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Biomarkers: a tool for reinforcing causal reasoning in the relationship of ambient air pollution and lung cancer

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Abstract

Prospective studies support a positive association between ambient air pollution and lung cancer but causality remains uncertain. We evaluated the contribution of intermediate biomarkers in epidemiological studies to ascertain whether it reinforces causal reasoning. We identified papers which evaluated the effects of ambient air pollution using biological markers of dose and effect. The evidence for each marker was evaluated using the Venice criteria which rate a group of studies from A (strong) to C (weak) on amount of evidence, replication of findings, and protection from bias. Biomarkers that scored A or B for all three criteria are included here. The markers that fulfilled these criteria are: 1-Hydroxypyrene, DNA adducts, chromosomal aberrations, micronuclei, oxidative damage to nucleobases, and methylation changes. These biomarkers cover the whole spectrum of progression from external exposure to tumor formation and some have also been suggested to be predictive for risk of future cancer, reinforcing causal reasoning.

Table 1 (below) summarizes recent prospective studies investigating the relationship between PM2.5 and lung cancer.

Lung cancer incidence studies, 2001-2011							
Study, country	**Author, Year (exposure period)**	**Follow-up period**	**Mean level of exposure**	**Sex**	**Age bands**	**Confounders**	**RR (95% C.I.)**
The NLCS-AIR Study, Netherlands	Brunekreef, 2009 (1987-1996)	1986-1997	28.3 +/-2.1(sd) µg/m3*	Both	55 to 69 in 1986	Age, sex, smoking, area level SES	0.81 (0.63-1.04)
*PM2.5 levels estimated from PM10 values							
Lung cancer mortality studies, 2001-2011							
Study, country	**Author, Year (exposure period)**	**Follow up period**	**Mean level of exposure**	**Sex**	**Age bands**	**Confounders**	**RR (95% C.I.)**
Cancer prevention study II, USA	Turner, 2011 (1979-1983; 1999–2000)	1982-2008	17.6 (3.7) µg/m3**	Both	>30 years	Age, sex, ethnicity, SES, smoking, BMI, Marital status, ETS, vegetable/fruit/fiber consumption, fat consumption, industrial exposures, occupation dirtiness index, and mean county-level residential radon concentrations	1.19 (0.97-1.47)
Three-prefecture Cohort Study, Japan	Katanoda, 2011 (1974-1983)	1993-1995 (10 years post recruitment)	16.8-41.9 µg/m3$	Both	>40 years	Age, sex, occupation, smoking, Family & childhood ETS, CHIL daily green and yellow vegetable consumption (yes/no), daily fruit consumption (yes/no), and indoor charcoal or briquette braziers used for heating (yes/no), daily consumption of vegetables other than green and yellow vegetables (yes/no), health insurance (4 categories), occupational exposures	1.23 (1.09-1.38)
The NLCS-AIR Study, Netherlands	Brunekreef, 2009 (1986-1996)	1987 -1996	28.3 +/-2.1(sd) µg/m3^	Both	55 to 69 in 1986	Age, sex, smoking, area level SES	1.06 (0.82-1.38)
Norway	Naess, 2006 (1992-1995)	1992-1998	15 µg/m3	Both (but analysed separately)	51-90	Age, education, occupation	1.07# (0.97-1.18) 51-70 men 1.07# (0.97-1.18) 71-90 men 1.27# (1.13-1.43) 51-70 women 1.16# (1.02-1.32) 71-90 women
**calculated as average of the periods of exposure assessment 1979-1982 and 1983-2000; $PM2.5 estimated from total solid particulate matter value ^PM2.5 levels estimated from PM10 values #OR calculated for quartile increases in PM2.5							

Table 1: Summary of prospective studies investigating the relationship between PM2.5 and lung cancer incidence and mortality, published between 2001 and 2011.

Systematic Review
Title:
Biomarkers of ambient air pollution and lung cancer: a systematic review

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ABSTRACT

Background

The association between ambient air pollution exposure and lung cancer risk has been investigated in prospective studies and the results are generally consistent, indicating that long-term exposure to air pollution may cause lung cancer. Despite the prospective nature and consistent findings of these studies, causality assessment can benefit from biomarker research.

Objectives

In the present systematic review we assess the contribution of intermediate biomarkers in epidemiological studies, to ascertain whether their measurement reinforces causal reasoning.

