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Mirlande Présumé, Angélique Simon-Deckers, Céline Tomkiewicz-Raulet, Béatrice Le Grand, Jean Tran van Nhieu, et al.. Exposure to metal oxide nanoparticles administered at occupationally relevant doses induces pulmonary effects in mice. Nanotoxicology, 2016, 10 (10), pp.1535-1544. 10.1080/17435390.2016.1242797 . hal-01496265

HAL Id: hal-01496265

https://hal.sorbonne-universite.fr/hal-01496265

Submitted on 27 Jul 2019

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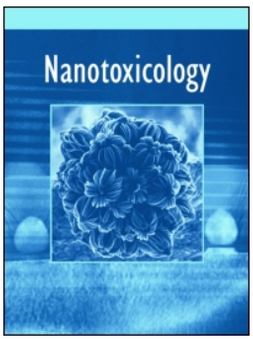

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: http://www.tandfonline.com/loi/inan20

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To cite this article: Mirlande Présumé, Angélique Simon-Deckers, Céline Tomkiewicz-Raulet, Béatrice Le Grand, Jeanne Tran Van Nhieu, Gregory Beaune, Olivier Duruphty, Jean Doucet, Xavier Coumoul, Jean-Claude Pairon, Jorge Boczkowski, Sophie Lanone & Pascal Andujar (2016): Exposure to metal oxide nanoparticles administrated at occupationally-relevant doses induces pulmonary effects in mice, Nanotoxicology, DOI: 10.1080/17435390.2016.1242797

To link to this article: http://dx.doi.org/10.1080/17435390.2016.1242797

Accepted author version posted online: 28 Sep 2016.

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Exposure to metal oxide nanoparticles administrated at occupationally-relevant doses induces pulmonary effects in mice

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Running title: Pulmonary effects in mice of metal oxide nanoparticles administrated at occupationally-relevant doses.

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ABSTRACT

In spite of the great promises that the development of nanotechnologies can offer, concerns regarding potential adverse health effects of occupational exposure to nanoparticle (NP) is raised. We recently identified metal oxide NP in lung tissue sections of welders, located inside macrophages infiltrated in fibrous regions. This suggests a role for these NP in the lung alterations observed in welders.

We therefore designed a study aimed to investigate the pulmonary effects, in mice, of repeated exposure to NP administrated at occupationally-relevant doses. We therefore chose 4 metal oxide NP representative of those found in the welder's lungs: Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ and CrOOH. These NP were administrated weekly, for up to 3 months, at 2 different doses: 5 µg, chosen as occupationally-relevant to welding activity, and 50 µg, chosen as occupationally-relevant to the context of a NP-manufacturing facility. Our results show that 3 month-repeated exposures to 5 µg NP induced limited pulmonary effects, characterized by the development of a mild peribronchiolar fibrosis observed for $MnFe_2O_4$ and CrOOH NP only. This fibrotic event was further extended in terms of intensity and localization after the repeated administration of 50 µg NP: all but Fe_2O_3 NP induced the development of peribronchiolar, perivascular, and alveolar fibrosis, together with an interstitial inflammation.

Our data demonstrate for the first time a potential risk for respiratory health posed by repeated exposure to NP at occupationally-relevant doses. Given these results, the development of occupational exposure limits specifically dedicated to NP exposure might therefore be an important issue to address.

Keywords: nanoparticles – occupationally-relevant dose – pulmonary effect – occupational exposure limit – welding fume.

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INTRODUCTION

Atmospheric pollution has long been known to potentially induce adverse health effects in the general population [1]. Particulate pollution represents one component of the atmospheric pollution that is associated with an increased morbidity and mortality, in particular at the cardiovascular and respiratory levels [2-5]. Nanoparticles (NP) constitute an important and still growing part of particulate pollution not only in the environmental context, but also in occupational settings.

At workplace, NP pollution can be emitted secondary to various industrial processes, such as arc-welding. During this process, welding fumes contain NP that represent up to 10% in mass and 80% in number of the total particles emitted [6-9]. NP can also be emitted intentionally at workplace, thereby generating manufactured NP. Exposure of workers to NP can occur during the initial production of NP, as well as during their further processing, dispersion and/or integration in secondary products. NP concentrations up to several mg/m^3 have been detected in workplaces manufacturing nanosized iron oxides, titanium dioxide, silver or nanostructured materials used for the electric industry for example [10-14].

A growing number of workers is estimated to be involved in work processes linked to the intentional or unintentional production of NP; up to 6 millions of workers will potentially be exposed to NP in 2020 [15]. In spite of the great promises that the development of nanotechnologies can offer, concerns regarding potential adverse health effects of such occupational exposure to NP is raised. However, very few data are currently available, probably because of the relatively recent development of nanotechnologies and production of manufactured NP. On the other hand, NP unintentionally emitted during an occupational activity such as welding is however not new, and we can, as such, take advantage of the literature available on this population of workers. Various adverse respiratory outcomes have been described in welders, among which inflammation and lung remodeling are largely

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reported [16, 17]. We recently identified metal oxide NP (Fe, Mn, Cr oxides essentially) in lung tissue sections of welders [18]. These metal oxide NP were located inside macrophages present in the alveolar lumen and infiltrated in fibrous regions, which suggests a role for these NP in the lung alterations observed in welders.

