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Inhalation Study for Cigarette-Smoke-related Lung Tumorigenicity in A/J Mice

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The A/J Mouse as a Lung Tumor Model

- Philip Morris International is committed to the development of Potentially Reduced-Risk Tobacco Products. This requires a state-of-the-art scientific approach to assess the disease risk of new products.

- Animal models with enhanced lung tumor formation after exposure to cigarette smoke are required to substantiate a reduced risk.

- The A/J mouse has been shown to respond to cigarette smoke exposure with enhanced lung tumor formation after a recovery period of several months (Witschi et al., 1997; D'Agostini et al., 2001; Stinn et al., 2005; Curtin et al., 2004).

A/J Mouse History and Strain Description

- A-strain mice inbred by Strong (1921) as a cross between CSH albino and Bagg albino random bred mice, thus most closely related to Balb/c
- Now approx. 270 generations, inbred
- Develop smoke-induced emphysema faster than other mice
- High percentage of mammary adenocarcinomas in multiparous females
- Spontaneous sarcoma development in skeletal musculature

- **High spontaneous incidence of lung adenomas (K-ras mutation)**
- **Lung tumors in response to carcinogens**

The A/J Mouse as a Carcinogenicity Model

Used for approx. 30 years as carcinogenicity model (Dragani et al., 1995)

- Application of carcinogens (~70 compounds tested): few i.p. administrations
- Tumors develop 16 to 24 weeks after carcinogen application
- All carcinogens positive in the A/J mouse are genotoxic (Pereira and Stoner, 1985; Maronpot et al., 1986).

Differences between Humans and Rodents

Adult Human Smoker	Smoke-exposed Rodent
– Cyclic inhalation of nearly undiluted smoke (bolus)	– Continuous inhalation (hrs) of highly diluted smoke
– Mouth breather	– Nose breather
– Long life span	– Short life span
– Genetically diverse	– Genetically identical copies (inbred)

Study Design

- Male A/J mice
  - bred under SPF conditions, animals from Jackson Laboratories (Maine, U.S.A.)
  - age at start of inhalation: 10 to 14 weeks
  - in total: 1040 mice

- Diluted mainstream smoke (MS) from the Kentucky Standard Reference Cigarette 2R4F

- Total particulate matter (TPM) target concentrations: 150 mg/m^3 (low MS) and 300 mg/m^3 (high MS)

- Whole-body exposure for 6 hours per day, 5 days per week for up to 18 months

Experimental Conditions

- Cigarettes and smoking conditions
- Cigarette smoke generation and exposure
- Animal housing conditions
- Overview of parameters analyzed in test atmosphere
- Test atmosphere data

Cigarettes and Smoking Conditions

CIGARETTE

Cigarette type:	2R4F
Source:	University of Kentucky
Number:	approx. 1,200,000
Storage:	temperature 5-10 °C

CONDITIONING

Duration:	>7 to <20 days prior to smoking, storage of conditioned cigarettes in airtight containers
Temperature:	22 ± 0.3 °C
Relative humidity:	60 ± 1%

