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Sidestream Cigarette Smoke Toxicity Increases with Aging and Exposure Duration

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Objectives: Determine the effects of aging on the toxicity of sidestream tobacco smoke, the complex chemical mixture that enters the air from the lit end of burning cigarettes and constitutes the vast bulk of secondhand smoke.

Design: Statistical analysis of data from controlled experimental exposures of Sprague Dawley rats to fresh and aged (for more than 30 minutes) sidestream smoke for up to 90 days followed by histological sectioning on the respiratory epithelium. The data were obtained from a series of experiments conducted at Philip Morris' formerly secret INBIFO laboratory in Germany.

Results: Using total particulate material as the measure of smoke exposure, that aging sidestream cigarette smoke for at least thirty minutes increases its toxicity fourfold for 21 day exposures and doubles the toxicity for 90 day exposures, relative to fresh sidestream smoke.

Conclusions: These results help explain the relatively large biological effects of secondhand smoke compared to equivalent mass doses of mainstream smoke.

WHAT THIS PAPER ADDS

Secondhand smoke, the smoke that enters the air when people are smoking tobacco products, kills about one nonsmoker for every eight smokers that active smoking kills. Fresh sidestream smoke (the smoke that comes from the lit end of the cigarette when it is smouldering) is 3-4 times as toxic to laboratory animals as the fresh mainstream smoke the smoker inhales. When sidestream smoke ages after it enters the air, it becomes 2-4 times more toxic to laboratory animals than fresh sidestream smoke. This result helps explain the relatively large biological effects of secondhand smoke compared to equivalent mass doses of mainstream smoke.

About one nonsmoker dies from secondhand smoke exposure for every eight smokers who die from smoking even though secondhand smoke doses (in terms of total mass inhaled) are substantially lower. In a previous analysis of unpublished sidestream cigarette smoke toxicity experiments done by Philip Morris, we showed that freshly generated sidestream cigarette smoke is 3-4 times more toxic to laboratory animals than mainstream smoke (the smoke the smoker inhales)(1). However, most secondhand smoke is not freshly generated. In typical indoor spaces secondhand smoke lingers for 1.5-2.0 hours. When sidestream smoke is released into the open air, it changes chemically and physically.(2). A large percentage of sidestream smoke consists of oils and waxes that are emitted as small particles. These volatile and semi-volatile organic compounds evaporate as the smoke is diluted, forming gases and smaller particles.(3) The vapors and small particles adsorb onto surfaces, then desorb over time, effectively increasing the exposure period.(2)

Though these changes in secondhand smoke chemistry are known, there are few publications that compare the toxicity of freshly-generated and aged sidestream smoke.(4, 5) The tobacco industry has been concerned with these effects since the early 1980's.(6, 7) We identified research projects at several tobacco companies but limit our analysis to experiments done by Philip Morris at their formerly secret laboratory the Institut für Biologische Forschung (INBIFO) in Germany because of the consistency of methods and quality of data. Our analysis of these data show that the acute toxicity of sidestream smoke increases by a factor of 2-4 as it ages.

Methods

Tobacco industry documents

We found reports documenting Philip Morris' *in vivo* experiments with sidestream cigarette smoke by searching the approximately 45 million pages of tobacco industry documents made public as a result of litigation against the tobacco companies. Between January and December of 2005, we searched the UCSF Legacy Tobacco Documents Library

(http://www.legacy.library.ucsf.edu), the UCSF British American Tobacco Documents Archive (http://bat.library.ucsf.edu/index.html), Tobacco Documents Online (http://tobaccodocuments.org), and Philip Morris documents (http://www.pmdocs.org), using standard strategies,(8) starting with keywords "sidestream," "aging" and "lifetime exposure." The initial searches yielded the identification numbers of projects and assays, which were then searched.