To deepen our findings and extend them to occupational settings where NP are intentionally emitted, we designed an *in vivo* study aimed to investigate the pulmonary effects, in mice, of repeated exposure to NP administrated at occupationally-relevant doses. Our underlying hypothesis was that repeated exposure to NP at occupational levels could lead to lung remodeling similar to that observe in lung tissue from welders. Therefore, 4 metal oxide NP in relationship with the occupational exposure of welding, and that we previously identified in lung tissue sections from welders [18] were selected: Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ and CrOOH. These NP were weekly administrated to mice up to 3 consecutive months, at 2 doses each one being relevant to occupational settings: 5 µg, chosen as occupationally-relevant to the welding activity, and 50 µg, chosen as occupationally-relevant to the context of a NP-manufacturing facility. Our results demonstrate that, while repeated exposures to 5 µg NP induced only limited pulmonary, administration of 50 µg NP induced the development of peribronchiolar, perivascular, and alveolar fibrosis, together with an interstitial inflammation in response to all but Fe_2O_3 NP. Overall, these data provide new evidence for a potential risk for health of repeated exposure to NP at occupationally-relevant doses.

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METHODS

Metal oxide NP

Four NP representative of welding occupational exposure (Fe_2O_3, Fe_3O_4, $MnFe_2O_4$, CrOOH) were chemically synthetized and characterized as powder and as suspension after sonication as previously described [18]. Stock solutions of NP suspensions (2 mg/mL) were prepared in ultrapure water and stored at -80°C. Just before use, the suspensions were sonicated for 10 min in ultrasonic bath (Elmasonic S30H). NP suspensions were observed using a JEOL1400-TEM at 120 kV. The observation of at least 200 NP per suspension showed that all NP are spherical and present an average diameter of 20-25 nm (Fe_2O_3, Fe_3O_4 and $MnFe_2O_4$) or 15 nm (CrOOH) (Supplemental Figure S1). Dynamic light scattering experiments demonstrate that all NP formed aggregates in solution, their size largely depending on the chemical nature of the NP considered (Supplemental Figure S2).

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Animal housing and handling

Animal housing

Male C57BL/6 mice 6–7 weeks of age were purchased from Janvier (Le Genest-St-Isle, France) and acclimated during 1 week. All mice were supplied with food (SAFE, Auguy, France) and tap sterilized water *ad libitum* in standard wire-topped cages in a controlled environment, with a 12 h light/dark cycle. All experiments were approved by our local Institutional Animal Care and Use Committee (Comité d'éthique - ComEth, ANSES/ENVA/UPEC, #C2EA-16).

Animal exposure to NP

To estimate the amount of NP to administrate in our study in order to be relevant for realistic occupational exposure, we utilized the following equation [17]:

Daily deposited dose of NP = occupational exposure limit (OEL) x NP mass fraction in occupational emission x minute ventilation x duration x NP deposition efficiency

with 1/ OEL for welding fumes at 5 mg/m^3, 2/ a mass concentration of NP in welding fumes at 10% of the total particle emitted 3/ a mean minute ventilation at 22.500 mL/min (tidal volume of 1500 mL and respiratory frequency of 15/min for a worker with moderate activity), 4/ a daily occupational exposure of 8 hours, and 5/ a mean deposition efficiency of NP in the alveolar region of 50% for an average NP aerodynamic diameter of 20 nm [18-21]. Importantly, these calculations do not account for particle clearance, but provide an estimate of the plausible worker exposure concentrations. Overall, the daily NP lung burden was estimated at 2.7 mg per worker per day. Given the average worker weight (70 kg) and the average weight of the mice (25 g), the daily deposited dose of NP for a mouse was estimated at 1 µg. Therefore, the dose of 5 µg weekly was considered as representative of occupational exposure of workers in the context of an unintentional process such as welding [8, 9]. The dose of 50 µg weekly was subsequently calculated for a NP only-containing aerosol, within the 5mg/m^3 OEL.

Once a week, mice received Saline (Control group), 5 or 50 µg NP (Fe_2O_3, Fe_3O_4, $MnFe_2O_4$, CrOOH groups) administrated by pharyngeal aspiration performed under anesthesia (1.6 mg ketamine (Virbac, Carros, France) plus 300 mg xylazine (Bayer®, Puteaux, France)). Mice were sacrificed as previously described 24h, 1 week, 1 or 3 months after the first exposure [22]. A schematic representation of the experimental protocol is given in Supplemental Figure S3.

Lung tissue collection

At the time of the sacrifice, mice were anesthetized and their right lung was inflated, fixed with 10% of OCT (Optimal Cutting Temperature) at a constant pressure of 20 cm H_2O and paraffin-embedded for further histological and immunohistochemical analysis. Before being

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frozen in liquid nitrogen and stored at -80°C, left lung was lavaged with twice with 1 mL of physiological saline, removed from the chest cavity, and immediately frozen at -80 °C for use in further experiments.

Histological and immunohistochemical analysis

Paraffin-embedded lung tissue sections (5 µm) were stained with hematoxylin-eosin-saffron (HES) for histological examination or with Sirius Red for collagen deposition. The occurrence, localization and severity of histological lesions were assessed using a semi-quantitative score adapted from Ashcroft [23]. At least 10 fields per lung tissue section were analyzed (magnification x200), and a scale of 4 semi-quantitative fibrosis scores was utilized: [0: normal; 1: minimal fibrous thickening; 2: moderate thickening of walls without obvious damage to lung architecture; 3: increased fibrosis thickening of walls with definite damage to lung structure and formation of fibrous bands or small fibrous masses] for peribronchial, perivascular or alveolar wall thickness extension.

A similar scale of 4 semi-quantitative scores was used for inflammatory infiltrates in the alveolar, bronchiolar or vessel lumen, peribronchiolar or perivascular walls, as well as in the interstitium [0: < 20 inflammatory cells/field; 1: 21 to 50; 2: 51 to 100; 3: > 100]. Finally, the number of NP agglomerate/aggregate per field was also quantified (magnification x200).

Additionally, immunohistochemical (IHCh) analysis was performed using specific antibodies directed against Heme oxygenase (HO-1) protein as previously described [18].

