A clearance period after soluble lead nanoparticle inhalation did not ameliorate the negative effects on target tissues due to decreased immune response

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ABSTRACT

Background: The inhalation of metal (including lead) nanoparticles poses a real health issue to people and animals living in polluted and/or industrial areas. In this study, we exposed mice to lead(II) nitrate nanoparticles [Pb(NO₃)₂ NPs], which represent a highly soluble form of lead, by inhalation. We aimed to uncover possible differences in the effects of lead exposure on individual target organs and to reveal potential variability in the clearance of lead from organs after inhalation of soluble and insoluble lead particles. Mice were exposed to Pb(NO₃)₂ NPs in whole-body inhalation chambers for up to 11 weeks. We examined (i) lead biodistribution in target organs using laser ablation and inductively coupled plasma mass spectrometry (LA-ICP-MS) and atomic absorption spectrometry (AAS), (ii) lead effect on histopathological changes and immune cells response in secondary target organs and (iii) the clearance ability of target organs (lung, liver, kidney, spleen, bone and blood).

Results: After exposure to Pb(NO₃)₂ NPs, the lead concentration was the highest in femur at all designated time points with the exception of 3-day inhalation. In contrast to other organs, the ability to clear the lead from the femur was very low. In the lungs, Pb(NO₃)₂ NP inhalation induced serious structural changes; lung damage was present even after a 5-week clearance period despite the lead being almost completely eliminated from the lung tissue. Furthermore, in secondary target organs (kidney and liver), Pb(NO₃)₂ NPs induced several morphological defects, with alterations that remained visible after the clearance period even though the tissue lead concentrations decreased to minimal values. The numbers of lung and liver macrophages were significantly decreased after 11-week Pb(NO₃)₂ NP inhalation; conversely, abundance of

alpha-smooth muscle actin (α -SMA)-positive cells, which are responsible for augmented collagen production, was increased in both tissues. Moreover, the expression of nuclear factor κ B (NF- κ B) and selected cytokines (tumour necrosis factor alpha [TNF- α], transforming growth factor beta 1 [TGF β 1], interleukin 6 [IL-6], IL-1 α and IL-1 β) displayed a tissue-specific response to lead exposure.

Conclusions: Taken together, diminished inflammatory response in lung and liver after Pb(NO₃)₂ NPs inhalation was associated with prolonged negative effect of lead on tissues, as demonstrated by enhanced pathological changes in target organs, even after a 5-week clearance period that contrasts to the stronger effect observed previously after the clearance of insoluble lead nanoparticles.

Keywords

clearance, lead nanoparticles, inhalation, LA-ICP-MS imaging, toxicity

BACKGROUND

Air pollutants are major factors that impact public health or diseases. Ground-level ozone (O₃), nitrogen dioxide (NO₂) or particulate matter (PM) are the most common air pollutants that negatively affect health [1]. In recent decades, risk assessment of air particulate contaminants has received more attention; many experimental and environmental epidemiological studies have confirmed the biological effects of fine PM (PM_{2.5} with an aerodynamic diameter ≤ 2.5 μ m). They cause negative changes in the respiratory and cardiovascular systems as well as pathological nervous system alterations [2]. Chronic diseases such as chronic obstructive pulmonary disease (COPD), asthma, lung cancer, and atherosclerotic cardiovascular disease are all closely associated with PM [1]. PM exhibits a long residence time in the air, and its effects can be widely spread by atmospheric flows [3].

Therefore, tracking PM in the air is not only a primary concern for local emission areas but it is of crucial interest around the world.

The main crustal elements – aluminium (Al), calcium (Ca), iron (Fe), magnesium (Mg) and potassium (K) – and also toxic elements from anthropogenic sources – zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), arsenic (As), cadmium (Cd) and antimony (Sb) – have been detected in the atmospheric PM_{2.5} and PM₁ (diameter $\leq 1.0 \mu\text{m}$) samples in Asian megacities [4] and large European cities [5, 6, 7]. Interestingly, Pb is the most common metal in the air, while Fe, Zn, Cu and Pb are the most abundant metals in PM_{2.5} [8]. Some studies have identified Pb as the third most common metal behind Fe and Zn [3, 5, 9] or as the second most common metal in others surveys [4]. While the limit for airborne Pb determined by the World Health Organization (WHO) is $0.5 \mu\text{g}/\text{m}^3$, it is regularly exceeded in many cities around the world. The measured concentrations of airborne Pb in some areas have reached $20 \mu\text{g}/\text{m}^3$ in urban background sites and even more than $70 \mu\text{g}/\text{m}^3$ in industrial areas [8].

