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A critical review of assays for hazardous components of air pollution

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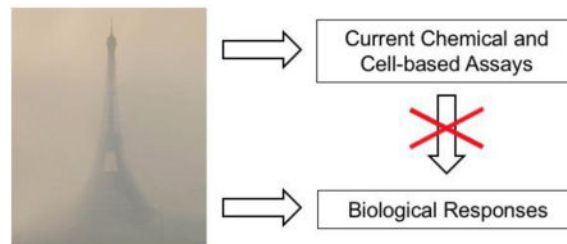
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Abstract

Increased mortality and diverse morbidities are globally associated with exposure to ambient air pollution (AAP), cigarette smoke (CS), and household air pollution (HAP). The AAP-CS-HAP aerosols present heterogeneous particulate matter (PM) of diverse chemical and physical characteristics. Some epidemiological models have assumed the same health hazards by PM weight for AAP, CS, and HAP regardless of the composition. While others have recognized that biological activities and toxicity will vary with components, we focus particularly on oxidation because of its major role in assay outcomes. Our review of PM assays considers misinterpretations of some chemical measures used for oxidative activity. Overall, there is low consistency across chemical and cell-based assays for oxidative and inflammatory activity. We also note gaps in understanding how much airborne PM of various sizes enter cells and organs. For CS, the body burden per cigarette may be much below current assumptions. Synergies shown for health hazards of AAP and CS suggest crosstalk in detoxification pathways mediated by AHR, NF- κ B, and Nrf2. These complex genomic and biochemical interactions frustrate resolution of the toxicity of specific AAP components. We propose further strategies based on targeted gene expression based on cell-type differences.

Graphical abstract



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Keywords

oxidants; nanoparticles; iron; signaling; air pollution; tobacco smoke

1. Introduction

Airborne particulate material (PM) from ambient air pollution (AAP), cigarette smoke (CS), and household air pollution (HAP) is globally associated with 15 million deaths per year (Table 1A). Synergies have also emerged for AAP and CS, which show super-additive effects (Table 1B, discussed below). The manifold components of AAP-CS-HAP aerosols present heterogeneous PM of diverse chemical and physical characteristics, including carbonaceous cores and minerals together with organic components. While carbonaceous particles may deliver exogenous toxicants, they may also effect biological responses by adsorbing endogenous compounds. Besides vehicular and industrial sources of fossil fuels, AAP often includes silicates of crustal origin [1][2].

The sizes of aerosol PM are identified by the aerodynamic diameter in micrometers: PM₁₀, PM_{2.5}, and PM_{0.1}; the larger size classes include all smaller PM. Ambient air pollution is continually monitored by the EPA, which reports PM_{2.5} throughout the US by density ($\mu\text{g}/\text{m}^3$ air); however, but PM composition is not regularly reported. The larger size PM₁₀–2.5 μ (coarse) are considered less toxic and are trapped in the upper airways; the PM_{0.1} and smaller (ultrafine) may be even more toxic than the PM_{2.5} [3][4], but is currently not monitored by the EPA. Cigarette smoke exposure is monitored by blood level of cotinine and other nicotine metabolites, and is rarely reported by size class.

Epidemiologic studies generally show robust associations of health hazards in proportion to ambient PM_{2.5}. Besides loss of life expectancy, specific disease associations include Alzheimer's, ischemic heart disease and stroke, lung cancer, and chronic obstructive pulmonary disease (Appendix 1). In recent U.S. Medicare data for older adults, mortality rates varied by 7.3% per 10 $\mu\text{g}/\text{m}^3$ of PM_{2.5} over the 90% range of 6.2–15.6 $\mu\text{g}/\text{m}^3$ [13]. This and a Canadian study [14] show health hazards into the lowest levels of PM_{2.5} [15]. Thus, for AAP as for CS, there is a no safe minimum exposure.

The highest regional North American exposures are 10-fold below some Asian cities where PM_{2.5} often exceeds 200 $\mu\text{g}/\text{m}^3$. Some studies suggest that the American PM_{2.5} hazard associations for AAP extend into the many-fold higher levels of PM_{2.5} [16][17]. Curvilinear associations are indicated for CS. Additional complexities arise from HAP of homes and other indoor sites in the developing world often include high levels of HAP from domestic fuels of wood and dried-dung, as well as CS. Airborne dust from deserts and agricultural fields (silicates of crustal origin) is also associated with health hazards, although less clearly than urban PM_{2.5} [18][19][20]. The mechanisms generating these associations are poorly understood.

To our knowledge, no studies with chemical or cell assays have compared ambient PM_{2.5} from multiple global urban samples. Because the heterogeneous sources of PM_{2.5} have widely divergent activities by chemical and cell-based assays, we argue that oxidants cannot

be directly estimated by the common assays. Furthermore, many publications refer to the oxidants in, or derived from cellular interaction with air pollution components as reactive oxygen species, usually abbreviated as 'ROS'. Indeed, by using 'ROS', it appears that this diverse group of molecules are treated as a single entity. Unfortunately, the actual oxidant has not often been identified or assays have been used that do not accurately measure what is purported for them.

Integrated epidemiological models have considered the total impact of AAP, CS, and HAP on these diseases for global data [16,17]. The shared pathologic associations AAP and CS go from 'lung to brain' across humans and rodent models (Appendix 1). The convergent chronic pathologies from AAP and CS suggest biochemical and genomic processes that shared widely across populations. Less is known about cell-type specific responses to various sources of HAP.

Two epidemiological assumptions warrant inquiry. Many studies assume the same toxicity by weight for all sources of PM_{2.5} and that different sources of PM_{2.5} (fossil fuel vs cigarette) are additive, i.e. do not synergize. While these are reasonable initial assumptions, other evidence convinces us that PM_{2.5} by weight cannot adequately represent its toxicity, because of synergies between AAP and CS. For example, AAP and CS have supra-additive effects in adult lung cancer and childhood obesity (Table 1B). These synergies are not well explained by the existing mechanisms that remain to be experimentally studied.

To approach these complex questions, Section 2 compares AAP, CS, and wood smoke for biochemical and cell responses. Section 3 evaluates chemical confounds in common assays for oxidative activity, and comments on misinterpretations of oxidant measurements that persist in the toxicology literature. Recent studies of dung smoke PM show the need to broaden the profile of inflammatory responses in other assays. Section 4 revisits the epidemiologic assumption of equal activity by PM mass, while Section 5 confronts gaps in understanding PM entry to cells and organ systems. Lastly, we propose development of assays for chemical activities and genomic responses to airborne PM with specific cell types. Our inquiry selected studies to best illustrate our main points. We hope to be fair in representing our conclusions about the uncertainties in some widely used assays, for which we suggest alternative approaches. We have not addressed the complexities of pathophysiology or attempted to review global sources. For a more detailed treatment of these complex topics, see the forthcoming monograph [1].

2. Ambient air pollution and cigarette smoke comparisons

The assumption of some epidemiological studies that PM_{2.5} from different sources has equivalent activity has received little evaluation. We found only one study to assay urban PM with CS and wood smoke with chemical composition by multiple assays with dose responses by Jin and colleagues [21](Table 2). PM₁₀ were collected from two Chinese mega cities and compared with PM from a laboratory diesel engine and from the combustion of cigarette, coal, and wood. The composition of PM₁₀ varied widely by source: up to 100-fold for water-soluble organic carbon (WSOC) and 10-fold for water-insoluble polyaromatic

hydrocarbons (PAH). Among metals, iron was the most prevalent by 10-fold or more. The 20-fold higher Fe content of PM₁₀ from Wuhan than Beijing suggests more desert dust.

Cell assays for inflammatory responses and what these authors referred to as “ROS production” gave divergent responses between these samples. For TNF α release by macrophage cells, urban PM₁₀ was >2-fold more active than the PM₁₀ from smoke PM₁₀ from diesel or cigarette, while wood smoke was inactive and without any dose response up to 400 μ g/ml. In contrast, the cell fluorescence assay using dihydroethidium (DHE) gave up to 100% higher values for CS and wood smoke than for urban PM₁₀; notably, the urban AAP PM₁₀ did not induce any DHE response. The authors concluded from the reversed order that “factors affecting ROS and TNF α release were rather different”, and that TNF α also positively correlated with metals (Cu, Fe, Pb), but negatively with WSOC. The oxidation of dithiothreitol (DTT) did not correlate with TNF α induction; e.g., diesel had the most DTT activity, but the least TNF α response. (Table 2B). These divergent outcomes may be in part understood by the chemical confounds in these assays as discussed below.

