

1 Lung Developmental Is Altered After Inhalation Exposure to Various Concentrations of Calcium  
2 Arsenate

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24 Short Title: Lung Developmental Changes

25 **Abstract**

26 Exposure to dust from active and abandoned mining operations may be a very significant  
27 health hazard, especially to sensitive populations. We have previously reported that inhalation of  
28 real-world mine tailing dusts during lung development can alter lung function and structure in  
29 adult male mice. These real-world dusts contain a mixture of metal(oid)s, including arsenic. To  
30 determine whether arsenic in inhaled dust plays a role in altering lung development, we exposed  
31 C57Bl/6 mice to a background dust (0 arsenic) or to the background dust containing either 3% or  
32 10% by mass, calcium arsenate. Total level of exposure was kept at 100  $\mu\text{g}/\text{m}^3$ . Calcium arsenate  
33 was selected since arsenate is the predominant species found in mine tailings. We found that  
34 inhalation exposure during *in utero* and postnatal lung development led to significant increases in  
35 pulmonary baseline resistance, airway hyper-reactivity, and airway collagen and smooth muscle  
36 expression in male C57Bl/6 mice. Responses were dependent on the level of calcium arsenate in  
37 the simulated dust. These changes were not associated with increased expression of TGF- $\beta$ 1, a  
38 marker of epithelial to mesenchymal transition. However, responses were correlated with  
39 decreases in the expression of Club cell protein 16 (CC16). Dose dependent decreases in CC16  
40 expression and increases in collagen around airways was seen for animals exposed *in utero* only  
41 (GD), animals exposed postnatally only (PN) and animals continuously exposed throughout  
42 development (GDPN). These data suggest that arsenic inhalation during lung development can  
43 decrease CC16 expression leading to functional and structural alterations in the adult lung.

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45 Key Words: Simulated Mine Tailings Dust, Lung Development, Lung Disease.

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## 48 **Introduction**

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50           The low humidity and strong winds found in the arid US Southwest make respirable dust  
51 levels potentially problematic. The incidence of high dust storms in the Southwestern US has been  
52 increasing in recent years and this is especially a serious concern in arid and semi-arid regions due  
53 to the increased susceptibility to windborne transport of metal-laden dust particles. This issue can  
54 be particularly acute just downwind from sites where dusts can contain high levels of metal(loid)s,  
55 including arsenic (Csavina et al., 2011). Global climate models predict warmer and drier  
56 conditions in the Southwest in the near future that will heighten windblown dust and the abundance  
57 of dust-borne particulates among other effects. Thus, there are immediate and growing needs to  
58 evaluate the toxicity of dusts and their components. Limited studies suggest that inhalation  
59 exposures are also relevant in certain environmental settings (Chan et al., 2011; Fedulov et al.,  
60 2008; Mauad et al., 2008; ). With the exception for a few studies on occupational exposures, little  
61 data exist concerning the risk in adults from exposure to arsenic containing dusts through  
62 inhalation. Our own studies following developmental exposure to real-world dusts containing  
63 arsenic are among the few that have examined the effect of inhalation of arsenic containing dusts  
64 during *in utero* and early life exposure (Witten et al., 2019).

65           Our data indicate that arsenic exposure through drinking water or inhalation of complex  
66 real-world dusts containing arsenic during early life development leads to alterations consistent  
67 with the development of chronic lung disease. Changes we have reported include increased airway  
68 reactivity, increased airway smooth muscle mass (most prominent in smaller airways), increased  
69 airway collagen deposition and altered expression of TGF- $\beta$ 1 and MMP-9. These data are  
70 consistent with alterations in lung function, increased inflammation and increased chronic

71 respiratory symptoms in children (ages 6 -12) that have been exposed to high levels of arsenic in  
72 their drinking water ( Recio-Vega et al., 2015; Smith et al., 2013; Olivas-Calderon et al., 2015;  
73 Farzan et al., 2013; Mazumder et al., 2007) and point to arsenic-induced epithelial to mesenchymal  
74 transmission (EMT) as a mechanism. Inappropriate EMT can lead to phenotypic alterations in the  
75 airway epithelium. These phenotypic alterations can include Club cells where a change in  
76 phenotype could lead to decreased expression of Club cell protein 16 (CC16: also called CC10,  
77 CCSP, Uteroglobin or SCGB1A1)

78           CC16 is a homodimeric pneumoprotein that was initially attributed to Club cells (a.k.a.  
79 Clara cells) in distal airways, but has since been shown to be expressed by many other non-ciliated  
80 epithelial cells in both proximal and distal airways (Coppens et al., 2007; Singh et al., 1988; Boers  
81 et al., 1999). CC16 is present in lung fluids and circulation (Lakind et al., 2007), and it was  
82 originally considered as a lung injury marker indicative of epithelial damage, for which epithelial  
83 leakage and impaired barrier function were speculated to contribute to the CC16 presence in the  
84 circulation. However, in chronic airway diseases such as COPD or asthma, although the epithelial  
85 barrier is compromised, low (but not high) CC16 in the serum has consistently been demonstrated  
86 to associate with decline of lung function (Lomas et al., 2008; Ye et al., 2004). In some of these  
87 studies, CC16 in lung fluids or sputum has also been significantly decreased (Braido et al., 2007).  
88 We have reported that cellular levels of CC16 could be severely downregulated by environmental  
89 smoke exposure (Zhu et al., 2015). The change of serum CC16 may reflect its altered production  
90 in the lung. We, and others, have demonstrated that low serum CC16 is a significant risk factor for  
91 impaired lung growth and function in childhood and for accelerated lung function decline and  
92 development of COPD and lung cancer in adults (Guerra et al., 2013; Guerra et al., 2015; Park et  
93 al., 2013). The molecular pathways through which CC16 exerts its function have been poorly

94 understood, but this molecule has demonstrated anti-inflammatory, anti-oxidant and anti-tumoral  
95 properties in the lung via inhibition of PLA2 activity (Levin et al., 1986), proinflammatory  
96 prostaglandins (Mandal et al., 2005), chemotaxis (Antico et al., 2006) and cytokine production  
97 (Hung et al., 2004).

98         Adult arsenic exposures via chronic ingestion or occupational inhalation can alter CC16  
99 levels in adults (Parvez et al., 2008). In a study from Bangladesh with water arsenic levels at 160  
100 ppb (WHO/USEPA recommended guideline < 10 ppb), serum CC16 was inversely associated with  
101 urinary arsenic levels in individuals with evidence of arsenic disease (skin lesions) (Parvez et al.,  
102 2008). Decrement in lung function was also associated with lower CC16 levels. In an occupational  
103 setting, inhalation of arsenicals at 22 ng/m<sup>3</sup> resulted in urinary arsenic level of 70 ppb and a  
104 decrement in serum CC16 levels (Halatek et al., 2014). Pulmonary diseases associated with arsenic  
105 exposure have been shown to be most severe when exposures occur during critical early life  
106 developmental times (Dauphine et al., 2011; Hamada et al., 2007). In children living downwind  
107 from a legacy mine tailing, urinary CC16 was associated with soil arsenic, suggesting that  
108 localized arsenic exposure in the lungs could affect the airway epithelium and predispose children  
109 for diminished lung function (Beamer et al. 2016).

110         In this report we examined the effect of using various concentrations of inhaled calcium  
111 arsenate to simulate-mine tailings dusts exposures during lung development. We have seen  
112 increased airway hyper-reactivity, increased baseline pulmonary resistance and increased collagen  
113 and smooth muscle around airways. These alterations do not appear to be the result of arsenic-  
114 induced EMT through activation of TGF- $\beta$ 1, but rather may be caused by arsenic-induced decrease  
115 in expression of CC16. The finding that developmental exposures can lead to decreased CC16  
116 levels and increased airway collagen suggest Club cells may be a target of arsenic exposure.

117 **Methods**

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119 *Animal Inhalation Exposures*

120 Following mating, plugged female C57BL/6 mice were exposed daily to either background  
121 Arizona road dust (Powder Technology, Arden Hills, MN, no arsenic or other metals), low (3%)  
122 calcium arsenate, or high (10%) calcium arsenate dust aerosol by the Vilnius dust generator (CH  
123 Technologies, Westwood, NJ) attached to 9 individual inhalation cages with an “in-line, real-time”  
124 aerosol particle measurement device (Casella Microdust Pro Sampler, Casella Measurement,  
125 Bedford, UK) with continuous negative feedback. Dust generation was at the flow rate of 2 L/min  
126 at a concentration of 4.8 mg/m<sup>3</sup> for 30 min/day. This exposure period of 30 min/day was chosen  
127 to reduce stress to the mice to achieve a 24-hour time weighted average exposure of 100  
128 micrograms/m<sup>3</sup>. The level of dust concentration inside the cages was also measured by a separate  
129 device, Dust Track II, which was placed inside the exposure system. The concentration of the dust  
130 exposure was adjusted by constant evaluation of the two dust measurement systems.