SMOKING CONDITIONS

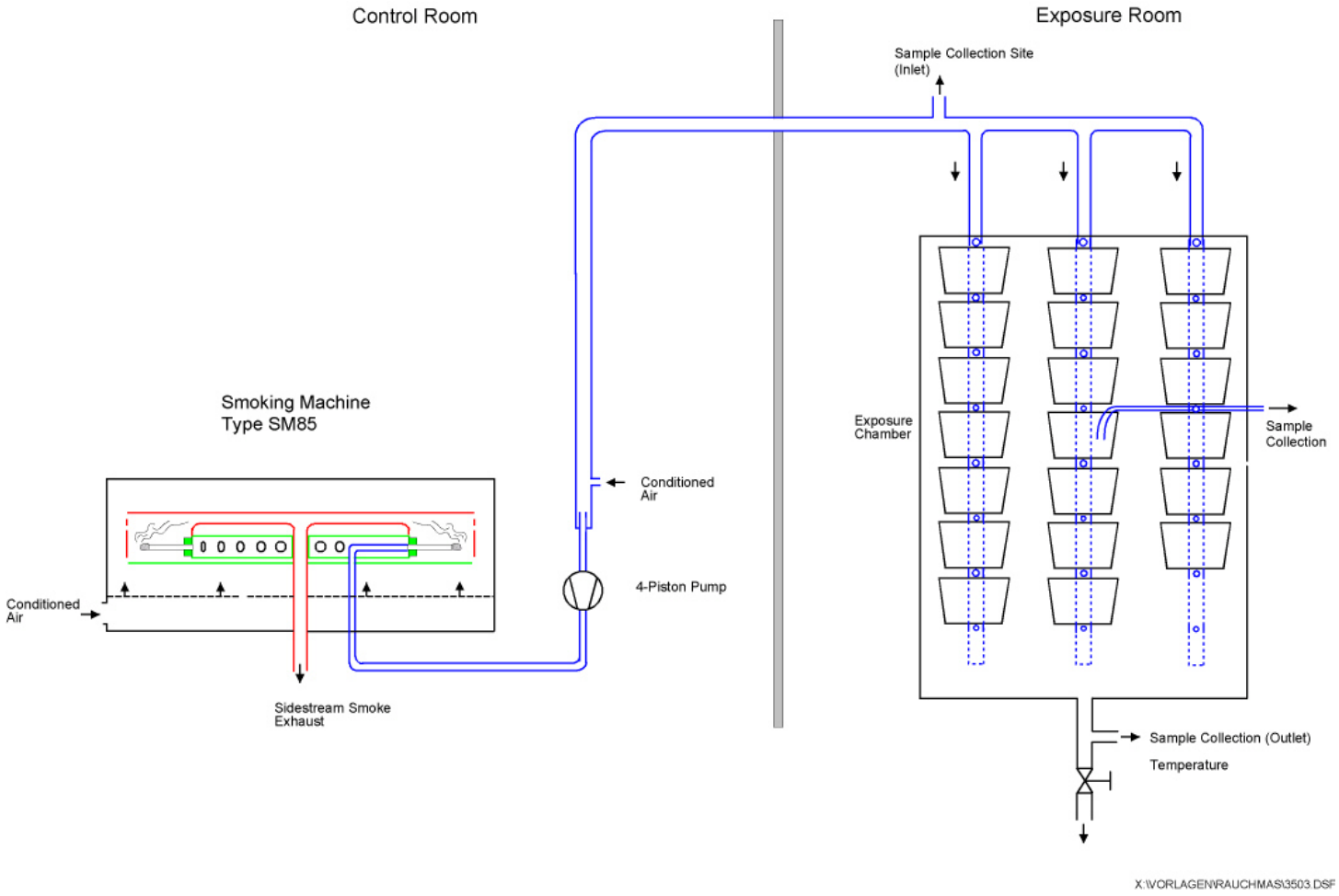
Basic conformity with ISO standards

Cigarettes smoked on carrousel (30 for high MS and 15 for low MS)

MS diluted with app. 80L filtered fresh air/min

Mean age of smoke in middle of the exposure chamber was ≤6 min

Cigarette Smoke Generation and Mice Exposure



Housing Conditions

Parameter	Specification
Air changes inside the exposure room	≥10 times/h
Room positive pressure	20 to 30 Pa
Temperature inside the exposure room	22 ± 3 °C
Rel. humidity inside the exposure room	between 30% and 70%
Light/dark cycle	12/12 h, light from 06:00 to 18:00
Animal housing	up to 8 mice/cage, cages changed ≥2 times/week
Bedding material	Changed ≥2 times/week
Food supply	ad libitum in all groups except during exposure
Water supply	ad libitum, also during exposure

Overview of Parameters analyzed in Test Atmosphere

Parameter	Frequency	Flow (l/min)	Duration (min)
TPM	3 filters / exposure day	1.5	60
Particle size	4 times / study	1	60
Carbon monoxide	every exposure day, online	1	360
Nicotine	Once / week during first 4 weeks; thereafter, once / 4 weeks	1	60
Aldehydes	Once / 4 weeks	1	30
Ammonia	Once / 4 weeks	1	30