The perplexing divergence in these findings are familiar to many investigators seeking the source attribution of AAP components for which the chemical and cell assays are inadequate or misleading. We respect these studies for providing both chemical composition and using multiple assays in their ‘en suite’ study of major ambient PM. We further note the need for direct chemical comparisons of CS, AAP, and wood smoke (HAP) by PM size class concurrently in the same assay.

CS may be considered less complex than AAP because it arises from immediate combustion of a single plant species directly enters the body; CS also includes multifarious soil contaminants and pesticides, as well as nicotine-specific toxicants and high levels of carbon monoxide that are atypical for AAP. Nonetheless, both AAP and CS include carbonaceous particles that derive from the incomplete combustion of organic material.

CS particle sizes and chemistry overlap with AAP: for both, the vast majority of particles is <0.2 μ m [22][23] [24]. CS has thousands of trace organic components including polycyclic aromatic hydrocarbons (PAHs) and many trace metals. The 5000+ organic compounds in CS include more than 50 carcinogens recognized by the International Agency for Research on Cancer (IARC), some of which are shared with AAP from near-roadway sites (traffic associated air pollution, TRAP).

To start this inquiry, we assembled data from TRAP and CS. Figure 1 shows the distribution of several metals and water insoluble organic compounds (WIOC) in TRAP vs primary CS: For both, a group of toxic elements (arsenic, chromium, cadmium, lead) was highest in the ultrafinePM; however, the larger PM are not equally represented in these samples. The larger size classes of CS appear to contain more WISOC than in TRAP, which has implications for pathophysiology. Direct comparisons are needed using the same sampling protocol and assays for WSOC and other components by the same size classes for AAP and CS.

The next section examines in detail chemical assays for PM activity that are widely used. Our discussion aims to illustrate mechanisms and confounds with examples, and does not

attempt to comprehensive review the extensive literature on diverse sources of PM with different chemistries.

3. Assays for ambient particles

A decade ago, Künzli et al. [31] provided a thorough comparison of the chemistry and biological responses to samples from multiple sites, “*PM oxidative activity varied significantly among European sampling sites. Correlations between oxidative activity and all other characteristics of PM were low, both within centers (temporal correlation) and across communities (annual mean). Thus, no single surrogate measure of PM redox activity could be identified. Therefore, it is important to define the appropriate methods to determine oxidative activity of PM.*” Attempts to find such an appropriate method have continued. Three prominent and commonly used chemical assays are discussed next, followed by cell-based assays.

3a. Chemical assays

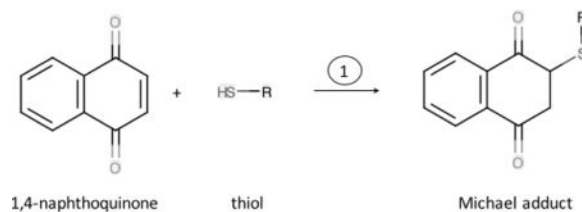
To evaluate the potential toxicity of PM_{2.5} with varying composition, many studies use chemical assays for the oxidative activity of PM_{2.5}. Widely used assay substrates include dithiothreitol (DTT) and ascorbate acid (AA)-glutathione (GSH), which we discuss in sequence.

Dithiothreitol—Findings for DTT are often reported as units of reactive oxygen species (ROS). However, as stated earlier this is not chemically valid because “ROS” is not a single molecular species. In fact, the DTT and other assays are sensitive to diverse agents: some are true free radicals with unpaired electrons (superoxide O₂⁻ and hydroxyl HO[•]). Additionally, these assays detect other redox active agents that are not free radicals (hydrogen peroxide, ozone, singlet oxygen, and peroxyxynitrite). Each differs markedly in physical, chemical, and biochemical properties. Contrarily, the *putative* values given for ROS by the DTT assay actually represent the *non-specific* production of undefined oxidation products. Thus, most reports using DTT cannot be considered as either quantitative or qualitative for the oxidants in the sample. Conclusions from the DTT assay do not serve well to predict APP-associated cell response (inflammatory and/or anti-inflammatory) and cell damage. Furthermore, the heterogeneity and variability of AAP frustrates simple interpretation of these measurements as end-points for oxidative activity.

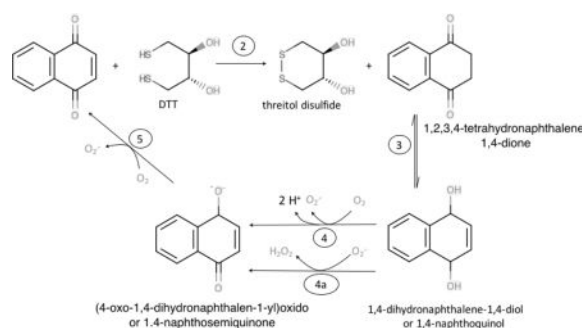
The DTT assay as initially reported assumed that quinones mediated DTT oxidation *by catalysis* [32]. While thermodynamically feasible, this reaction scheme cannot account for the DTT oxidation observed in the assay (see Appendix 2). We suggest a more plausible mechanism.

Quinones mediate many AAP and CS oxidative mechanisms, and occur in the water soluble organic fraction (WSOC) of both TRAP [33] and CS tar [34][35][36]. Quinones are defined as aromatic ring compounds with two carbonyl groups at various locations. During combustion, quinols (the reduced form of quinones) are oxidized to semi-quinone free radicals which can propagate oxidation by redox re-cycling [36]; Reactions 1-5 below.

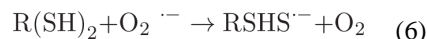
As described 80 years ago, quinones (1,2- or 1,4) readily combine with thiols in a Michael addition reaction [37]. 1,4-naphthoquinone (NQ), is a component of AAP [33]. Here we show it forms an adduct with thiols (RSH), such as cysteine or GSH in Reaction 1:



The reaction of GSH with quinones is a potential contributor to GSH depletion observed in interactions of AAP with lung-lining fluid components [31]. In contrast, because DTT has two thiol groups in close proximity that readily form a stable six membered ring when oxidized, DTT reacts in a concerted reaction with NQ in which the NQ is reduced (Reaction 2). NQ then rearranges to form the 1,4-naphthoquinol (Reaction 3).



Quinols can then react either with O_2 (Reaction 4) to generate superoxide ($O_2^{\cdot-}$) or with $O_2^{\cdot-}$ (Reaction 4a) to generate hydrogen peroxide (H_2O_2). In either case, a semiquinone is formed, which is a free radical. The semiquinone then is restored to the original quinone by reacting with another O_2 (Reaction 5). The key point is that quinones can cycle through oxidation and reduction, *but* catalyzing DTT oxidation without being consumed. The $O_2^{\cdot-}$ generated could also react with DTT to produce the thiyl radical, as reported [32]:

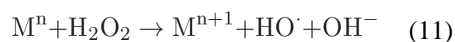
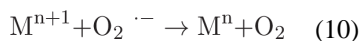
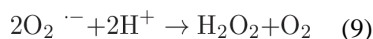


The thiyl radical will then react with another molecule of O_2 , thus accounting for



the observed acceleration of DTT consumption by O_2 . Consistent with recycling of the quinone, 10-fold more DTT can be consumed than the quinone that is lost. The loss is most likely through adduct formation with one of the thiols of DTT in Reaction 1.

Transition metals (M , below), the most abundant of which is iron, catalyze the oxidation of DTT even more easily than quinones because of their larger reduction potential and reaction rates.



The hydroxyl radical generated in Reaction 11 (Fenton chemistry) will oxidize rapidly oxidize DTT to sulfinic and sulfonic acid forms.