BALF analysis

Cellularity

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Free alveolar cells were recovered from the lavage fluid by centrifugation at 400g for 15 min at 4 °C. The cellular pellet was suspended in 150 µL of physiological saline. An aliquot of the cell suspension was then examined using a hemocytometer to evaluate the total white cell number. For differential counts, the cell suspension was cytospun (cytospin-2, Shandon Products Ltd.), fixed in methanol, and stained with Diff Quick solution (Medion Diagnostics, Plaisir, France).

Alveolar epithelial barrier integrity and cytotoxicity evaluations

Total protein content and LDH concentrations in BALF were determined by the Bio-Rad protein assay (Bio-Rad, Marne-La-Coquette, France) and by the Cytotoxicity Detection Kit (Roche®), respectively.

Lung inflammation

In order to evaluate lung inflammation, BALF content in Transforming growth factor beta (TGFß), Tumor Necrosis Factor alpha (TNFα) and Interleukin 1beta (IL1β) proteins was quantified by DuoSet® ELISA as per the manufacturer's instructions (R&D Systems, Lille, France).

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Quantification of mRNA expression in lung homogenates

mRNA were isolated from frozen lung tissue samples using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France). mRNA expression of genes related to inflammatory (TNFα, Monocyte Chemoattractant Protein 1 - MCP-1, Interleukin 6 - IL-6, Prostaglandin-endoperoxidase synthase 2 - PTGS, Regulated upon activation normal T-cell express, and presumably secreted - RANTES) and oxidative responses (NAD(P)H/quinone oxidoreductase - NQO-1, Cytochrome P450-family 1 subfamily A-polypeptide 1 - CYP1A1) was measured by relative quantification method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the

housekeeping gene and performed with a PCR ABI 7700 apparatus (Applied Biosystems, Courtaboeuf, France) with SybrGreen technologies (Applied Biosystems). Primer sets are shown in Table 1S.

Statistical analysis

Eight animals per experimental group were used for each time-point and each dose, except for one week exposure at 50 µg where 10 mice per experimental group were used. Taking into account the possibility of non-normal distribution in the hypothetic mice population, and the number of mice (lower than 30 independent mice), non-parametric tests (Kruskal-Wallis statistical test followed by Dunn's multiple comparison test) were used (GraphPad Prism software, version 5.01, USA). For all statistical tests, p values smaller than 0.05 were considered as significant. Values are expressed as the mean or median and interquartile range for histological semi-quantitative scores.

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RESULTS

Histological analysis of lung tissue sections

Figure 1 shows representative HES (Panel A) and Sirius Red (Panel B) images of lung tissue sections obtained after weekly exposure of mice to 5 µg NP during 3 months. Semi-quantification of the histological alterations showed a small but significant increase in peribronchiolar thickness in response to $MnFe_2O_4$ or CrOOH NP, but not in response to Fe_2O_3 or Fe_3O_4 NP (Figure 1, Panel C). These modifications were not present at shorter exposure durations (data not shown).

At the dose of 50 µg administrated weekly during 3 months, all NP induced a pronounced lung fibrotic remodeling characterized by the thickening of alveolar, peribronchiolar as well as perivascular walls, together with the presence of interstitial lung inflammation (Figure 2). These modifications were not present at shorter exposure durations (data not shown). Whatever the experimental condition, neither granuloma formation nor the development of emphysema could be observed. Moreover, no bronchiolar hyperplasia could be detected.

The presence of microscopically observable NP aggregates/agglomerates was not detected in 5 µg-exposed animals, but was detectable in all animals exposed to 50 µg weekly, essentially in alveolar and bronchiolar lumens, with a significantly higher number of aggregates/agglomerates present in animals weekly exposed to 50 µg $MnFe_2O_4$ or CrOOH NP as compared to those exposed to Fe_2O_3 or Fe_3O_4 NP (Figure 3).

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Alveolar epithelial barrier integrity

We next addressed the integrity of the epithelial barrier by assessing the total protein content and LDH release in the BALF of NP-exposed mice. As shown in Figures 4 and 5, there was a significant increase of BALF total protein content only in the $MnFe_2O_4$-exposed mice after 1

month of weekly exposure to 5 µg NP (Figure 4, Panel A). No significant modification of BALF total protein content was observed for the other experimental conditions. Moreover, no significant modification of LDH release was observed, whatever the exposure duration, the dose or the chemical nature of the NP administered (Figures 4, 5, Supplemental Figure S4).

Pulmonary inflammatory and oxidative responses

As the presence of NP in the lungs has been related to the induction of inflammatory and oxidative responses [24, 25], we next explored the total cellularity of BALF. As shown in Figures 4, 5, and Supplemental Figure S4, BALF total cellularity was significantly increased only in CrOOH-exposed mice as compared to unexposed animals, whatever the dose received (5 or 50 µg weekly), after a 3 months-exposure. The cell differential was kept unchanged, whatever the experimental condition; only macrophages and rare neutrophils were detected in BALF samples (data not shown).

We next evaluated the release in the BALF of NP-exposed mice, of 3 inflammatory cytokines known to represent important markers of the pulmonary response to NP exposure: TNFα, TGFß and IL1ß [24-26]. For the dose of NP relevant to welding activity (5 µg), only CrOOH-exposed animals present a transient but significant increase in TNFα secretion 24 hours after the first exposure as compared to unexposed mice (Supplemental Figure S4, Panel C). For the highest dose of NP (50 µg weekly), all but Fe_2O_3 NP induced significant increases in BALF TNFα content after 1 day (Supplemental Figure S4, Panel D). This upregulated secretion could still be observed after 1 month (for Fe_3O_4- and CrOOH-exposed animals) or 3 months (for $MnFe_2O_4$- and CrOOH-exposed mice, Figures 4 and 5) repeated exposure. Interestingly, TGFß secretion was significantly increased in $MnFe_2O_4$-exposed mice, after 3 month repeated

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exposure to 50 µg NP (Figure 5). IL1ß BALF content was not modified by NP exposure, whatever the chemical nature of the NP, the dose or the exposure duration studied.