Position: inside animal cage, center of the chamber

Test Atmosphere Data

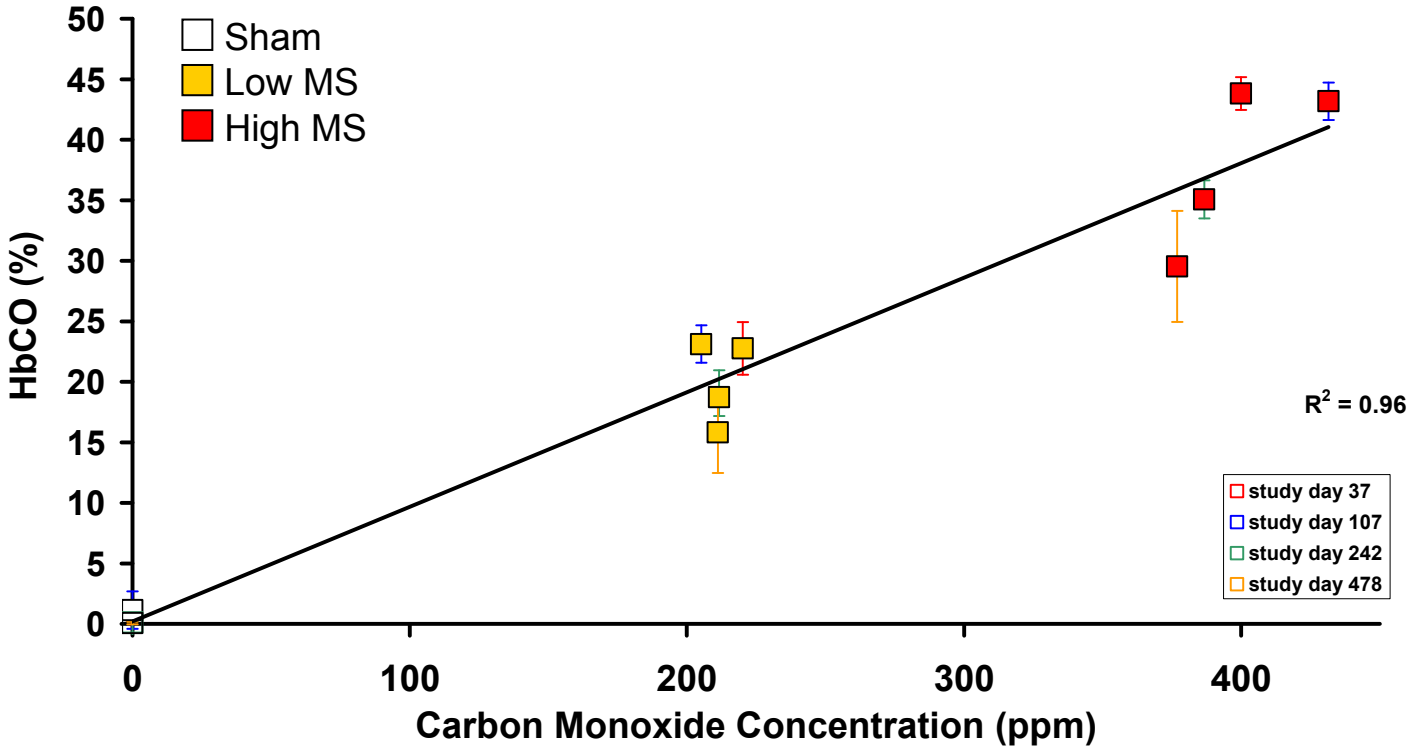
- Target concentrations were met
- Particle size indicates that the particles were respirable for the mice

Parameter	N	Sham	Low MS	High MS
TPM (mg/m^3)	389	< DL	153 ± 8	298 ± 15
Particle size (µm)	4	-	0.67	0.72
CO (ppm)	388	< DL	210 ± 20	375 ± 28
Nicotine (µg/l)	21	< DL	5.78 ± 0.99	12.78 ± 2.31
Formaldehyde (µg/l)	26	-	0.19 ± 0.05	0.29 ± 0.05
Acetaldehyde (µg/l)	26	-	12.00 ± 1.65	22.34 ± 2.15
Acrolein (µg/l)	26	-	1.20 ± 1.65	2.25 ± 0.22
Ammonia (µg/l)	20	< DL	0.30 ± 0.11	0.40 ± 0.14