Due to their size, inhaled nanoparticles (NPs) can invade the lower respiratory tract more easily than corresponding larger forms and are predominantly deposited in the alveolar regions of lungs. Although the inhalation of metal NPs, including those that contain Pb, poses a real threat to humans, only a few studies have evaluated the potential toxicity of Pb in different nanoforms [10, 11, 12, 13]. Different approaches have been used for the applications of metal nano-suspensions into animal models such as intraperitoneal injections [11], intravenous injections [12, 14], intratracheal instillation [15] or by using specific inhalation devices for rodents [16] to study systemic intoxication.

Pb NPs in the air can come into contact with various parts of the body, such as the respiratory system, the olfactory region, the skin, the hair or the conjunctiva of the eyes. Moreover, all inhaled NPs exhibited extrapulmonary effects because they exposition twice: first time during inhalation and the second time during the clearance from the lung through

mucociliary transport [17]. Therefore, here we exposed animals to Pb NPs in whole-body inhalation chambers to simulate natural conditions. We used very low Pb NP concentrations in inhaled air to reflect complex real situations of how humans are exposed to metal NPs in the ambient atmosphere. We exposed the animals to lead(II) nitrate nanoparticles [Pb(NO₃)₂ NPs] at a mass concentration of 68.6 µg/m³ (i.e. 42.9 µg Pb/m³), corresponding to the exposure of city inhabitants during pollution peaks.

In our previous study [18], we evaluated the ability of organs to clear the Pb load of lead oxide nanoparticles (PbO NPs), which are insufficiently soluble and pass the lung barrier to be transported into secondary target organs in the NP form. Here, we instead evaluated tissue response upon exposure of animals to a highly soluble form of Pb, specifically Pb(NO₃)₂ NPs. We examined the Pb biodistribution, toxicological effects and clearance ability of target organs (lung, liver, kidney, spleen, bone and blood) following sub-chronic inhalation of Pb(NO₃)₂ NPs using mice as a model organism. Our results revealed that highly soluble Pb(NO₃)₂ NPs evoke distinct effects on target organs than those observed for less soluble PbO NPs used in our previous study.

RESULTS AND DISCUSSION

There are three typical routes – food, water and air [19] – of human exposure by metal contaminants. Given that approximately 8–10% of Pb is absorbed through ingestion, compared with 50% absorption through respiration [20], the inhalation pathway of this metal has recently gained attention. For this reason, we selected NP inhalation in whole-body chambers as the delivery method that represents the most typical exposure condition in humans. Specifically, we tested the effect of sub-chronic (11 weeks) inhalation of Pb(NO₃)₂ NPs dispersed in the air (**Fig. 1, Table 1**). First, we analysed the influence of these highly soluble NPs on animals with

a focus on their behaviour inside living organisms, with mapping the ability of individual organs to eliminate the Pb burden after a clearance period.

Figure 1: The characterization of $Pb(NO_3)_2$ NPs in the inhalation chambers, and the scheme of overall experimental design.

A, B) The characterization of $Pb(NO_3)_2$ NPs analyzed in the scanning transmission electron microscope (STEM). **C)** The mass concentration of $Pb(NO_3)_2$ NPs throughout the inhalation experiment. **D)** Design of the inhalation experiment. The groups of animals inhaled clean air for a period of up to 11 weeks (ctr), the other groups inhaled air with $Pb(NO_3)_2$ NPs for the same period ($Pb(NO_3)_2$ NP), and one group inhaled six weeks air with $Pb(NO_3)_2$ NPs and thereafter five weeks of clean air (clearance group - Pb/cl). **E)** The number size distribution of $Pb(NO_3)_2$ NPs in the inhalation chambers measured by Scanning Mobility Particle Sizer (SMPS). **F)** Surface area of $Pb(NO_3)_2$ NPs size distribution ($dS/d\log D_p$). The surface area of

fractions of $Pb(NO_3)_2$ NPs deposited in the extrathoracic (S_{ET}), tracheobronchiolar (S_{TB}) and alveolar region (S_A) of lungs, S_T - the total surface area of generated $Pb(NO_3)_2$ NPs, S_{TB+A} - the lung-deposited surface area.