Methods

We have reviewed 524 papers which described the relationships between ambient air pollution and biological markers of dose and early response. The evidence for each marker was evaluated using assessment criteria which rate a group of studies from A (strong) to C (weak) on amount of evidence, replication of findings, and protection from bias. Biomarkers that scored A or B for all three criteria are included here.

Results

The markers that fulfilled the inclusion criteria are: 1-Hydroxypyrene, DNA adducts, chromosomal aberrations, micronuclei, oxidized nucleobases to nucleobases, and methylation changes. These biomarkers cover the whole spectrum of disease onset and progression from external exposure to tumor formation and some have also been suggested as risk predictors of future cancer, reinforcing causal reasoning. However, methodological issues such as confounding, publication bias and use of surrogate tissues instead of target tissues in studies on these markers are of concern.

Conclusions

The identified biological markers have potential to shed light on the pathways of carcinogenesis, thus making the association more stringent for public health interventions.

AIR POLLUTION AND LUNG CANCER: STRENGTH OF EVIDENCE

The association between exposure to ambient air pollution and the risk of lung cancer has been evaluated in a number of prospective studies, which are summarized in Web only material Table 1. The evidence linking exposure to urban air pollutants, mainly particulate matter ($PM_{2.5}$ or PM_{10}), and lung cancer is generally consistent albeit formal statistical significance was often not always reached. Cohorts from the United States as well as from Europe have found increased risks of lung cancer with higher exposure to PM and other substances present in polluted air, with statistically significant Risk Ratios (RRs) ranging from 1·14 to 5·21 (Table 1 – Web only material for references).

The main strength of the studies above resides in their prospective nature, with exposure being assessed long before disease ascertainment. However, causality is still uncertain, as a recent document by the Health Effects Institute has stressed.[1] In the present systematic review we evaluate the contribution of biological markers of internal dose, biologically effective dose, and early effect in epidemiological studies on air pollution, to ascertain whether such contribution reinforces causal reasoning.

Biological markers of dose and effect can improve the investigation of health effects of various exposures, including air pollution, not only through the improvement in exposure assessment, but also because they increase the understanding of mechanisms and thereby provide biological plausibility; they may also allow the investigation of individual susceptibility.[2]

This review aims to identify biological markers of dose and effect for which there is consistent evidence in the literature, to support the results of epidemiological studies on the effects of ambient air pollution. Epidemiological evidence from the selected studies has been assessed using a set of criteria that have been developed elsewhere.[3] These account for (i) the total number of subjects investigated, (ii) the degree of replication of findings across

studies, and (iii) the potential protection from bias and/or confounding. PRISMA guidelines were also used to structure the analyses and to report the results.[4]

METHODS

Search Strategy, Selection Criteria

Online databases PUBMED and OvidSP were searched to identify papers that evaluated the effects of ambient air pollution using biological markers until January 2012. This encompasses studies on subjects that have been exposed to environmental air pollution at their residence or at work, including traffic related air pollution. As illustrated in Figure 1(Web only material),[5] corresponding search terms included “ambient” and “traffic-related air pollution”, “particulate matter”, “polycyclic aromatic hydrocarbons” (PAHs), “benzene”, “NO_x”, and “SO_x”. References within each paper found from that initial search were also investigated and relevant papers identified. The resulting papers evaluated exposure using a variety of methods: personal air sampling, ambient pollution data from monitoring sites close to the place of residence or workplace, or traffic density in the place of residence. Only papers published in English were reviewed. The final reference list was generated on the basis of relevance to the broad scope of this review, excluding papers without relevant exposure or outcome, studies on animals or in vitro studies, as well as perspectives and opinion reviews.

Papers were categorized according to the type of biological marker under investigation. As illustrated in Figure 1,[6] biomarkers can in principle represent each step in a causal pathway from exposure to disease. They are usually grouped as biomarkers i) of internal dose, ii) biologically effective dose, indicating how much of the exposure has damaged the molecules in the body and has possibly been removed by metabolic or repair mechanisms, iii) biological effects indicating changes in function or permanent alterations, iv) disease, and v) susceptibility, which can modify transition rates at each step. Based on the figure, the biomarkers in this review were defined as: biological markers (i) of internal dose, which included 1-hydroxypyrene (1-OHP), (ii) of effective dose, which included DNA adducts and oxidized nucleobases, (iii) of early effect, which included chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), micronuclei (MN), as well as mutations in the