In-life Observations

- Blood carboxyhemoglobin
- Body weight development
- Mortality

Blood Carboxyhemoglobin

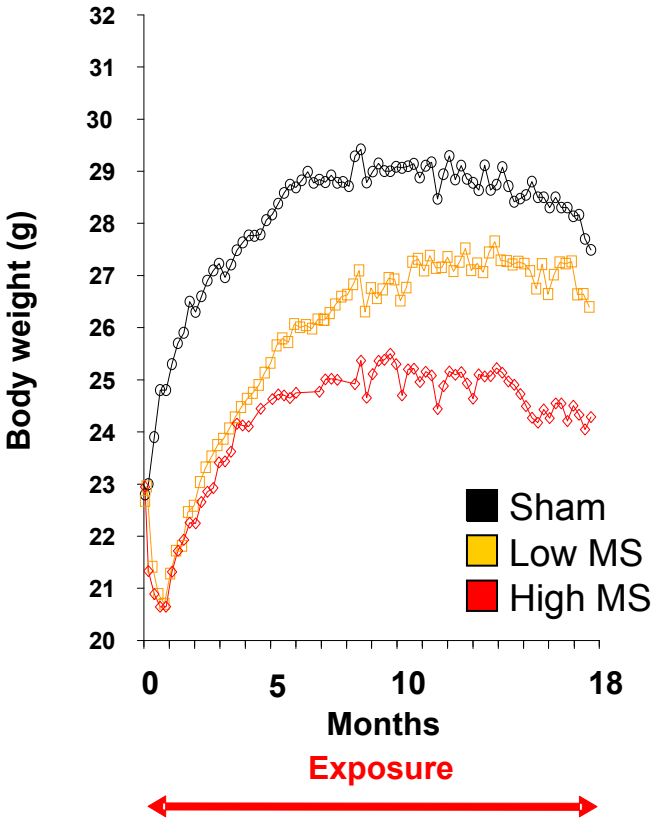


Data represent mean + SE

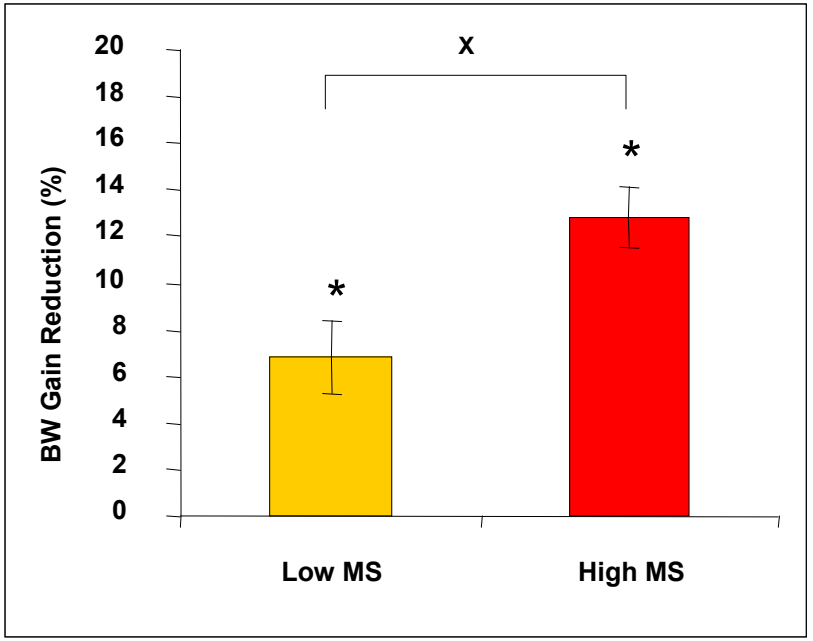
7 to 8 mice per group and determination

Body Weight Development

18 Months Exposure



BW Gain Reduction



Data for BW gain reduction represents mean ± SE.

* Statistically significant difference compared to sham, p≤0.05

X Statistically significant difference compared to low MS, p≤0.05

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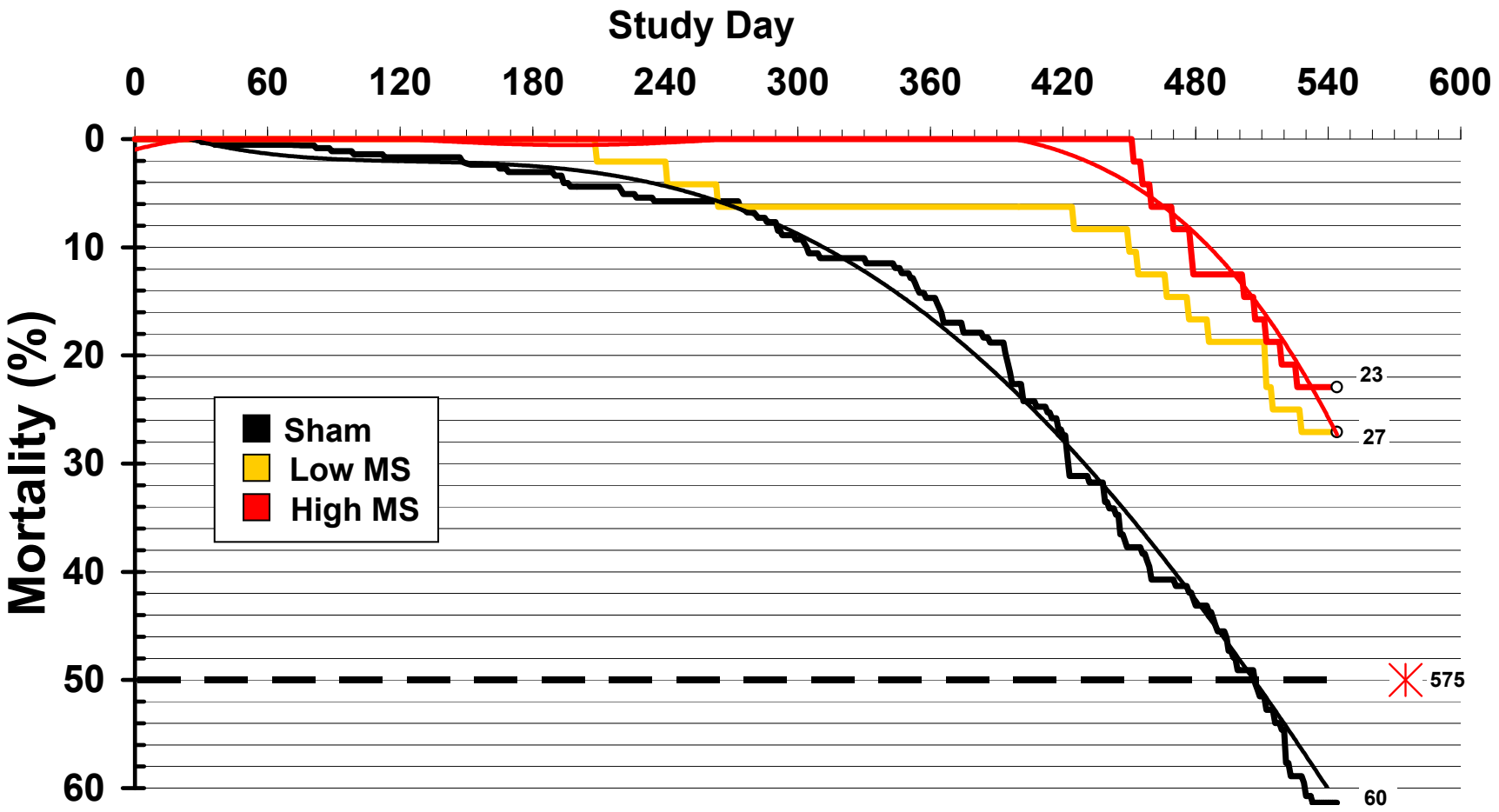
Mortality after 18 Months Exposure



