

## **PUBLIC COMMENT FOR CASAC OZONE MEETING**

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**SEPTEMBER 11 2012**

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In a previous public comment to CASAC (January 9, 2012), I commented on the ozone chamber results of Kim et al. (2011). In that paper, the authors found a statistically significant decrease in forced expiratory volume in one second (FEV1) due to exposure at 0.06 ppm ozone; however, the result did not appear to reach the level that is generally considered clinically significant. I also presented some regression analyses which showed how the FEV1 response to ozone depended on other variables, in particular pre-exposure FEV1 adjusted for height. One member of the panel suggested that Kim's results were more consistent with clinical significance in the case of another variable, percent change in polymorphonuclear neutrophils in sputum (%PMN), which is a measure of inflammatory and immunomodulatory effects in the airways. The present contribution is concerned with those measurements. I appreciate the assistance of Dr. Kim and Dr. Neil Alexis who have provided me their raw data and helped me with the interpretation.

There are in fact two measures of inflammation: %PMN, which is a measure of relative proportion, and total PMN cell count itself, which some researchers consider a better measure of impact. Alexis et al. (2010), in an experiment conducted at 0.08 ppm ozone, reported a statistically significant rise in PMN cell count post-exposure compared with pre-exposure. They also found, but did not report, a statistically significant rise in %PMN.

Kim et al. (2011) repeated the experiment at 0.06 ppm ozone, but also used a superior study design, the controls in this case being post-exposure measurements in clean air; this allows for a possible exercise effect. They reported that they did not find a significant rise in total PMN cell count, which may be due to the considerable variability of that measurement, but they did find a significant increase in %PMN. That is the first point I would like to make to CASAC: they did find a statistically significant effect at 0.06 ppm, but in what is arguably the less interesting of the two measures.

My second point is to return to the question of clinical significance. I am not myself an expert in these kinds of responses, but my impression is that there is no agreement on what rise in %PMN is clinically significant. Perhaps I could pose this as a question to CASAC: in the context of what could possibly be an expensive new regulation, what exactly is the health effect this measure would be protecting against?

For the third part of my discussion, I use regression analysis in an attempt to gain a better understanding of the sources of variability in these experiments, in similar fashion to my earlier results on FEV1. The covariates include physical characteristics (sex, age, height, weight etc.) and the control measurements of FEV1 and %PMN. The results are a mixed bag that is hard to interpret: at 0.06 ppm, the only significant covariate is sex, whereas at 0.08 ppm, sex is not significant but several of the baseline

measurements are (see Technical Appendix). I got yet another regression equation when analyzing total PMN cell counts at 0.08 ppm.

So let me come to what is possibly the most critical question here: what is the comparison between the 0.08 ppm and 0.06 ppm results? Despite concerns about the two different methods of deriving a control sample, a simple t-test comparison between the two sets of control measurements shows negligible difference. Based on that, I decided to combine all the data into a single regression analysis for all 39 subjects. The results again confirm an overall statistically significant result for the rise in %PMN following ozone exposure, but none of the tested covariates, including ozone level itself, is statistically significant. There is no dose-response relationship that we can determine on the basis of these experiments.

My bottom line: there is much of potential scientific interest in these results, but they are not definitive enough to justify basing a new regulation upon them.

## References

Alexis, N.E. and others (2010), Low-level exposure induces airways inflammation and modifies cell surface phenotypes in healthy humans. *Inhalation Toxicology* 22(7), 593-600.

Kim, C.S. and others (2011), Lung function and inflammatory responses in healthy young adults exposed to 0.06 ppm ozone for 6.6 hours. *Am J Respir Crit Care Med* 183, 1215-1221`.

Smith, R.L. (2012), Public comment to CASAC. [http://yosemite.epa.gov/sab/sabproduct.nsf/A39FB1582778272E8525797C0048F9B5/\\$File/smith+oral+statement.pdf](http://yosemite.epa.gov/sab/sabproduct.nsf/A39FB1582778272E8525797C0048F9B5/$File/smith+oral+statement.pdf)

## Technical Appendix

### Analysis of %PMN data at 0.06 ppm

Kim et al. (2011) reported that the difference in %PMN (Ozone-CA) at 0.06 ppm exposure to ozone is 15.7 (standard error: 3.1). Subdivided into men and women, then corresponding numbers were 24.2 (4.3) for men and 8.5 (3.7) for women. All of these are statistically significant at the 0.05 level. The difference between men and women is also statistical significant. However, analysis using GSTM1 as a predictor did not show a statistically significant effect. Dr. Kim has kindly provided me with the individual %PMN numbers used for these comparisons and I can confirm the correctness of the numbers in Kim's Table 4.