Sidestream inhalation studies at Philip Morris

Each of the experiments done at INBIFO had a unique identifying number, which we use. In 1989, after completing 35 biological assays of freshly-generated sidestream smoke,(1, 9) Philip Morris invented a sidestream smoke aging system.(10) They piped hot sidestream smoke via a large cross-section duct into a 30 m^3 room. Air in the room was circulated with a slowly rotating ceiling fan and a temperature of 26°C was maintained with 2 heat exchangers.(11-14) Between 1984 and 1998 Philip Morris did five (3149(10), 3195(12, 13), 3169(5, 14, 15), 3216 (16, 17), 3248 (18, 19); Table 1) inhalation experiments at INBIFO to test the effects of aged sidestream smoke on rat respiratory epithelium. Experiments 3216 and 3248 were excluded from the analysis because data on individual animals was not available, study 3149 was excluded because it did not include complete assessment of histopathological damage to the vocal cords.

We included in our analyses experiments 3123, 3125 and 3127 (Table 1) in which the rats were exposed to sidestream cigarette smoke that was piped directly from the smoking machine to the animals and was approximately 10 seconds old. We refer to this smoke as "fresh" sidestream. In experiments 3195 and 3169 the rats were exposed to sidestream cigarette smoke that had been held in a 30 m^3 chamber with continuous air exchange rates of 0.75 or 2 air changes/hour before it was piped to the rats. We refer to these smokes as "aged" sidestream. (In their publications on aged sidestream smoke(5, 15, 17, 19-23) Philip Morris referred to these smokes as "room aged sidestream" or RASS.) Studies 3123, 3125, 3127 and

3169 used Kentucky Reference cigarette 2R1 and study 3195 used Kentucky Reference 1R4F. All five experiments included sham exposures where rats were placed in clean exposure chambers and exposed to clean, HEPA filtered air as controls.

In experiment 3169 Philip Morris tested the effects of a 90 minute aging period with furnishings placed in the aging room (Table 2). Adding the furnishings appears to have resulted in greater adsorption of TPM and nicotine onto surfaces in the aging chamber. In experiment 3195 they used a 30 minute aging and no furnishings. These differences are reflected in the ratios among CO and TPM and nicotine (Table 3).

We combined data from experiments 3123, 3125 (21 day exposures) and 3195 (28 day exposures). Experiments 3127 and 3169 were 90 day exposures. The methods of exposure varied in the five experiments (Table 1). In whole-body exposure the animals were held in standard cages and the smoke was piped into the cages. In head-only exposure and nose-only exposure the animals were held in snug tubes, which were then mounted in holes in a smoke-filled duct so that only the head or nose of the animal projected into the smoke.

Exposure Calculations

We normalized exposures either on the basis of concentration-hours of total particulate matter (TPM) measured at INBIFO as the mass of solids deposited on a glass fiber filter (Gelman #6004300) or on the basis of concentration-hours of carbon monoxide (CO) measured using nondispersive infrared photometry.(14) The glass fiber filter was rated by Gelman Company to retain 99.7% of particles greater than or equal to 300 nm. Samples for all chemical determinations were taken from the breathing zone in the animal exposure chambers. Exposure times ranged from five hours a day, seven days a week to seven hours a day, seven days a week (Table 1). To provide a common metric for exposure, we multiplied the TPM concentrations the animals were exposed to by the number of hours per day and number of days per week (TPM mg/m^3 x hours/day x days/week) to obtain weekly exposure rates in TPM mg-hrs/m^3- week. CO ppm-hrs/ week were calculated the same way.

Histopathological scoring

Fixation and sectioning protocols were consistent through the five experiments. The larynx was sectioned transversely, according to Lewis(24) The trachea was sectioned longitudinally at the tracheal bifurcation. The nose was sectioned transversely according to Young(25) to obtain tissue slices immediately posterior to the upper incisor teeth (nasal 1) and at the incisive papillae (nasal 2).

All tissue slices were embedded in Paraplast, cut at 5-6 μm, and stained with hematoxylin and eosin. In addition, some sections were stained with alcian blue/periodic acid Schiff's reagent to identify goblet cells. All slides were read by a veterinary pathologist at INBIFO.