We further evaluated inflammation at the level of the whole lung, by qPCR analysis. As shown in Figures 6, 7 and Supplemental Figure S5, a transient increase in MCP-1 mRNA expression was observable after 1 day of exposure to 5 µg Fe-based NP (Fe_2O_3, Fe_3O_4 and $MnFe_2O_4$). This increase was also observed after 1 day and 1 month weekly repeated exposure to CrOOH NP. Moreover, TNFα mRNA expression was increased in response to all NP after 3-months weekly repeated exposure to the occupationally-relevant dose of 5 µg (Figure 7). This increase in TNFα mRNA expression was also detectable in the 50 µg-exposed groups, either transiently (Fe_2O_3 and $MnFe_2O_4$) or steadily (Fe_3O_4, starting after 1 month, and CrOOH, at all exposure durations). Moreover, in Fe_3O_4- and CrOOH-exposed mice, MCP-1 and PTGS mRNA expressions were increased as compared to that measured in unexposed mice after 1 and 3 months. The same was true for IL-6 mRNA expression after 1 month (Figure 6, Panel B).

As for the oxidative response, no major modification of NQO1 or CYP1A1 expression could be detected in the 5 µg-exposed animals, except for a significant increase in CYP1A1 expression after 1 day of exposure to Fe_3O_4 NP (Supplemental Figure S5). However, in 50 µg-exposed animals, the antioxidant NQO1 mRNA expression was significantly increased in Fe_3O_4-, $MnFe_2O_4$- and CrOOH-exposed mice after 1 month repeated exposure as compared to unexposed animals, and was still significantly increased after 3 months exposure duration in CrOOH-exposed mice (Figures 6 and 7). Neither modification of CYP1A1 mRNA expression nor that of HO-1 protein was detected, whatever the experimental condition (Figures 6, 7, 8 and Supplemental Figure S5).

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DISCUSSION

The general aim of our study was to investigate the pulmonary effects, in mice, of repeated exposure to NP administrated at occupationally-relevant doses. To achieve this aim, we developed an experimental protocol designed to mimic at best an occupational exposure in 2 working contexts: welding (unintentional NP production) and NP-manufacturing facility (intentional NP production).

Our results demonstrate that repeated exposure of mice to metal oxide NP at doses relevant to welding activity induce no (after Fe_2O_3 and Fe_3O_4 NP exposure) or only limited (after $MnFe_2O_4$ and CrOOH NP exposure) lung remodeling; in these latter animals, only a slight although significant peribronchiolar fibrosis could be observed after 3 months, without any major sign of inflammation. To the best of our knowledge, these results are the first to address the specific issue of the pulmonary effects of repeated exposures to metal oxide NP administrated at doses relevant to the occupational activity of welding. Indeed, although the relevance of the exposure dose is a long-time ongoing issue in the literature dedicated to NP exposure [27], the majority of the studies published so far have been conducted using doses that are largely over the calculated doses relevant to the welding activity: at least 1 mg/kg (corresponding to 25 µg per animal for a 25 µg mouse), and up to 500 µg per animal. Moreover, these doses were most often administrated as a single bolus, which dampens the overall relevance of the results obtained in these studies [28, 29]. Our results are therefore difficult to compare with data from the literature. However, considering the results obtained with the lowest doses used in previously published studies, the effects of weekly pulmonary exposures to Fe or Cr oxide NP can be described as limited; absence of acute inflammatory cell recruitment in the BALF in most cases, low levels of inflammatory cytokines secretion, and no histological modification after 1 month (longest time point analyzed in these studies) [26, 30-32, 33 6264]. Our results are in accordance with these data, and further extend them,

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not only to doses relevant to the occupational activity of welding, but also to longer exposure durations (up to 3 months) and to a more realistic scenario of repeated exposures instead of administration of NP as a single bolus. Interestingly, lung TNFα mRNA expression was increased in response to all NP after 3 months weekly exposure to 5 µg. Given the implication of this cytokine in the early events of inflammation, one could infer that longer exposure duration to doses of NP relevant to welding activity could lead to more pronounced lung alterations. We are fully aware that welding fumes cannot be summarized to only metal oxide NP, as a complex mixture of particles (micrometric and nanometric) together with a number of potentially toxic gases (e.g. ozone, nitrogen oxide, nitrogen dioxide) are generated during the welding process [34]. However, the relevance of our findings is supported by the fact that we were recently able to link the presence of NP aggregates/agglomerates in lung tissues samples of arc-welders to the presence of similar fibrotic lesions [18]. Various adverse outcomes have been described in welders, and are not limited to pulmonary endpoints [34]. Therefore, although we found only limited pulmonary effects of repeated exposure to occupationally-relevant doses of metal oxide NP, extra-pulmonary adverse effects could have been detected. This could be particularly true for the brain for example, as exposure to Mn is associated with neurotoxicity in welders [34]. Moreover, an increase in the incidence of lung cancer has been described particularly in those exposed to Cr [34]. However, as the maximum exposure duration was 3 months in our study, this was probably not enough to evaluate the carcinogenicity of the exposure, even for a mouse presenting a 2.5 years life expectancy. As Mn- and Cr-based NP were the most potent to induce lung remodeling when utilized at doses relevant to welding, their extra-pulmonary effects could have been particularly interesting to evaluate. These outcomes should deserve further studies.