Table 1. The characterization of generated $Pb(NO_3)_2$ NPs

Parameter	$Pb(NO_3)_2$
Number concentration	1.94×10^5 NPs/cm ³
Surface area	1.68×10^3 $\mu\text{m}^2/\text{cm}^3$
Mode	37.2 nm
Geometric mean diameter	31.3 nm
Geometric standard deviation	2.15
Mass concentration	68.6 $\mu\text{g}/\text{m}^3$
Estimated deposited dose (after 11w)	0.774 $\mu\text{g}/\text{g}$

Adult female mice divided into control (air) and exposed [$Pb(NO_3)_2$ NPs] groups were placed into inhalation chambers, where they were exposed to modified air for the entire experimental period. For each designated time point (3 days, 2 weeks, 6 weeks and 11 weeks), 10 biological replicates were evaluated in both control and $Pb(NO_3)_2$ NP-exposed groups (**Fig. 1**). In addition, one group of mice inhaled air with $Pb(NO_3)_2$ NPs for 6 weeks, and thereafter clean air for 5 weeks (this group is further referred to as the clearance group: Pb/cl). Thus, at the end of the inhalation period (11 weeks), 10 biological replicates of control group, each $Pb(NO_3)_2$ NP-exposed group and the Pb/cl group were examined in the same manner as the previously described experimental groups (**Fig. 1D**).

To assess the toxicological impacts of NPs and their possible risks to human health, information about NP deposition in different human lung regions is required. Therefore, we simulated the deposition of inhaled $Pb(NO_3)_2$ NPs in different parts of the human respiratory

tract using deposition fractions calculated by the International Commission on Radiological Protection (ICRP) deposition model for the extrathoracic, tracheobronchial and alveolar regions [21]. The total surface area of generated $\text{Pb}(\text{NO}_3)_2$ NPs (i.e. $1.68 \times 10^3 \mu\text{m}^2/\text{cm}^3$; S_T) was calculated by SMPS spectrometer software from measured particle size distribution. Based on the surface area of the NPs deposited in the extrathoracic region (S_{ET} ; the anterior and posterior nasal passage, larynx, pharynx and mouth), tracheobronchial region (S_{TB} ; trachea, bronchi and bronchioles) and alveolar region (S_A ; respiratory bronchioles and alveolar ducts), the highest $\text{Pb}(\text{NO}_3)_2$ NP surface area (25.3% of the total) is deposited in alveolar region of lungs, followed by the extrathoracic (7.4 %) and tracheobronchial (3.3 %) regions (**Fig. 1F**).

Lung-deposited surface area (LDSA) concentration, another relevant metric for health effects of aerosol particles [22], combines the surface area of the NPs deposited in the alveolar and tracheobronchial regions. Therefore, we evaluated the particle size distribution of S_T , S_{ET} , S_{TB} and S_A as well as the LDSA ($S_{TB} + S_A$) (**Fig. 1F**). The LDSA corresponds to 28.6% of total surface area of prospectively inhaled $\text{Pb}(\text{NO}_3)_2$ NPs, while the sum of S_{ET} , S_{TB} and S_A is 36.0% of total surface area of inhaled $\text{Pb}(\text{NO}_3)_2$ NPs (**Fig. 1F**).

Pb accumulates in all analysed target organs upon $\text{Pb}(\text{NO}_3)_2$ NP inhalation

In animals exposed to $\text{Pb}(\text{NO}_3)_2$ NPs, the Pb concentration in the organs continuously increased with the increasing exposure time (**Table 2**). After a 5-week clearance period, the Pb concentration in the lungs and spleen then decreased to the limit of detection (LOD). This was not the case for the femur, kidney and liver, in which Pb was clearly detectable even after a 5-week clearance period.

It needs to be said that because of the technical reasons the $\text{Pb}(\text{NO}_3)_2$ NPs concentration in the inhaled air was not absolutely constant and slightly fluctuated during the experiment (**Fig. 1C**). This fact was determined by collecting NPs on the filters and the evaluation of the mass

concentration of $\text{Pb}(\text{NO}_3)_2$ that was found to be lower in comparison to the earlier timepoints (Fig. 1C). Still, these small irregularities in the mass concentration of inhaled $\text{Pb}(\text{NO}_3)_2$ were not reflected by the total Pb concentrations detected in individual organs and in the blood (Tables 2 and 3) with exception of kidney where stagnation of Pb level between the 2nd week and 6th week timepoint was observed.