To assess the effects of smoke inhalation, INBIFO scientists fixed and sectioned the upper respiratory tract tissues and examined them for pathological changes. Figure 1 shows the section locations, cell types and pathological changes that the INBIFO pathologists evaluated in at least one experiment; we based our analysis on those scored in all the INBIFO experiments we examined. All pathological changes were scored according to a subjective severity scale from 0 to 5: 0 = no visible lesion, 1 = slight lesion, 2 = slight to moderate lesion, 3 = moderate lesion, 4 = moderate to marked lesion, and 5 = marked lesion. The exact definitions of slight, moderate, and marked lesions are not available, but the same veterinary pathologist oversaw all seven experiments so the criteria can be assumed to be consistent. We summed the histopathology scores from nasal section one through the trachea to create a total respiratory epithelium histopathology score for each animal. Thus, each animal had a total score from 0 (no lesions) to a maximum of 85 (17 locations x 5 [maximum score]). We excluded data from 2 obvious outliers: animal 007 in 3123, and animal 505 in 3169 and from any animal with scores missing for any section, cell type or pathology.

Statistical analysis

We tested the effects of TPM μg/m^3-hrs-week or ppm/hrs-week, together with exposure duration and aging using a multiple regression implementation of an analysis of covariance on total respiratory epithelium histopathology score. We constructed this analysis by defining dummy variables using reference coding with the 21/28 day exposure to the fresh smoke condition as the reference condition:

$$\text{Aged} = \begin{cases} 0 & \text{if Fresh smoke} \\ 1 & \text{if Aged} \end{cases}$$

and

$$\text{Duration} = \begin{cases} 0 & \text{if 21 or 28 day exposure} \\ 1 & \text{if 90 day exposure} \end{cases}$$

We began with the regression equation :

Histopathology score = b_0 + b_{Exposure} Exposure

+ b_{Aged} Aged + $b_{\text{Exposure x Aged}}$ Exposure x Aged

+ b_{Duration} Duration + $b_{\text{Exposure x Duration}}$ Exposure x Duration + $b_{\text{Aged x Duration}}$ Aged x Duration +

$b_{\text{Exposure x Aged x Duration}}$ Exposure x Aged x Duration

where Exposure is TPM mg-hrs/m^3-week or CO ppm-hrs/week. Preliminary analysis revealed that the terms associated with changes in the intercept (b_{Duration} , b_{Aged} , $b_{\text{Duration x Aged}}$) were not significantly different from zero (P >0 .2), so they were dropped from the final model. We also did a separate analysis including how long the smoke was aged, the presence of furnishings in the aging chamber, and exposure method. Calculations were done using SigmaStat version 3.1.1.

Results

Using TPM mg-hrs/m^3-week as the measure of smoke exposure and aging (vs. fresh) smoke and exposure duration (21/28 vs 90 days) as variables, demonstrates that aging sidestream cigarette smoke increases the slope of the respiratory histopathology dose-

response relationship by a factor of 4.0 for 21 day exposures ([0.00386+0.01160]/0.00386) and by a factor of 2.1 for 90 day exposures ([0.00386+o.00751+0.0116+0.00129]/[0.00386+0.00751]) (Table 4, Figure 1). Increasing exposure duration from 21/28 days to 90 days increases the damage to the respiratory epithelium by a factor of 3.0 for fresh smoke ([0.00386+.00751]/0.00386) and a factor of 1.6 for aged smoke ([0.00386+0.01160+0.00751]/[0.00386+0.01160]). The effects of aging the smoke and exposure duration on the slope are additive; the interaction term is not significant (Table 4, Figure 1).

Using CO ppm-hrs/week as the measure of smoke exposure demonstrates that aging sidestream cigarette smoke increases the slope of the respiratory histopathology dose-response curve by a factor of 3.8 for 21/28 day exposures, but decreases it by a factor of 0.68 for 90 day exposures (Table 4, Figure 2). Longer exposures increase the slope of the dose-response curve for damage to the respiratory epithelium by a factor of 2.8 for fresh smoke but for aged smoke the damage after 90 days exposure is 0.5 times that of the 21/28 day exposure. There is a significant interaction between aging and duration, with the effects being less than additive. We also tested the inclusion of exposure method (head-only, nose-only or whole-body), the length of time the smoke was aged, and the presence of furnishings or carpet in the aging chamber in the model, allowing for effects on both the intercept and the slope in the full regression model. Including the exposure method produced a statistically significant improvement in the fit, but the effect was small, with the R^2 increasing from only 0.874 to 0.881 for TPM and from 0.875 to 0.881 for CO. Including the length of time that the smoke was aged (30 to 90 minutes, as a continuous variable) and dummy variables for the presence of furnishings in the aging chamber did not significantly improve the fit. Because there is a limited number of experiments under each combination of conditions, these results need to be interpreted with caution.

