

Department of Health Commissioned Review

Is there a threshold for ozone-induced pulmonary responses in healthy adults?

Ian S. Mudway & Frank J. Kelly

School of Health & Life Sciences, Franklin-Wilkins Building, 150 Stamford Street

Kings College, London, London SE1 9NN, UK

Address correspondence to:

Professor Frank J. Kelly

Environmental Research Group

School of Health & Life Sciences

King's College, London

150 Stamford Street

London SE1 9NN

Tel (44) 207 848 4004

Fax (44) 207 848 3891

Email: frank.kelly@kcl.ac.uk

ABSTRACT

Objectives: Exposure of healthy subjects to ozone results in a range of transient symptoms including lung function decrements and airway neutrophilia. Whilst the relationship between ozone dose and lung function decrements has been well defined, that for ozone-induced inflammation remains unresolved. To address whether a simple exposure-response relationship exists we performed a meta-analysis of the ozone-induced inflammatory responses that have been reported in human controlled chamber studies. Our aim in doing so was to determine whether a threshold dose of ozone exists below which inflammation is insignificant.

Methods: Analyses were based on a comparison of the percentage of neutrophils recovered by bronchoscopy based lavage in response to a set dose of ozone, defined as the product of ozone concentration (ppm), exposure time (minutes) and minute ventilation ($L/min/m^2$) – CVT. Analysis included data from 21 peer-reviewed publications. All subjects were healthy non-smoking adults (18-40 years) exposed to ozone (0.08-0.6ppm) and filtered air. Exposures varied between 1.0-6.6h, with exercise performed at minute ventilation rates (V_E) of 14.8-35.0 $L/min/m^2$. Exercise duration varied between 48.2-100% of the total exposure period.

Results: Following air challenge the proportion of BAL fluid neutrophils across all studies at the late (18-24h post exposure) time point was found to be 2.2 (1.5-2.9%) {mean (95%CI)}. A significant linear association ($r^2=0.42$, $p<0.05$) was observed between the ozone-induced neutrophilia and inhaled dose (CVT) with the regression line cutting the upper confidence interval of the control neutrophil level at a CVT of 623.3. A similar association was also observed when the early acute responses were

examined in the distal lung lavage samples: CVT1 vs %PMN ($r^2=0.64$, $p<0.01$ – threshold 512).

Conclusions: These data suggest a threshold for ozone-induced inflammation at a CVT of 500-620. This threshold is below that reported for lung function decrements, but still within the current legislative limits (0.08ppm, 8h average), which at a ventilation rate of 18 L/min/m² gives a CVT of 536. These data suggest that advice aimed at regulating outdoor activity during air pollution episodes will be more beneficial than further reductions in peak concentrations.

INTRODUCTION

Ozone is both a source of protection and risk for all life forms on Earth. The majority of ozone is found in the stratosphere where it plays an important role in preventing harmful ultraviolet radiation from reaching the surface of the earth. In contrast, ozone present within the lower troposphere (ground level to 10 km) is detrimental to health as breathing it can cause a range of deleterious responses in the lung. Tropospheric ozone is formed from a complex mixture of oxidant compounds derived from the interaction of primary pollutants (volatile hydrocarbons, halogenated organics, oxides of nitrogen) predominately derived from motor vehicles with sunlight irradiation and high temperatures. Baseline concentrations of ozone vary between 20-40 ppb depending on seasonal and geographical factors. Importantly, epidemiological studies have demonstrated measurable effects at, or just above, background concentrations. In contrast, the magnitude of difference between baseline and toxic levels of other air pollutants such as sulphur dioxide and nitrogen dioxide are in the order of 1000 to 10,000-fold.

Air quality standards are set based on the integration of data from epidemiological, clinical and animal toxicological disciplines. Epidemiological studies demonstrate strong associations between health endpoints (eg hospital admissions for respiratory disease, and primary care consultations for asthma) and elevated daily ozone concentrations. However such studies have important limitations as they cannot establish causation or identify threshold exposure concentrations. Indeed, many epidemiological studies suggest no threshold exists (a concept difficult to understand in toxicological terms) and demonstrate measurable effects just above ambient

background concentration. Whilst background concentrations of ozone, due to mixing between the stratosphere and the troposphere, have increased marginally over the last hundred years, human evolution continues against this natural background. It therefore seems illogical to suggest that ozone at these concentrations is detrimental to health. Therefore the issue of whether a threshold concentration exists, below which the pollutant has no effect on population health, is of key importance when considering ozone. If such a threshold could be identified, no additional public-health benefits would be expected from bringing ozone concentrations below this level.

Whilst the literature contains a wide range of controlled human exposures to ozone, the vast majority of these studies have focused on the acute changes in lung function seen immediately after ozone inhalation [1, 2]. Because these measurements are easy to perform large numbers of subjects have been included in these studies making it possible to examine the relationship between lung function decrements with ozone concentration and total inhaled dose in considerable detail [1-3]. Although often considered as a detrimental response to ozone, lung function decrements if acute and reversible are actually protective as they reduce the dose of ozone entering the lung. In contrast, ozone-induced pulmonary inflammation is a more damaging response as repeated exposures do not appear to resolve tissue inflammation [4] which may result in accumulative damage. Unfortunately, there are considerably fewer studies where ozone-induced pulmonary inflammation has been assessed. Further, as these studies have been fairly heterogeneous in design little attempt has been made to investigate dose-response relationships, beyond those made by specific groups who have examined responses at two doses [5, 6].

In this meta-analysis we have integrated findings from 21 controlled human ozone exposure studies in which bronchoscopy based lavage was employed to assess acute airway inflammatory responses. Using this approach we have sought to establish whether an exposure-response (E-R) relationship exists, as has been demonstrated for acute lung function decrements [2, 3] and hence whether it is possible to identify a non-inflammatory or 'safe' dose of ozone.

METHODS

Study selection criteria: Only randomised, air controlled studies were considered eligible for inclusion. Double-blinded studies were preferred, but single-blinded and open studies were also reviewed for possible inclusion.

Study populations: All data used in the current analysis was based on the exposure of healthy human subjects (18-40y – with a clear male to female bias) free of acute and chronic respiratory disease. All subjects included in these studies gave full and informed consent and underwent detailed medical exams prior to inclusion. All subjects used in this meta-analysis were non-smokers (predominately non-allergic) and in all studies subjects were asked to refrain from taking antioxidant supplements and painkillers for the duration of the study. Where subjects were exposed on two separate occasions (control air and ozone) intervals of between 2-4 weeks between successive exposures were the norm, to limit carry-over effects due to the exposure, and / or the bronchoscopy procedure.

One study included in these analysis addressed inflammatory responses in subjects classified as responders (>15%), or non-responders (<5%) based on the magnitude of their FEV1 decrement following ozone exposure [7, 8]. These two groups were otherwise healthy subjects and their data has been included in these analyses. However on the basis of their pre-selection their data are treated separately. Smokers [9], asthmatics [7, 10, 11], subjects taking antioxidant supplements [12] were excluded from these analyses but healthy control data from these studies were included where paired ozone and air exposure data was available. In the case of study 13 (c. **Table 1**) the control group had received a placebo compound for a 14 day

period, following a period of dietary restriction. However, as neither intervention had a significant impact these data were included in the full analysis.