The mild pulmonary alterations detected at doses relevant to the occupational activity of welding were further extended both in terms of intensity and spatial distribution after repeated

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exposure of mice to doses relevant to the context of a NP-manufacturing facility; not only a more pronounced thickening of the peribronchial wall could be observed, but also that of perivascular and alveolar walls, together with the presence of an interstitial inflammation. These findings were observed in response to all but Fe_2O_3 NP, given at the same mass concentration. In an attempt to decipher the underlying molecular mechanisms of these fibrotic lesions, the expression pattern of MCP-1 mRNA in lung homogenates could be of interest, as MCP-1 expression was increased after 1 and/or 3 months of repeated exposure to 50 µg of all but Fe_2O_3 NP. Indeed, MCP-1 is a chemokine synthetized by a large variety of cells including macrophages and fibroblasts, and which exerts chemotactic and activating effects on macrophages [35]. Moreover, it has been recently described that pulmonary fibroblast-derived MCP-1 drives fibroblast proliferation and migration in response to silicon dioxide [35]. It is also important to note that the secretion of TGFß was increased only in the BALF obtained from $MnFe_2O_4$-exposed animals during 3 months. Although we did not evaluate longer exposure durations, and given the essential role of this cytokine in the development of lung fibrosis, this result suggests that $MnFe_2O_4$-exposed animals could be particularly prone to lung remodeling, thanks to the implication of molecular mechanisms specific to this speciation of NP. This is also suggested by the fact that $MnFe_2O_4$-exposed animals develop peribronchiolar wall thickening after 3 months weekly exposure at doses relevant for welding activity. However, such remodeling was also present in animals repeatedly exposed to 5 µg CrOOH NP, which indicates the occurrence of other, yet unidentified, molecular mechanism(s) that could be responsible for lung remodeling in response to these NP.

Our data showed that Fe_2O_3 NP were overall less reactive than Fe_3O_4 NP. This difference could be due to different degrees of solubility between NP. However, previous data from our lab using the same metal NP as those used in the present study indicate a very small if no

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solubility at all whatever the NP tested [18]. Another explanation for this difference could be the different accumulation profiles between Fe_2O_3 and Fe_3O_4 NP. However, the number and localization of NP aggregates/agglomerates were similar for these NP, thus underlying the importance of chemical speciation in NP's effects. Interestingly, previous data from our team demonstrated that Fe_2O_3 NP were more reactive than Fe_3O_4 NP in terms of pro-inflammatory cytokines secretion by macrophages [18]. This suggests that macrophages might not be the essential or only target cell type to drive lung remodeling in response to iron oxide NP. Interestingly, although aggregates/agglomerates of metal oxide NP could be detected in all groups of animals in the present study (at the 50 µg dose), yet all but Fe_2O_3 NP were able to induce fibrotic lesions. However, the number of aggregates/agglomerates observed 3 months after repeated exposure to Fe_3O_4 NP, which was lower than after exposure to $MnFe_2O_4$ or CrOOH NP, didn't affect the intensity of the remodeling, which was similar for the 3 NP. It must be noted that, as our study was dedicated to investigate the pulmonary effects of repeated exposure to NP administrated at occupationally-relevant doses, the critical endpoint we focused on was therefore the initial mass concentration of the NP, not their size or agglomeration state immediately after sonication, i.e., just after administration to mice. Finally, although we cannot rule out the technical difficulty of observing and characterizing such aggregates/agglomerates with a regular optical microscope, this result confirms the multifactorial origins potentially linking the presence of NP to any biological effect [36].

Our results overall demonstrate that repeated exposure to NP at doses relevant to occupational exposure represents a potential risk for respiratory health. There is currently no international consensus regarding which OEL can be applied to NP, although they could represent a major component of the occupational pollution [37, 38]; the proposed OEL for NP is 10% of the exposure limits established for the respective micro-scale industrial aerosols [39]. Indeed, with the exception of nanometric TiO_2 for which a recommended exposure limit (REL) at 0.3

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mg/m^3 has been proposed by the US, OEL have been defined for respirable particles only i.e. most of them in the micrometric range [37, 40, 41]. Some NP-specific values have been proposed, but they differ according to the different proposing countries or agencies, in terms of the metric used to quantify the relative exposure to NP or the categorization of NP in various risk groups, background upon which these OEL have been developed [37], which makes these proposed values highly provisional. Moreover, the current OELs don't consider the speciation of NP, although it represents an important determinant of their biological effects.

CONCLUSION

Overall, our data demonstrate for the first time a potential risk for respiratory health posed by repeated exposure to NP at occupationally-relevant doses. Given these results, the development of OEL specifically dedicated to NP exposure might therefore be an important issue to address in the near future.

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ACKNOWLEDGMENTS

This work was supported by funds from INSERM, Agence Nationale de la Recherche (grant ANR-09-CESA-017), Université Paris Est-Créteil, Chancellerie des Universités de Paris (Legs POIX), Fonds de Dotation-Recherche en Santé Respiratoire (SPLF), and C'Nano (grant AAP09-NanoSoud). Mirlande Présumé was a fellow from Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) and Agence de l'Environnement et de la Maîtrise de l'Énergie (ADEME). Angélique Simon-Deckers and Grégory Beaune were both fellows from ANR. Sophie Lanone and Jorge Boczkowski were both recipients of a Contrat de Recherche Translationnelle, between Inserm and CHI Créteil (SL), or CHU Mondor (JB). This work also received the support of Labex SERENADE 11-LABX-0064 and DHU A-TVB (Département Hospitalo-Universitaire Ageing-Thorax-Vessel-Blood).

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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LEGEND TO FIGURES

Figure 1: Histological analysis of lung tissue sections after repeated exposure to 5 µg NP. Representative optical microscopy images of lung tissue sections from a control mouse and mice weekly exposed during 3 months to 5 µg Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ or CrOOH NP. Sections were stained with hematoxylin-eosin-saffron (HES) (Panel A) or Sirius Red (Panel B). Scale bar: 100 µm. Quantification of histological alterations in terms of alveolar, peribronchiolar, perivascular wall thickness or interstitial lung inflammation (Panel C). *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control.