131 This study was carried out in strict accordance with the recommendations in the Guide for  
132 the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was  
133 approved by the Committee on the Ethics of Animal Experiments of the University of Arizona  
134 (Protocol Number 07-140). All surgery was performed under sodium pentobarbital anesthesia,  
135 and all efforts were made to minimize suffering. Four groups of mice were utilized in the study;  
136 a control group exposed to ambient air combined with background Arizona road dust (Control), *in*  
137 *utero* (GD) or post-natal only (PN) exposures and a combined *in utero* and post-natal exposure  
138 group (GD + PN). Same day pregnant mice were placed in groups and exposed to the dust until  
139 they reached gestational time of 18 days and then were divided into two groups; *in utero* only

140 group that had their dust exposure stopped at 18 days and a combined *in utero* and post-natal  
141 exposure group that kept undergoing the dust inhalation exposure. The post-natal group was  
142 housed in the animal facility until they reached gestational day 18 and then were transferred to the  
143 inhalation cages whereby the dust exposures commenced on the day of birth of the mouse pups.  
144 The mouse pups were exposed on a daily basis until the pups reached 28 days old when the  
145 inhalation exposure was stopped. The mice underwent pulmonary function tests, were sacrificed,  
146 and their lungs harvested for pathological study.

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#### 148 *Cytokine Analyses*

149 Eve Technologies (Calgary, Canada) Multiplex 32 cytokines and chemokines analysis  
150 were measured in plasma and BALF fluids for this study as an initial screen for alterations in  
151 expression in plasma and BALF fluids. Alterations seen with the multiplex were validated using  
152 ELISA from R&D Systems (Minneapolis, MN).

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#### 154 *Pulmonary Function Tests*

155 Airway responsiveness and respiratory mechanics were assessed by the Flexivent system  
156 by SCIREQ. Following sodium pentobarbital anesthesia (90 mg/kg body weight), the mouse  
157 trachea was surgically exposed, and a cannula was inserted into the trachea and tied off with  
158 surgical string. The mouse was then paralyzed with pancuronium bromide, 4 mg/kg, IP and then  
159 connected to a small animal ventilator with a respiratory rate set at 150 breaths/min, tidal volume  
160 of 10 ml/kg body weight, and 2.5 cm H<sub>2</sub>O PEEP for 5 min with 0, 0.5, 1.0, 2.0, and 2.5  
161 microgram/g body weight methacholine delivered sequentially via a nebulizer. After each dose,  
162 the Flexivent system would automatically perform a force oscillation cycle and acquire various

163 lung mechanical data including dynamic resistance, elastance, and compliance of the respiratory  
164 system. Each methacholine dose delivery and its measurement were completed within a 3 minutes  
165 time frame. The mouse was under continuous thermal support using a heat lamp throughout the  
166 pulmonary function test protocol.

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#### 168 *Lung Immunohistochemistry and Stereology*

169 Immunohistochemistry was utilized to localize alpha smooth muscle actin (SMA) (ab  
170 5694, Abcam, MA), TGF- $\beta$ 1 (Ab215715, Abcam, MA) SNAIL1 (sc28199, Santa Cruz, CA.),  
171 TWIST (sc15393, Santa Cruz, CA) and CC16 (ab40873, Abcam, MA). Lungs were fixed by  
172 intratracheal instillation of buffered formalin at a constant pressure of 20 cm H<sub>2</sub>O. Paraffin  
173 embedded sections (5 microns) were baked for 1 hour at 65 degrees C then subjected to a series of  
174 de-paraffinization (3 times of 5 min in xylene) and dehydration. The microwave antigen retrieval  
175 method was performed by boiling in 10mM sodium citrate buffer, pH of 6, for 10 min, cooled for  
176 20 min after the slide was washed with PBS. The slide was then incubated in 1% H<sub>2</sub>O<sub>2</sub> to quench  
177 peroxidase activity for 10 min, washed with PBS, and then incubated in 2% normal secondary host  
178 serum for 30 min. The slides were incubated with primary antibodies diluted in PBS 0.05% Tween  
179 20 for one hour at room temperature. The antibodies dilutions utilized were: Alpha SMA at 1:500,  
180 TGB- $\beta$ 1 at 1:100, SNAIL1, at 1:200, TWIST at 1:100 and CC16 at 1:100. After three washes of  
181 3 min each of buffer (PBS with 0.1% Tween 20), the slides were incubated with biotinylate HRP  
182 secondary antibodies for one hour using ABC Vector Elite kits (Vector Laboratories, Burlingame,  
183 CA). The slides were again washed 3 times for 3 min each wash, incubated with Avidin-Biotin  
184 complex for 30 min, and finally administered another 3 washes with buffer solution. The color  
185 development was done using Vector VIP detection solution following the manufacturer's

186 instructions. Airway collagen was stained using pico-sirius red dye special stain and visualized  
187 by polarized light microscopy. Elastin was stained using Miller's stain.

188         The amount of smooth muscle, elastin and collagen around the airways was quantitated by  
189 analyzing digital images collected using SimplePCI software (Pittsburgh, PA). Sections of lung  
190 tissue were scanned, and all airways cut in cross section (the ratio of maximum to minimum  
191 diameter was less than 2) were analyzed. Diameters were determined by filling of the area inside  
192 of the airway epithelium. SimplePCI was able to obtain the minimum and maximum diameters of  
193 a region of interest. Basement membrane perimeter was obtained by tracing. The area of smooth  
194 muscle was determined by thresholding the images to detect only the antibody staining and  
195 measuring the number of pixels detected (Camateros et al., 2007). The area of collagen staining  
196 was obtained using polarized light microscopy of picosirius red stained sections (Last et al., 2004)).  
197 Area of smooth muscle actin, elastin and collagen staining were then normalized to the square of  
198 the basement membrane perimeter ( $BM^2$ ) (Camateros et al., 2007). Data were analyzed for all  
199 airways in cross section on the chosen slides and also were subdivided by airway diameter (small  
200 airways, diameter < 100 microns versus large airways, diameter > 100 microns). The minimum  
201 diameter was used as a measure of the airway diameter (Weibel, 1979).

202         For TGF- $\beta$ 1, SNAIL, TWIST and CC16, we were only interested in the level of expression  
203 in the airway epithelium. Digital imaging allowed us to isolate the airway epithelium from the  
204 remainder of the lung tissue. Levels of expression were then determined in the epithelium by  
205 thresholding the image and measuring the number of positive pixels. This was then normalized to  
206 the area of the airway epithelium giving us a volume density in the airway epithelium for each of  
207 the proteins analyzed.

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*Statistical Analyses*

In order to ensure that data were collected in an unbiased manner, for each animal, tissue slides to be analyzed were selected using a systematic random sampling method (Weibel, 1979). Once a slide was selected, all airways that appeared in cross-section on that slide (maximum to minimum radius of 2 or less) were analyzed. The person collecting the data was blinded to the exposure group being analyzed. In addition, data were collected by a single person to eliminate inter-individual variation. Sample collection continued until intra-animal coefficient of error was 10% or less. Animal means were calculated using values from all airways analyzed for that animal. Analyses of whether statistical differences occurred between exposure groups (dust, Lo CA and Hi CA) was determined using ANOVA, requiring  $p < 0.05$  for statistical significance (Winer et al., 1991). Student-Newman-Keuls procedure was used *post hoc* to determine which groups were significantly different from each other. The “N” number listed in results is the number of animals.

224 **Results**

225           Developmental inhalation exposure to dust containing various levels of calcium arsenate  
226 (CA) caused physiological changes in the lungs of male mice. Our previous results had indicated  
227 that the combined *in utero* and postnatal exposure to dusts, as would be encountered in the real-  
228 world, led to the most significant physiological and structural changes in the lung. We therefore  
229 concentrated our initial analysis on continuous developmental exposure. Baseline and  
230 methacholine challenged (airway reactivity) values of pulmonary resistance were measured in  
231 male (Figure 1A) and female (Figure 1B) mice.

232           Continuous developmental exposure to the dust resulted in a significant increase in airway  
233 resistance after methacholine challenges in male mice. In 28-day-old animals, the degree of  
234 airway reactivity depended on the amount of calcium arsenate in the simulated dust. Lo CA  
235 exposures (3% calcium arsenate in dust) caused significant increases in airway hyper-reactivity  
236 (AHR) compared to dust alone (no arsenic). Exposure to Hi CA dusts (10% calcium arsenate in  
237 dust) led to greater AHR compared to the Lo CA exposures. Airway baseline resistance was also  
238 significantly increased as a function of CA levels in the dust. Baseline resistance in male mice  
239 exposed to dust alone was 0.55 +/- 0.02 cm H<sub>2</sub>O/ml. Lo CA exposure did not result in an increase  
240 in baseline resistance (0.56 +/- 0.02 cm H<sub>2</sub>O/ml). However, the baseline resistance in male mice  
241 exposed to the Hi CA was increased compared to the other two exposure levels (0.67 +/- 0.02 cm  
242 H<sub>2</sub>O/ml). Female mice did not show a similar response. Females did not have a significant increase  
243 in AHR for any of the exposure groups (females exposed to the Hi CA dust continuously  
244 throughout development is shown in Figure 1B). Since the females did not demonstrate any  
245 pulmonary function alterations, subsequent data were only collected in the males.