Exposure conditions: Ozone concentrations ranged from 0.08 – 0.6 ppm, with exposure duration's of 60-396 min. The majority of exposures were conducted in whole body chambers maintained at room temperature (approximately 20-25°C) and supplied with humidified air, though ozone was also supplied through mouth pieces in a number of studies [9, 13] and also via a helmet assembly [4]. With the exception of the mouthpiece exposures subjects were allowed to follow their normal breathing patterns. Work rate ranged from 14.8-35L/min/m² body surface area. In the majority of studies subjects were given no instructions to breath nasally or orally, In this study we have classified exercise as: rest <10L/min/m², light, 20-30 L/min/m², light and 30-40 L/min/m², heavy. In all studies, with the exception of that by Schelegle et al and Hazbun et al [14, 15], subjects alternated between exercise and rest, however the duration of exercise within the studies varied considerable, c. Table 1. For the analysis of E-R relationships the data were separated into early (0-6hPE) and late responses (18-24hPE). There are, to the best of our knowledge, no studies covering the intermediate period.

Dose: Two separate dose metrics were used in this study: CVT1, and a CVT2. The former was based on the exposure concentration of ozone, indexed to exposure duration and the breathing rate of the exercising subjects: concentration (ppm) x time (minutes) x minute ventilation (L/min/m²) – CVT1. The second expression was based on the first expression corrected for variations in the proportion of exercise performed by subjects during the exposure period. These two values for each of the studies cited

are illustrated in Table 1. Both of these expressions are effectively dimensionless but the CVT1 expression may be of some use in comparing responses to theoretical exposures. The CVT2 expression should not be used in this way. It is always likely to be half of CVT1 as in the vast majority of chamber studies exercise is performed for 50% of the exposure period. It's use here is only to demonstrate whether the duration of exercise is a significant modifier of the CVT1 expression.

Bronchoscopy procedure: A number of groups have performed multiple human exposure studies with bronchoscopy based lavage and whilst internally within these studies the lavage technique is highly consistent, between groups there is much variation. In the exposure studies carried out by the US EPA (studies 2,3,6,13, 14, and 16 in Table1) the lavage technique is as first outlined by Koren et al, 1989 [16]: instillation of 50ml x 6 sterile saline into the segmental/sub-segmental bronchus of the lingula, followed by a repeat procedure in the right lobe (total instilled volume 600ml). The returns from both sets of installations were pooled to generate one large distal lung BAL fluid sample, with a reported recovery efficiency of approximately 75%. None of these studies have addressed responses in the more proximal airways. The Group of Professor Thomas Sandstrom at Umea, Sweden, has also performed multiple exposure studies (18, 19, 20, and 23 in Table 1) with a consistent lavage method as outlined: proximal airway sampling by instillation of 2x 20ml of sterile saline in either the lingula lobe bronchus or middle lobe bronchus (the return of the first instillation being used for cell counts), followed by a distal airway lavage of 3 x 60ml, with alternating sampling sites between exposures [17] (total instilled volume 220ml). Proximal and distal airway samples are also obtained by the group of Professor Mark Frampton (study 7 in Table 1) labelling the return of the first 50ml

saline instillation of 4, the bronchial fraction at both the inferior segment of the lingula and the right middle lobe (total instilled volume 400ml). A similar approach is also adopted by the group of Balmes (studies 8 and 12), but with the innovation that in certain studies [7, 18] a peripheral airway lavage is performed using a modified balloon catheter to isolate specific airway segments. Here the PAL sample is considered separate from the bronchial fraction, usually the first 10ml return of the 4x60ml saline installations. All of the remaining studies can be broadly classified into those in which a large volume of saline is instilled (into the lung segments outlined above) in aliquots and then pooled to obtain a representative distal lung sample [4, 6, 13, 19, 20]; or those in which a small (and variable) initial volume of the first instillation is retained separately from the remaining aspirations [14, 21]. In this later case this small volume sample is thought to reflect a more proximal airway sample.

Assessment of airway neutrophilia: Total and differential cell counts were performed in all studies using standard methods, usually based on counts of not less than 300 cells per slide. To avoid complications with the variable dilution we elected to use the percentage of lavage fluid neutrophils as our response endpoint. In all cases the % of neutrophils was relative to total leukocytes, not total cells. Lavage fluid may contain significant numbers of airway epithelial cells.

Assessment of altered epithelial permeability: Total protein and albumin were determined using a range of standard methodologies.

Data abstraction: For studies where the original data (or a useful data summary) was not presented, when possible they were extracted from graphical presentation using

image capture and scaling software. Where data was only alluded to in text, attempts were made to contact the study authors to obtain data directly. All data were expressed as mean (SE). As above where data was summarised using non-parametric expressions raw data was obtained and re-analysed.

Data Analysis: Data was classified as being obtained from either the early or late acute response, and also with respect to the generic airway sampled, proximal or distal. To identify threshold concentrations, plots of mean (SEM) neutrophil percentages after both air and ozone versus the CVT1 and two dose expressions were produced. Using the mean control data in each of the selected studies, an overall post air %PMN was derived which was represented as the overall mean and 95% confidence intervals. A linear regression was then performed only on the post ozone data with the intercept point between this regression line and the upper 95% CI of the overall control post air %PMNs thought to reflect the upper boundary of a significant effect at the 5% level. It was assumed that whilst significant increases in neutrophil numbers may have occurred at CVT doses below this point, if the increased value post-ozone fell within the overall control range it was not biologically significant. To investigate whether elevated post air %PMNs could effect these interactions, i.e. proportionate overall responses at different doses which are given an inappropriate weighting as they occur from an enhanced baseline, correlation were performed using the absolute change in PMNs (ozone-air) versus dose. The strength of these interactions was tested using Pearson's correlation.

RESULTS

TEMPORAL PROFILE OF ACUTE OZONE-INDUCED NEUTROPHILIA

As is shown in **Table 1** bronchoscopy with distal and proximal lavage was performed at a range of time points ranging from immediately post exposure (in reality, given lung function testing and pre-medication for the bronchoscopy procedure between 0.5 – 1.0h) to 24h post challenge. Given that airway inflammatory responses are progressive, including both induction and resolution phases, we felt it was important before segregating studies for dose-response analysis that we had an understanding of the temporal profile of the response. This issue has been investigated in four studies [9, 14, 22, 23], although each only considered a limited number of time points. The findings of these studies indicate that in terms of neutrophilic inflammation there is a rapid induction between 1 and 6h, with persistent inflammation still detectable at 18-24h post exposure. To address this issue in a meta-analysis we used the CVT1 expression to divide the data into high (>1000) and low dose (<1000) studies. The data expressed as the absolute change (mean % post ozone minus the mean % post air) in the proportion of neutrophils due to ozone challenge are shown in **Figure 1** for both distal (BAL fluid) and proximal lavage (bronchial/airway lavage) samples. Proximal airway lavage (PAL) samples [8, 18] were not included in this analysis. The distal lavage neutrophil data, which included information from all of the 23 ozone exposures in **Table 1** indicated a rapid induction of neutrophilia in the high dose studies, peaking at 3h (the time point being based on a single study only [6]). A similar pattern was observed in the low ozone dose studies with a peak at three hours though again this was based on a single study [20]. The temporal response in the distal airways was less clear, compounded by the lack of proximal airway data. The

low ozone dose studies seemed to suggest a peak at the 6h post challenge time point, with a slower resolution of the neutrophilia at the 18 and 24h time points compared with the distal airway response. The response at the high ozone dose in the upper airways in contrast appeared very rapid, with evidence of a significant response at the 0-1h time point. This level of response persisted through to the 18-20h time point and indeed there was some evidence that the response was still increasing at this time. At 24h, the neutrophilic response appeared to attenuate, although again this was in the context of the data at this time being derived from a single study [21]. On the basis of these results we decided it was rational to examine the ozone dose – neutrophil response over two defined periods in both the upper and lower airway samples. First the early, defined as between 0 and 6h post exposure, and second as the late response, between 18-24h after the end of the challenge period. There are to the best of our knowledge no human ozone exposure studies that have examined responses between these two periods.