Table 2: The concentration of Pb (ng/g) in organs following $\text{Pb}(\text{NO}_3)_2$ NP inhalation determined by AAS

		ctr/3d,2w, 6w,11w	Pb/3d	Pb/2w	Pb/6w	Pb/11w	Pb/cl
lung	range mean SD	< LOD*	137-246 184 40	185-295 239 39	215-312 262 47	552-1130 752 225	< LOD*
femur	range mean SD	< LOD*	282-372 336 36	1095-1423 1269 123	2397-3120 2640 297	4042-5576 4750 551	2523-3941 3097 584
kidney	range mean SD	< LOD*	368-655 454 118	488-760 599 102	560-669 597 43	1259-1559 1433 109	48-91 72 15
liver	range mean SD	< LOD*	46-80 60 13	60-80 68 8	70-87 77 6	159-281 211 49	4-10 7 3
spleen	range mean SD	< LOD*	< LOD*	< LOD*	42-174 102 51	171-293 234 49	< LOD*

Comparison of Pb concentration in lung, femur, kidney, liver and spleen at different time points.
* Limit of detection in lung, femur, kidney, liver, and spleen was 26, 21, 9, 3, and 29 ng/g Pb, respectively.

Table 3: Pb concentration in blood determined by AAS

		ctr/3d,2w, 6w,11w	Pb/3d	Pb/2w	Pb/6w	Pb/11w	Pb/cl
erythrocytes (ng/g)	range mean	< LOD*	54-92 71	63-92 79	72-114 88	93-213 168	10-43 19

	SD		15	13	17	48	14
proteins (ng/g)	range mean SD	< LOD*	< LOD*	< LOD*	< LOD*	< LOD*	< LOD*
serum (ng/g)	range mean SD	< LOD*	< LOD*	< LOD*	< LOD*	< LOD*	< LOD*
blood (ng/g)		< LOD*	31	40	47	85	10
blood (µg/dl)		< LOD*	3.1	4.0	4.7	8.5	1.0

The concentration of Pb in blood at different time points.

* Limit of detection in erythrocytes in control animals, in proteins at all data points, in serum at all data points and in the whole blood in control animals was 6, 11, 4 and 3 ng/g Pb, respectively.

The response of individual organs studied here in animals exposed to Pb(NO₃)₂ NP also slightly varied and caused the changes of their weight coefficients (**Fig. S1**). After 2 weeks, the liver weight coefficient was significantly increased ($p < 0.001$), while the kidney coefficient was decreased ($p < 0.05$ for left; $p < 0.01$ for right) and the spleen coefficient was decreased ($p < 0.01$) compared with control mice. After 11 weeks, there was a decrease in the liver weight coefficient in the Pb/cl compared with the control group ($p < 0.05$). Some contribution to organ weight variability could be due to organ dissection; however, we observed similar alterations of organ weight coefficients after metal NP exposure, as was described previously in other studies [23, 24].

Pb becomes evenly distributed in the lungs upon Pb(NO₃)₂ NP inhalation

The lungs are a major portal for the entrance of inhaled particles. Owing to their size, metal NPs including Pb nanoforms can invade the lower respiratory passages down to alveolar region more easily than larger forms. Thus, the final effect of metal NPs can be more damaging to the lung tissue than microparticle forms. We analysed the presence of Pb in lungs after their exposure to Pb(NO₃)₂ NPs at different time points; there was a continuous increase in Pb as the

NP inhalation period increased (**Fig. 2, Table 2**). A 5-week clearance period positively affected Pb clearance from the lung; indeed, the Pb concentration decreased almost to the LOD (26 ng/g).

Figure 2: The distribution of selected metals at designated time points after $Pb(NO_3)_2$ NP inhalation in the lung.

A) Distribution of Pb, Na, K, Ca, Fe and Zn in lung samples using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Scale bar in all panels = 5 mm. **B)** The graph of Pb level in the lungs at designated time points. **C)** The graphs of Na, K and Ca level in the lungs at designated time points. The graphs values denote average $\pm$ SD for 5 mice/group, ** $p < 0.01$, *** $p < 0.001$ compared with the corresponding control group, and †† $p < 0.01$ compared with the corresponding $Pb(NO_3)_2$ NP group by unpaired t -test. Limit of detection (LOD) for Pb in the lungs was 26 ng/g.