Biological Outcome

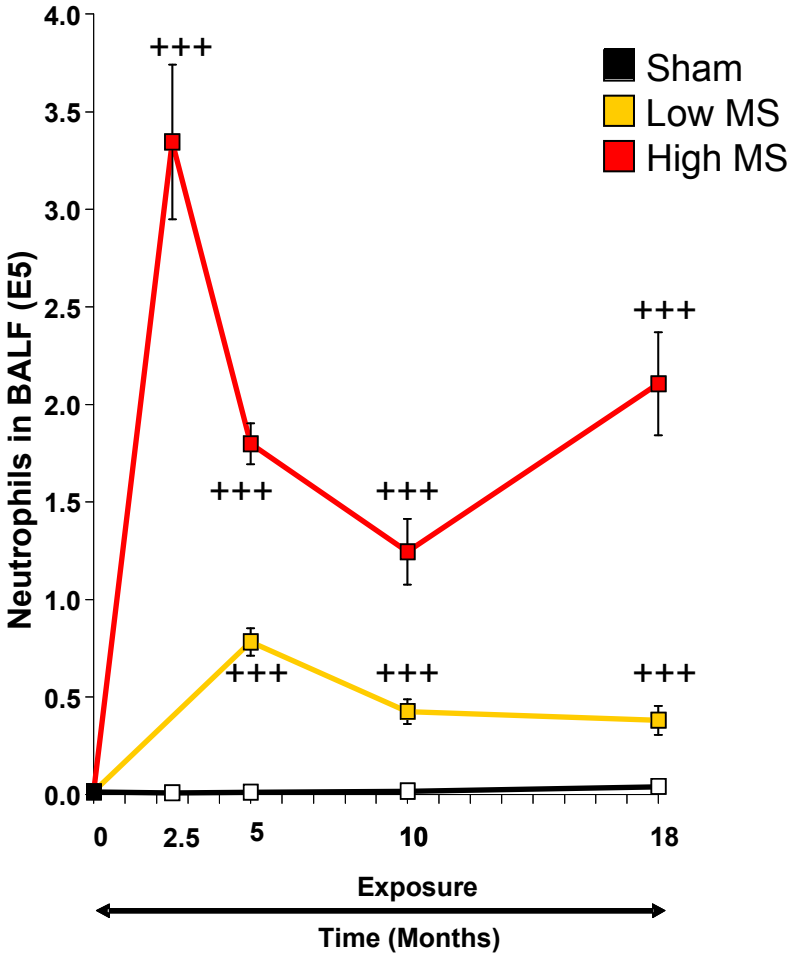
- Inflammation markers in the lung
- Lung tumor formation