Hypoxanthine phosphoribosyltransferase (HPRT) gene and changes in methylation patterns. As justified in the discussion, we have not examined markers of genetic susceptibility related to gene variants or markers of inflammation. Figure 1 also shows the location of each of these biological markers in the pathway to disease. The response and step transition time can vary at each step with half-lives counted in hours for e.g. 1-OHP, oxidized nucleobases, and gene expression, whereas bulky adducts show half-lives of weeks and for CAs and MN the half-life can be years. In lung cancer pathogenesis, damage to DNA in the form of bulky adducts and base oxidation from biotransformed PAHs and oxidative stress, as well as inflammation, are considered central with resulting chromosome damage and mutations. These changes, together with altered gene regulation, can lead to loss of cell cycle control and genomic instability.[2]

Evaluation Criteria

For each biological measure, epidemiological evidence from the corresponding papers was assessed by generalizing the Venice criteria, which were initially developed in the context of genetic association studies.[3] These criteria are based on a scoring strategy according to three characteristics: (i) amount of evidence (sample size), (ii) results replication, and (iii) protection from potential bias and/or confounding. As detailed in Table 1, biological markers of dose and effect with a large amount of evidence (total sample size >1000) were scored with A for amount of evidence.[3] Similarly, markers extensively replicated among studies scored A for replication, provided that at least one well conducted meta-analysis with limited between study heterogeneity was available. However, meta-analyses being rare in this field, some markers were scored A only on the basis of clear result replication (i.e. unambiguous agreement in showing or not showing a significant association). Biological markers were finally scored A for protection from bias if potential bias could affect the magnitude but not the presence of the association, with B if there was no obvious bias that could affect the presence of the association but there was considerable missing information concerning

possible bias, and with C if the studies demonstrated potential for bias that could affect the presence or absence of the association. Confounding and publication bias are two important limitations of the studies we assessed, to which we have devoted a specific section. In particular, we have assessed publication bias separately at the end of the results section. The analytical methodology as well as reporting were based on the PRISMA guidelines.

Table 1 – The grading criteria for the evaluation of cumulative evidence on the relationship between air pollution and biomarkers

Criteria	Categories	Proposed operationalization
Amount of evidence	A: Large-scale evidence B: Moderate amount of evidence C: Little evidence	Thresholds may be defined based on sample size, power or false-discovery rate considerations. As a simple rule, we suggest that category A requires a sample size over 1000 (total number in cases and controls assuming 1:1 ratio) evaluated in the least common genetic group of interest; B corresponds to a sample size of 100–1000 evaluated in this group, and C corresponds to a sample size <100 evaluated in this group
Replication	A: Extensive replication including at least one well-conducted meta-analysis with little between-study inconsistency B: Well-conducted meta-analysis with some methodological limitations or moderate between-study inconsistency C: No association; no independent replication; failed replication; scattered studies; flawed meta-analysis or large inconsistency	Between-study inconsistency entails statistical considerations (e.g. defined by metrics such as I^2, where values of 50% and above are considered large and values of 25–50% are considered moderate inconsistency) and also epidemiological considerations for the similarity/standardization or at least harmonization of phenotyping, genotyping and analytical models across studies.
Protection from bias	A: Bias, if at all present, could affect the magnitude but probably not the presence of the association B: No obvious bias that may affect the presence of the association but there is considerable missing information on the generation of evidence C: Considerable potential for or demonstrable bias that can affect even the presence or absence of the association	A prerequisite for A is that the bias due to phenotype measurement, genotype measurement, confounding (population stratification) and selective reporting (for meta-analyses) can be appraised as not being high plus there is no other demonstrable bias in any other aspect of the design, analysis or accumulation of the evidence that could invalidate the presence of the proposed association. In category B, although no strong biases are visible, there is no such assurance that major sources of bias have been minimized or accounted for because information is missing on how phenotyping, genotyping and confounding have been handled. Given that occult bias can never be ruled out completely, note that even in category A, we use the qualifier 'probably'.

Adapted from Ioannides J et al. Int. J. Epidemiol. (2008) 37 (1): 120-132. (21)

It is recognized that the studies are heterogeneous within the specific exposure circumstances that they evaluate, and this may contrast with the application of a single score for the assessment of causality. However, the general exposure studied in this review is ambient air pollution and all the reviewed studies can be grouped under this broad category.

In the present review, only biological markers of internal dose, biologically effective dose, and early effect that scored A or B for all three criteria are included: 1-OHP, DNA adducts, CAs, MN, oxidized nucleobases, and methylation changes. SCE and HPRT mutations were not considered further as they failed to score A or B for the three criteria. Data from relevant studies were extracted and are summarized in Tables 1-10 (in Web only Material). Quantitative meta-analyses were not performed, owing to the large heterogeneity between the included studies. However, for clarity, the results for DNA adducts, oxidized nucleobases, and CAs were summarized as standard mean differences (SMD) in forest plots.