246           Increases in airway reactivity and baseline resistance seen could be due to interactions with  
247 developmental processes leading to structural alterations to the airways. We have previously  
248 shown that continuous *in utero* and postnatal exposure to arsenic in drinking water resulted in  
249 increased airway reactivity associated with alterations in smooth muscle and extracellular matrix  
250 around airways (Lantz et al, 2009). Alterations in smooth muscle and extracellular matrix around  
251 airways was also seen following exposure to real-world dusts containing a mixture of metals  
252 (Witten et al., 2019). These alterations could result from induction of epithelial to mesenchymal  
253 transition (EMT) leading to alterations in developmental programming. Alterations in EMT can  
254 result in altered smooth muscle and extracellular matrix. We first examined levels of collagen  
255 around airways in 28-day-old male mice to detect airway remodeling in response to the arsenic  
256 containing dust exposures (Figure 2). Tissue sections were stained with picosirius red and were  
257 visualized under polarized light microscopy. Areas of positive staining were then counted in the  
258 adventitia around airways. As can be seen from the micrographs (bright areas) and the quantitative  
259 image analysis, increases in airway collagen occurred following continuous developmental  
260 exposure to the Hi CA dust. This increased expression was seen in both small (<100  $\mu$ m) and  
261 large (>100  $\mu$ m) airways. While collagen levels around airways was increased by HI CA  
262 exposure, elastin levels around airways were not significantly altered (Figure 3). Similarly, to  
263 collagen, exposure to Hi CA dusts lead to increased smooth muscle around both small and large  
264 airways (Figure 4).

265           It is accepted that EMT plays a critical role in airway remodeling, (Lamouille et al, 2014;  
266 Xu et al, 2009) and that TGF- $\beta$ 1 is a master regulator of EMT. We have previously seen alteration  
267 in EMT markers in animals exposed to arsenic in drinking water or to real-world dusts during  
268 development. To determine if inhalation exposure to our simulated dust induced EMT, we

269 evaluated changes in EMT markers TGF- $\beta$ 1, SNAIL1 and TWIST. Continuous developmental  
270 exposure to Hi CA levels in 28-day-old male mice did not result in increased expression of TGF-  
271  $\beta$ 1 or TWIST in airway epithelium. However, we did see significant increases in airway epithelial  
272 expression of SNAIL1. (Figure 5). (Figure 5 shows representative IHC stained micrographs of  
273 each of these EMT markers as well as quantitative image analysis. Arrows indicate that image  
274 analysis was only performed on airway epithelium).

275 Alterations in EMT during development can potentially alter the phenotype of airway  
276 epithelial cells. Levels of CC16 expression have been associated with alterations in lung function  
277 and increased expression of matrix and smooth muscle around airways. We therefore determined  
278 whether inhalation exposures to arsenic containing dusts during development could be associated  
279 with decreased expression of CC16 in the airways, leading to alterations in lung function and  
280 increased deposition of collagen and smooth muscle around airways.

281 Using IHC, we determined the level of CC16 expression in the lungs was decreased in a  
282 dose dependent manner following continuous developmental exposure to dusts containing calcium  
283 arsenate. Figure 6 shows representative micrographs of CC16 stained tissue. Arrows indicate that  
284 image analysis was performed on airway epithelium. Image analysis shows that CA exposure  
285 reduced CC16 expression in a dose dependent manner.

286 A broad screening multiplex analysis identified several cytokines, chemokines and EMT  
287 markers that were potentially altered by exposure to the arsenic containing dusts. Six of these  
288 were validated in BALF and plasma using ELISA (Table 1). Exposure to the Hi CA dust resulted  
289 in significant increases in expression for IL-13, CXCL9 and CXCL10 in BALF. MMP-9, CXCL9  
290 and CXCL10 were also increased in the plasma. TGF- $\beta$ 1 and eotaxin were not increased.

291 We have previously shown that the continuous exposures from conception through early  
292 postnatal development led to the most severe changes in lung structure. We were interested to see  
293 if *in utero* or early postnatal exposures alone could reproduce the alterations seen with the  
294 continuous exposures. Figure 7 shows the IHC image analysis results when exposure to dust, Lo  
295 C and Hi CA were carried out *in utero* only (GD), postnatally only (PN) or exposures occurred  
296 both *in utero* and postnatally (GDPN). With the Lo CA exposures, significant alterations in CC16  
297 exposure were only seen with the continuous developmental exposures (Figure 7). However,  
298 exposure to the Hi CA levels resulted in decreased CC16 expression regardless of the  
299 developmental time of exposure.

300 These changes correlated to changes in collagen expression around the airways. Hi CA  
301 exposures during both gestation and early postnatal development resulted in increased collagen  
302 expression around airways (Figure 8).

303 Loss of CC16 positive cells following inhalation of calcium arsenate could contribute to  
304 the alterations we are seeing in lung function and structure. We have previously seen similar  
305 alterations in lung function and structure following inhalation of real-world dusts during  
306 development. We, therefore, wanted to see if inhalation of mine tailing dust also resulted in loss  
307 of CC16 positive cells. Figure 9 shows that, similarly to the calcium arsenate results, inhalation  
308 exposure to mine tailing dust during development also lead to decreased expression of CC16.  
309 Similar to the Hi CA exposures, exposure to the real-world dusts resulted in decreased CC16  
310 expression when exposure occurred during any of the developmental times tested. Arrows indicate  
311 that analysis was carried out on airway epithelium only.

312 **Discussion**

313           Using a mouse model, we have examined the effect(s) of inhalation of arsenic containing  
314 dusts that occur during *in utero* and early postnatal development. Our results indicate that  
315 inhalation of dusts containing calcium arsenate resulted in increased airway hyper-reactivity and  
316 increased expression of collagen and smooth muscle around airways. The levels of alterations in  
317 lung structure and function were a function of the levels of calcium arsenate in the inhaled dusts,  
318 indicating a dose-response for the inhalation exposure to calcium arsenate. When we examined  
319 whether there was a sensitive developmental window that resulted in greater alterations, we found  
320 that gestational exposure alone, postnatal exposure alone or continuous gestational and postnatal  
321 exposures all resulted in increased collagen expression after exposure to Hi levels of calcium  
322 arsenate containing dusts and that the level of response depended on the level of calcium arsenate  
323 exposure.

324           Our results also show that only males exposed to the inhaled arsenic containing dusts  
325 showed significant alterations in pulmonary function. Females from the same litters did not show  
326 differences from controls. These results are similar to previously reported alterations following  
327 early life exposures to PM<sub>2.5</sub> (Jedrychowski et al., 2009; Hsu et al., 2015), to real-world dusts from  
328 mine tailings (Witten et al., 2019), or ingested arsenic (Raqib et al., 2009).

329           We have previously shown that inhalation exposure to real-world metal containing dusts  
330 during sensitive developmental times can lead to alterations in lung function and structure.  
331 Because the real-world dusts were a mixture of several different metals, we wanted to test whether  
332 arsenic was contributing to the alterations that we were seeing after exposure to real-world dust.  
333 In order to test whether arsenic inhalation could contribute to the alterations, we added calcium  
334 arsenate at several concentrations to a background dust that did not contain metals. We used

335 calcium arsenate since arsenates are the predominant arsenic species found in the real-world mine  
336 tailing dusts we have used in previous studies (Thomas et al., 2018). We kept the total exposure  
337 level of dusts at a constant level, only changing the arsenate concentrations. We found that  
338 alterations in pulmonary function and airway extracellular matrix following inhalation exposure  
339 to calcium arsenate dusts are similar to alterations we have seen following exposure to real-world  
340 dusts. Similarities between the two exposures includes alterations in airway hyper-reactivity and  
341 pulmonary baseline resistance, increased levels of smooth muscle and collagen around airways  
342 and increased expression of EMT transcription factor SNAIL1.

343         There were some interesting differences between the responses to exposure to real-world  
344 dusts and to our synthetic calcium arsenate dusts. First, the levels of arsenic in the dusts were quite  
345 different. In the real-world dusts, arsenic made up less than 0.5% of the mass, while the calcium  
346 arsenate dusts required that arsenic be 10% of the total mass to see the same effects. This  
347 difference could be due to having arsenic as the only metal(oid) in the current exposures, while  
348 the real-world dusts contain other metals that could contribute to the responses seen. Our previous  
349 results following ingestion of arsenic or inhalation of real-world dusts have indicated that the  
350 alterations we have seen may be due to activation of epithelial to mesenchymal transition. In those  
351 reports, significant increases in EMT markers TGF- $\beta$ 1 and MMP9 were seen. TGF- $\beta$ 1 was not  
352 significantly increased following calcium arsenate exposures. However, SNAIL1 and MMP9,  
353 EMT markers, were increased. This may indicate that the calcium arsenate is still leading to  
354 increased EMT, but through a pathway that does not require activation by TFG- $\beta$ 1, such as the  $\beta$ -  
355 catenin pathway (Kim et al., 2019).

356         The levels of calcium arsenate needed to produce alterations in lung structure and function  
357 are more than 10 times higher than the levels in the real-world dusts we have previously used.