In sum, the widely used DTT oxidative assays are confounded by facts that both the quinone and the metal reactions are neither completely catalytic, nor first order reactions driven by substrate concentration.

Ascorbate acid (AA)-glutathione (GSH)

The oxidative activity of PM2.5 in AAP is also often assayed by AA and GSH, the normal reductants in lung fluids [31]. We focus on relationships of oxidative activity to metal content.

Many studies show associations of oxidative activity with the iron content of AAP. Positive correlations of AA and GSH oxidation with PM2.5 iron content were reported for Paris [38]; for 8 of 9 sites in the Los Angeles Basin [39] and for a Dutch train station [40]. In contrast, PM2.5 from the Barcelona Metro showed *negative* correlations of iron levels with oxidative activity (Table 3) [41]. The electrified Barcelona Metro PM2.5 are notably rich in metals from mechanical abrasion of metal surfaces and of brake linings, but have minor contribution from fossil fuels. When iron or other transition metals fail to cause DTT or AA oxidation, we suggest this is due to their location within the particle or their chelation which prevents their redox cycling. Studies discussed below illustrate this for surface iron in silica particles.

For iron to participate in cell responses to particles, it must be accessible to interact. Thus, the iron must be on the surface rather than buried within the structure. It also must be chemically reactive. Thus, metallic iron (no charge) or iron that is complexed in such a way as to make it chemically inert, will not participate in reactions. Indeed, iron being part of the composition of the internal structure or inert, is largely the reason its content is so poorly correlated with biological responses. When iron participates in biological responses to particles, it is usually through initiation of lipid peroxidation. In reactions 10 and 11 above, iron cycles between the ferric (3+) and ferrous (2+) state while generating hydroxyl radicals that then initiate lipid peroxidation. But, we also note that AA can reduce iron from ferric to ferrous and may thereby act, seemingly counterintuitively, as a pro-oxidant. This pro-oxidant effect of AA and other reductants that is due to their reduction of iron has been known since the 1960s [42].

We note that some correlations would be considered anomalous and misleading by biochemists. Oxidative activity with AA and GSH was positively correlated with antimony and lead in these studies (Table 3) and by [38]. However, neither antimony nor lead *can* directly oxidize glutathione, although these metals are bound ionically by GSH and other thiols (RSH) and may thereby facilitate their oxidation. Nickel and zinc did not correlate with oxidative activity. This is reasonable because under physiologic conditions, these metals also may, under some conditions, facilitate but *cannot* oxidize ascorbate, glutathione, or other organic compounds. The potential in vivo toxicity of nickel, zinc, and other trace metals is indisputable, but cannot be evaluated by these and other in vitro chemical assays.

3b Cell assays

Cells will react with both quinones and metals both at the cell plasma membrane and internally if taken up, with differences by cell type. Cell types also vary in reactivity depending on handling of metals and whether quinones are reduced and/or conjugated. Cell response to PM also differ in production of O_2^- and H_2O_2 by activating NADPH oxidases, which mediate signaling pathways (Section 3e below). Next, we discuss cell- based assays for oxidative activity for three widely used markers: DCF, DHE, and MTT.

Dichlorofluorescein (DCF)—The substrate 2',7'-dichlorofluorescein diacetate (DCFH-DA) is hydrolyzed within cells to DCFH that, in turn, may be oxidized to the fluorophore 2',7'-DCF. The assay described for H_2O_2 originally required the presence of a peroxidase catalyst [43]. Mistakenly, the assay began to be used for H_2O_2 and other oxidants without regard for the need for catalysis. Rigorous studies showed that dye oxidation was primarily due to iron-catalyzed reaction with H_2O_2 . [44][45][46]. Moreover, DCFH is oxidized by diverse agents that lack specificity for H_2O_2 or other reactive species [47]. The DCF assay may be even more problematic because the DCF radical generates superoxide and H_2O_2 from spontaneous free radical chain reactions [48]. By neglecting this established chemistry, erroneous conclusions continue. For example, one study employed microfluidic electrophoresis with fluorescence of DCF enhanced by plasmon-resonance, from which it was calculated that each puff of CS had 8 pmol of "ROS" [49]. Despite the elegant plasmon technology for directly assaying ambient CS, the DCF response was not defined chemically for any of the oxidants in the assay. As described above, calculation of ROS per mole has no

chemical meaning. Furthermore, because of chain reactions intrinsic to DCFH chemistry, the fluorescence DCF lacks chemical specificity.

Dihydroethidium (DHE) can be a specific assay for superoxide—The substrate hydroethidine is oxidized to 2-hydroxyethidium by superoxide, but also by many other agents. To identify superoxide requires separation of the products by HPLC [50]. Thus, DHE is superior to DCF for superoxide if properly conducted. However, superoxide formed in the media away from the cell surface would dismutate to oxygen and H_2O_2 (see Reaction X), which could affect cells. Regardless, formation of superoxide is dependent on particle surface reactions and will not correlate with bulk measurements of metals, quinones or other particle components.

Ferricytochrome c—The spectrophotometric measurement of SOD-inhibitable reduction of ferricytochrome c remains among the simplest real-time assays [51]. However, PM may interfere with the assay by physically blocking ferricytochrome c, a 12 kDalton protein, from accessing the site of $O_2^{\cdot -}$ production. Alternatively, the smaller substrate nitro-blue tetrazolium can be used with appropriate controls [52].

MTT—The reduction of MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its purple formazan product gives a measure of mitochondrial activity. However, large decreases of the MTT substrate do not necessarily indicate cell death or irreversible cell stress: depending on the toxicant, cell death may be overestimated [53] or *underestimated* [54].

Reliable assays for cell death include release of the easily measured lactate dehydrogenase (LDH) and the uptake of propidium iodide which binds to DNA, [55]. Cell damage may also be assessed by cell exclusion of the vital dyes, Trypan Blue and Erythrosine, or binding of fluorescently tagged Annexin V. While all of these assays may indicate cell damage by particles, none of them can show that the damage is specifically *due to* oxidative stress. Although airborne PM may include agents that potentially cause oxidative damage, it difficult to prove causality of one agent among a suite of candidates in a complex composition.

Lastly we note two major studies which used expanded arrays of multiple chemical and cell responses to PM_{2.5}, but reached the same frustrating conclusions as did Kunzli et al. [31]. Crobeddu et al. [38] included DTT, AA, and GSH; a plasmid scission assay, and DCFH; expression of two antioxidant enzymes and pro-inflammatory IL-6. They also measured a large group of metals and multiple PAH species. They concluded that AA and GSH oxidation correlated best with both DCFH oxidation and expression of selected genes. However, Visentin et al. (2016)[54]?? did not find correlations of DTT and ascorbate assays; contrarily, the content of zinc was highly correlated with DTT oxidation, which makes sense to biochemists because Zn is not oxidizable under these conditions (Section 3, Table 2). The state-of-the-art for source apportionment of disease risk assessment for based on these assays looks more like the randomness of a Jackson Pollack painting than the orderliness of a Piet Mondrian.

3c. Ambient free radicals

Could persisting free-radicals in ambient particles also be factors in these assays? We observed free radical signals from re-aerosolized ultrafinePM collected a month earlier and stored in frozen aqueous suspension (from co-author Nicos Petasis) [57]. These EPR spectra are consistent with carbon-based free radicals in the graphitelike particles [33]. The long-term stability of free radicals in TRAP suggests that these radicals are 'buried' within the particle matrix and thus inaccessible for reaction.

Long-lived carbon-based free radicals also occur in coal dust [35]. Because the half-life of CS-tar derived free radicals in solution is short (seconds) [35], it is likely that the soluble free radicals are rapidly quenched by ascorbate and other antioxidants that are enriched in the lung surfactant fluids [58]. Although CS tar can stimulate production of hydroxyl radicals (HO^\bullet) in chemical and cell assays, this activity critically depends upon the presence of iron and other metals. If the hydroxyl radical HO^\bullet is produced in fluids covering cells, its effect would be minimal because its reactivity would be quenched by ascorbate and glutathione, together with lipids and proteins in the fluids lining the airways and alveoli, even before any contact with cell surfaces. HO^\bullet reacts promiscuously with neighboring molecules with near 100% efficiency, causing *in vivo* half-lives of nanoseconds [59]. Because iron and other transition metals are essential to HO^\bullet formation, this radical can *only* be involved when the metal is in direct proximity. Thus, most if not all free radicals in ambient PM measured in these assays cannot contribute to oxidative signals in cells. In contrast, H_2O_2 formed in the media may last long enough to interact at the cell membrane or enter cells to mediate redox signaling [60].