We have focused on genotoxic and epigenotoxic effects as markers of biologically effective dose and biological effect directly related to carcinogenesis. Although chronic inflammation is considered relevant to particle-induced lung carcinogenesis,[7] at least one mechanism of action is thought to involve oxidative stress-induced DNA damage, which is addressed here.[8] While exposure to air pollutants has been associated with acute inflammation in the airways and to elevated levels of systemic markers of inflammation, such as C-reactive protein and fibrinogen, this has so far mainly been associated with the risk of cardiovascular diseases.[9]

RESULTS

Biological Markers of Exposure and Internal Dose: 1-Hydroxypyrene (1-OHP)

1-OHP is a useful marker for occupational exposure and it has also become the biomarker most commonly used to assess exposure to traffic-related air pollution and particularly to PAHs. It is a urinary excreted metabolite of pyrene and can be measured as a marker of systemic absorption of PAHs.[10,11]

Based on our inclusion criteria, eight papers and one review studied the association between exposure to air pollution or chemicals in polluted air and the levels of 1-OHP excretion in the urine of exposed individuals. Tables 2 and 3 (Web only Material) summarize the associations reported in these studies. Some of the studies suggested positive associations in adults, e.g. mail carriers and bus drivers,[12–14] and other studies showed higher 1-OHP levels in exposed children.[15–19]

Confounding

Among the studies on 1-OHP, five adequately adjusted for confounders including smoking data, where relevant.[13,15–17,19] One of the studies only adjusted for smoking,[14] one did not mention confounding, [[18]] and one was a review.[12]

Grading: Evidence - A, Replication - A, Bias - B

Though the overall number of subjects is large (n=1708) and findings have been replicated several times, it is not completely clear whether confounding from smoking, occupational exposures, or environmental tobacco smoke can be ruled out; this justifies a B for the third grading criterion.

Biological Markers of Exposure and Effective Dose: DNA Adducts

DNA adducts are formed when carcinogens, or metabolites of carcinogens, react with sites in DNA, resulting in the formation of a covalent bond between the carcinogen and DNA. Even though adducts can be removed by repair proteins, some can persist. This can result in nucleotide substitutions, deletions, and chromosome rearrangements during replication, contributing to cancer development.[20] Numerous studies have considered DNA adducts as a biomarker of exposure to genotoxic carcinogens. The studies reported here (N=25) are cross-sectional and case control studies, some of which were nested in prospective cohorts. Some studies carried out correlation and regression analyses on all subjects (Table 4- Web only Material) while others compared the mean DNA adduct levels in individuals with estimated high or low external exposures (Table 5 - Web only Material). As illustrated in figure 2, most studies (including two reviews) suggested positive associations between exposure to air pollution or chemicals in polluted air and the formation of DNA adducts in exposed individuals. Subjects in these studies included, among others, policemen in Bangkok,[14] schoolchildren in Thailand,[17] policemen in Genova,[21] and in Prague,[22] residents in an industrial area and rural controls in Poland,[23] bus and taxi drivers in Stockholm,[24] bus drivers in Copenhagen,[25] students in Denmark and in Greece,[26] as well as street vendors, taxi drivers, gasoline salesmen and road side residents in Benin[27]. Fetal exposures and DNA adducts in newborns also showed positive associations.[28–30] Only two studies reported no association.[31,32]

Confounding

Fourteen studies investigating DNA adducts adjusted for a number of confounders, seven of which adjusted for PAHs in diet. One study adjusted only for smoking, and one only for various risk alleles. For six studies there is no information on confounding. Two publications were reviews and for one study confounding was not mentioned by the authors as the measurements were from the same subjects before and after a change in working

conditions, within a 3 month interval, during which exposure to potential confounder(s) can be assumed as constant (Table 6a - Web only material).