358 However, because the responses are dependent on the level of calcium arsenate, we believe that  
359 the responses are being caused by the arsenic exposure. While the arsenic species in the real-world  
360 dust we used is predominantly arsenates, the bioavailability of arsenic may be different between  
361 the real-world dusts and calcium arsenate. There may be differences in the solubility between  
362 arsenic compounds seen in the real-world dusts and the calcium arsenate used in these experiments.  
363 Estimates of solubility of arsenic in real-world mine tailing dusts is around 10 – 50% of that of  
364 soluble forms of arsenic (sodium arsenate) (Arizona Department of Health Report on Iron King  
365 Mine & Humboldt Smelter, March 26, 2009). However, calcium arsenate is 1000 times less  
366 soluble than sodium arsenate, indicating that calcium arsenate exposure may lead to soluble arsenic  
367 levels that are equivalent or even less than arsenic from real-world mine tailing dust.

368 Arsenic exposures have been shown to decrease the levels of CC16 expression and this  
369 decrease may lead to alterations in lung function and structure. We therefore examined the effect  
370 of inhalation of arsenic or real-world dusts on the expression of CC16 in the lung. We found that  
371 both calcium arsenate and real-world dusts led to significant decreases in the expression of CC16.  
372 These decreases were associated with increased collagen deposition around the airways. Changes  
373 in lung function and structure that we have seen following exposure to calcium arsenate are similar  
374 to those seen in CC16 knockout mice. Both show alterations in AHR and baseline resistance as  
375 well as alterations in smooth muscle and collagen around airways. These changes occur in the  
376 absence of increased TGF- $\beta$ 1 expression, both in our experiments and in CC16 KO mice (Zhai et  
377 al., 2019). These similarities lend support to the idea that inhibition of CC16 following calcium  
378 arsenate or real-world dust exposure contributes to alterations in lung structure and function.

379 Decreases in CC16 expression occurred during all the developmental stages we examined.  
380 With both calcium arsenate and real-world dust exposures, decreases in CC16 expression and

381 increases in collagen expression occurred with *in utero* exposure only, postnatal exposure only or  
382 combined *in utero* and postnatal exposures. Decreases in the expression levels could be caused by  
383 several different mechanisms. First, we have shown that arsenic may directly be affecting the  
384 ability of the club cells to produce CC16. This may occur through arsenic suppression of retinoic  
385 acid signaling (Liu et al, 2020) by reducing retinoic acid receptor levels. In addition, we have  
386 reported that retinoids increase CC16 secretion in human bronchial epithelial cells from both  
387 normal individuals and patients with COPD; that these effects are mediated mainly through  
388 retinoic acid receptors, RAR $\alpha$  and RAR $\gamma$ . *In vivo*, vitamin A treatment results in a significant  
389 increase in circulating CC16 levels in individuals with no COPD (Chen et al 2017).

390 A second possible mechanism for reduction of CC16 expression following calcium  
391 arsenate exposure is a reduction in the number of CC16 producing cells. An et al., (2005), have  
392 previously shown that dimethylarsenic can target club cells presumably through oxidative stress.  
393 This could be due to cyclical pattern where arsenic reduces production of CC16, leading to  
394 increased response to oxidative stress, resulting in additional sensitivity of club cells to oxidative  
395 stress (Johnston et al, 1997; Mango et al., 1998; Plopper et al, 2006). Selective depletion of club  
396 cells can lead to lung inflammation (Reynolds et al, 2004).

397 Finally, we and others have shown that arsenic exposures can lead to EMT (Tang et al.,  
398 2017), potentially resulting in a change of club cell phenotype and reduction of CC16 expressing  
399 cells. While not as strong as we have seen in our previous reports, arsenic-induced EMT is still  
400 evident following calcium arsenate inhalation exposures.

401 The depletion of CC16 levels in the lung is an important contributor to the alterations in  
402 lung structure and function that we have seen. In comparing our results with those of CC16  
403 knockout mice (Zhai et al., 2019), we see that in both situations, lack of CC16 expression leads to

404 altered lung function and altered collagen and smooth muscle expression in the lungs. These  
405 changes occur without increases in TGF- $\beta$ 1 expression. These results lend support for a central  
406 role of CC16 in our responses.

407 Our experiments were designed to determine sensitive exposure times during development  
408 when arsenic exposure would have a major impact on adult function. We have previously shown  
409 that for real-world dusts, continuous exposures during *in utero* and postnatal periods is needed to  
410 show significant structural and functional changes. Based on previous reports that showed that  
411 CC16 levels do not reach adult levels until several weeks after birth in the mouse, we expected  
412 that postnatal exposures would be the most sensitive time (Coppens et al., 2009). However,  
413 decreases in CC16 expressing cells occurred even when exposures were only carried out during *in*  
414 *utero* development. Suppression of CC16 expression occurred after *in utero* alone exposures, after  
415 postnatal only exposures and after continuous *in utero* and postnatal exposures.

416 We have previously shown that increased airway reactivity after developmental exposure to  
417 soluble arsenicals and real-world dusts is associated with increased levels of IL-13, eotaxin,  
418 CXCL-9 and CXCL-10 (Zheng et al., 2012; Tao et al., 2013; Witten et al., 2019). Following  
419 calcium arsenate exposure, we have found increases in IL-13, CXCL-9 and CXCL-10 in the lung  
420 BALF. Increased expression of these inflammatory cytokines/chemokines maybe due to a  
421 decrease in CC16 levels. CC16 KO mice have increased expression of IL-13 (Chen et al, 2001).  
422 CC16 down regulates T<sub>H</sub>2 differentiation (Johansson et al, 2007).

423 Our aim has been to determine whether arsenic compounds inhaled during *in utero* and  
424 early postnatal life can result in altered adult lung function and structure. We have found that  
425 inhalation of calcium arsenate results in alterations in airway reactivity and changes in the collagen  
426 content around airways. These changes were dependent on the dose of inhaled arsenic, lending

427 support for the premise that these changes are a result of arsenic inhalation. Both arsenic exposure  
428 and decreased levels of CC16 in childhood have been associated with decreased adult lung  
429 function (Beamer et al, 2016; Lantz et al, 2009; Parvez et al, 2010; Smith et al, 2013). CC16  
430 appears to be a key protecting protein that is reduced in response to inhaled toxicants. In addition,  
431 alterations are associated with a decline in CC16 expression in the lungs, indicating that club cells  
432 may be a target for the inhaled arsenicals.

433

#### 434 **Acknowledgements**

435 This work was supported by National Institutes of Health, National Institute of  
436 Environmental Health Sciences R01ES027013 to RCL and YC, by the Southwest Environmental  
437 Health Science Center (P30ES006694) and by the University of Arizona Superfund Program  
438 (P42ES004940). The Cellular Imaging Core of the Southwest Environmental Health Science  
439 Center was essential for assisting us with the capture and analysis of tissue images.

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711

712 Figure Legends

713

714 Figure. 1. Airway reactivity following methacholine challenge. 28-day-old male (Fig. 1A) and  
715 female (Fig. 1B) mice were assessed for their baseline and methacholine challenged pulmonary  
716 resistance. Animals were exposed via inhalation to 100  $\mu\text{g}/\text{m}^3$  Arizona road dust with no arsenic  
717 (dust), or Arizona road dust containing, by weight, 3% (Lo CA) or 10% (Hi CA) calcium arsenate.  
718 Animals were exposed during both *in utero* and postnatal developmental periods. In males,  
719 continuous exposure to dusts containing calcium arsenate throughout development led to  
720 significant increases in airway reactivity compared to dust alone (no arsenic). The level of AHR  
721 increased as a function of the amount of calcium arsenate in the inhaled dust. Females did not  
722 show any increase in airway reactivity. For males N=9 for dust, N=10 for Lo CA and N=6 for Hi  
723 CA. For females, N=3 for both dust and Hi CA. A = significantly different from dust animals.  
724 B=significantly different from dust and Lo CA animals ( $p < .05$ ).

725

726 Figure 2. Airway collagen expression in 28-day-old male mice that had been exposed continuously  
727 throughout *in utero* and postnatal development. Slides were stained with picosirius red and  
728 collagen was imaged using polarized light microscopy. Micrographs show Arizona road  
729 dust/control 100  $\mu\text{g}/\text{m}^3$ , Lo calcium arsenate exposure (3% of total mass) and Hi calcium arsenate  
730 exposure (10% of total mass). The bright areas in the representative micrographs are collagen.  
731 Increases in collagen expression can be seen in the Hi CA exposure. Level of collagen expression  
732 were measured using image analysis and normalized to basement membrane area. N=9 for dust, 3  
733 for Lo CA and 12 for Hi CA. Collagen was significantly increased in both small and large airways  
734 of animals receiving the Hi CA exposure. \* = Significantly different from dust and Lo CA animals  
735 ( $p < 0.05$ ).

736

737 Figure. 3. Elastin expression around airways. Airway elastin expression in 28-day-old male mice  
738 was not significantly changed around airways from animals that had been exposed continuously  
739 throughout *in utero* and postnatal development. N = 4 for dust, N=16 for Lo CA and N=10 for Hi  
740 CA. Dust = Arizona road dust with no arsenic, 100  $\mu\text{g}/\text{m}^3$ . Lo CA= 3% calcium arsenate; Hi Ca  
741 = 10% calcium arsenate.