3d. Particle content: solubility and accessibility

Another major issue is the solubility and accessibility of prospective toxicants in PM to the assay, as shown for iron in silica PM. Iron has well documented roles in pro-inflammatory responses and injury to cells [61]. In macrophage responses to natural silica PM, Forman and colleagues showed that the surface iron was essential for induction of $\text{TNF}\alpha$ and $\text{IL1}\beta$ [62]. For a 100 nm spherical particle composed of a uniform iron-containing material, only 6% of the iron would be on the surface. Silica PM from which iron was leached by acid treatment failed to induce $\text{IL-1}\beta$ and $\text{TNF}\alpha$ in macrophages, indicating that an iron-particle-cell interaction was essential for these cytokine effects. As further evidence, adding an equal amount of iron to that on the particle surfaces (0.5 nM) in the absence of particles did not alter expression of $\text{IL1}\beta$ or $\text{TNF}\alpha$. Thus, iron chemistry and availability may vary considerably dependent on whether it is bound to carbonaceous particles, or present as multinucleate ferric hydroxide on the surface of silicate PM, or in a nano-chunk of steel.

The accessibility of iron and other metals may underlie assay complexities shown for PM from five Canadian industrial sites including smelters and petrochemicals [63]. This study is exemplary for its detailed dose responses in cell assays by source and PM size class, but did not include chemical assays. PM size classes (<0.1 to >10 μm) were characterized for metals and polycyclic aromatic hydrocarbons (PAH) in assays of cytotoxicity and cytokine responses with cell lines representing macrophages (J774A.1) and lung epithelia (A549, type II alveolar cells). As expected, endotoxin levels strongly correlated with cytokine release,

which gives an important validation. Smaller PM varied in metal content between sampling sites more than PM >2.5 μm , especially for water-soluble iron and other metals. For all PM classes, cytokine release correlated weakly with iron and other metals, as well as with PAH. In dose responses, PM_{0.1} had lower cytotoxicity and cytokine (IL6) release than larger PM, contrary to many reports [39].

In considering, the role of metals in CS toxicity, iron is often discounted as a major contributor [64]. This is counter-productive, as iron is the most prevalent metal in combustion products from coal, fly ash, wood, diesel exhaust, and AAP. Iron is also enriched in the humic-like substances (HLS) of CS PM_{0.2} [22][65][66]. The HLS occur globally in AAP [67][68]. The iron bound to HLS largely comes from the microbial degradation residues found in soil, peat, and coal [69]. Its complex composition includes aromatic rings and aliphatic chains with multiple hydroxyl and carboxyl moieties that can bind metals, particularly iron. The association of HLS with oxidant generation could be a key to toxicity of combustants from fossil fuels, forest fires and cigarettes [70]. In rat lung, HLS from CS caused deposits of iron with inflammatory responses that resembled iron-rich foci in smokers' lungs [65][71]. Long-term deposits of HLS are associated with sequestered iron and focal disruption of iron homeostasis [22].

Ghio and colleagues [69] also showed that the production of the inflammatory mediator LTB₄ by macrophages could be stimulated by iron on silica in the presence of ascorbate, which is present in the airway and alveolar fluids. While iron is the most studied transition metal for oxidative stress, other transition metals including copper, manganese catalyze the same reactions, which raises the same issues regarding bulk content versus surface availability.

3e. Signaling pathways for gene expression

Most toxicology assays have examined a small number of oxidative or inflammatory markers. The value of a broader genomic-based approach is illustrated in pioneering studies of smoke from burning dried dung (DS) [72][73]. Dung is common household fuel of impoverished households of Asia and Africa, with undefined contributions to the mortality associated with household air pollution (Table 1). Based on their size of $0.57 + 0.15 \mu\text{m}$, DS particles are predicted to deposit in bronchioles, with limited penetration to alveoli. Accordingly, these experimenters chose to model cell responses with human small airway epithelial cells (SAEC), obtained from adult donors with no smoking history. In a novel air-liquid culture model, SAEC cells were exposed to *ambient* DS at $1000 \mu\text{g}/\text{m}^3$ density for 60 minutes to simulate household cooking times. Surprisingly, after 24 hours, only two cytokines, IL8 and GM-CSF responded with several-fold increase, while there was no response by IL6, TNF α or more than 30 other cytokines, or of NF- κ B, a key inflammatory transcription factor. Cell type may be underlie the lack of NF- κ B activation, because epithelial cells are unresponsive to TLR2 agonists [74]. Nonetheless, DS induced Cox-2 via the JNK-AP-1 and aryl-hydrocarbon receptor (AhR) pathways, which also mediate inflammatory responses to urban AAP. DS from six herbivores including foregut and hindgut fermenters all activated AhR and AP-1. Notably, DS impaired the interferon response to a viral analog (poly I:C), suggesting a molecular mechanism for the lung

infections associated with household air pollution. These findings indicate that cell responses to DS may have major differences from the smokes of fossil fuel and wood, which were not tested in parallel. These pathways are summarized in Fig. 3.

Together with data of [21] (Table 2), these findings suggest that smoke PM from wood and dung has less inflammatory activity than from AAP and CS in cell assays, and that these chemical assays did not strongly predict cell responses. Further direct comparisons of dung and wood smoke with PM_{2.5} from CS and AAP are needed.

NF- κ B is recognized as a key regulatory of inflammatory responses to AAP in vivo, shown in rodent models [75]. Most of inhaled PM initially disperse onto tracheal bronchial epithelial cells and alveolar macrophages, which receive the largest per cell dose. Activation of NF- κ B in response to AAP in macrophages and epithelial cells [76][77][78] stimulated transcription of TNF α , IL-1 β , and other pro-inflammatory cytokines.

Using the model of natural silica from which surface iron was removed and replenished, we showed that iron-dependent lipid peroxidation at the surface of the human THP-1 macrophages resulted in with lipid raft disruption [62]. This disruption led to activation of the phosphatidylcholine specific phospholipase C (PC-PLC) pathway that, in turn activated NF- κ B, with following induction of TNF α and IL1 β . Air pollution PM-stimulated production of cytokines by human airway epithelial cells is also metal-dependent [78]. NF- κ B activation by TNF α through the PC-PLC pathway was also proposed for human airway epithelium [79][80], but remains untested. The PC-PLC pathway was activated by other agents in epithelial cells in several studies [81][82][83]. Nonetheless, the PC-PLC pathway is not considered as a classical pathway to NF- κ B activation by oxidants.

We recently compared glial genomic responses of ultrafinePM with LPS, a widely used model for endotoxin-induced inflammation, by whole genome microarray [84]. Both agents induced TNF α and NF κ B through the shared TLR4 pathway. These responses were verified in the hippocampus of mice chronically exposed to ultrafinePM. Because these ultrafinePM had minimal LPS activity, the activation of TLR4 suggests additional signaling pathways for AAP components.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is a master regulator of transcriptional responses to stresses from electrophiles including the oxidants in, or generated by, exposure to PM from both air pollution and cigarettes (Figure 2). Nrf2 activation is mediates increased transcription of groups of antioxidant enzymes, of detoxification enzymes that catabolize xenobiotic compounds, proteasome subunit expression [85].

Nrf2 is continuously synthesized and then rapidly destroyed through interaction with Keap1. These dynamics facilitate its rapid degradation by the ubiquitin-proteasome pathway. In a simplified view of these mechanisms, modification by alkylation or oxidation of specific cysteines in Keap1 inhibits Nrf2 degradation, thereby allowing newly synthesized Nrf2 to reach the nucleus. Electrophiles, including quinones in nPM [33] and acrolein [86] and benzene-derived quinones [87] in tobacco smoke, modify Keap1 and thereby activate Nrf2. H₂O₂ is also an activator of Nrf2 [88]. H₂O₂ can be generated by activation of NADPH oxidases or from redox cycling of quinones, a common pathway for PM from AAP and CS.