Grading: Evidence - A, Replication - A, Bias - B

The association between ambient air pollution and DNA adducts has been shown in a large number of subjects (n = 3075) and replicated. Confounding is unlikely in the studies that included only never and ex-smokers such as Peluso et al (2005). However, publication bias cannot be entirely excluded (see below, publication bias section), and a major determinant of DNA adducts is diet[33] (which was not ascertained in most studies), so we rate B for the third grading criterion. It is important to mention that the levels of DNA adducts in white blood cells (WBC) have been shown to predict the risk of lung cancer in cohort studies and recently in a prospective pooled analysis.[34]

Biological Markers of Exposure and Effective Dose: Oxidized nucleobases

Concerning oxidized nucleobases to nucleobases, there is more data available (N=34 publications) as this is one of the most plausible mechanisms by which air pollutants may affect lung pathophysiology (Figure 2 – Web only Material). Oxidized nucleobases refers to modified purine and pyrimidine bases formed when reactive oxygen species (ROS) react with DNA or the nucleotide pool. Substances such as PM can generate ROS directly or through enzymatic reactions in target or inflammatory cells;[35] ozone and NO_2 are themselves reactive species, and benzene metabolism can also generate ROS.[10] Oxidatively modified DNA bases have potential to damage the integrity of the genome. For example, 8-oxo-7,8-dihydroguanine (8-oxoGua), one of the most critical lesions, leads to GC to TA transversion unless repaired prior to DNA replication. Cell levels of 8-oxoGua are usually measured as its 2'-deoxyribonucleoside equivalent 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). The most relevant repair in terms of base excision results in 8-oxoGua, which, however, is difficult to measure in urine, whereas 8-oxodG resulting from

other putative repair pathways and nucleotide pool sanitization can be readily measured. Lastly, formamidopyrimidine DNA glycosylase (FPG) sensitive sites in mononuclear blood cells (MNBC) are a marker of oxidative damage. FPG is a base excision repair enzyme which recognizes and removes oxidized purines, including 8-oxoGua.

Effects of air pollution on oxidized nucleobases have been studied in controlled exposure scenarios, in panel and in cross-sectional studies(Tables 6 and 7 – Web only Material). The results of studies comparing mean levels of markers of guanine oxidation (8-oxodG or 8-oxoGua) are summarized in a forest plot (Figure 3) which illustrates that for the majority of studies, biomarker levels are higher in exposed subjects compared to controls (positive SMD).

The effect of controlled exposure to air pollution (mainly traffic generated ultrafine particles with diameter less than 100 nm), has been investigated in healthy humans showing usually a higher level of FPG sensitive sites in MNBC in the exposed subjects than in the unexposed.[36–38] In addition increased urinary excretion of 8-oxoGua was observed in studies where subjects were exposed to exhaust in traffic-intense areas.[39,40]

A number of panel studies have also investigated effects of air pollution in the general population showing contradictory effects of air pollution on oxidized nucleobases.[31,41,42]

The cross-sectional studies investigating the effect of air pollution on oxidized nucleobases can be grouped into two main categories, according to their design. A first group of studies investigated the effect of air pollution among subjects with different occupational exposures. We refer here only to investigations in which the occupational exposure was qualitatively similar to the exposure of the general population (e.g. we excluded categories with special exposures such as gasoline workers). Using job titles as the basis for stratification of

exposure, subjects characterized by having jobs with high exposure to traffic emissions showed increased level of oxidized nucleobases. A second group of residential studies generally showed positive associations between living and/or working in highly polluted areas and oxidized nucleobases. Studies using benzene as a marker of urban air pollution exposure also showed associations with markers of oxidized nucleobases.[27,43–46]

A formal meta analysis of the effects of air pollution on DNA base oxidation (measured in MNBC) and excretion of repair products in urine, as well as an integrated analysis incorporating the endpoints of oxidatively damaged nucleobases in cultured cells, experimental animal models and humans, has been carried out.[47,48] Despite large heterogeneity between studies, the analysis showed highly significant effects with a standardized mean difference between exposed and unexposed subjects of 0.53 in blood (95% CI: 0·29-0·76) and 0.52 in urine (0·22-0·82). Based on the studies included in the current review, we have replicated these findings (results not shown).

Confounding

Among publications on oxidized nucleobases (N=34), 23 adjusted for a number of confounders including smoking. Five studies adjusted only for metabolic genes and four studies were cross over studies in short time frames, and confounding was therefore not relevant. For two studies there is no mention of confounders(Table 7a - Web only material).

Grading: Evidence - A, Replication - A, Bias - A/B

Altogether, there is consistent and strong evidence that exposure to ambient air pollution leads to increased levels of biomarkers of oxidation damage to nucleobases, both in observational and experimental studies. High urinary excretion of 8-oxodG or 8-oxoGua has been associated with increased risk of lung cancer in one prospective and several case-control studies.[6]

Biological Markers of Early Effect: Chromosome aberrations (CAs)

CAs are defined as modification of the normal chromosome complement due to deletion, duplication, or rearrangement of genetic material.