742

743 Figure 4. Smooth muscle actin (SMA) expression around airways. Airway smooth muscle  
744 expression in 28-day-old male mice was significantly increased around both small and large  
745 airways from animals that had been exposed continuously throughout *in utero* and postnatal  
746 development to Hi CA levels. Arrows indicate location of the smooth muscles around the airways.  
747 Greater expression is seen in the animals receiving the Hi CA exposure. Dust = Arizona road dust  
748 with no arsenic, 100  $\mu\text{g}/\text{m}^3$ . Lo CA= 3% calcium arsenate; Hi CA = 10% calcium arsenate. N=6  
749 for dust, N=6 for Lo CA and N=9 for Hi CA. \* = significantly different from dust and Lo CA  
750 animals ( $p < .05$ ).

751

752 Figure 5. Expression of EMT markers TGF- $\beta$ 1, SNAIL1 and TWIST in airway epithelium of 28-  
753 day-old mice. Representative micrographs show expression of EMT markers. Arrows indicate  
754 that measurements were made from airway epithelium. Arrows indicate that image analysis was  
755 performed on airway epithelium. As seen in the representative micrographs and the image analysis  
756 results, TGF- $\beta$ 1 and TWIST levels were not increased by exposure to Hi CA. Only SNAIL1

757 expression was significantly increased. N=4 for TGF- $\beta$ 1, N=5 for SNAIL1 and N=6 for TWIST.  
758 N=4 for Dust \*=significantly different from Dust (p<0.05)

759  
760 Figure 6. Levels of expression of club cell protein 16 (CC16). 28-day-old male mice were exposed  
761 to dust, Lo CA (3% calcium arsenate) or Hi CA (10% calcium arsenate) through *in utero* and  
762 postnatal development (GDPN). Apparent decrease in CC16 as a function of CA exposure levels  
763 can be seen in the representative micrographs. This was substantiated by image analysis that found  
764 a CA dose dependent significant decrease in the CC16 positive cells in airway epithelium (N=5  
765 for dust, Lo CA and Hi CA. A= significantly different from dust. B=significantly different from  
766 dust and Lo CA. (p < .05).

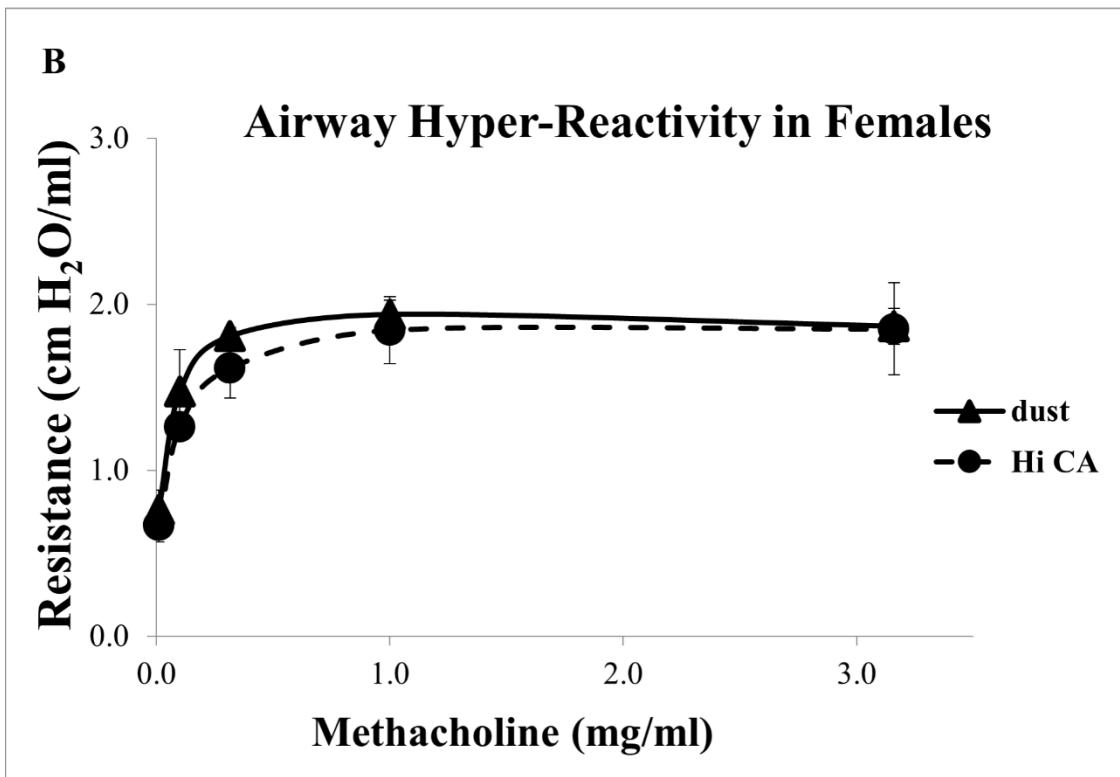
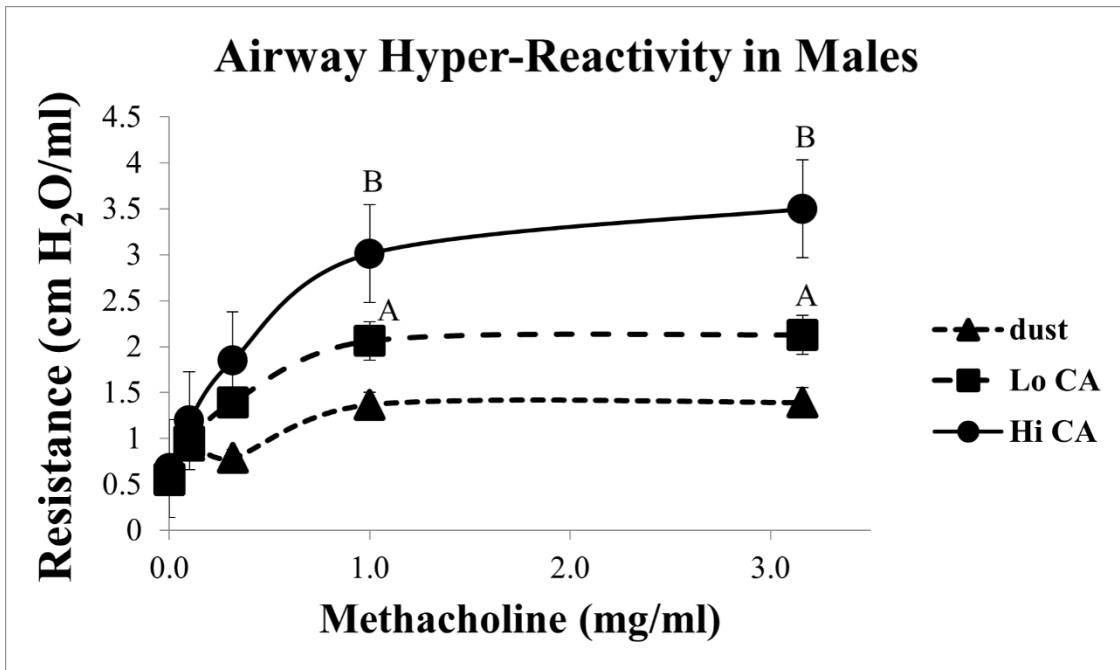
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768 Figure 7. Levels of CC16 expression in airways of 28-day-old male mice exposed either *in utero*  
769 only (GD), postnatally only (PN) or both (GDPN). Animals were exposed to Arizona road dust  
770 (dust) alone or road dust containing Lo CA (3% calcium arsenate) or Hi CA (10% calcium  
771 arsenate). Exposures to Hi CA during each of these developmental times led to significant  
772 decreases in the CC16 positive cells in the airways of the 28-day-old animals. Lo CA exposure  
773 lowered CC16 expression only when exposure was continuous throughout development (GDPN)  
774 N=3 to 5 for all groups. A = significantly different from dust. B = Significantly different from  
775 dust and Lo CA. (p < .05).

776  
777 Figure 8. Levels of collagen expression around airways of 28-day-old male mice exposed either  
778 *in utero* only (GD) or postnatally only (PN). Animals were exposed to Arizona road dust (dust),  
779 Lo CA (3% calcium arsenate) or Hi CA (10% calcium arsenate). Exposures to Hi CA during these  
780 developmental times led to significant increases in the collagen around both small and large  
781 airways. Increases in collagen are dose dependent and are similar to those seen in animals that  
782 had continuous *in utero* and postnatal exposures (Figure 2). N=9 for dust, N=3 for Lo Ca and N=7  
783 for Hi CA. \* = significantly different from dust and Lo CA (p < .05).

784  
785 Figure 9. CC16 expression in airways of 28-day-old male mice exposed to real-world mine tailing  
786 dust. Animals were exposed *in utero* only (GD) or postnatally only (PN) or both. Control animals  
787 were not exposed to the real-world dusts. As seen in the micrographs and the image analysis,  
788 exposures during any of these developmental times led to significant decreases in the CC16  
789 positive cells in the airways of the 28-day-old animals. N=5 for all groups. \* = significantly  
790 different from controls (p < .05).