Responses to air pollution PM as three tiers proposed by Nel and colleagues [89] a decade ago extended the concept of a balance between induced antioxidant defenses and oxidative stress resulting from inflammation to the realm of signaling involving Nrf2 for antioxidant defense and NF- κ B for inflammation. However, recent research shows that these two signaling systems are not dichotomous and interact at multiple levels. For example, activation of NF- κ B by PM can result in activation of Nrf2 signaling several hours later [90].

The Nrf2 pathway also mediates body-wide responses to AAP. In our mouse model, chronic exposure to ultrafinePM, induced robust Nrf2-dependent responses in lung, liver and brain of young mice, specifically for glutamate-cysteine ligase (GCL) which is one of the classic phase II xenobiotic detoxifying enzymes (Figure 2). Surprisingly, the size of GCL induction (50-60%) was equally large in brain (cerebellum) as in lung and liver, We discuss this puzzle further in Section 5.

CS extract also induced Nrf2 in epithelial cells more rapidly and at lower levels than HO-1 and other components of the phase 2 detoxifying pathway [91] The oxidative stress associated with CS extract was alleviated by repression of an Nrf2 inhibitor KEAP1 [92]. Systemic and local cell responses to direct contact with inhaled particles are discussed below.

There is a complex interaction between Nrf2 and NF- κ B signaling. At one level, the gene products of the two pathways affect the expression of the other. NF- κ B can be activated by endogenously produced H₂O₂ [93]. Nrf2-regulated enzymes, and production of GSH, and thioredoxin, and NADPH, which all contribute to the elimination of intracellular H₂O₂, should also affect the H₂O₂-dependent activation of NF- κ B even when cells are not stimulated [94]. Thus, increases in antioxidant genes through Nrf2 activation by AAP, CS, or dung smoke should attenuate pro-inflammatory gene induction. There are also direct interactions between the NF- κ B and Nrf2, with reciprocal negative feedback loops that inhibit transcription dependent upon the other [95]. At a third level, the proteins produced by NF- κ B activation further stimulate the activation of Nrf2. Most recently, we used iron-coated silica nPM to investigate the second and third levels [90]. Within the first six hours after nPM exposure of macrophages, pro-inflammatory cytokines were induced by NF- κ B activation, but without increase in the mRNAs of four Nrf2-dependent genes. However, at 18 hours post exposure, the four Nrf2-dependent mRNAs were all elevated through an NF- κ B-dependent mechanism Interestingly, one Nrf2-dependent gene, glutamate cysteine ligase catalytic subunit, was depressed at 6 hours in an NF- κ B-dependent manner.

The aryl-hydrocarbon receptor (AhR) pathway catabolizes polycyclic aromatic hydrocarbons (PAH) and is a major mediator of responses to airborne toxicants from AAP, CS, and HAP. The AhR is a ligand-activated transcription factor residing in a cytoplasmic complex with HSP90 and other chaperones. After ligation, AhR binds to xenobiotic response elements of de-toxifying genes, as well as genes mediating other functions. The broad pleiotropies of AhR extend to inflammatory cytokines, cell proliferation, apoptosis, and stem cells. The NF- κ B module also mediates CS-induced apoptosis through the AhR [96]. The AhR protein can also dimerize with RelB, with cross-over regulation of NF- κ B regulated genes [97].

CS induced AhR and NF- κ B, in vitro and in vivo [98]. Dung smoke (DS) also induced the AhR, but without induction of NF κ B in both studies, discussed above. This is the first indication that DS and CS could activate different subcellular pathways. CS also alters DNA methylation (DNAm) of the *AhRR* (aryl hydrocarbon repressor) [99], a tumor suppressor gene for xenobiotics that we suggest also could influence vulnerability to air pollution.

4. The epidemiological quandary: how to evaluate different sources of PM

Lung cancer, known for strong CS associations, is also linked to AAP with increased risk of 8% per 10 $\mu\text{g}/\text{m}^3$ PM_{2.5} [100][101][102]. These findings led the International Agency for Research on Cancer in 2013 to classify both outdoor air pollution and airborne PM as carcinogenic. Synergies of AP and CS for lung cancer are shown in a benchmark analysis from the Cancer Prevention Study II [9] (Table 1B): The combined mortality from lung cancer for CS with high PM_{2.5} exposure was 2-fold over simple additive effects of high PM_{2.5} or CS, and accounted for 14% of lung cancer mortality. While lifetime CS exposure can be reasonably estimated, the total PM_{2.5} exposure is less certain. The authors commented “*Potential biological mechanisms for greater-than-additive effect remain unclear*”. No animal model studies have included both AAP/TRAP and CS (Appendix 1).

Beyond lung cancer, AAP-CS synergies have not been widely considered in epidemiological studies. A baseline for further studies is the integrative dose-response model [16][17], which includes these diverse sources. Lung cancer shows linear increases across a 10,000-fold range of PM_{2.5}, while ischemic heart disease reaches a plateau at much lower levels. These studies assumed an inhaled dose of PM_{2.5} of 12 mg per cigarette, equivalent to a daily intake of ambient PM_{2.5} at 667 $\mu\text{g}/\text{m}^3$. However, the assumed equivalent toxicity by weight for all-source PM_{2.5} is challenged by a little noted gap between initial nicotine content and resulting blood levels.

Using careful pharmacokinetics, Benowitz and Jacob [103] showed that 1 mg of nicotine per cigarette reaches the circulation, which is 90% less than the 8-9 mg of nicotine in the unsmoked cigarette. Because nicotine is evenly distributed across particle sizes [29] and is soluble, we suggest that this 10% also approximates CS delivery of soluble toxicants associated with CS PM_{2.5}. Thus, the effective dose of PM_{2.5} per cigarette would be 10% of the initial 12 mg of PM_{2.5} per cigarette, or 1.2 mg. This deduction still allows conclusions from many studies that lungs retain the majority (60-90%) of particles inhaled by smokers [104]. However, none of these studies considered the solubility of potential toxicants, many of which are water soluble, e.g. metals and oxidized PAH and other carbon compounds [26]. These adjustments of delivered PM_{2.5} would recalculate these data with a left-ward shift by about 10-fold for values above 10,000. This recalculation would not alter the main conclusion that the IHD risk reaches a plateau, while lung cancer increases linearly with at higher levels of PM_{2.5} exposure.

Second-hand smoke (SHS) also interacted with AAP in two studies of childhood obesity. In the Children’s Health Study of Los Angeles, the body mass index (BMI) was higher in adolescents exposed to SHS, with further increases in those living near roadways [10](Table 1B). The net impact was 3.0 BMI units above those with low PM_{2.5} and no SHS exposure

by age 18, and scaled with the number of household smokers. Similarly, children of the National Health and Nutrition Survey (NHANES) also showed super-additive synergy of SHS and traffic exposure for obesity [105]. Urinary PAH was used as a measure of exposure to fossil fuel exhaust, with correction by serum cotinine, a marker for SHS. Compared with the lowest exposures, children with high SHS and PAH exposure had 30-fold more obesity. The strongest PAH-obesity associations were with naphthalene metabolites (2-benzene rings). Consistent with these findings, German adolescents [106] and adults [23] showed strong association of insulin resistance with PM_{2.5}. The recognition of AAP as an endocrine disruptor for insulin resistance [107] must now be expanded to include these synergies with CS.

As an approach to synergies of airborne toxicants, urban PM from a Canadian city (EHC-93) were eluted by sonication from filters followed by centrifugation and passage through 0.2 μ filters, yielding water soluble (WS) and water insoluble fractions (WIS) [108]. Protein changes and cytotoxicity of A459 lung cells and in vivo exposures marked differed in response between the WIS and WS, including a group of inflammatory proteins that were increased by WS and increased by WIS. In vivo installation of the WS fraction caused mild lung injury in rats, whereas WIS was less toxic, unlike in vitro cell responses. It was concluded that the response to the combination of the two fractions was the result of both synergistic and antagonistic interactions. However, the delivery of water-soluble agents to cells in this method cannot reflect the much higher local concentrations of an agent as it diffuses from particles on the cell surface or as it taken up by endocytosis into cells.