The studies on CAs (N=10) (Table 8 – Web only Material) are not all supportive of a positive association with exposure to air pollution, or its constituents in adults. As illustrated in Figure 4, some studies found a higher frequency of CAs with exposure to heavy air pollution,[49–55] others did not find statistically significant associations,[56,57] and others produced contradictory results.[58]

Confounding

Only 6 of the studies investigating chromosomal aberrations have adequately adjusted for confounders such as age, sex and smoking habits.[49–52,54,57] Three did not adequately adjust as they controlled only for age or only for sex.[53,55,58] One study did not mention adjustment for any confounders.[56]

Grading: Evidence - A, Replication - B, Bias - B/C

Even though not all studies agree, there is some evidence to support the association between exposure to air pollution and chromosome aberration frequencies. Confounding and publication bias cannot be ruled out.

Biological Markers of Early Effect: Micronuclei (MN)

MN are nuclei, separate from and additional to the main nucleus of a cell. During cell division, DNA replicates and divides equally between the two daughter cells. If the process is

disrupted, or the chromosomes are broken or damaged by chemicals or radiation, then the distribution of genetic material between the two daughter nuclei may be affected and pieces or entire chromosomes may fail to be included in either of the two daughter nuclei. The genetic material that is not incorporated into a new nucleus may form its own "micronucleus".[59] Thus, MN are a marker of chromosomal damage.

Four recent studies [21,55,60,61] and a review [62] have looked at the association between ambient air pollution or its constituents, and MN in the cells of exposed individuals (Table 9 – Web only Material) finding positive associations.

Confounding

There is one review and four studies on MN, two of which have adjusted for smoking and gender,[60,61] and one included some polymorphisms.[61] Two studies only adjusted for sex.[21,55] The study on newborns also adjusted for a number of relevant confounders.[60]

Grading: Evidence - A, Replication - B, Bias - B/C

Given the replication of results between the studies, there is some evidence to support the association between exposure to air pollution and MN. However, confounding and publication bias cannot be entirely ruled out.

Biological Markers of Early Effect: Methylation patterns

DNA methylation refers to the addition of methyl groups to nucleotides. The genome has a well-established pattern of methylation. Increase or decrease of the methylated sites in DNA affect gene expression and can also lead to genomic instability. The degree of methylation is passed on to daughter strands at mitosis by maintenance DNA methylases. DNA methylation and the associated repressed or activated transcription of genes have been implicated in carcinogenesis.[63] Five reports (from four studies) have recently investigated

the effects of air pollution exposure on methylation patterns[30,64–67] mostly focusing on Long Interspersed Element-1 (LINE-1) and Alu elements methylation as measures of whole genome methylation (Table 10 – Web only Material). LINE-1 and Alu elements are retrotransposons, repetitive and mobile sequences in the genome. LINEs make up a large proportion of the genome and LINE-1 as well as Alu methylation correlates with overall level of DNA methylation in the cell. LINE-1 methylation was frequently found to be altered by exposure to air pollution,[64–67] Alu methylation was also significantly altered in one study.[67] One study investigated global methylation in cord blood samples with the use of an assay kit and found that it was altered in response to prenatal PAH exposure.[30] These epigenetic changes can contribute to carcinogenesis at least as much as genetic changes.

Confounding

The five reports investigating methylation patterns have adequately adjusted for a number of clinical and environmental confounders, including smoking.

Grading: Evidence - B, Replication - B/C, Bias - B

The results above suggest that LINE-1 methylation levels may be affected by exposure to air pollution or its constituents. Even though only few studies were available, the replication between them was fairly good, thus supporting the B/C grading for replication. Alu methylation levels were less consistently affected. It is also relevant to note that LINE-1 methylation levels were found to increase with the level of exposure to some constituents of air pollution, for example PM_{10}, but to decrease with exposure levels to other constituents such as $PM_{2.5}$. Therefore, further evidence is needed to determine which constituents in air pollution affect methylation levels and in which direction, before we can more confidently conclude about the effect of exposure to air pollution on methylation levels.