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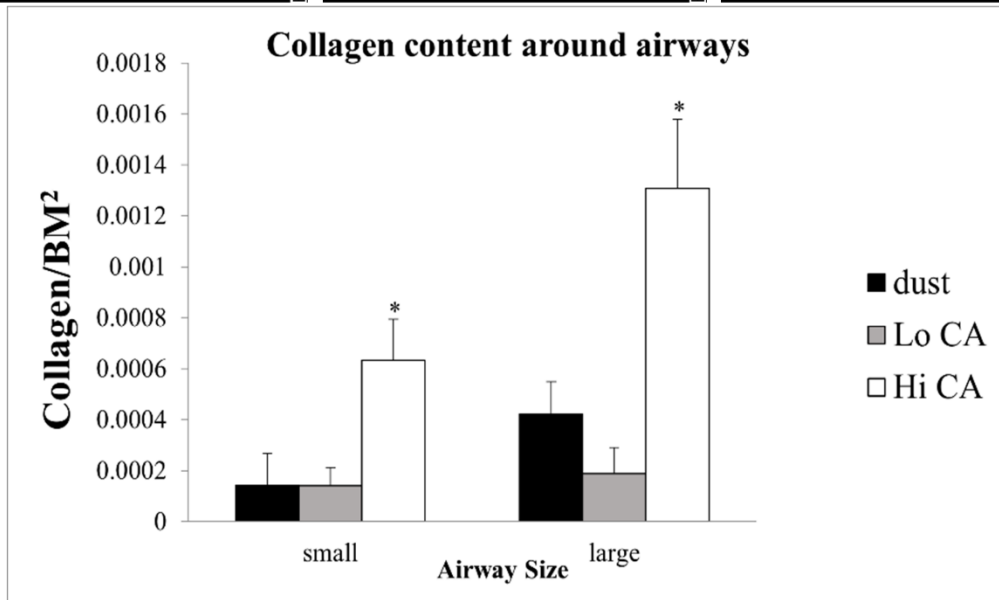
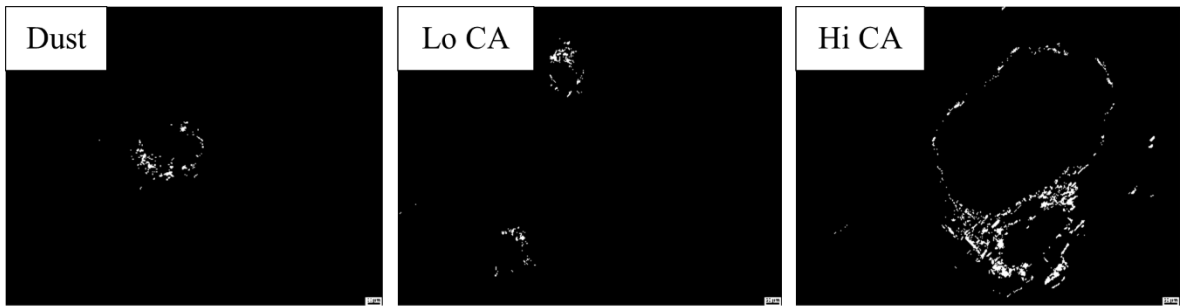
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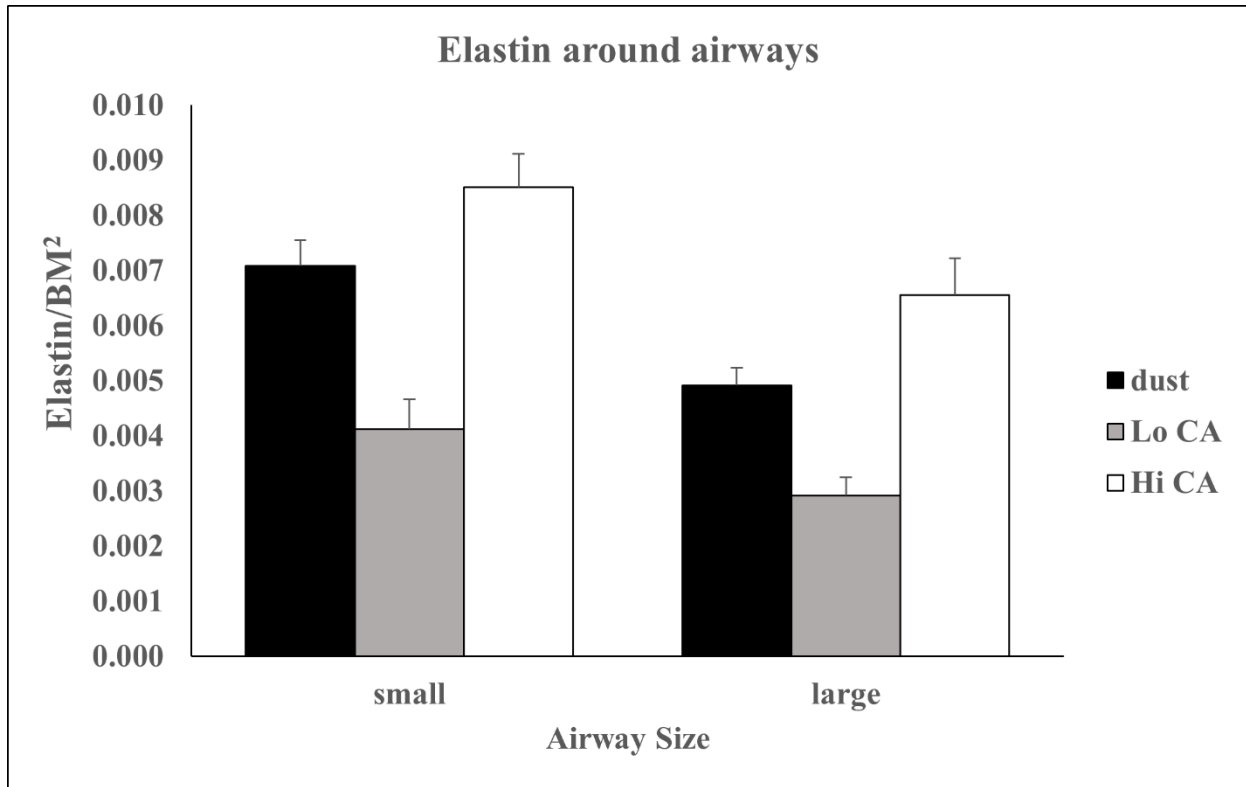
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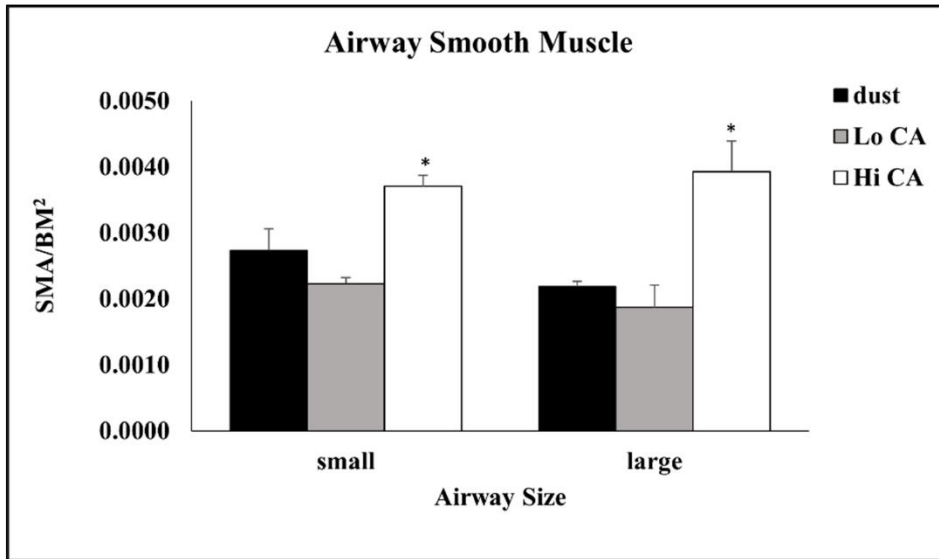
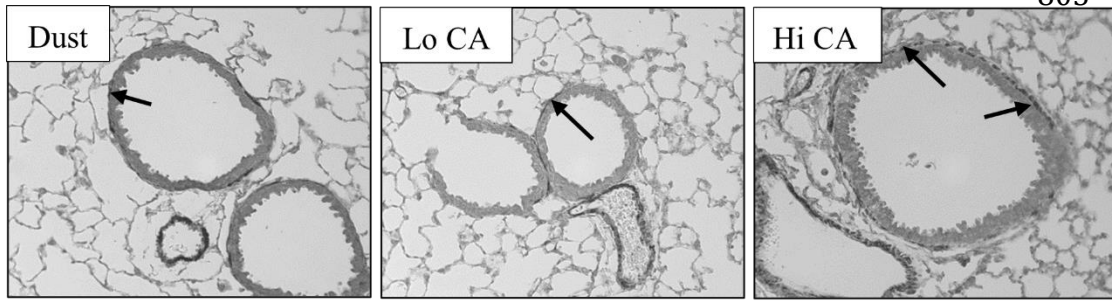
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Figure 2