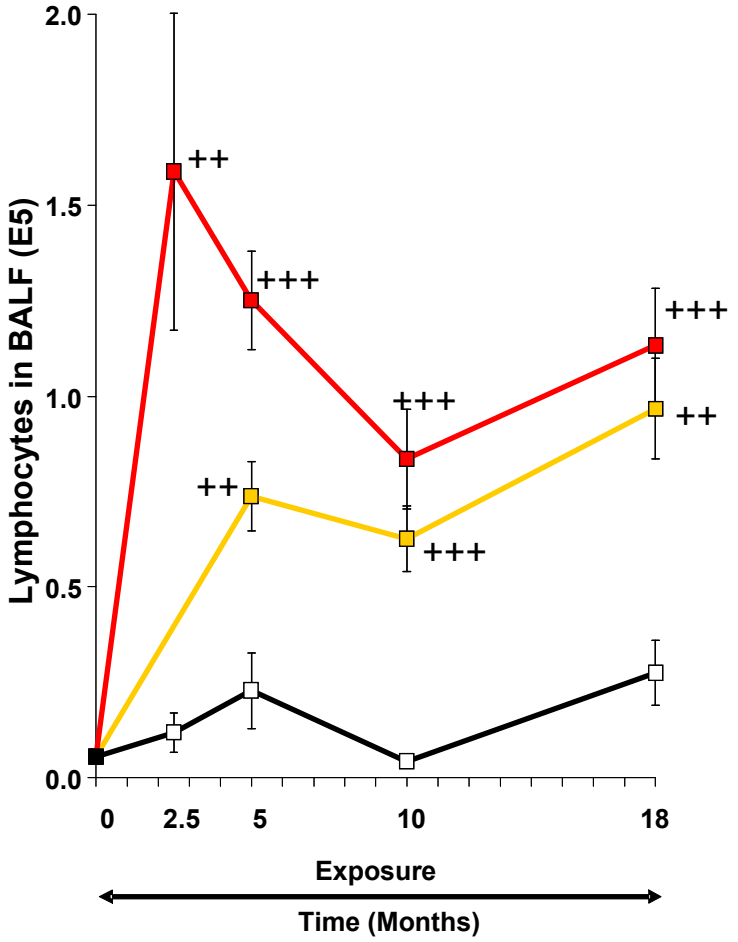
Inflammation Markers in Lungs of Mice

Bronchoalveolar Lavage Fluid (BALF)

Leukocytes	Inflammatory Mediators
Neutrophiles	IL1-α
	MCP-1
Lymphocytes	MIP-2
	MMP-9

Inflammatory Cells in BALF of Mice





mean ± SE;
+++, p <0.001; ++, p <0.01;
ANOVA plus Dunnett posthoc test

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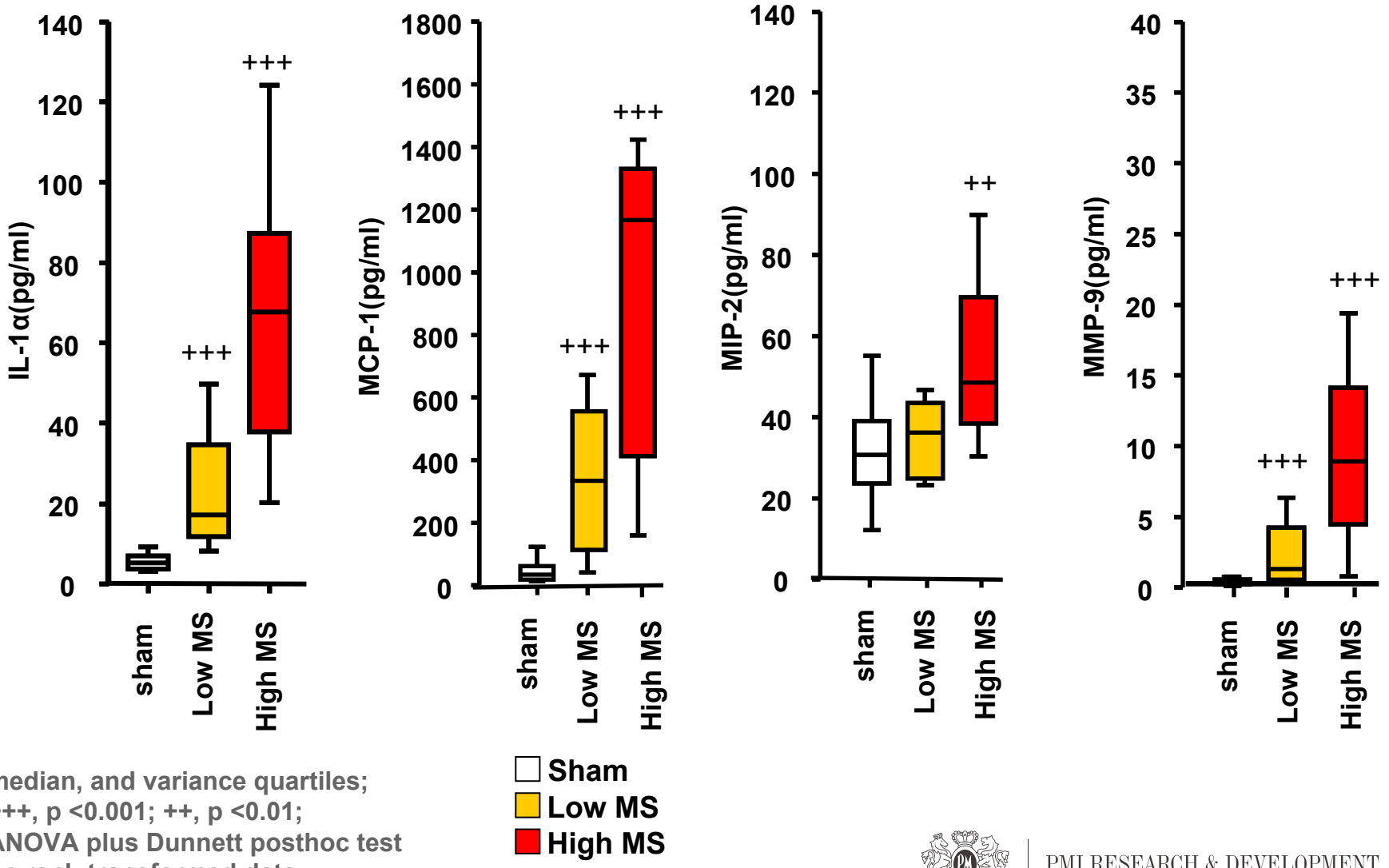
Inflammatory Mediators in BALF of Mice