Figure 2: Histological analysis of lung tissue sections after repeated exposure to 50 µg NP. Representative optical microscopy images of lung tissue sections from a control mouse and mice weekly exposed during 3 months to 50 µg Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ or CrOOH NP. Sections were stained with hematoxylin-eosin-saffron (HES) (Panel A) or Sirius Red (Panel B). Scale bar: 100 µm. Quantification of histological alterations in terms of alveolar, peribronchiolar, perivascular wall thickness or interstitial lung inflammation (Panel C). *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control. ***: $p \leq 0.001$ vs Control.

Figure 3: Analysis of agglomerates/aggregates accumulation in lung tissue sections. Representative optical microscopy images of lung tissue sections from a control mouse and mice weekly exposed during 3 months to 50 µg Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ or CrOOH NP (Panel A). Sections were stained with HES. Scale bar: 30 µm. Quantification of agglomerates accumulation (Panel B). Data are given as mean number of agglomerates/aggregates per field observed in at least 10 fields per animal. *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control. ***: $p \leq 0.001$ vs Control.

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Figure 4: BALF analysis of mice exposed to NP for 1 month. BALF total protein content, LDH release and total cellularity of mice weekly exposed for 1 month to 5 µg (Panel A) or 50 µg (Panel B) NP. BALF levels of TNFα, TGFβ and IL1β in mice weekly exposed for 1 month to 5 µg (Panel C) or 50 µg (Panel D) NP. n: 8 mice per condition. *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control.

Figure 5: BALF analysis of mice exposed to NP for 3 months. BALF total protein content, LDH release and total cellularity of mice weekly exposed for 3 months to 5 µg (Panel A) or 50 µg (Panel B) NP. BALF levels of TNFα, TGFβ and IL1β in mice weekly exposed for 3 months to 5 µg (Panel C) or 50 µg (Panel D) NP. n: 8 mice per condition. *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control.

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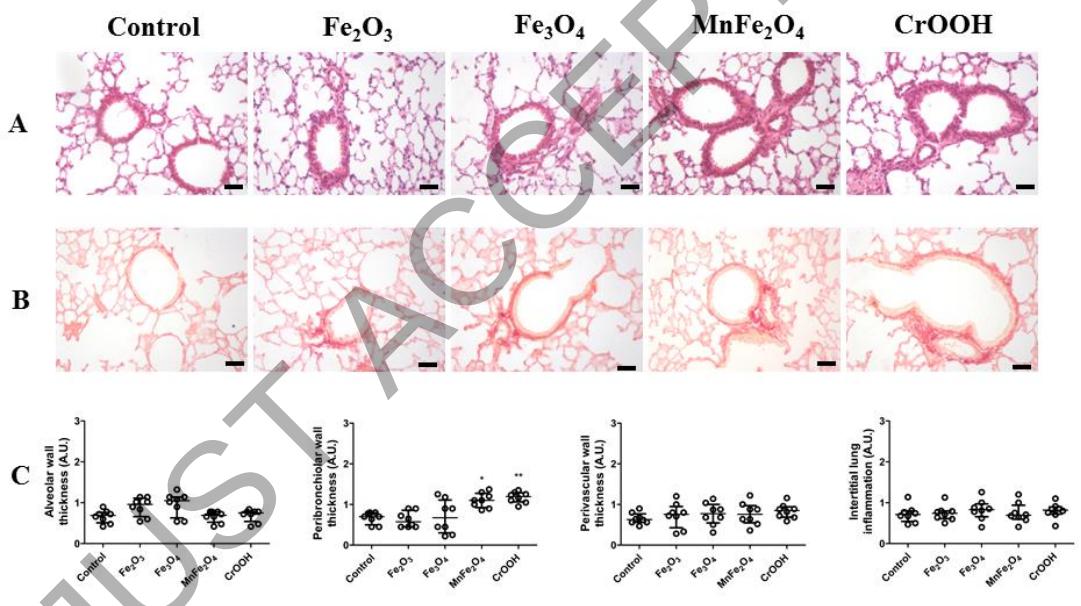
Figure 6: Total lung mRNA analysis of mice exposed to NP for 1 month. Quantification of TNFα, MCP-1, IL-6, PTGS, RANTES, NQO-1 and CYP1A1 mRNA expression in lung sample from mice weekly exposed for 1 month to 5 µg (Panel A) or 50 µg (Panel B) NP. n: 8 mice per condition. Data are given as the ration between mRNA of interest's expression to that of GAPDH, and normalized to the Control condition. *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control. ***: $p \leq 0.001$ vs Control.

Figure 7: Total lung mRNA analysis of mice exposed to NP for 3 months. Quantification of TNFα, MCP-1, IL-6, PTGS, RANTES, NQO-1 and CYP1A1 mRNA expression in lung sample from mice weekly exposed for 3 months to 5 µg (Panel A) or 50 µg (Panel B) NP. n: 8 mice per condition. Data are given as the ration between mRNA of interest's expression to

that of GAPDH, and normalized to the Control condition. *: $p \leq 0.05$ vs Control. **: $p \leq 0.01$ vs Control. ***: $p \leq 0.001$ vs Control.

Figure 8: Heme-Oxygenase-1 (HO-1) expression in lung tissue sections. Representative optical microscopy images of lung tissue sections stained for HO-1 protein from a control mouse and mice weekly exposed during 3 months to 50 µg Fe_2O_3, Fe_3O_4, $MnFe_2O_4$ or CrOOH NP. Scale bar: 100 µm.