LATE ACUTE OZONE RESPONSES IN THE DISTAL LUNG

The relationship between inhaled ozone dose and the neutrophilic response in the distal airways at the ‘late’ time point is illustrated in **Figure 2**. This analysis is based on 12 published studies. Using the CVT1 dose expression, uncorrected for variations in exercise duration during exposure, a significant linear relationship was observed with the proportion of neutrophils observed after ozone challenge: $r^2=0.42$, $P=0.02$. This relationship was strengthened when the CVT2 expression was substituted: $r^2=0.55$, $P<0.01$. In the data set used only one of the selected studies gave results that deviated significantly from the overall trend line. The study by Christian et al [19] investigated the impact of multiple day exposures on a range of ozone endpoints, but

gave a remarkably high level of neutrophilia after the single day ozone challenge ($16.4\pm 3.0\%$). This data was therefore omitted from the analysis, a decision also based on the absence of a randomised air control exposure in this study. Interestingly, the attenuated neutrophilic response observed in this study after 4 consecutive days of ozone challenge fell very near ($7.6\pm 1.3\%$) to the regression line.

In both of these analyses the proportion of neutrophils appearing in the airways after ozone exposure was compared against the parallel air exposure data. It should be noted at this point that we saw no evidence that control neutrophil numbers were affected by exercise duration during the air exposures. Using these mean data we calculated the overall air control neutrophilia across all of the studies. For distal lavage samples at the 18-24h time point this gave a control neutrophil value of 2.2% of total leukocytes with a 95% confidence interval of 1.5 – 2.9%. We reasoned that the point at which the upper 95% CI was cut by the CVT1 – ozone induced neutrophilia regression would therefore represent the threshold of the ozone-induced response. This analysis revealed the CVT1 threshold to be 623. When the overall neutrophil response was examined (%PMNs after ozone minus the % post air) the significant associations with ozone dose remained indicating that differences in post air challenge neutrophilia were not biasing the response.

EARLY ACUTE OZONE RESPONSES IN THE DISTAL LUNG

The early (0-6h) neutrophil responses in the distal airways are summarised in **Figure 3** and this is based on the results of 13 peer reviewed studies. Given the data presented in **Figure 1**, demonstrating the rapid induction of neutrophilia over this time course, it did not appear likely that a strong relationship between ozone dose and response

would be seen. Despite this, significant associations were observed using both the CVT1 ($r^2=0.55$, $P<0.01$) and CVT2 ($r^2=0.38$, $P<0.01$) expressions. As the majority of these studies actually examined airway responses at 1-2h post exposure we repeated these analysis without the data from the few studies with lavage performed between 3-6h [6, 11, 13, 14, 20], for the reasons outlined above. As two of these studies included time points less than 3h [14, 20] the number of studies considered in this reduced analysis was 9. Removal of data from these later time points did not significantly affect the strength of the underlying CVT-response relationship: for CVT1 ($r^2=0.64$, $P<0.01$) and CVT2 ($r^2=0.41$, $P<0.01$). Consequently CVT threshold value was calculated using the full data set and an average air control neutrophil percentage of: 1.5 (0.9-2.1)% [mean with 95CI], as 512 (CVT1). As with the late responses in distal lavage samples the underlying relationship between ozone dose and neutrophil response remained when the ozone-induced neutrophilia was corrected for the control air values.

LATE ACUTE OZONE RESPONSES IN THE PROXIMAL LUNG

The number of human chamber studies where inflammatory responses in the proximal airways have been considered are limited. In addition, where such studies have been undertaken the range of techniques employed are considerably more heterogeneous than for distal airway lavage. With this limitation in mind, the analysis outlined above was repeated on this more limited data set and the results are summarised in **Figure 4**. In these figures all of the available data are shown, irrespective of whether the upper airway lavage fluid sample consisted only of the first return from the first lavage instillation or was obtained using proximal airway lavage to isolate specific airway segments. The analysis on these data sets however excluded such samples as they

have considerably higher air control neutrophil numbers and also because the number of such studies are very limited. Of note, the data derived from the study by Aris et al [18] are based on the BAL1 bronchial sample, not the PAL sample, which is described as being very similar to their BAL2 (distal lung) sample. Initial comparison of the CVT1 – neutrophil relationship did not reveal any strong relationship at this late time point: $r^2=0.26$, $P=0.16$. Overall neutrophil numbers post air were 6.02 (2.24-9.79)% [mean with 95CI]. It was clear from this data that the bronchial wash neutrophil number observed in the study by Stenfors et al [24] post air was abnormally high. The analysis were therefore repeated omitting this study but this did not effectively improve the strength of the association: $r^2=0.40$, $P=0.13$. Notably, correcting the response to allow for the post air neutrophilia did not reveal any underlying association between CVT and ozone-induced neutrophilia in these studies. Using the regression through the CVT1 data set, omitting study 20, gave a dose threshold of 576. The use of the CVT2 dose expression also revealed the absence of a simplistic relationship.

EARLY ACUTE OZONE RESPONSES IN THE PROXIMAL LUNG

Only 5 studies were available which examined neutrophilic responses in the proximal lung between 0-6h post exposure. The data from these studies are illustrated in **Figure 5**. Considering the relationship with the CVT1 dose expression first, there appeared to a qualitative, though not significant relationship ($r^2=0.29$, $P=0.21$) between dose and neutrophilia. To ascertain whether any underlying association was blunted by the range of responses observed over the six hour post exposure period these analysis were repeated focusing on studies sampling the airways less than 3h post exposure. Whilst this did not yield a statistical significant result the strength of the association

did improve: $r^2=0.41$, $P=0.23$. A similar improvement was seen when the neutrophil response was corrected for post air values, with a highly significant association in this restricted group of studies: $r^2=0.86$, $P<0.05$. Similar, though less robust observations were made using the CVT2 dose metric. If these regressions were used to calculate CVT dose thresholds, with the proviso that the number of studies limits the utility of such analysis, then the CVT1 threshold would be: 378, based on a control neutrophil level of 5.05 (2.27-7.83)%.