Discussion

We found only two previous publications on the effects of aging on sidestream toxicity.(4, 5) Sonnenfeld and Wilson(4) tested the toxicity of whole sidestream smoke on monolayer cultures of L-929 cells by measuring cell death. They found that toxicity decreased rapidly in the first 30 seconds of aging and predicted that the smoke would lose all toxicity to the cells after seven minutes aging. The differences in assessed toxicity between our analyses and their experiments may be because the INBIFO studies did not examine changes in smoke toxicity during the first 30 seconds of aging or because they looked at a different measure of toxicity.

Philip Morris published the results of experiment 3169 in 1998.(5) They compared the effects of aging on the histopathology scores at single sites in the respiratory tract and found that fresh and aged sidestream smoke induced approximately equal levels of damage when compared on the basis of TPM and that "most of the effects seen were less expressed in RASS-[aged sidestream] than in FSS [fresh sidestream]-exposed rats when compared on the basis of the CO concentrations"(5, 26). The figures in the Philip Morris publication present data normalized on the basis of CO exposure which emphasizes the loss of TPM with aging. They tested for differences between aged and fresh sidestream at single sites in 142 rats using two-way analysis of variance, whereas we examined effects on the entire respiratory tract in 253 rats, yielding much higher power to detect an effect. There is scatter in the data (Figure 2), which may have obscured the conclusions that we were able to draw based on the much larger data set. There is no evidence that Philip Morris ever did the cross-experiment, multi-site statistical comparison that we have done. They appear not to have realized that aging actually increases the toxicity of sidestream cigarette smoke when normalized on the basis of TPM. Their paper (5) emphasizes the loss of TPM from secondhand smoke over time but does not misrepresent the conclusions we find in their internal scientific reports.

Although there is a some debate about the best markers to use to quantify secondhand smoke,(27, 28) particulate material and nicotine are most commonly used. Thus our finding that sidestream smoke becomes more toxic after it is diluted and aged when measured by TPM is especially important. Secondhand smoke is the primary source of particulates in most spaces where people are smoking(29) and the particulate phase contains many of the most toxic and carcinogenic components of sidestream smoke.(27). Though aging results in the loss of 30-70% of the airborne TPM, most of the "missing" TPM is adsorbed onto surfaces in the environment(2) which desorbs back into the air over time.(30)

The experiments we analyzed do not reveal the mechanism for the increased toxicity of aged sidestream smoke compared on the basis of equal exposures to TPM. Whatever the cause, the change in toxicity appears to happen in the first 30 minutes, because smoke aged 90 minutes was not significantly more toxic than smoke aged 30 minutes. It may be that because airborne TPM is lost to adsorbtion over time, equalizing exposures on the basis of TPM increases the proportion of toxic gaseous components of sidestream smoke. Earlier Philip Morris experiments(1) showed that the gas/vapor fraction of fresh sidestream smoke was more toxic to the respiratory epithelium than the particulate fraction. It is also possible that chemical reactions occur in secondhand smoke over time, producing compounds with higher toxicity.

The finding that aging increases toxicity 2.8-fold in 21/28-day exposures but decreases it by 0.5 for 90 day exposure when carbon monoxide was used as the exposure metric (Figure 2C and D) is remarkable because carbon monoxide is a marker for the components of sidestream that are not lost to adsorbtion. We do not know why the relative toxicities of aged and fresh smokes were different for 21/28 and 90 day exposures when exposure was measured with CO. Regressions using data from experiment 3149, which used the Kentucky 2R1 cigarette, give similar results (data not shown), so potential differences between toxicity of Kentucky 2R1 and Kentucky 1R4F sidestream are not the cause.

Limitations

These experiments were conducted on Sprague Dawley rats, a well-established animal model. The criteria for the histopathological damage scoring system are not known. Because the same person supervised all of the scoring, we have assumed that the criteria were constant over time and consistent between studies. If the criteria were not constant and consistent, the pooled comparison we made may not be valid.