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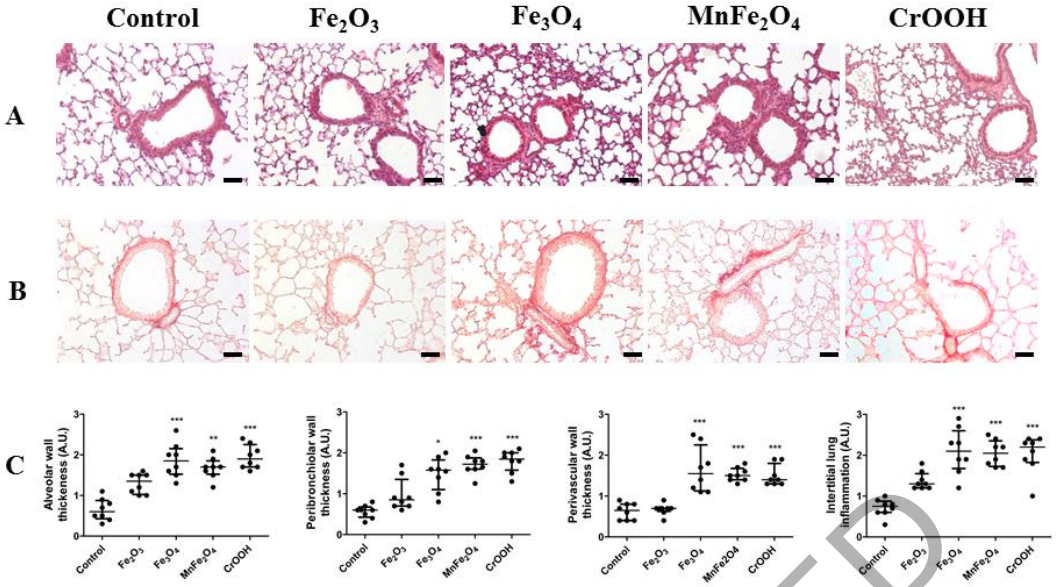

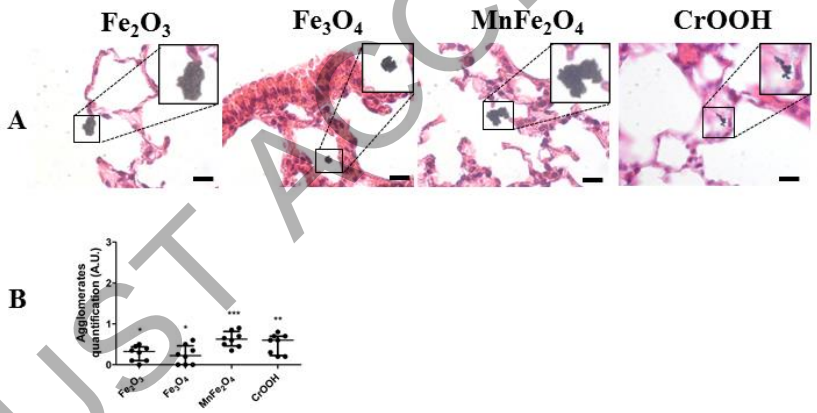

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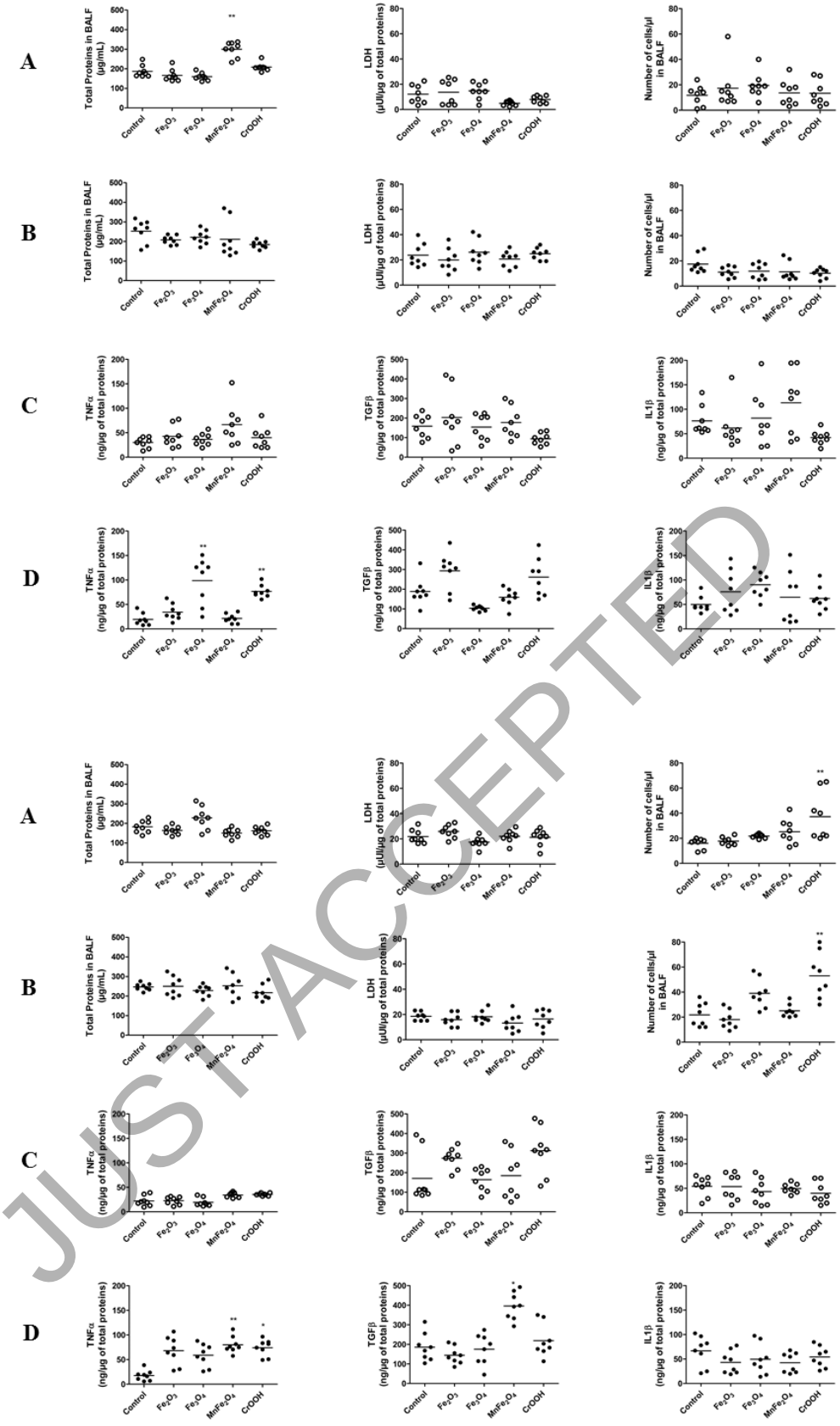