DISCUSSION

Ozone is unique as an air pollutant as it appears to have biological effects at, or a little above, background concentrations. Given this, there is uncertainty if a true threshold concentration exists below which no additional public-health benefits would be expected from bringing ozone concentrations below this level. To consider this issue we examined a cross section of the human studies performed over the period of the early 1980s to the present day. Prior to comparing these studies we felt it important to identify a measure of the total delivered dose of ozone breathed by a subject.

Inflammatory responses have been observed in studies employing low ozone concentrations (0.08 and 0.10 ppm) [5]. Notably though, although the ozone concentration was low, both the duration of exposure and the work rate performed by the exposed subjects were high. Consequently, when considering responses across different studies an expression for the cumulative dose is required. As the dose of ozone reaching the lung tissue cannot be measured surrogate measures must be employed to estimate the inhaled dose including factors for the actual ozone concentration used (C), the duration of exposure (T) and the rate of subject ventilation (V) [25]. Variations in the CTV expression, giving different weightings to the various components have been proposed [26, 27] but in this study we used the former expression to permit comparison of the effects across different studies with or without a weighting for the duration of exercise in each of the exposure periods. Clearly this represents an over simplification, making little allowance for individual differences in delivered dose due to the variations in subject breathing pattern, exercise tolerance or method of breathing e.g. nasal versus oral breathing. It does however permit some comparison of dose that is more informative than simply comparing responses at absolute concentrations. The value was based upon 'C', expressed as ppm, 'T', in

minutes, and 'V', expressed as L/min/m² body surface area. Recent studies have attempted to estimate the fractional uptake (the amounts of ozone absorbed) of ozone during a single breath in healthy human subjects (0.2, 0.4 ppm for 0.5 and 1h whilst performing light to moderate work loads 10 and 20 L/min/m² BSA). The concentration of ozone, exposure duration and minute ventilation all have been found to have a marked effect on ozone absorption.

To consider this problem in more detail we examined a cross section of human studies and related their CVT to the pattern of response observed (**Table 1**). At the lowest dose examined (CVT, 288[28]) subjects were largely asymptomatic. To our knowledge this study represents the lowest dose delivered to human subjects in a controlled chamber, single pollutant exposure study. As stated above lower concentration studies have been performed at 0.08 ppm [29] [5]) and 0.1 ppm [5]) but here the CVT values were greater 480, 634 and 792 respectively, for the reasons outlined previously.

The relationship between lung function decrements and dose has already been studied in some detail by McDonnell and co-workers [68; 72; 63]. In these studies initial lung function decrements have been observed down to a dose of 585 [1] and projected in modelling experiments to occur below this level [2, 3]. Indeed decrements and evidence of significant FEV1 decrements as well as small airway narrowing has been observed experimentally at a CVT of 480 [17], though were absent at a CVT of 360 and 288 [13, 28]. This would suggest a dose threshold for lung function decrements and increased airway resistance at a cumulative dose between 360 and 480 in normally responsive subjects. As these studies relate to changes in lung function they have not been considered in any further detail in this report.

Evidence to support the existence of a threshold level for the inflammatory and permeability responses is considerably less clear. The majority of studies addressing these responses have sampled the airways at 6-18 hours post exposure and have used relatively high cumulative doses (CVT 540-1680), either as short duration, high O₃ concentration [6, 16, 23], or long duration low O₃ concentration exposures [5, 18]. To address this issue we therefore undertook a meta-analysis of the controlled human exposures that have been performed to date, focusing on those studies where pulmonary inflammation and epithelial injury have been assessed in lavage fluid samples obtained from bronchoscopy. Whilst there are many studies which have addressed pulmonary inflammation using a range of indirect methods we choose not to include these as we felt that these methodologies were insufficiently established.

In preparing this report a number of important caveats and assumptions have been made.

1. We have focused solely on bronchoscopy based human studies and not utilised data from other human studies where pulmonary inflammation has been assessed using less invasive methodologies such as, induced sputum, exhaled NO and breath condensate [30]. The decision to exclude these studies was based upon the difficulty of integrating their data into our analysis. We believe that bronchoscopy-based lavage still represents the gold standard for assessing distal and proximal airway inflammation. Whilst, some studies have attempted to validate indirect methods against bronchoscopy based lavage data these are in our opinion rather preliminary and under-powered. Whilst these less invasive methods are easier to perform and would permit, once validated against traditional methods, detailed dose-inflammatory response relationships

to be drawn, to date, they have only been used in high dose studies where bronchoscopy based data is already available.

2. Bronchoscopy-based lavage is a very invasive technique. Consequently in all of the studies cited only a single time point could be studied following both ozone and filtered control air exposures. Thus one is presented with a snapshot of the inflammatory response in the lung at that time, typically either early or late post exposure. This presents a problem in terms of allowing for the temporal progression of the response in any E-R relationship. Stated simply, the absence of an effect in the immediate post exposure period does not exclude the possibility of a delayed response. In order to overcome this problem we adopted two approaches. First, in the E-R analysis the available data set has been separated into early (0-6h PE) and late responses (12-24h PE). Second, whilst it is true that only a single time point can be examined, in many studies a range of inflammatory markers have been measured (pro-inflammatory cytokines protein and mRNA and adhesion molecules) permitting the observed responses to be placed into some form of overall inflammatory context.
3. A further problem in this type of analysis is that different research laboratories have adopted widely differing lavage procedures. Consequently there is a range of issues relating to differing dilution effects as well as differences in the sampled sites within the lung to be considered. To overcome dilution issues, as well as differences in the methodologies utilised to assess various inflammatory parameter we have focused on examining the proportion (%) of PMNs recovered in lavage fluid after both control air and ozone challenges as

our inflammatory index. We have also considered lavage fluid total protein and albumin concentrations as a measure of altered epithelial permeability, though in this case we have expressed the response as the fold increase/decrease after ozone relative to post air values. Although there are wide differences in the lavage techniques employed in the different studies, the samples obtained can be viewed as reflecting the proximal or distal airway surface. The differences between these two samples will be addressed later in the discussion, but we have treated inflammatory data from these two compartments separately when examining the E-R relationship.

4. As the dose of ozone reaching the actual tissue cannot be easily determined surrogate measures have been employed to estimate the inhaled dose including factors for the actual O₃ concentration used (C), the duration of exposure (T), and the rate of subject ventilation (V) [25]. Variations in the CTV expression, giving different weightings to the various components have been proposed [26, 27] but we have used the former expression to permit comparison of the effects across different studies, with or without a weighting for the duration of exercise in each of the exposure periods. Clearly this represents an over simplification, making little allowance for individual differences in delivered dose due to the variations in subject breathing pattern, exercise tolerance, nasal vs. oral breathing etc. It does however permit some comparison of dose that is more informative than simply comparing responses at absolute concentrations. The value is based upon 'C', expressed as ppm, 'T', in minutes, and 'V', expressed as L/min/m² body surface area.

5. Human volunteer studies overcome problems arising in animal exposure models where there are always issues of interspecies extrapolation. However, human volunteer studies may not permit examination of responses in the most sensitive individuals due to ethical considerations. As a consequence the findings of this report relate only to healthy subjects within the population. There are also limitations with controlled chamber studies themselves. Subjects tend to be exposed to a single pollutant, mostly on a single occasion, not really modelling the 'real world' cumulative exposure to the cocktail of pollutants that are addressed in epidemiological studies. Whilst each of these approaches has inherent strengths and weaknesses the data from controlled human exposures do provide a unique resource and guidance on the health impact of individual pollutants.

