in blood supply to the myocardium (the muscle of the heart wall) caused by a reduction in blood flow through the coronary arteries.
kg	Abbreviation for kilogram
Longitudinal studies	Epidemiological techniques that involve following populations or individuals over a period of time.

Low-density lipoprotein (LDL)	Molecule that is a combination of lipid (fat) and protein. The main function is to transport cholesterol from the liver to the tissues of the body. Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) is known to be a key molecule in the pathogenesis of atherosclerosis.
Mean arterial pressure (MAP)	The average blood pressure in a cardiac cycle.
Meta-analytical techniques	Statistical techniques that allow the results of studies (commonly epidemiological) to be combined
Metalloproteinases	A large group of enzymes that play an important role in controlling the amount of connective tissues in the body.
Micrometre or micron (μm)	A unit of length. 1 μm = 1 thousandth of a millimetre or 10^{-6} metres.
Microvascular function (MVF)	Is the functional properties of the microvasculature. Is an important predictor of more widespread endothelial dysfunction.
mg	Abbreviation for milligrams
Millimetres of mercury (mmHg)	Units used to measure blood pressure
Mitogen activated kinases (MAKs)	Group of related kinases with a wide variety of cellular responses. Comprise the extracellular signal-regulated kinases1/2 (ERK1/2) and c-Jun NH_2-terminal kinases
Myocardial infarction	Death of part of the muscular wall of the heart caused by impairment of its blood supply.
Myocardium	The muscular tissue comprising the great majority of the mass of the heart
NAC	*N*-acetylcysteine: known to have specific anti-inflammatory and anti-oxidant roles.
NADPH	Nicotinamide adenine dinucleotide phosphate, used as a reducing agent in

	anabolic reactions.
Nanometre (nm)	A unit of length. One nanometre = 1 millionth of a millimetre or 10^{-9} metres
Nitric oxides (NOx)	A mixture of gases that are composed of nitrogen and oxygen. NOx in ambient air consist primarily of nitric oxide (NO) and nitrogen dioxide (NO_2)
Nitric oxide synthase (NOS)	Family of enzymes catalysing the production of nitric oxide from L-arginine.
Nitrogen dioxide (NO_2)	A gas produced during combustion by the oxidation of atmospheric nitrogen
Nitroglycerin-mediated dilation (NMD)	Method used to induce vasodilation of arteries.
Oxidative potential (OP)	The capacity of particles to cause damaging oxidative reactions
Oxidative stress (OS)	Reflects an imbalance between reactive oxygen species and anti-oxidant defences.
Ozone (O_3)	An oxidant gas. Ozone is produced by the photochemical breakdown of nitrogen dioxide to nitric oxide and an activated oxygen atom that reacts with oxygen to form ozone.
Panel studies	A study of a generally small and closely monitored group of individuals.
Particulate matter (PM)	A minute portion of matter – frequently a very small solid or liquid particle (or droplet) of micrometre or nanometre dimensions.
PM_{10}, $PM_{2.5}$	The concentrations (expressed in $\mu g/m^3$) of particles of generally less than 10 μm and 2.5 μm in the ambient air.
ppm	Abbreviation for parts per million
Primary air pollutants	Pollutants emitted directly into the air from sources. They can have effects both directly and as precursors of

	secondary air pollutants (chemical formed through reactions in the atmosphere).
Pulse pressure (PP)	Difference between the systolic and diastolic blood pressure readings.
Reactive hyperaemia index (RHI)	Used as a measure of endothelial function
Reactive oxygen species	Chemically reactive chemical species containing oxygen. Examples include, superoxide (O_2^{*-}) and hydroxyl radical (OH^{*-}).
SDNN	Standard deviation of the N-N (normal-normal) interval. Used to assess heart rate variability.
Soluble intercellular cell adhesion molecule sICAM	See cell adhesion molecule
Soluble vascular cell adhesion molecule sVCAM	See cell adhesion molecule
Sulphur dioxide (SO_2)	An acidic gas formed by the oxidation of sulphur found in fossil fuel.
Superoxide anion radical ($O2^{*-}$)	See free radical
Superoxide dismutase (SOD)	Metal-containing antioxidant enzyme that reduces potentially harmful free radicals of oxygen to oxygen and hydrogen peroxide.
Randomised control trial	An experimental design in which treatments (or exposures in the case of toxicology) are allocated to a subject on a random basis. Commonly, a number of subjects are randomly allocated to a small number of treatment groups.
Resistance artery	Small diameter blood vessel that contributes to the creation of the resistance to and regulation of blood flow.
RMSDD	Time domain tool used to assess heart rate variability. It is the mean of the sum of the squares of differences between adjacent N-N intervals.
Total anti-oxidant capacity (TAC)	Analyte frequently used to assess the