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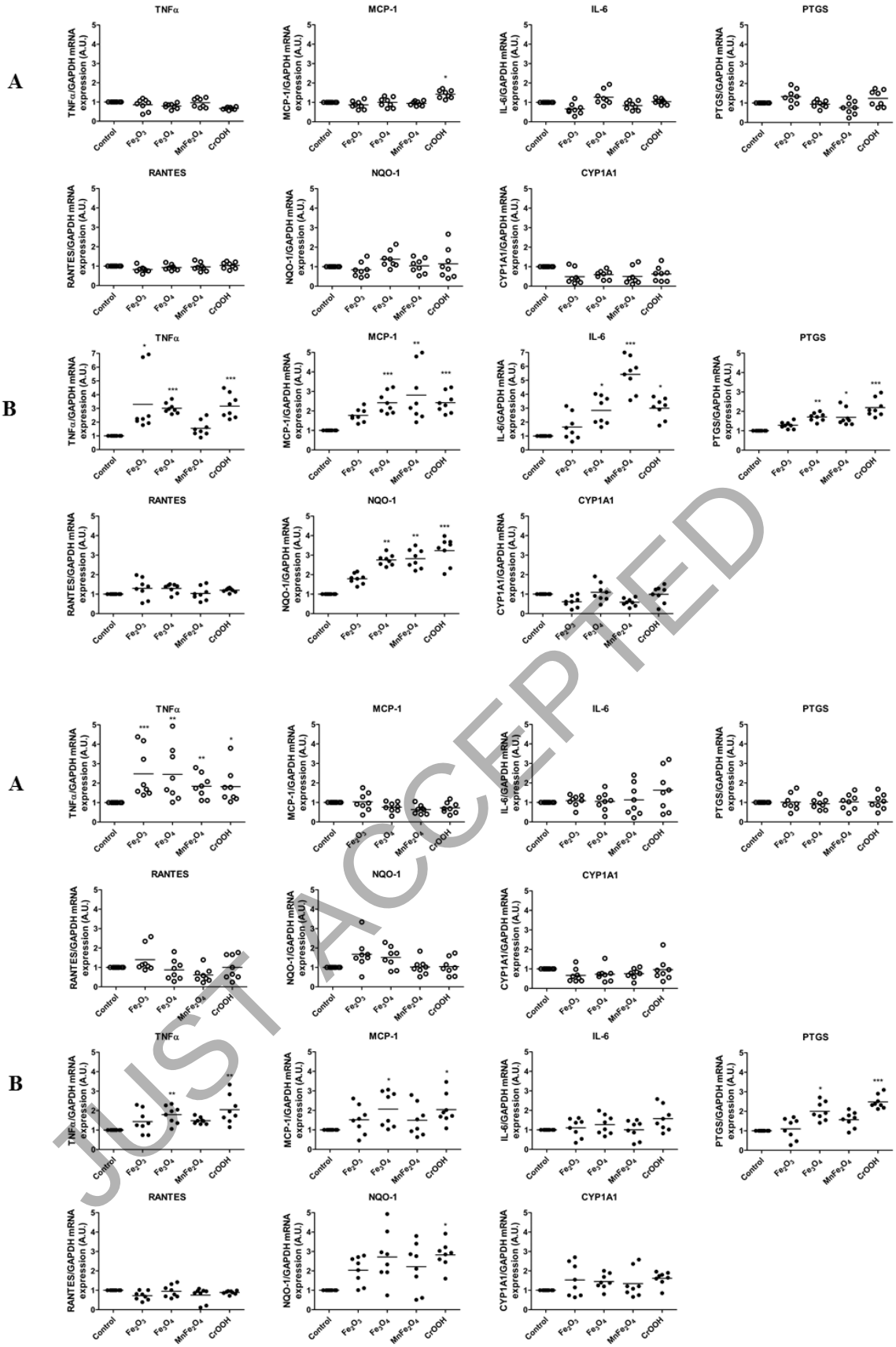

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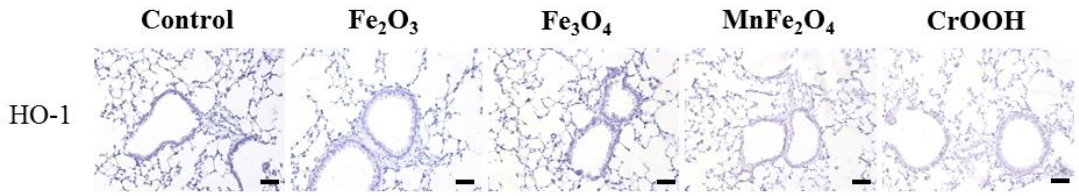

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