In an attempt to go further using regression analysis, I constructed a covariate matrix consisting of the following ten predictors:

X1: sex (=1 for male, =0 for female)

X2: GSTM1 (=1 if positive, =0 if null)

X3=X1\*X2 (this is used to test for a possible interaction effect between sex and GSTM1)

X4: age

X5: %PMN after clean air experiment

- X6: Height
- X7: Weight
- X8: Body surface area
- X9: Pre-exposure FEV1 in clean air
- X10: Pre-exposure FEV1 in 0.06ppm ozone

The intention behind including the variables X5, X9 and X10 was to see whether variables that might indicate the prior health of the subject had an influence on the change on %PMN due to ozone. The other variables are general “personal characteristic” variables that may be relevant in determining vulnerability. Note that all the subjects were young (age range 20-33), so we would not expect age to have a major influence.

Best subset regression was used to select the best model of each model order, followed by an examination of the selected models to determine which variables were statistically significant. The result was clear-cut: sex is the only significant covariate among the ones listed above. The final fitted model is as shown in the Table 1.

This essentially confirms the result of Kim et al.’s Table 4 – the only statistically significant variable was sex, with an estimated coefficient (male minus female response) of 15.5, standard error 5.5, significant at  $p < 0.01$ .

**Table 1: ANOVA table for Regression**  
**Dependent Variable: %PMN(Ozone-CA) at 0.06 ppm**

Residuals:

Min	1Q	Median	3Q	Max
-33.782	-10.410	1.417	8.415	26.218

Coefficients:

	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	8.485	3.719	2.282	0.03254 *
Sex	15.697	5.493	2.858	0.00915 **

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Signif. codes: 0 \*\*\* 0.001 \*\* 0.01 \* 0.05 . 0.1 1

Residual standard error: 13.41 on 22 degrees of freedom

Multiple R-squared: 0.2707, Adjusted R-squared: 0.2376

F-statistic: 8.167 on 1 and 22 DF, p-value: 0.009147

## Analysis of %PMN data at 0.08 ppm

Now let us consider the data of Alexis et al. (2010). In this experiment, there was only one ozone level of 0.08 ppm, with no control experiment in clean air, a point which the authors acknowledged was a deficiency of their study design. In place of a clean air measurement, the authors measured %PMN and a second variable, total cell count (in Cells/mg), both before and after the experiment. These two variables measure different things with cell count being possibly the better measure of impact (Dr. Neil Alexis, personal communication) but it is also subject to more variability; indeed, for the experiment at 0.06 ppm, the difference in total cell count was not statistically significant as reported by Kim et al. (2011).

For the present analysis, I have repeated the same form of analysis as was done with the %PMN data at 0.06 ppm but using the pre-exposure value of %PMN as the control variable. Note that, for this result to be comparable with the previously reported results in 0.06 ppm ozone, we would effectively be assuming that there is no difference in %PMN in clean air due to exercise alone; this is not certain but is very likely correct (Dr. Neil Alexis, personal communication).

**Table 2: ANOVA table for Regression**  
**Dependent Variable: %PMN(Post-Pre) at 0.08 ppm**

Residuals:

Min	1Q	Median	3Q	Max
-14.0944	-6.6883	-0.5837	3.3436	22.7282

Coefficients:

	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	72.4755	19.1605	3.783	0.00303 **
Pre-exp %PMN	-0.5933	0.1609	-3.687	0.00358 **
Pre-exp FEV1 at 0.06ppm	60.4067	24.0749	2.509	0.02903 *
Pre-exp FEV1 at 0.08ppm	-67.9447	23.1347	-2.937	0.01352 *

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Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 11.14 on 11 degrees of freedom  
Multiple R-squared: 0.6529, Adjusted R-squared: 0.5583  
F-statistic: 6.898 on 3 and 11 DF, p-value: 0.007032

The variables used in the regression in this case were:

X1: Pre-exposure %PMN

X2: sex (=1 for male, =0 for female)

- X3: age
- X4: Height
- X5: Weight
- X6: Body surface area
- X7: Pre-exposure FEV1 in clean air
- X8: Pre-exposure FEV1 in 0.06ppm ozone
- X9: Pre-exposure FEV1 in 0.08ppm ozone

The significant variables in this case were X1, X8 and X9, which are all pre-exposure measures, but it is hard to explain why these particular variables turned out to be statistically significant. The ANOVA table and related statistics in this case are in Table 2.