Conclusion

Philip Morris' toxicological experiments using rats may help explain the epidemiological observation that approximately one nonsmoker dies due to secondhand smoke exposure for every eight smokers who die of smoking.(27) If aged sidestream smoke is approximately three times more toxic than fresh sidestream, and fresh sidestream smoke is approximately four times more toxic than mainstream smoke,(1, 31) then aged sidestream smoke is approximately twelve times more toxic than mainstream smoke. While the mass of smoke that nonsmokers inhale is far lower than that which smokers inhale, the smoke itself appears to be substantially more toxic.

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Competing Interests

All authors declare that the answer to the questions on your competing interest form [http://bmj.com/cgi/content/full/317/7154/291/DC1] are all No and therefore have nothing to declare.

Ethical Approval

Not required.

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Table 1. Summary of INBIFO experiments used in this paper

Year	ID #	Days	Hr/day	Days/ week	Exposure Method	Smoke type	TPM mg/m^3	CO ppm	TPM mg-hr/ m^{3}-week	CO ppm-hr/ week	Average smoke age (minutes)	# of animals
21 and 28 Day Exposures												
1986	3123(32-34)	21	7	7	Whole-body	Sham	0	1	0	49	-	8
			7	7		Fresh	15	51	735	2499	<1	9
			7	7		Fresh	51	153	2499	7497	<1	8
1986	3125(35)	21	7	7	Nose-only	Sham	0	1	0	49	-	12
			7	7		Fresh	2	9.5	98	465	<1	12
			7	7		Fresh	6.2	25.7	304	1259	<1	9
1992	3195(12, 13)	28	7	5	Head-only	Sham	0	1	0	35	-	8
			7	5		Aged	3	10	105	350	30	9
			7	5		Aged	10	33	350	1155	30	7
			7	5		Aged	30	95	1050	3325	30	6
90 Day Exposures												
1986	3127(36, 37)	90	7	7	Nose-only	Sham	0	1	0	49	-	11

			7	7		Fresh	2.1	9.1	103	445	<1	9
			7	7		Fresh	6.0	21.8	294	1068	<1	7
1991	3169(14)	90	6	7	Head-only	Sham	0	1	0	42	-	22
			6	7		Fresh	1.5	5.6	63	231	<1	22
			6	7		Fresh	3.6	12.6	151	529	<1	21
			6	7		Fresh	8.7	27.8	365	1168	<1	20
			6	7		Aged	0.6	5.6	25	231	90	21
			6	7		Aged	1.2	12.2	50	512	90	19
			6	7		Aged	2.6	28.7	109	1205	90	22

Table 2. Experimental variables in secondhand smoke generation

	Mean smoke residence time, aging chamber (minutes)	Aging chamber surface area (m^2)*	Aging chamber wall surface	Furnishings	Mean smoke residence time, exposure chamber (minutes)	Exposure chamber surface area (m^2)
INBIFO standard for fresh sidestream (38)	0	-	-	-	<1	0.06
3169(5)	90	118	Painted wallpaper	wool carpet, wool curtain, wooden bookshelf, books, magazines	<1	0.06
3195(12, 13)	30	118	Painted wallpaper	vinyl floor tile	<1	0.06

* Includes surface area of heat exchanger

Table 3. Effects of aging on chemical composition of sidestream						
	Fresh (3169)(14)		Aged (3169)(14)		Aged (3195)(13)	
TPM mg/m^3	1.5	8.7	1.2	2.9	2.6	9.9
CO ppm	5.5	27.8	12.2	10.5	28.7	30.1
Nicotine $\mu g/m^3$	410	2210	240	400	520	1590
TPM/CO	0.28	0.31	0.098	0.28	0.091	0.33
TPM/Nicotine	0.0037	0.0039	0.005	0.0072	0.005	0.0062
CO/Nicotine	0.013	0.013	0.051	0.026	0.055	0.019
Particle mass median aerodynamic diameter μm	0.37	0.35	0.42	0.48	0.41	0.48