median, and variance quartiles;
+++, p <0.001; ++, p <0.01;
ANOVA plus Dunnett posthoc test
on rank-transformed data

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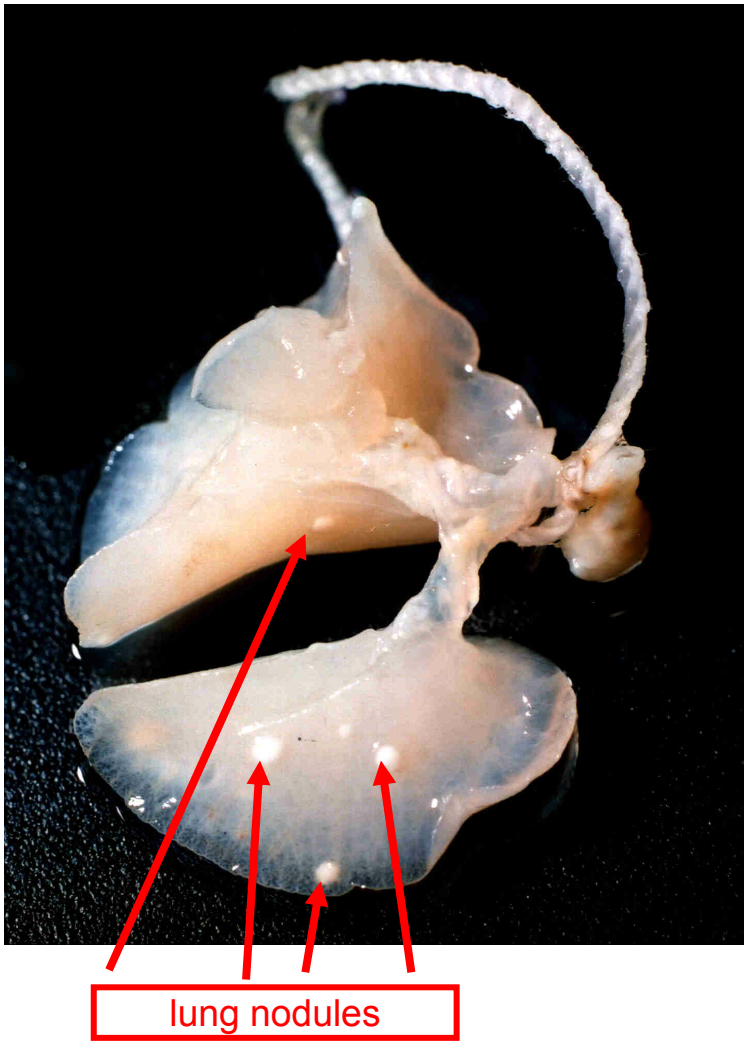
Nodules in the A/J Mouse Lung