These limitation aside we believed that there was sufficient data available from the human exposure studies to date to perform a limited analysis to try and address whether there is evidence of a clear threshold dose of ozone where inflammatory responses are attenuated.

In summary, these data arising from this meta-analysis suggest a threshold for ozone-induced inflammation at a CVT of 500-620 in healthy subjects. This threshold is below that reported for lung function decrements, but still within the current legislative limits (0.08ppm, 8h average), which at a ventilation rate of 18 L/min/m² gives a CVT of 536. We therefore conclude that advice aimed at regulating outdoor activity during air pollution episodes will be more practical and beneficial than introducing measures to further reduce peak ozone concentrations.

REFERENCES

1. McDonnell, W.F., et al., Pulmonary effects of ozone exposure during exercise: dose-response characteristics. *J Appl Physiol*, 1983. **54**(5): p. 1345-52.
2. McDonnell, W.F., et al., Ozone-induced respiratory symptoms: exposure-response models and association with lung function. *Eur Respir J*, 1999. **14**(4): p. 845-53.
3. McDonnell, W.F., et al., Prediction of ozone-induced FEV1 changes. Effects of concentration, duration, and ventilation. *Am J Respir Crit Care Med*, 1997. **156**(3 Pt 1): p. 715-22.
4. Jorres, R.A., et al., The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. *Am J Respir Crit Care Med*, 2000. **161**(6): p. 1855-61.
5. Devlin, R.B., et al., Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am J Respir Cell Mol Biol*, 1991. **4**(1): p. 72-81.
6. Seltzer, J., et al., O₃-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J Appl Physiol*, 1986. **60**(4): p. 1321-6.
7. Balmes, J.R., et al., Effects of ozone on normal and potentially sensitive human subjects. Part I: Airway inflammation and responsiveness to ozone in normal and asthmatic subjects. *Res Rep Health Eff Inst*, 1997(78): p. 1-37; discussion 81-99.
8. Balmes, J.R., et al., Ozone-induced decrements in FEV1 and FVC do not correlate with measures of inflammation. *Am J Respir Crit Care Med*, 1996. **153**(3): p. 904-9.
9. Torres, A., et al., Airway inflammation in smokers and nonsmokers with varying responsiveness to ozone. *Am J Respir Crit Care Med*, 1997. **156**(3 Pt 1): p. 728-36.
10. Basha, M.A., et al., Bronchoalveolar lavage neutrophilia in asthmatic and healthy volunteers after controlled exposure to ozone and filtered purified air. *Chest*, 1994. **106**(6): p. 1757-65.
11. Mudway, I.S., et al., Differences in basal airway antioxidant concentrations are not predictive of individual responsiveness to ozone: a comparison of healthy and mild asthmatic subjects. *Free Radic Biol Med*, 2001. **31**(8): p. 962-74.
12. Samet, J.M., et al., Effect of antioxidant supplementation on ozone-induced lung injury in human subjects. *Am J Respir Crit Care Med*, 2001. **164**(5): p. 819-25.
13. Krishna, M.T., et al., Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. *Eur Respir J*, 1998. **11**(6): p. 1294-300.
14. Schelegle, E.S., A.D. Siefkin, and R.J. McDonald, Time course of ozone-induced neutrophilia in normal humans. *Am Rev Respir Dis*, 1991. **143**(6): p. 1353-8.
15. Hazbun, M.E., et al., Ozone-induced increases in substance P and 8-epi-prostaglandin F2 alpha in the airways of human subjects. *Am J Respir Cell Mol Biol*, 1993. **9**(5): p. 568-72.

16. Koren, H.S., et al., Ozone-induced inflammation in the lower airways of human subjects. *Am Rev Respir Dis*, 1989. **139**(2): p. 407-15.
17. Blomberg, A., et al., Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. *Eur Respir J*, 1999. **13**(6): p. 1418-28.
18. Aris, R.M., et al., Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. *Am Rev Respir Dis*, 1993. **148**(5): p. 1363-72.
19. Christian, D.L., et al., Ozone-induced inflammation is attenuated with multiday exposure. *Am J Respir Crit Care Med*, 1998. **158**(2): p. 532-7.
20. Coffey, M.J., et al., Increased 5-lipoxygenase metabolism in the lungs of human subjects exposed to ozone. *Toxicology*, 1996. **114**(3): p. 187-97.
21. Weinmann, G.G., et al., Ozone exposure in humans: inflammatory, small and peripheral airway responses. *Am J Respir Crit Care Med*, 1995. **152**(4 Pt 1): p. 1175-82.
22. Koren, H.S., et al., Time-dependent changes of markers associated with inflammation in the lungs of humans exposed to ambient levels of ozone. *Toxicol Pathol*, 1991. **19**(4 Pt 1): p. 406-11.
23. Devlin, R.B., et al., Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: a comparison of mediators found in bronchoalveolar lavage fluid 1 and 18 hr after exposure. *Toxicol Appl Pharmacol*, 1996. **138**(1): p. 176-85.
24. Stenfors, N., et al., Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects. *Respir Med*, 2002. **96**(5): p. 352-8.
25. Silverman, F., et al., Pulmonary function changes in ozone-interaction of concentration and ventilation. *J Appl Physiol*, 1976. **41**(6): p. 859-64.
26. Hazucha, M.J., Relationship between ozone exposure and pulmonary function changes. *J Appl Physiol*, 1987. **62**(4): p. 1671-80.
27. Hazucha, M.J., L.J. Folinsbee, and E. Seal, Jr., Effects of steady-state and variable ozone concentration profiles on pulmonary function. *Am Rev Respir Dis*, 1992. **146**(6): p. 1487-93.
28. Krishna, M.T., et al., Short-term ozone exposure upregulates P-selectin in normal human airways. *Am J Respir Crit Care Med*, 1997. **155**(5): p. 1798-803.
29. McDonnell, W.F., et al., Respiratory response of humans exposed to low levels of ozone for 6.6 hours. *Arch Environ Health*, 1991. **46**(3): p. 145-50.
30. Nightingale, J.A., D.F. Rogers, and P.J. Barnes, Effect of inhaled ozone on exhaled nitric oxide, pulmonary function, and induced sputum in normal and asthmatic subjects. *Thorax*, 1999. **54**(12): p. 1061-9.

FIGURE LEGENDS

Figure 1: Temporal profile of neutrophilia observed in human proximal and distal airways following exposure to ozone. Data are presented as the overall change in lavage neutrophils (%post ozone – %post air) following high or low dose ozone challenges at various time points post challenge. Dose is defined using the CVT1 expression. Data represent the mean change (\pm SD) observed from a range of studies either at the same time point, or individually, where data is available from one study. Responses are classified as either early (0-6h post exposure) or late (18-24h).