Table 4: Effects of Aging on Sidestream Smoke Toxicity (Linear Regression Results)			
TPM			
Variable	Coefficient	Standard error	P
Constant	0.200	0.151	0.185
Exposure	0.00386	0.000238	<0.001
Exposure x Aged	0.01160	0.000661	<0.001
Exposure x Duration	0.00751	0.000975	<0.001
Exposure x Aged x Duration	0.00129	0.00344	0.708
CO			
Constant	0.0380	0.154	0.805
Exposure	0.00129	0.000783	<0.001
Exposure x Aged	0.00362	0.000207	<0.001
Exposure x Duration	0.00228	0.000293	<0.001
Exposure x Aged x Duration	-0.00477	0.000424	<0.001

The regression equation is

$$\text{Score} = b_0 + b_{\text{Exposure}}\,\text{Exposure} + b_{\text{Exposure x Aged}}\,\text{Exposure x Aged} + b_{\text{Exposure x Duration}}\,\text{Exposure x Duration} + b_{\text{Exposure x Agedx Duration}}\,\text{Exposure x Aged x Duration}$$

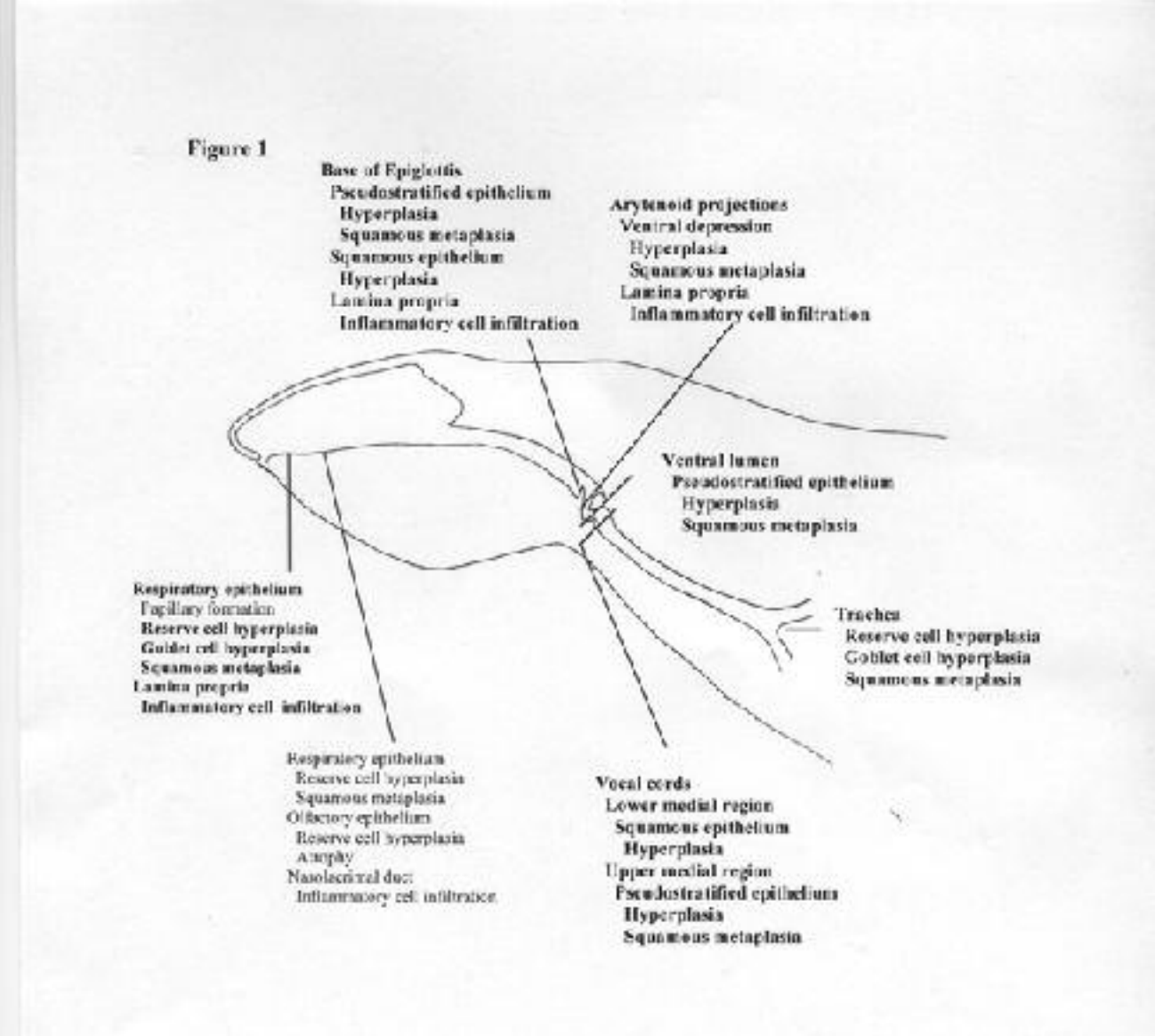

Figure 1. Diagram and list of section sites, cell types and cell pathologies scored at INBIFO in the studies we analyzed. The sites included in our analysis are indicated in bold type.

Figure 2

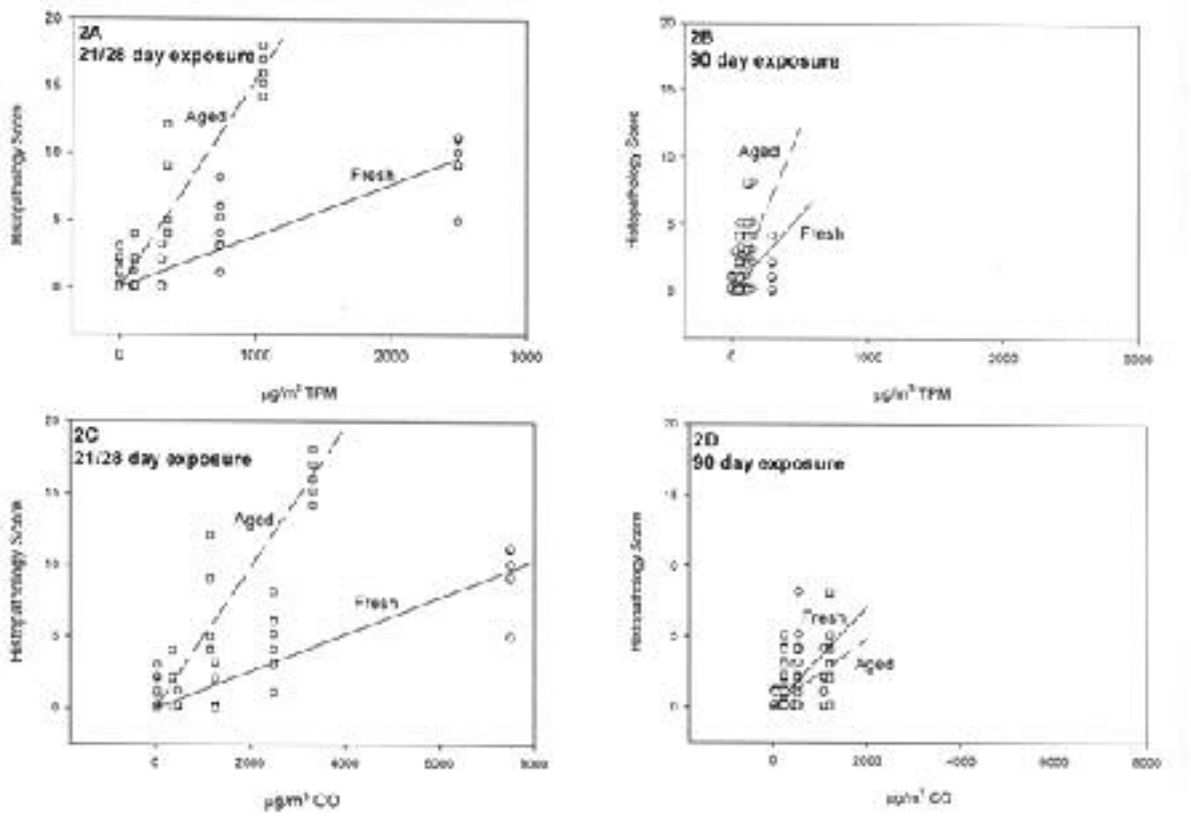

Figure 2. Raw data and regression fits for the histopathology score as a function of exposure, measured with TPM (A and B) or CO (B and C). Scores for animals with the same score are staggered by adding random numbers between 0 and 0.25 to make individual points more apparent. The actual values were used in the multiple regression analysis.