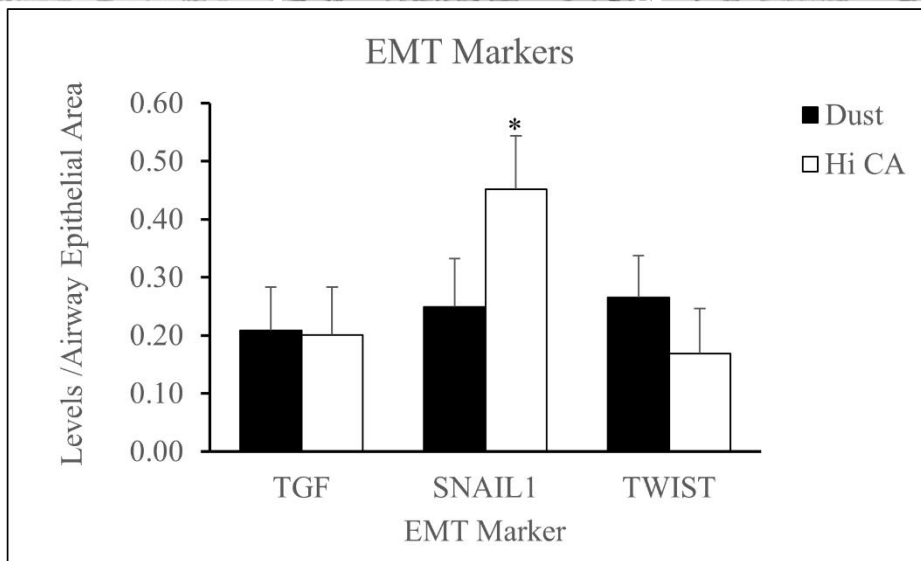
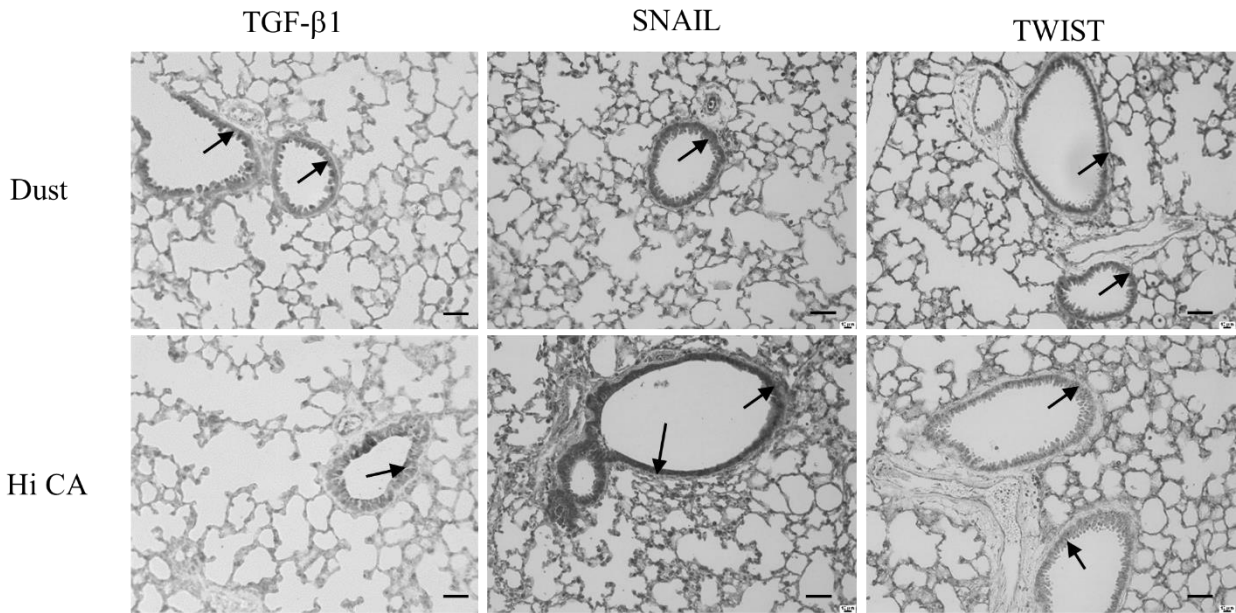


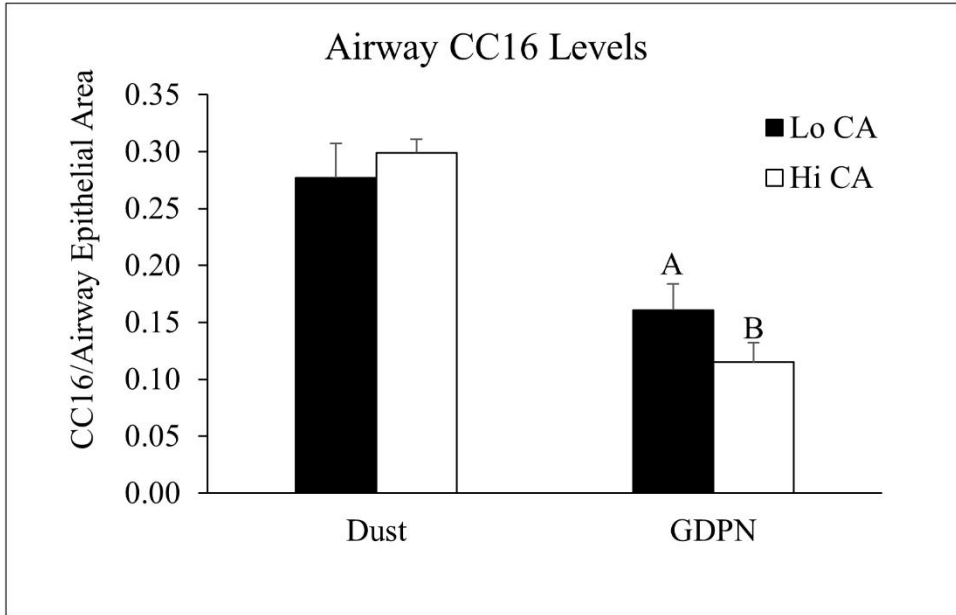
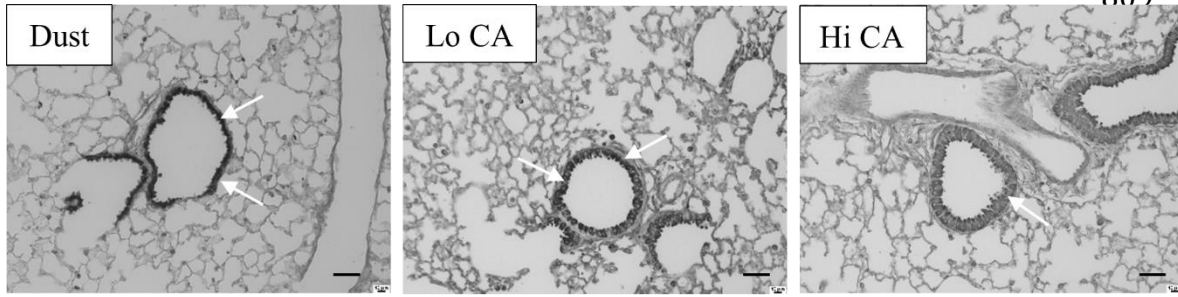
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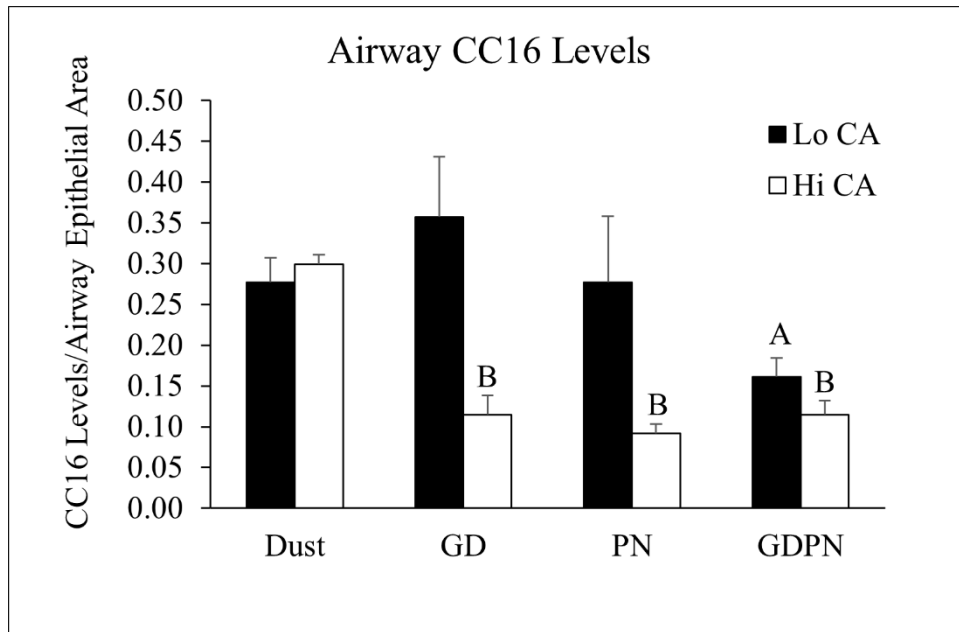