hyperplasia

↓

bronchioloalveolar adenoma

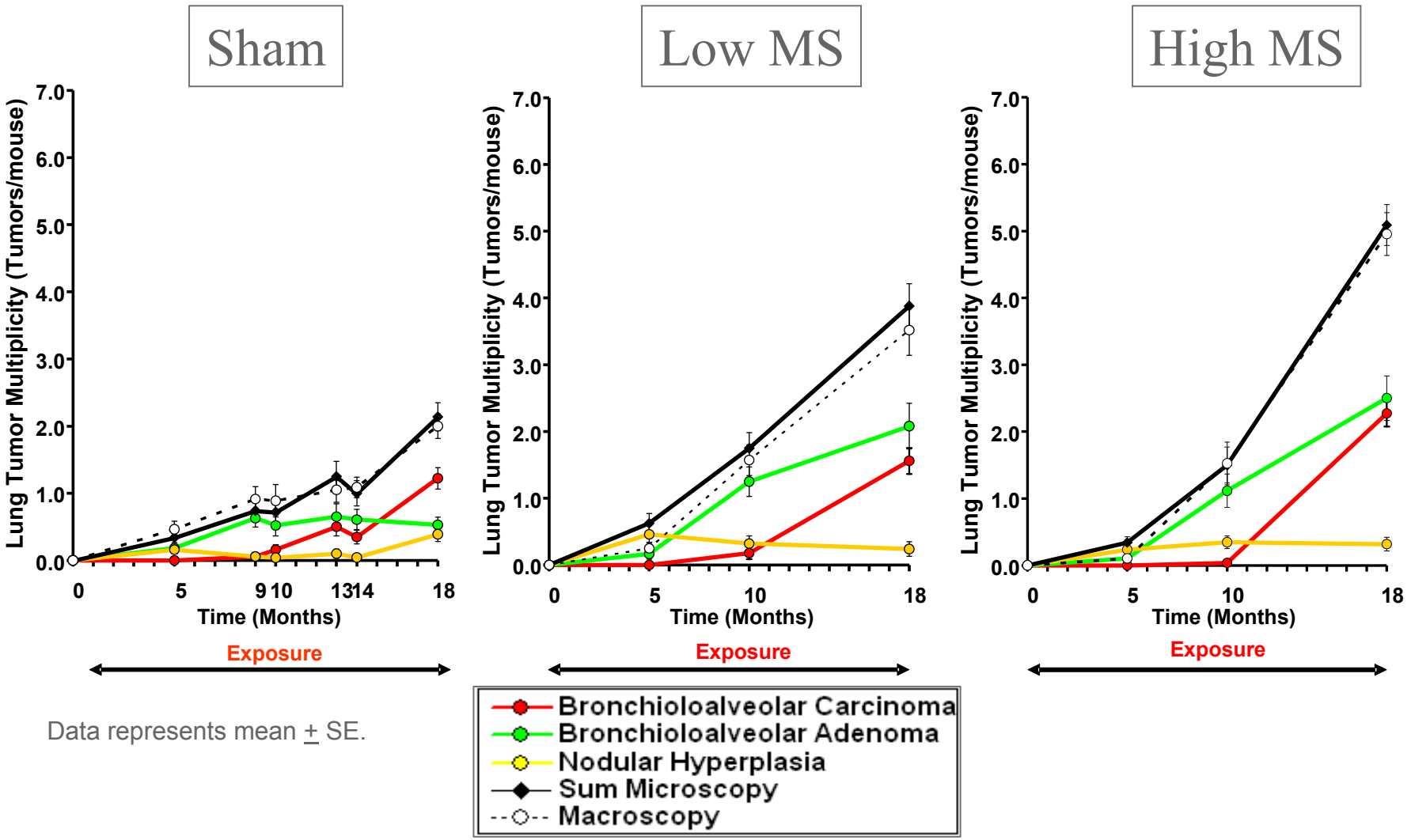
↓

bronchioloalveolar adenocarcinoma

Evaluation of Lung Tumor Response

- Macroscopic counting of nodules after immersion in Tellyesniczky's fixative for 1 day followed by fixation in ethanol
- Histopathological evaluation of serial lung sections cut at a distance of 300 µm
- Lung tumor classification in accordance with WHO guidelines (2001)

Time Course of Lung Tumor Multiplicity



Data represents mean ± SE.

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Summary and Conclusions

- Under long-term, carefully controlled conditions, the A/J mouse is a promising model for cigarette-smoke-related lung tumorigenicity

  - Severe inflammation in the bronchoalveolar lavage of smoke-exposed mice

  - Significant and concentration-dependent enhancement of lung tumors, i.e., adenomas and adenocarcinoma was observed

- The relevance of the A/J mouse model for cigarette-smoke-induced lung tumors in humans requires further validation

Acknowledgements

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