Publication Bias and Heterogeneity

One of the factors determining the third grading criterion is publication bias. As discussed, publication bias cannot be ruled out for most of the biological markers mentioned above. Funnel plots are a useful tool for checking the existence of publication bias, and a symmetric inverted funnel plot typically indicates that publication bias is unlikely. In this review, funnel plots could only be constructed for DNA adducts and oxidized nucleobases, where enough studies were available. Also, because of the diversity in effect estimates for each biological marker, only studies comparing mean levels of markers in cases and controls could be used. Moreover, different sample types (WBC, MNBC or urine), analytical methods and units were used for each marker. The funnel plots of standardized mean differences (Web only Material - Figures 3 and 4), were fitted using a fixed effects model and using the inverse variance as weight. The asymmetrical inverted funnels thus obtained demonstrate that publication bias may be a concern when investigating the available evidence on biological markers of dose and effect and the relationship with air pollution. However, despite the asymmetry in the plots, the Egger's regression asymmetry test did not demonstrate significant presence of publication bias for studies on DNA adducts or oxidized nucleobases (P values: 0.376 and 0.576 respectively).

DISCUSSION

On the basis of the evidence from recent large cohort studies in the U.S. and in Europe (Web only Material Table 1), it has been suggested that ambient air pollution may increase lung cancer risk.

Overall, existing biological markers of dose and effect seem to contribute to reinforce the causal nature of the association between air pollution and lung cancer, though the markers in this review are not all specific to lung carcinogenesis. DNA adducts, CAs, MN, and oxidized nucleobase markers have been suggested to be predictive for the risk of future cancer [34]. The biological markers discussed in this review cover the whole spectrum of progression from external exposure to tumor formation (Figure 1). 1-OHP is an excellent

marker of internal dose, DNA adducts and oxidized nucleobases are markers of the biologically effective dose, whereas MN, CA, and DNA methylation are good markers of early biological effect. The multilevel evidence adds to the plausibility of a causal association between exposure to ambient air pollution and lung cancer. The available evidence is stronger for oxidized nucleobase markers, and the mechanisms supported by these biological markers are likely to be central in the biological process of air pollution induced lung cancer (Figure 1).

However, certain aspects of biological markers used in epidemiological studies need to be clarified. These include their reliability, the extent to which markers interact with genetic susceptibility, and inter-laboratory as well as inter-technique variation. Adequate adjustment for confounding factors needs to be considered. In the studies summarized above, body mass index, physical exercise, consumption of charcoal-broiled food, consumption of fresh fruits and vegetables, and seasonal variations were rarely controlled for (Tables 2-10 Web only Material). All these factors have been reported to influence bulky DNA adducts. Most studies have controlled for smoking, one of the most relevant confounders regarding exposure to air pollution and biological markers. Finally, the association between air pollution and biological markers of dose and effect depends on the level of exposure, with low levels of exposure often leading to weak and non-significant associations.

An issue difficult to tackle in studies utilizing biological markers, which are usually small in size, is publication bias. Funnel plots (Figures 3-4 Web only Material) do not show extensive publication bias. However, there is some asymmetry of the plots and there are only few large studies showing positive effects, implying some bias (Tables 2-10 Web only Material).

We have focused on genotoxic and epigenotoxic effects as markers of biologically effective dose and biological effect directly related to carcinogenesis, while we have not included markers of inflammation. Although chronic inflammation is probably relevant to particle-

induced lung carcinogenesis, the overall evidence is still relatively scanty. Exposure to air pollutants has been associated with acute inflammation in the airways and to elevated levels of systemic markers of inflammation, such as C-reactive protein and fibrinogen. A recent study found that medium-term exposure to traffic-related air-pollution may induce an increased inflammatory/endothelial response, especially among diabetics.[68] So far the inflammatory response has mainly been associated with the risk of cardiovascular diseases rather than cancer.[9]

The main limitations we identified in our review are related to control of confounding and publication bias. Also, almost none of the studies investigated more than one mechanistic pathway. It is conceivable, therefore, that the next generation of studies could address confounding in a more systematic way (e.g. by measuring cotinine), and will include markers that refer to more than one pathway (e.g. inflammation and epigenetics). Publication bias is a general problem of epidemiology and requires a concerted action by journal editors.

Another important, and probably largely unavoidable, limitation of these studies is that they are based on surrogate tissues e.g. WBC, that do not necessarily reflect changes in the target tissues. Due to the difficulties associated with obtaining lung tissue samples, surrogate tissues are used to estimate a measure of the damage caused in the target tissue. In the case of air pollution and lung cancer, lung tissue is the first point of contact with the carcinogen and therefore damage in this tissue is likely to be more pronounced than damage in surrogates such as WBCs.