Figure 2: Relationship between ozone-induced neutrophilia in the distal airways during the late acute response and ozone dose defined using the CVT1 and CVT2 expressions. Data are illustrated in the main figure (left hand) as the mean (SEM) % neutrophils after ozone (down triangles) or air (circles). Numbers above these data refer to the designated study number in **Table 1**. The overall average % neutrophils seen across all of the considered studies control air exposures is illustrated with a dotted line. The shading bordering this mean value represents the 95%CI. The solid lines represent the linear regression line passed through the % neutrophils after ozone only. The results of the linear regression are illustrated in the main body of the figure. Study ‘9’ was omitted from the regression analysis as the reported response deviated markedly from the overall trend line. The smaller figures offset to the right show the over neutrophil response corrected for the air controls (%PMNs post ozone - air) versus CVT1 and CVT2, with regression lines and results inset.

Figure 3: Relationship between ozone-induced neutrophilia in the distal airways during the early acute response and ozone dose defined using the CVT1 and CVT2 expressions. Details of the figure format are as outlined in the legend to Figure 2 with

the following amendments: The data highlighted in green indicate that more than one time point was considered (1 and 6h in study 17 and 0, 2 and 4h in study 5). The results of the regression analysis given in red are based only on the data with lavage performed prior to the 3h post exposure time point. The regression results in black refer to the whole data set.

Figure 4: Relationship between ozone-induced neutrophilia in the proximal airways during the late acute response and ozone dose defined using the CVT1 and CVT2 expressions. Details of the figure format are as outlined in the legend to Figure 2 with the following amendments: Although the PAL data is illustrated, the regression line and the analysis (black box) have excluded this data for the reasons outlined in text. Study 20 was excluded from the second round of regression analysis due to the high levels of neutrophils in the air control sample. The results of these analyses are given in red.

Figure 5: Relationship between ozone-induced neutrophilia in the proximal airways during the early acute response and ozone dose defined using the CVT1 and CVT2 expressions. Details of the figure format are as outlined in the legend to Figure 2 with the following amendments: The data highlighted in green indicates that study 17 examined responses at more than one time point: 1 and 6h. The results of the regression analysis given in red are based only on the data with lavage performed prior to the 3h post exposure time point. The regression results in black refer to the whole data set.

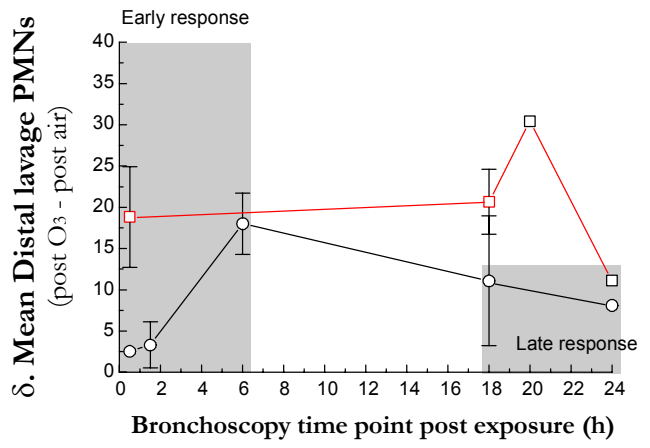
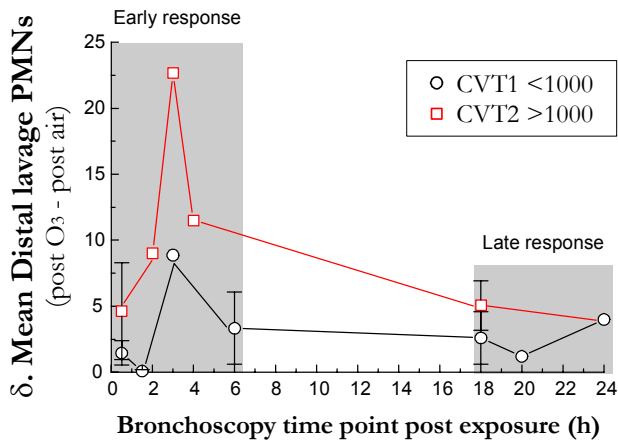
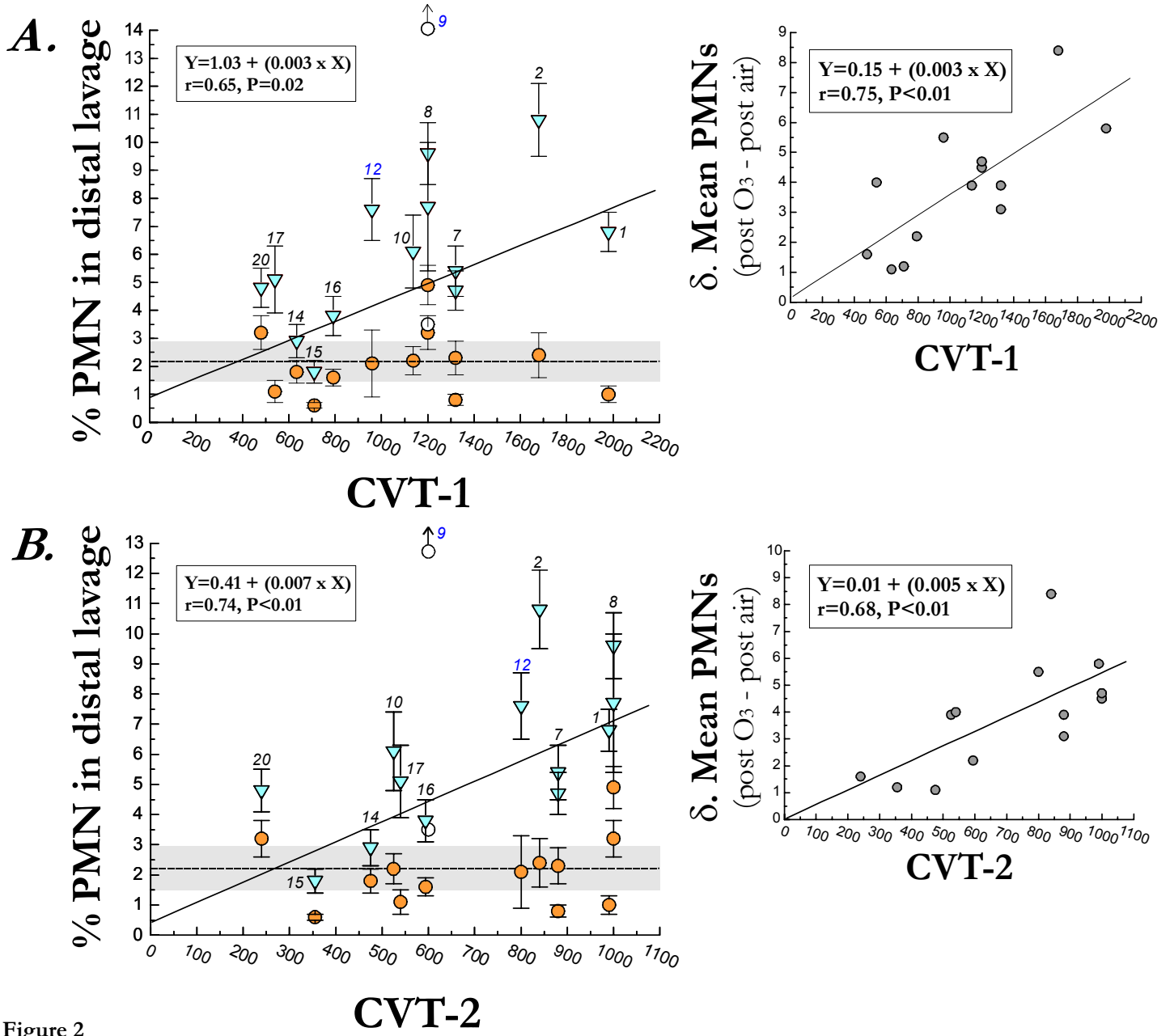