5. How do particles enter the cell and our body?

We briefly discuss major gaps in how inhaled PM enters cells, and impacts organs systemically in adults and during development.

5a. Cell entry

PM may enter cells by endocytosis, in which part of the plasma membrane surrounds the particle and then buds off into the cytosol [109]. Four pathways are described for endocytosis of ultrafinePM; caveolar-mediated endocytosis dependent upon clathrin, phagocytosis, macropinocytosis, and pinocytosis [110]. PM components could leak from the endosomes. However, because endosomal membranes are similar to the plasma membrane from which they originate, only those materials that enter from the extracellular fluid would likely escape the endosome into the cytosol. This route could enable delivery of soluble components of AAP into the cytosols, as proposed for drug delivery by nanoparticles [111]. Zinc and other metal ions from AAP may escape from endosomes, particularly if they fuse with lysosomes in which the acidity may solubilize them [112]. It seems unlikely that particles larger than small molecules can escape endosomes to enter mitochondria or nuclei.

PM entering a cell from the apical surface can form an endosome, which may cross the cell to the basolateral surface and fuse with the plasma membrane thereby releasing a particle into the extracellular fluid on the basolateral side. This trans-tissue transport can also work in reverse. Translocation across tissues depends on both the physico-chemical properties of the PM and the mechanism of endosome formation [110][113]. Independently of

endocytosis, cell signaling processes may be initiated by interactions at the plasma membrane, including receptor binding in macrophages [114] or disruption of membrane lipid rafts [62]. Thus, cell internalization PM is not required for toxicity.

5b: Inhalation

Inhalation is our main exposure to airborne pollutants. While lungs receive the bulk of PM_{2.5}, some is ingested after mucociliary transport into the throat, discussed below. There is definitive evidence that *some* particles enter the brain, but we lack consensus on the extent of ‘nose-to-brain’ transport by human olfactory neurons [115]. Humans breathe mostly by nose, with increasing mouth air intake during exertion. In contrast, rodents are ‘obligate nose breathers’ [116]. For humans and rodents, some of the nasal PM hits olfactory nerve endings, which *potentially* transport them directly into the forebrain. In mice, we showed inflammatory responses of the olfactory neuroepithelium and olfactory bulb within 24 hours [117].

‘Nose-to-brain’ passage of inhaled ultrafine PM was shown in classic studies with radio-tracers by Oberdorster, Elder, and colleagues. Their initial study exposed rats to aerosolized nano-scale [¹³C]-graphite PM (37nm ± 1.7 dia.) [118]. In several brain regions, the [¹³C] peaked at day 1, with modest if any decline by day 7, whereas lung [¹³C] sharply declined after day 1. Notably, levels of [¹³C] in olfactory bulb (OB) approximated that of cerebellum, which is several synapses from the olfactory input. Similarly, manganese oxide PM of 30 nm yielded the same levels in the frontal cortex and cerebellum after 6 and 12 days [119]. This equivalence suggests a systemic input of [¹³C] throughout the brain.

The importance of PM size for neuronal transport was shown by larger Mn of 1.9 μm, which restricted Mn elevations to the OB, without elevation in cerebellum [120]. A possible anatomical basis for size exclusion is the small diameter of olfactory neurons, which for smaller mammals, are among the smallest diameter brain neurons. In the most detailed analysis, guinea pig olfactory neurons had 0.28μm mean axon diameter with range 0.2-0.45 μm [121]. We have not found diameter measurements for human olfactory neurons. Calduron-Garciduenas et al. [122] described “abundant particulate material in the cytoplasm” of OB neurons from a Mexico City teenager, but did not give PM size estimates.

Moving into the respiratory tract, the PM contacts the epithelial cells lining our airways, nose and throat to lung. The ‘mucociliary escalator’ conveys particles, particularly the coarse PM, into the throat where they are swallowed. The portion of inhaled PM that reaches the gut is not well-defined. Gut delivery of PM is also used in some studies [123][124]. Fine and ultrafine PM from CS reach deep lung alveoli with high efficiency, 50-90% [116][104]. Deeper in the lung at alveolar surfaces, particles are phagocytosed by macrophages, with increasing efficiency <0.2 μm [125]. Ambient PM inhalability measured by radioactive tracers was similar for rodents and humans up to 5 μm [126]. For artificial aerosols of 2.5 μm, children had less efficient nasal filtering than adults, suggesting greater alveolar deposition [127]. This age-difference is relevant to interactions of household smoking with freeway exposure of that increase obesity risk of children [10].

Particle translocation from lung to blood is very inefficient. In human and rodents, 1-25% of inhaled artificial ultrafinePM reaching the lung was detected in blood cells [125]. Nonetheless, the above tracer studies definitively show that some PM reaches the brain from the lung, as well as from the nose. The fetal brain is also adversely impacted by vehicular exhaust PM as shown by experimental studies from three labs [128][129].

For inhaled PM to act directly on the fetus requires passage across at least three barriers: I, maternal air-lung interface into the maternal circulation; II, phagocytosis by circulating macrophages and by the liver; III, the placenta. Placental transfer of artificial PM depends on particle size and chemistry [130].

Human placentas can transport nanoscalePM into the fetal side of circulation [131][132], which is mediated by endocytosis at placental villi [132]. This research was motivated by concerns about the largely unregulated nanoparticles in cosmetics, and as food additives, baby food to beer. We anticipate adverse synergies of cosmetic and food nanoPM additives with air pollution in brain development.

System-wide cell reactions suggest a ‘lung-to-brain’ route [133]. Serum from ozone exposed rats increased production of TNF α and H₂O₂ when added to cultured microglia [133]. Because ozone is quenched *immediately* on contact with respiratory tract fluids, we anticipate that these ‘ozone effects’ represent chemically modified blood lipids or proteins, or from innate immune responses. The inhaled PM may cause oxidative damage to circulating blood cells, proteins, and lipids, as well as inducing innate immune responses.

The time course of inflammatory response to PM_{0.2} in a mouse model also suggest systemic factors. We did not see increased TNF α or other inflammatory responses in the cerebral cortex or cerebellum until after 3 weeks of exposure to PM_{0.2}, when the increases were as large in the olfactory epithelium as in the remotely connected cerebellum [134]. Recall from above that the radioactively-labelled graphite also yielded same levels in cerebellum as forebrain [118]. Moreover, we found that Nrf2 induction and other genomic detoxification responses to chronic PM_{0.2} were as large in cerebellum as in lung or liver (Fig.2). Because the lung traps most of the inhaled particles, it is hard to see how the liver and brain could have equal responses, without considering systemic down-stream effects from the lung adducts formed in the lung, as postulated for ozone [133].

6. Recommendations for alternative approaches

The prediction of epidemiological effects from lab assays remains problematic, as illustrated in examples of weak correlations and divergent outcomes in Sections 2 and 3. Readers may share our consternation, and wonder if *any* assays are more meaningful. In principle, free radicals can be detected by spin-trapping in cells exposed to AAP [136] or CS [36] [40]. However, spin-trapping requires electron paramagnetic spectrometers that are not commonly available. High concentrations of spin probes that do not interfere with metabolism can be used to assess by spin-trapping for semi-quantitative direct estimates of free radicals production by cells. Some of the more accurate bioassays for free radicals, e.g. by boronates, dihydroethidium (DHE) [46,137], require specialized expertise in redox biology. Peroxide-

activated reporter genes can also be included in genomic responses, discussed below. For assay of airborne activity, a major caveat is that most radicals have extremely short lifespans and are unlikely to reach the cell surface.

Other accessible assays include eicosanoid metabolites that are end-products of enzymatic lipid peroxidation produced by cyclooxygenase or lipoxygenases. Conveniently, the TBARS assay evaluates production of thiobarbituric acid reacting substances from the oxidation of lipids, as well as polysaccharides, nucleic acids, and small sugars. Other lipid oxidation products (prostaglandins, leukotrienes, compounds derived from arachidonic acid) are best measured by mass spectrometry, or more conveniently in some immunoassays [138]. The non-enzymatic oxidation of polyunsaturated fatty acids can produce unique products, including the F2-isoprostanes, also measured by mass spectrometry [139].