	anti-oxidant status.
Time-series study	An epidemiological method that focusses on the relationship between outcome (e.g. number of deaths or hospital admissions in a population) and explanatory variable (e.g. pollutant concentrations) using measures of these variables at regular time intervals, for example days..
Tumour necrosis factor-alpha (TNF-α)	See cytokine
Ultrafine particles	Particles of less than 100 nm diameter
Vascular dysfunction	A disorder of the vascular system of the blood vessels.
Ventricle	The right and left ventricles of the heart pump blood into the pulmonary artery and aorta respectively.
Ventricular fibrillation (VF)	Abnormal, rapid and uncontrolled ventricular electrical activity preventing the normal pumping of blood by the ventricle.
Ventricular tachycardia (VT)	Abnormal fast heart rate driven from a ventricular focus.

Appendix 4
Membership lists

Membership of the Committee on the Medical Effects of Air Pollutants

Chair

Professor Frank Kelly BSc PhD FRSB FKC

Members

Professor H Ross Anderson MD MSc FFPHM FRCP FMedSci (*until August 2016*)
Dr Richard Atkinson BSc MSc PhD PG Cert HE
Professor Alan R Boobis OBE PhD CBiol FSB FBTS
Dr Nicola Carslaw BSc MSc PhD
Ms Ruth Chambers MA MSc
Dr Beth Conlan BSc MSc and PhD
Professor Jonathan Grigg BSc MBBS MRCP MD FRCPCH
Professor Roy Harrison OBE PhD DSc CChem FRSC FRMetS HonFFOM HonMFPH FRS (*co-opted*)
Dr Mike Holland BSc PhD
Mr J Fintan Hurley MA
Professor Debbie Jarvis MBBS MRCP MD FFPH
Dr Jeremy Langrish BA MA MB BCh MRCP PhD (*until 2015*)
Professor Robert L Maynard CBE FRCP FRCPath FFOM (*co-opted*)
Dr Mark Miller BSc, PhD
Dr Brian G Miller BSc PhD CStat (*until May 2017*)
Professor Gavin Shaddick BSc MSc PhD (*co-opted*)
Mr John Stedman BA
Dr Heather Walton BSc DPhil
Professor Paul Wilkinson BA BM BCh MSc MFPHM FRCP

Secretariat

Dr Sotiris Vardoulakis BSc MSc PhD (Scientific) (*until May 2017*)
Ms Alison Gowers BSc MSc (Scientific)
Miss Inga Mills BSc MSc (Scientific) (*until June 2016*)
Dr Karen Exley (Scientific) BSc MSc PhD
Dr Sani Dimitroulopoulou BSc PhD (Scientific) (*until May 2017*)
Dr Sarah Robertson BSc MSc PhD(Scientific)
Dr Christina Mitsakou BSc MSc PhD(Scientific) *(from May 2017)*
Mrs Isabella Myers BSc MSc (Scientific) (*until June 2015*)

Member ship of the Committee on the Medical Effects of Air Pollutants CV Mechanisms Working Group

Chair	Professor Robert L Maynard CBE FRCP FRCPath FFOM
Members	Professor Frank Kelly BSc PhD FRSB FKC
	Dr Mark Miller BSc PhD
	Professor Alan R Boobis OBE PhD CBiol FSB FBTS
	Dr Jeremy Langrish BA MA MB BCh MRCP PhD (*until 2015*)
Secretariat	Dr Sarah Robertson BSc MSc PhD
	Ms Alison Gowers BSc MSc