Figure 1



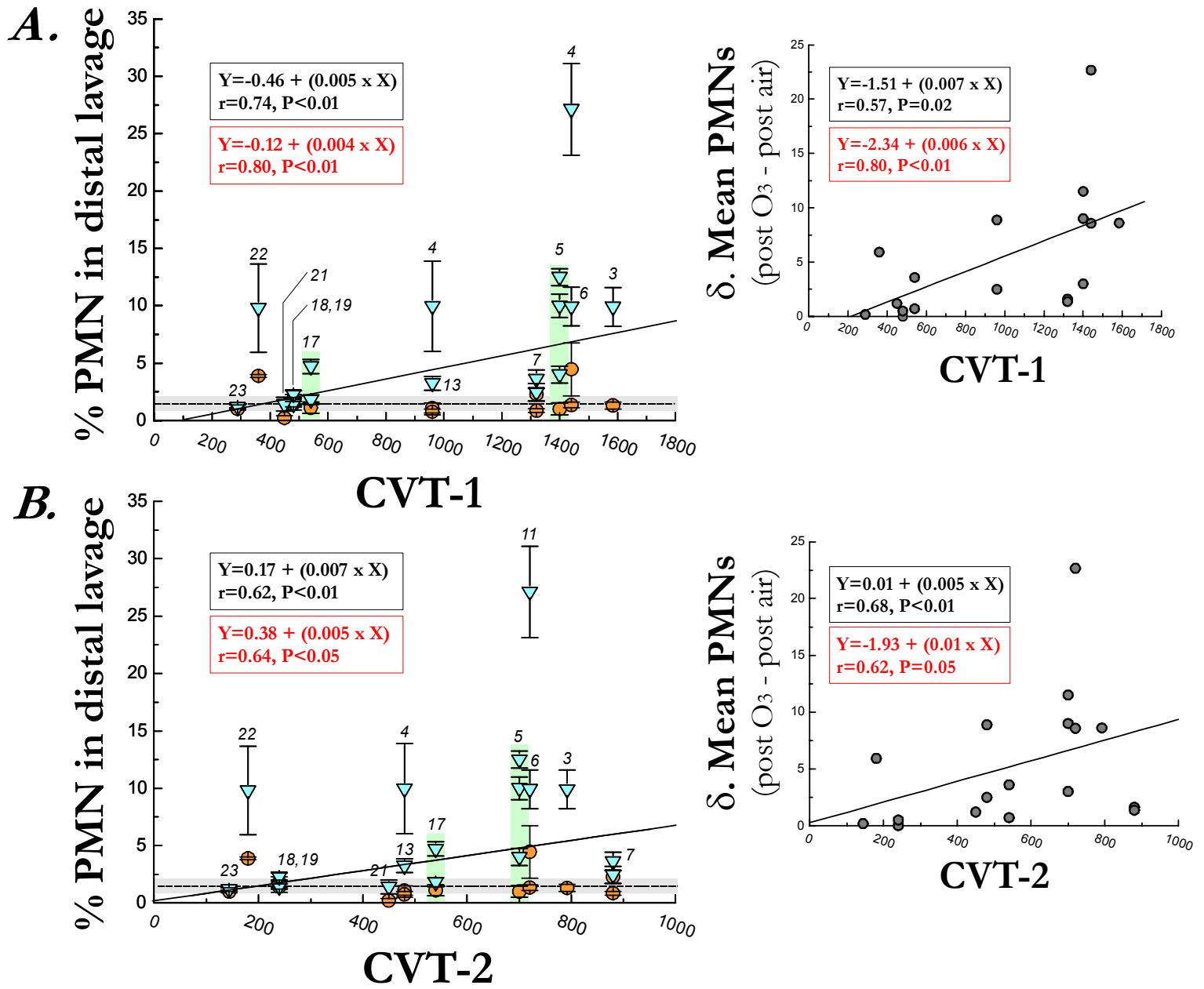


Figure 3

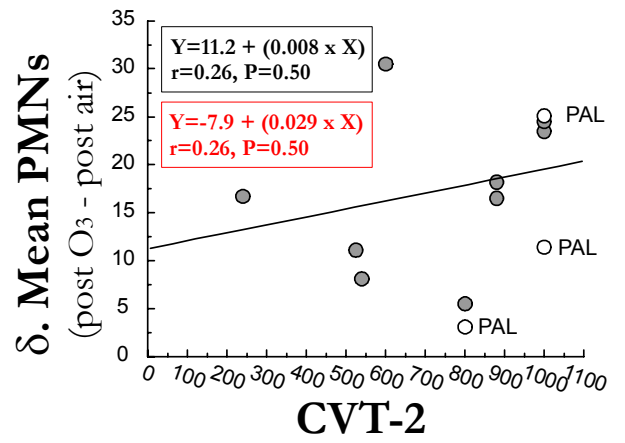
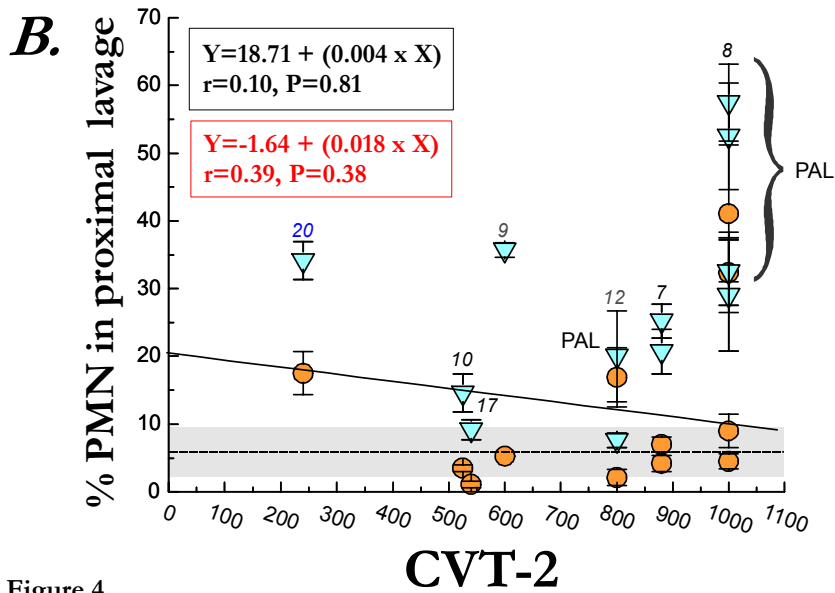
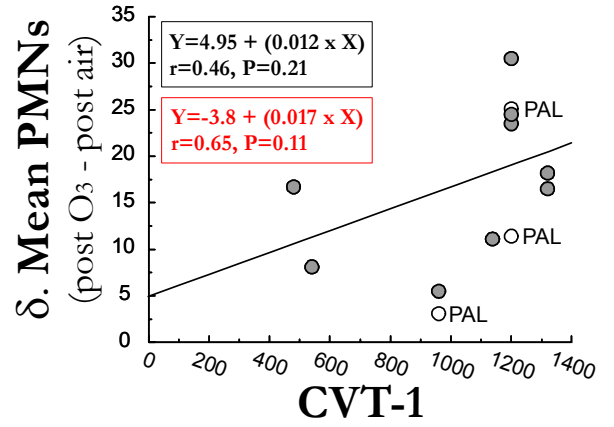
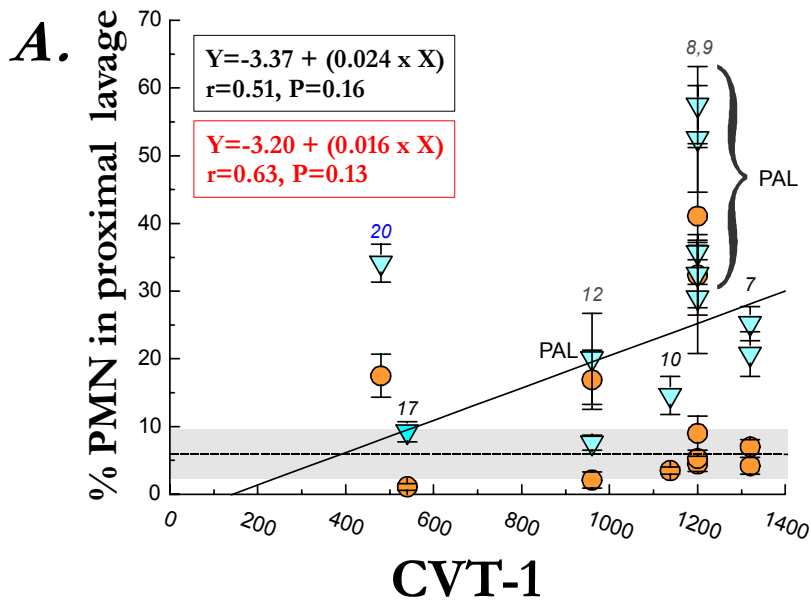