4-hydroxy-2-nonenal (HNE) is another useful marker of oxidative damage to lipids by forming protein carbonyl adducts, as studied for exposure to ozone, AAP, and CS [140][134] [141]. HNE adducts are readily measured by immunoassay, while protein carbonyls requires reaction with dinitrophenylhydrazine to form adducts that can be measured spectrophotometrically [142].

Recognizing the immense heterogeneity and variability by place and over time, new approaches are needed to understand how air pollution and cigarettes share so much convergent pathology (Table 1B and Appendix 1). We see two major goals in analyzing the human impact of airborne PM. *First*, better information is needed to formulate policy on air quality control. *Second*, we must better understand cell-type specific and systemic responses to various PM components. Some suggest that more comprehensive profiles of cell responses using the ever-expanding ‘omics’ to better define short-term and long-term consequences of exposure. This approach will certainly give a massive amount of new data, but seems unlikely to us to yield actionable hypotheses. The half century of efforts to identify ‘the leading carcinogens and toxicants in CS shows that the bioactivities of individual chemicals does not predict body-wide responses. We propose three systematic and mechanistic approaches to assay the epidemiological associations of AAP, CS, HAP and potential synergies:

I, Cell assays for signaling mechanisms should use the cell types that are the first to encounter inhaled PM. Airway and alveolar epithelial cells, and macrophages seem the best choices. Nasal epithelium includes olfactory neurons that can be cultured. Analysis could include an informed ‘omics’ approach that targets pro- and antiinflammatory responses, rather than a broad undefined search. The lung epithelial cell responses to dung smoke in Section 3 document the value of an expanded panel of inflammatory and redox gene expression responses. We suggest that cell inflammatory responses will prove more fruitful for comparing PM activities than some chemical assays. Synergies (Table 1A) urgently warrant study because of the global importance of household air pollution (Table 1A). For comparison with collected ambient PM, we suggest exposure of cells to PM of known surface composition that represents the prime suspects for toxicity, particularly PAH and transition metals. The composition of the defined particles should include assessment of its surface composition. Ambient PM should be chosen from diverse sites to represent a range

of surface particle components. We recommend against reporting correlations that have no biologically relevant chemical basis, as discussed for non-reactive metals in oxidative assays.

II, The animal models should be expanded from the rodent base to include small primates, such as the marmoset which are closer to humans in patterns of aging [143]. New in vivo studies are needed to address the key gap of how ambient PM can impact brain and the embryo, despite the small traces of particles or chemical components that leave the lungs, skin or eyes. We ask, could preventing the transmission beyond the lung be a practical approach defense against air pollution for people trapped in highly polluted, poorly regulated environments.

III, Air quality regulations should also consider the demonstrated pro-inflammatory effects of the PM surface components, rather than only PM mass or bulk composition. We advise against reporting assay associations with elements that have no chemical reactivity. The irreducible conclusion remains: we must globally reduce exposure to airborne toxicants.

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Appendix 1

Hazardous associations of Ambient Air Pollution (AAP) & Cigarette Smoke (CS)

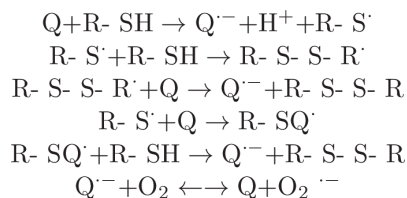
A. Human			
	AAP	CS	AAP-CS interactions; also see Table 1B
Atherosclerosis			
carotid	[144][145]	[148][149]	
coronary	[146][147]	[150][151]	[8]
Cancer			
lung	[152][153]	[154][155]	[17][9]
Metabolism			
insulin sensitivity, adult	[23][106]	weak or no association	
BMI, children	[10]		[105] [10]
Neurodegeneration of normal aging			
grey matter atrophy myelin atrophy	[156][157]	[159] [160]	
cognitive decline	[12][158]		[12]

A. Human			
	AAP	CS	AAP-CS interactions; also see Table 1B
Alzheimer's disease	[154][161][162]	[163][164][165]	
Stroke	[166][167]	[168]	

C. Rodents			
	AAP	CS	AAP-CS interactions
Atherosclerosis, aorta	[156][169][170][171][172]	[173][174]	No reports
Metabolism			
insulin sensitivity	[175]	[176]	
Neurodegeneration			
Neurite atrophy	[177]	[178]	
Alzheimer's transgenic mouse	[158]	[179]	
Stroke	[180]	[181][182]	

Appendix 2

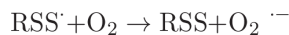
The proposed reaction scheme for the oxidation of DTT through catalysis by quinones [32].



where Q is a quinone, R-SH is a thiol and Me₂SO is dimethylsulfoxide. Superoxide then rapidly dismutates to hydrogen peroxide and oxygen:



As the authors showed, the series of reactions actually requires proximal thiols to proceed. Indeed, reaction 2 would rapidly occur within a single DTT molecule, R(SH)₂, far more rapidly than between two separate thiols. More importantly, reaction 3 would be very slow compared with the reaction of RSS[•] with O₂ [183]:



Thus, the authors own evidence suggests that a decrease in DTT by a quinone does not occur through this mechanism.

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Highlights

- Multiple pollution sources show synergies in disease risk, but assays are lacking
- Assays for air pollution toxicants may misinterpret oxidant activity
- Chemical and cell-based assays often show poor correspondence
- New strategies are needed for cell responses to complex toxicants

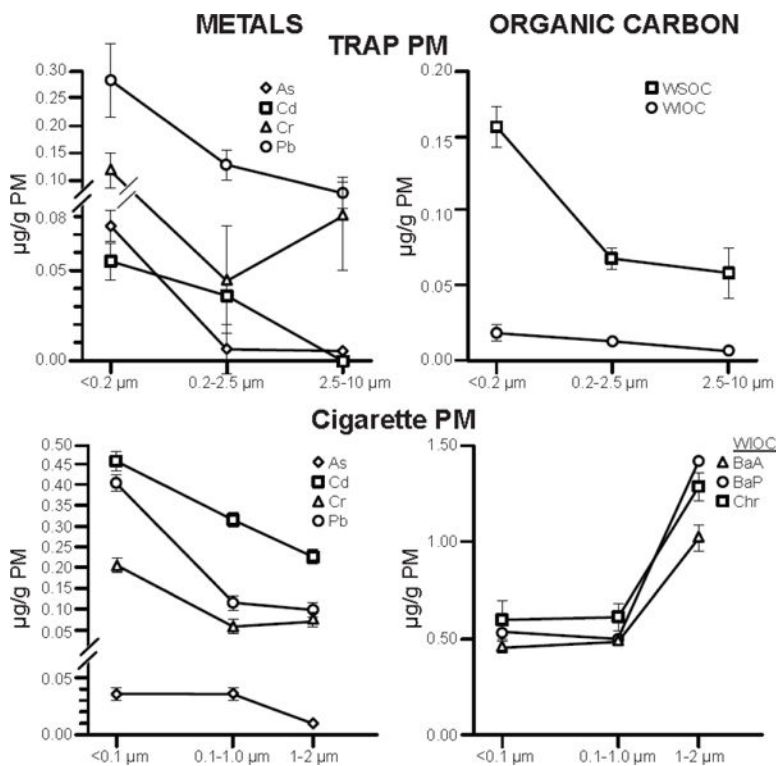


Figure 1.

Chemical composition of traffic related air pollution (TRAP) vs cigarette smoke (CS) by PM size class. Note differences between the larger size classes of TRAP vs CS.

PAH, polycyclic aromatic hydrocarbons; BaA, benz(a)anthrocene; BaP, benzo(a)pyrene; Chr, chrysene; WSOC, water-soluble organic carbon; WIOC, water-insoluble OC.