In spite of methodological limitations, there is overall good evidence concerning the genotoxicity of air pollution. Applying grading criteria for causal assessment we concluded that the cumulative evidence supports that air pollution affects some of the biological markers related to carcinogenesis, particularly 1-OHP, DNA adducts and 8-oxodG and other oxidized nucleobases. Some markers of genotoxicity have also been found to be associated

with lung cancer (DNA adducts and 8-oxodG/8-oxoGua in urine). Development of lung cancer occurs through a series of progressive pathological changes in the respiratory epithelium. Molecular alterations such as loss of heterozygosity, gene mutations, and gene promoter methylation have emerged as mechanisms of lung carcinogenesis.[2]

Though biomarkers seem to complement our knowledge coming from prospective epidemiological studies of the effects of air pollution, the overall evidence is still incomplete and fragmented. Not only for several markers the evidence is still equivocal, but we are far from being able to reconstruct the full pathogenetic pathway that leads from external exposure to the outcome. Few studies have been conducted on epigenetic and non-genotoxic changes, so that the evidence is skewed in favour of genotoxicity biomarkers. We propose that future efforts should be directed not only towards reducing uncertainty on the role of specific biomarkers, but also towards filling the gaps in the supposed pathogenetic pathways.

ADDITION TO SCIENTIFIC KNOWLEDGE AND CONCLUSIONS

Our review evaluated the data available on some of the most relevant biomarkers of air pollution exposure, and used well accepted criteria to grade the cumulative evidence on each biomarker with respect to amount of evidence, replication, and protection from bias. Several biological markers of dose and effect related to carcinogenic mechanisms, and especially oxidized nucleobases, have been found to be associated with exposure to ambient air pollution, and some of these markers have also been associated with risk for lung cancer.[34] These biological markers, that mark the continuum of progression from external exposure to cancer outcome, have potential to shed light on the pathways of carcinogenesis, thus making the association more stringent for public health interventions. To our knowledge, this is the first time a systematic evaluation of the topic was undertaken. Our review adds biological support to the relationship between air pollution and lung cancer. Nonetheless, future research to fill in gaps in our knowledge of supposed pathogenetic pathways is needed.

Author Contributions

Christiana Demetriou –

Authorship of most sections of the manuscript and review of the whole document, guarantor

Steffen Loft –

Authorship of the section on oxidized nucleobases, revising manuscript critically for intellectual content

Petter Moller –

Authorship of the section on oxidized nucleobases, revising manuscript critically for intellectual content, contribution of figures

Ole Raaschou-Nielsen –

Revising manuscript critically for intellectual content, critical comments on conclusions and stated ideas

Roel Vermeulen –

Revising manuscript critically for intellectual content, critical comments on conclusion and stated ideas

Marc Chadeau-Hyam –

Revising manuscript critically for intellectual content, critical comments on conclusion and stated ideas

Wei Xun –

Help in producing the funnel plots, revision of manuscript

Palli Domenico –

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Paolo Vineis –

Revision of the entire manuscript, substantial contribution to conception and design, analysis and interpretation of data, guarantor

Conflicts of Interest

"All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work."

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Figure 1 – Biological Markers of Exposure and Effects of Air Pollution

Adapted from: Loft, S and P. Møller (2006) Oxidative DNA damage and human cancer: Need for cohort studies, Antioxid Redox Signal 8:1021-1031.

Figure 2 –Standardized Mean Difference Forest Plot of Studies on DNA adducts reporting difference in means

☆ Weight was derived using the inverse of the variance in a fixed effects model. Forest plots are presented for clarity in data presentation. However, formal meta-analysis was not performed due to the heterogeneity of the studies included in the review

O Total refers to Total Sample Size in the experimental (exposed) and control groups.

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Figure 3 –

Standardized Mean Difference Forest Plot of Studies on Oxidized nucleobases reporting difference in means of 8-oxod or 8-oxoGua

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Weight was derived using the inverse of the variance in a fixed effects model. Forest plots are presented for clarity in data presentation.

O Total refers to Total Sample Size in the experimental (exposed) and control groups.

Figure 4 – Meta-analyses: Fixed Effect Model. Standardized Mean Difference Forest Plot of Studies on CAs reporting difference in means of CAs

☆Weight was derived using the inverse of the variance in a fixed effects model. Forest plots are presented for clarity in data presentation. However, formal meta-analysis was not performed due to the heterogeneity of the studies included in the review

O Total refers to Total Sample Size in the experimental (exposed) and control groups.