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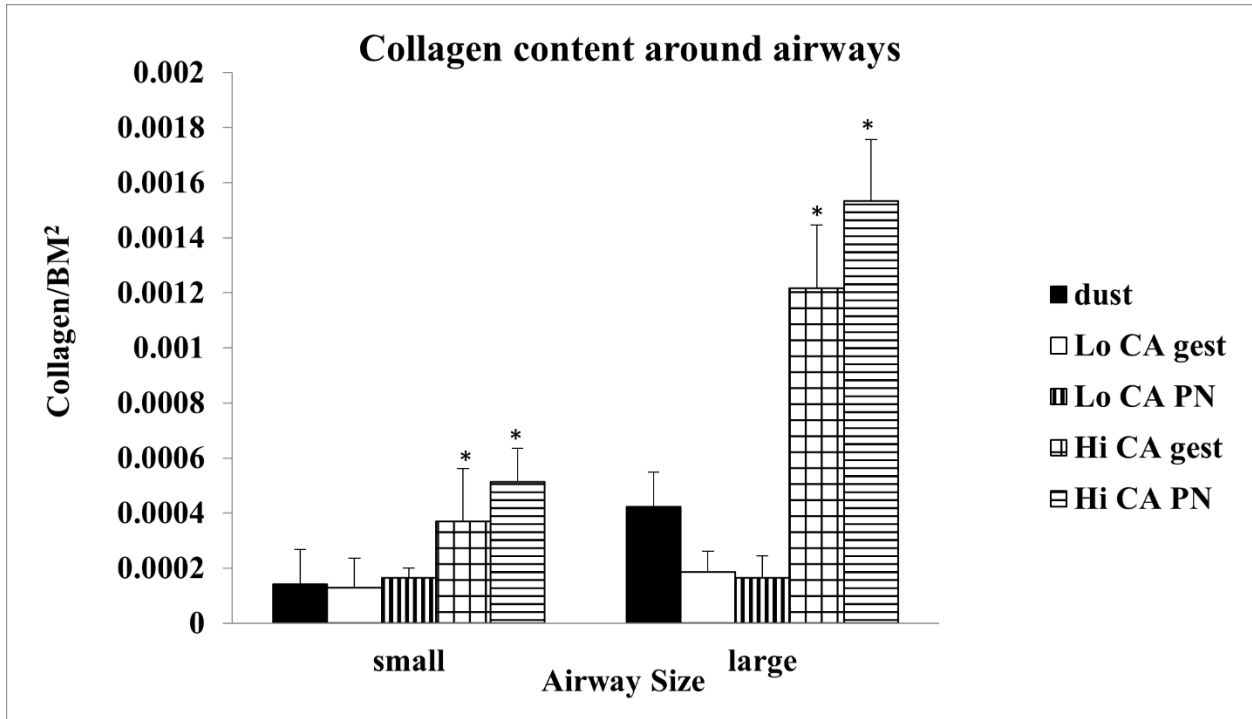




810 Figure 7  
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812 Figure 8



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814 Figure 9  
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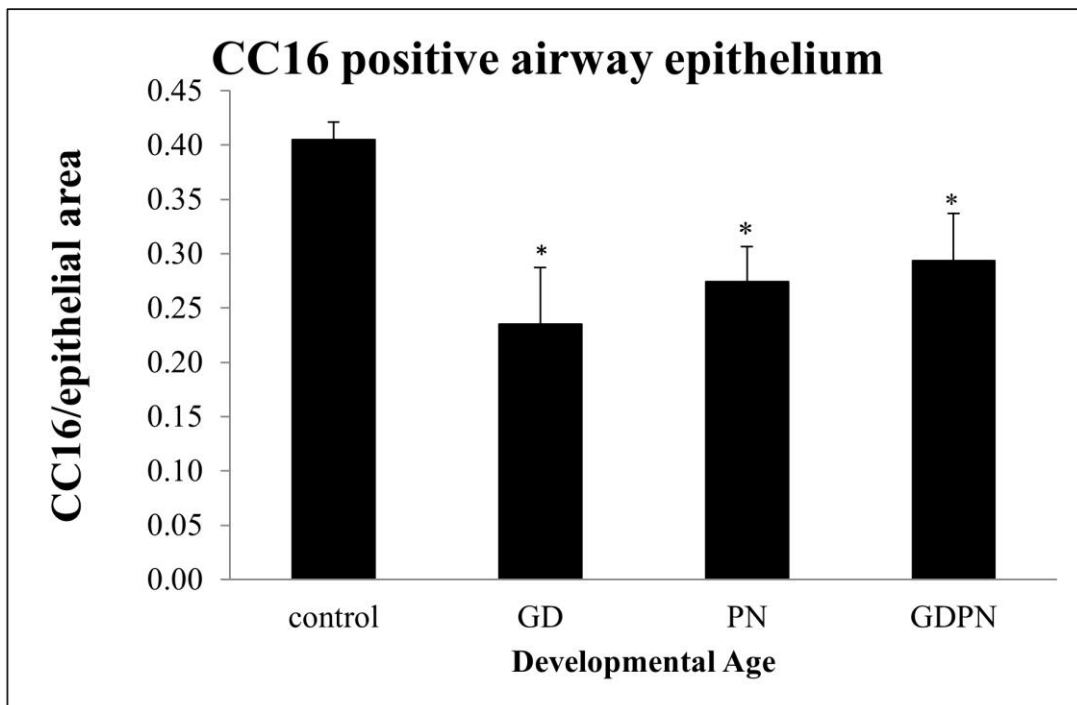
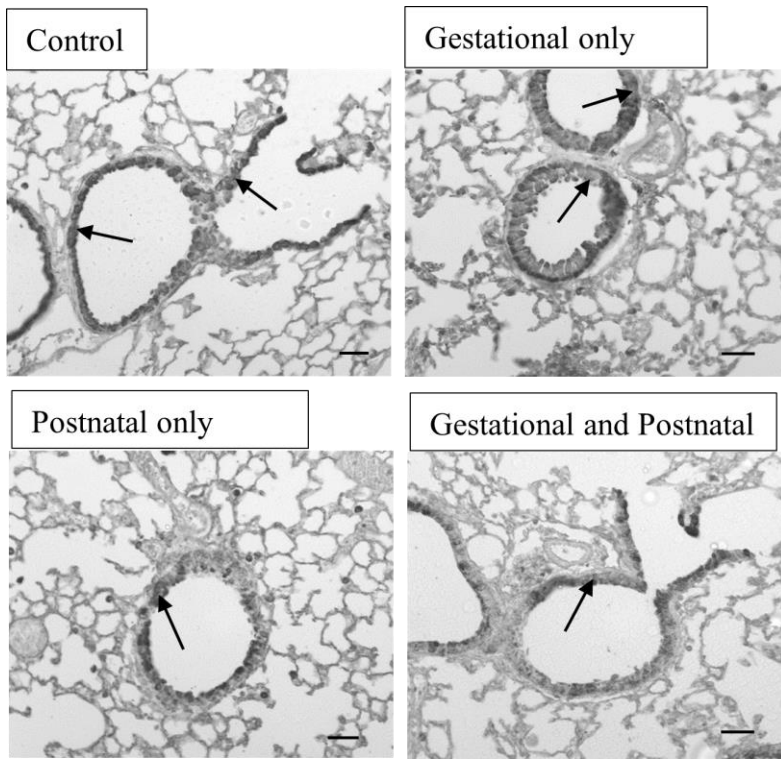


Table 1. EMT Markers and Cytokines				
	BALF		Plasma	
	Control	Hi CA	Control	Hi CA
TGF- $\beta$ 1	0.498 $\pm$ 0.067	0.313 $\pm$ 0.055	8.26 $\pm$ 2.88	7.86 $\pm$ 2.2
MMP-9	ND	ND	15.6 $\pm$ 0.9	25.7 $\pm$ 6.6*
IL-13	5.60 $\pm$ 0.90	9.04 $\pm$ 3.10*	26.6 $\pm$ 2.5	35.5 $\pm$ 12.7
Eotaxin	8.5 $\pm$ 1.6	10.0 $\pm$ 1.7	354 $\pm$ 26	438 $\pm$ 73
CXCL9	3.75 $\pm$ 0.10	5.52 $\pm$ 0.60*	26.4 $\pm$ 7.7	39.7 $\pm$ 11.3*
CXCL10	5.13 $\pm$ 0.31	7.63 $\pm$ 1.42*	47.2 $\pm$ 9.9	103 $\pm$ 23*

817 Values are mean $\pm$ SEM. Units are ng/ml except for IL-13 which is pg/ml. \*=significantly  
818 different from control dust exposure (p<0.05). Values are from ELISA. ND-not  
819 determined. N=3 for each determination.