TRAP PM: From Los Angeles County, 2013-2014; WSOC and WIOC are averaged across sampling sites from Fig 2 of Hu et al. [25]; metal levels obtained by Dr. Arian Saffari from data of [26][27]. WIOC includes PAHs, n-alkanes, hopanes, and steranes, WSOC includes n-alkanoic acids, resin acids. These data resemble Tokyo samples which had 2-fold greater PAH densities in ultrafine PM than fine PM [28].

Cigarette PM: From 3R4F reference cigarette; PM per cigarette, 10.5 mg. Unimodal distribution (mean 0.26 μm , range 0.02-5.9). Tobacco-specific nitrosamines and heavy metals are enriched in smaller PM; heavy metals, larger; nicotine evenly distributed. Redrawn from [29]. The size distribution confirms Li et al [30].

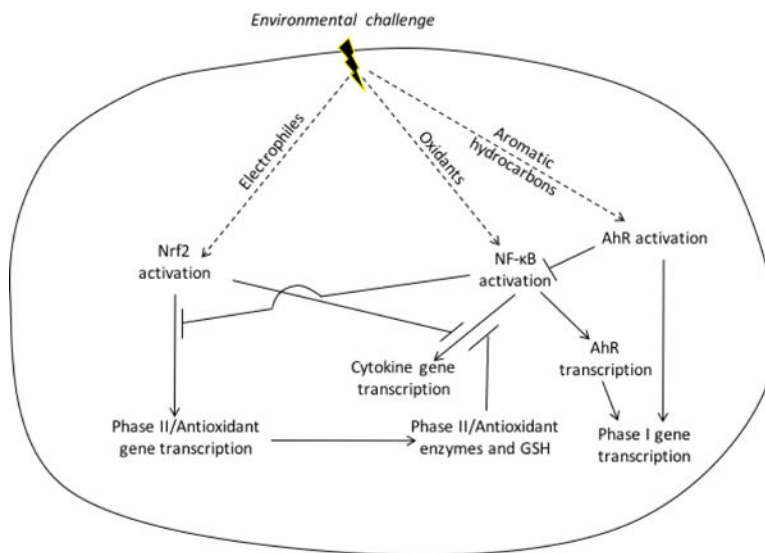


Figure 2. Schema of Nrf2, NFκB, AhR responses to airborne toxicants. AAP, DS, and CS induce antioxidant enzymes through electrophilic Nrf2 activation and NF-κB activation through oxidative signaling. Nrf2 activation is inhibited by c-Myc and Bach1, while NFκB activation is opposed by Nrf2-dependent antioxidant enzymes and glutathione. Also, there is non-redox dependent crosstalk by which NFκB inhibits Nrf2-dependent signaling and Nrf2 inhibits NF-κB-dependent transcription. This cross-talk includes antagonistic binding to DNA and interference with transcription through interaction with co-activators and repressors. AhR also forms a heterodimer with RelB, which enables cross-over regulation of NF-κB regulated genes.

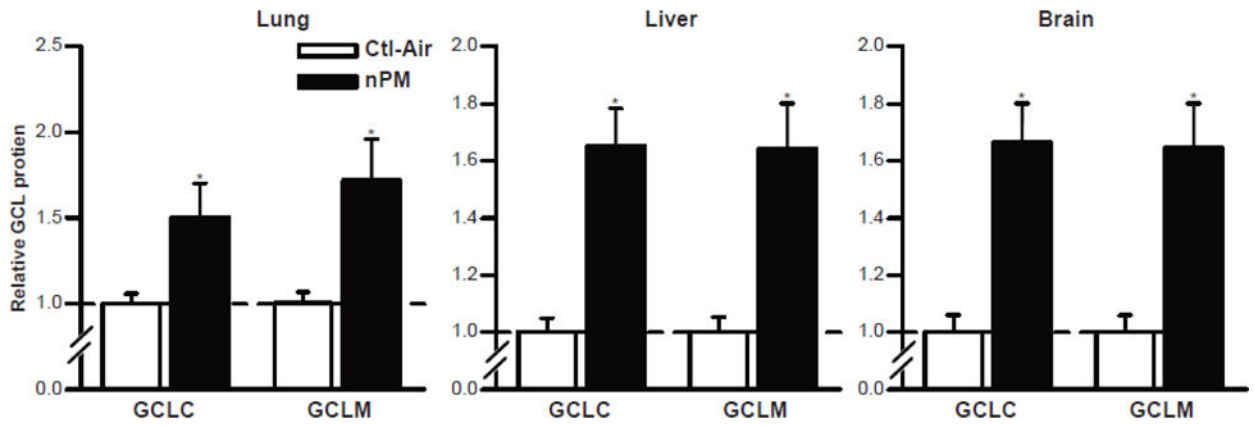


Figure 3.

Induction of Nrf2-dependent phase II detoxifying enzymes in young mice exposed chronically to defined levels of nPM, a water-soluble subfraction of ultrafinePM [135]. The graph shows protein levels of glutamate-cysteine ligase (GCL) subunits GCLC (catalytic) and GCLM (modifier), induced to 50-60% in all three organs.

Table 1

Airborne PM Hazards

A. Global premature mortality attributed to airborne PM	
	Annual excess mortality, millions (M)
Ambient air pollution (AAP)	4.2M [5]
Household air pollution (HAP) includes smoke from coal, dried dung, and wood	4.3M [6]
Cigarette smoke (CS)	
Direct	6.4M [7]
Second-hand	0.65M
Total premature deaths from airborne toxicants	15 M

B. Synergies of AAP and CS		
	Study	Synergy (fold-excess above additivity)
Cardiovascular mortality	ACS Prevention Study II: 429,406 adults [8]	1.1-fold excess
Cancer of lung	ACS Prevention Study II: 1.2 million adults [9]	2.2-fold excess
Body mass index (BMI)	Southern California Children's Health Study: 3318 children, ages 10-18 y [10]	1.3-fold excess
	Meta-analysis of 12 studies: 109,838 mother child pairs [11]	1.6-fold excess
Neurodegeneration cognitive decline	Health and Retirement Survey, 2004: 18,575, age 50 years [12]	1.9-fold excess

Table 2

PM10 of Chinese cities vs smoke from cigarette, fossil fuels, & wood

A. Composition						
	Beijing AAP	Wuwei AAP	Cigarette	Coal	Diesel	Wood
WSOC, mgC/g PM10	3.9	1.5	220	11	32	250
WIOC, µg/mL anthracene, benzo[a]pyrene, chrysene	0.8	31	140	3.9	3.8	0.5
	0.24	0.56		7.1		
	1.1	7.2	4.8	22	5.3	0.25
endotoxin (LPS), µg/g PM10	1.2	8.3	1.5	1.2	1.3	0.8
Cu, µg/g PM10	20	91	16	32	31	4
Fe	800	17,000	1,200	2,000	1,700	100
Ni	3	44	7	16	10	1.5
Pb	70	98	7	85	31	6.3
Zn	236	378	184	195	178	35

B. Oxidative and Cytokine Responses						
	Beijing	Wuwei	Cigarette	Coal	Diesel	Wood
Chemical assay	60	40	60	40	130	60
DTT oxidation, µmol/min µg PM10						
Cell assays ED50						
DHE, fluorescence	500	500	750	550	550	1000
TNFα, ng/ml media	11	8	4	4	3.8	1.8

From [21]; numbers are rounded. Coal, anthracite; pine wood, burned in cast iron stove; endotoxin by *Limulus* amoebocyte assay; cell assay ED50 estimated from Figs 1 & 2; DHE, dihydroethidium mean fluorescence intensity (MFI) in mouse macrophages (RAW264.7); TNFα, assayed in media of RAW264.7 cells.

Table 3

Correlation of metals vs oxidative activity in assays of biological reductants

	Ascorbic acid assay (AA)	Glutathione assay (GSH)
Antimony	0.27	0.05, p<0.01
Copper	0.29	0.64, p< 0.01
Iron	-0.56, p <0.01	-0.14
Nickel	-0.05	-0.12
Lead	0.40, p<0.05	0.34, p<0.05
Zinc	0.05	0.21

Adapted from Moreno et al [39]. Entries without P values were not significant.

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