Figure 4

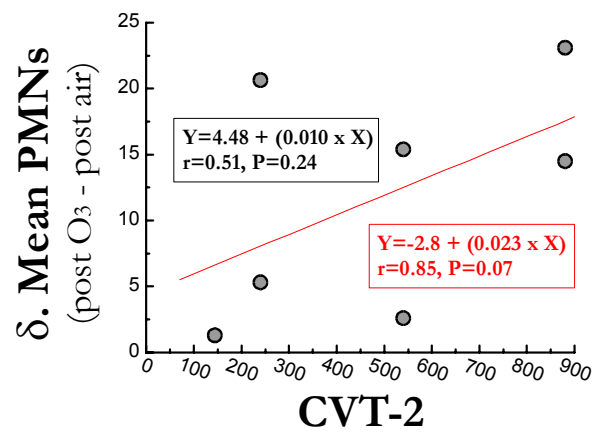
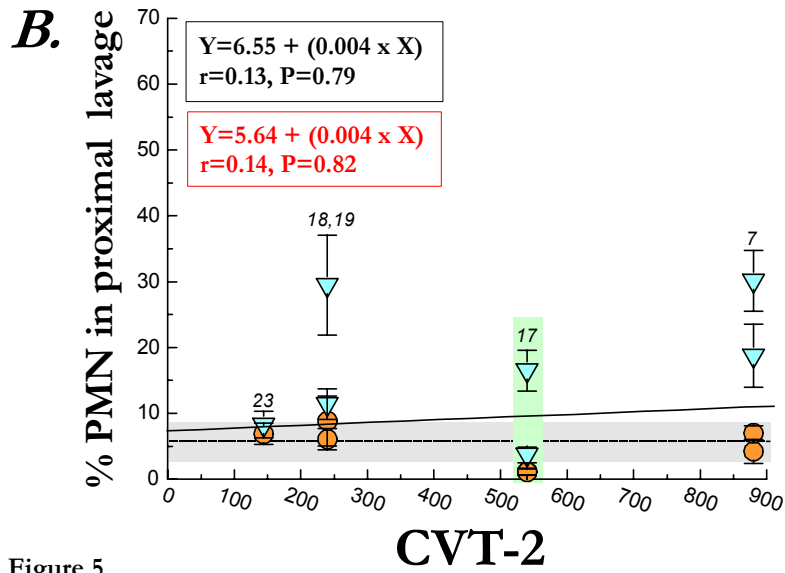
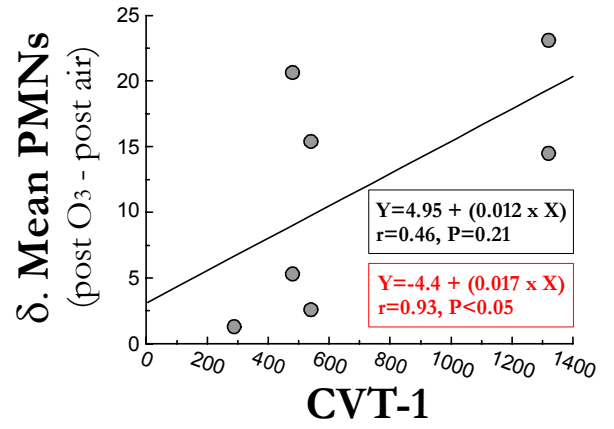
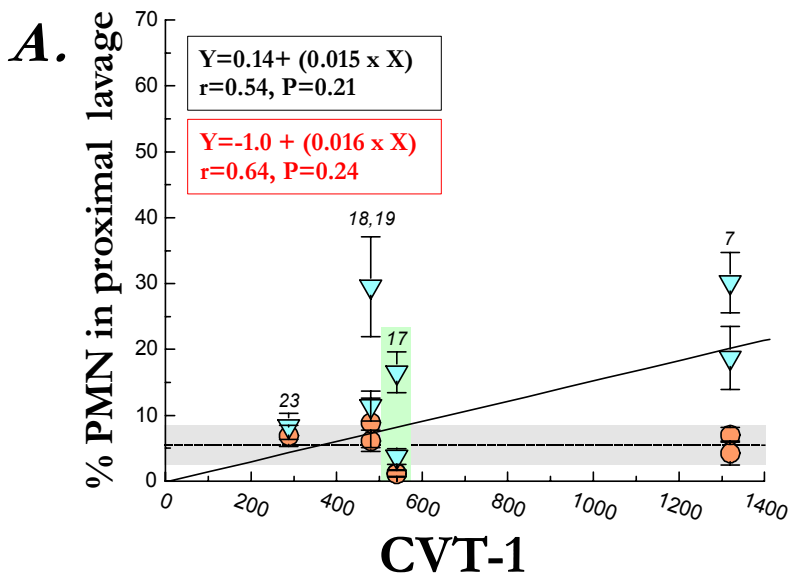


Figure 5