### Analysis of Total Cell Count at 0.08 ppm

In this case we took the logarithm of total cell count as the variable of interest, since the data are highly right-skewed and taking logarithms give a closer fit to the normal distribution. By analogy with the %PMN analysis, the difference (post-exposure minus pre-exposure) in log total cell count was taken as the dependent variable in a linear regression, while the variable X1 in the %PMN analysis was replaced by the pre-exposure total cell count. The optimal model in this case is as shown in Table 3. Here, X3, X4 and X7 were the significant variables.

**Table 3: ANOVA table for Regression**  
**Dependent Variable: Log total PMN cell count (Post-Pre) at 0.08 ppm**

Residuals:

Min	1Q	Median	3Q	Max
-0.9920	-0.3572	-0.1253	0.2635	1.2611

Coefficients:

	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	-16.14098	7.35641	-2.194	0.05060 .
Age	-0.13801	0.04906	-2.813	0.01688 *
Height	0.15903	0.05479	2.902	0.01438 *
Pre-exp FEV1 in CA	-1.71558	0.51494	-3.332	0.00669 **

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Signif. codes: 0 \*\*\* 0.001 \*\* 0.01 \* 0.05 . 0.1 1

Residual standard error: 0.6964 on 11 degrees of freedom

Multiple R-squared: 0.5732, Adjusted R-squared: 0.4568

F-statistic: 4.924 on 3 and 11 DF, p-value: 0.02086

### Differences in %PMN values between 0.06ppm and 0.08ppm

Now we turn to what may possibly be the most critical of the various statistical analyses, which is the comparison between the results at 0.06 ppm ozone and 0.08 ppm ozone. As noted already, the two

experiments are not strictly comparable because of the different control measurements, but there is actually very little evidence that they are different. Note that the two experiments were based on distinct groups of subjects, so the comparison is of the “two-sample t-test” type, not a paired comparison.

For pre-exposure %PMN in the 0.08 ppm experiment, the mean was 36.6 and the standard error 5.00

For the post-exposure %PMN in clean air, that was part of the 0.06 ppm experiment, the mean was 38.3 and the standard error 3.71.

For the difference, the mean was 1.70 and the standard error 6.23 ( $t=0.27$ , clearly not significant).

This confirms that there is no difference (in this analysis) between the pre-exposure reading at 0.08 ppm and the clear air reading with the 0.06 ppm cohort.

From now on, we assume that there is no difference between these two control measurements, and combine the two sets of data in a single regression analysis. The available covariates for this are

- X1: Control level of %PMN
- X2: Ozone concentration (coded 0 for 0.06 ppm, 1 for 0.08 ppm)
- X3: sex (=1 for male, =0 for female)
- X4: age
- X5: Height
- X6: Weight
- X7: Body surface area
- X8: Pre-exposure FEV1 in clean air
- X9: Pre-exposure FEV1 in 0.06ppm ozone

The variable X9 needs some explanation. It appears that the subjects who participated in the Alexis et al. (2010) experiment at 0.08 ppm also participated in the Kim et al. (2011) experiment at 0.06 ppm This is why X9 is available for subjects in both experiments. However, it seems that sputum measurements were only taken for the new subjects, not the ones who participated in the earlier experiment. A better comparison between the 0.06 ppm and 0.08 ppm ozone experiments could have been made if the measurements were repeated on the same subjects.

Nevertheless, I have conducted another linear regression analysis using the data as available. Again, the regression strategy was to use all-subsets regression to determine the best model of each order, following by checking the individual regression models for statistical significance of the coefficients. In this analysis, none of the covariates in any of the regression analysis (except for the intercept) was statistically significant at the 0.05 level. As an illustration, Table 4 shows the analysis with just ozone level as covariate.

The result confirms the statistical significance of the intercept, which represents the average rise in %PMN over all 39 subjects in the two experiments (15.7 with a standard error of 3.2). However, the difference between the two ozone levels (represented by X2) is not statistically significant. As a result, it is not possible to confirm a dose-response effect for this experiment.

**Table 4: ANOVA table for Regression**  
**Dependent Variable: %PMN(Post-Pre), Combined Experiment**

Residuals:

Min	1Q	Median	3Q	Max
-27.957	-13.212	1.121	11.207	34.721

Coefficients:

	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	15.679	3.246	4.830	2.38e-05 ***
Ozone level	6.198	5.234	1.184	0.244

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Signif. codes: 0 \*\*\* 0.001 \*\* 0.01 \* 0.05 . 0.1 1

Residual standard error: 15.9 on 37 degrees of freedom

Multiple R-squared: 0.03652, Adjusted R-squared: 0.01048

F-statistic: 1.402 on 1 and 37 DF, p-value: 0.2439