Elemental imaging of lungs after $Pb(NO_3)_2$ NP inhalation revealed Pb being regularly distributed in all parts of the lung (**Fig. 2**). After a 5-week clearance period, there was an

obvious reduction in the Pb level (**Fig. 2, Table 2**). Chemical analysis of other selected elements (sodium [Na], K and Ca) in the lungs revealed significant differences between control animals and those exposed to Pb(NO₃)₂ NPs for 11 weeks (**Fig. 2**): Na and K levels were increased after 11 weeks compared with controls ($p < 0.001$ and $p < 0.05$, respectively). The Ca level in the lungs was also altered during the experiment; however, due to high variability among animals (observed by both chemical and elemental analysis), the values were not statistically significant. Laser ablation and inductively coupled plasma mass spectrometry (LA-ICP-MS) revealed higher Fe and Zn concentrations in the Pb(NO₃)₂ NP-exposed animals compared with controls (**Fig. 2**).

Inhalation of Pb(NO₃)₂ NPs causes pulmonary injury

Histological analyses revealed numerous signs of inflammation and focal destruction of lung parenchyma in animals exposed to Pb(NO₃)₂ NPs; the damage depended on the exposure time (**Fig. 3A, Table S1**). These findings were similar to what has been described in the inhalation experiment with other metal NPs: PbO [13], titanium oxide (TiO₂) [25] and cobalt (Co) [26]. The damaged lung parenchyma included destroyed alveolar septa, with alveolar emphysema initiated in some areas. The lungs of exposed animals exhibited several signs of inflammation, such as hyperaemia; thickened alveolar septa with increased cellularity; regions with peribronchial and perivascular lymphocyte infiltrates; and areas of atelectasis and focal bronchiolitis. All of these distinct histopathological changes in the lungs were evaluated and scored for individual groups (**Table S1**). The extent of histopathological changes in lungs was statistically significant after 11 weeks ($p < 0.01$).

Figure 3: The effect of $Pb(NO_3)_2$ NP inhalation and its clearance on the lung.

A) The lungs in control animals, and after 2-week $Pb(NO_3)_2$ NP inhalation were without serious pathological changes. The lungs after 6 and 11-week $Pb(NO_3)_2$ NP inhalation exhibited remodeling in bronchiolar (b), and alveolar areas (a). The serious damage of lung parenchyma with destroyed alveolar septa and alveolar emphysema detected in some areas (em). Alteration of lung tissue was still detected after a 5-week clearance period. The graphs values refer to average $\pm$ SD; ** $p < 0.01$, and *** $p < 0.001$ compared with the corresponding control group (ctr) by unpaired t -test. B) The expressive lung secretion was found in bronchioles, and alveolar

spaces by electron microscopy analysis. The higher amount of surfactant (su), lamellar bodies (lb), cell detritus (de), and enormous macrophages (ma) were in the alveoli. Arrow points to the damaged membranes of alveolar cell, arrowheads indicate high amount of collagen fibrils in the alveolar septa. The nanoparticles were not present in lung tissue. **C)** Collagen fibers (red) were around blood vessels (bv), and bronchioles (b) in controls. After 11-week $Pb(NO_3)_2$ NP inhalation, collagen fibers were in alveolar regions (arrowheads). **D)** Immunohistochemical detection of α -SMA positive cells in the walls of bronchioles (br) or blood vessels (ve) in controls (arrows), and after 11-week of $Pb(NO_3)_2$ NP inhalation in the walls of bronchioles (br), and in the alveolar regions (al, arrows). **E)** Immunofluorescent detection of α -SMA positive cells in the walls of bronchioles (br) or blood vessels (ve) in controls, $Pb(NO_3)_2$ NP and Pb/cl inhaled groups. α -SMA positive cells aggregated in some alveolar regions (al, arrows, in box) after $Pb(NO_3)_2$ NP inhalation. Scale bar in panels A, C, D, E = 100 μm .

While chemical and LA-ICP-MS detection documented the absence of Pb in the lungs after a 5-week clearance period, the histopathological analysis uncovered persistent inflammatory changes in the lungs ($p < 0.001$, **Fig. 3A**, **Table S1**). The lungs of Pb/cl animals still exhibited several signs of inflammation: hyperaemia, thickened alveolar septa with increased cellularity, higher number of lung macrophages, regions with peribronchial and perivascular mononuclear infiltrates and focal bronchiolitis (**Fig. 3A**).

We next performed ultrastructural analysis of lung tissue by transmission electron microscopy (TEM) to identify the localisation of $Pb(NO_3)_2$ NPs in lungs (**Fig. 3B**). There were no NPs in bronchioles or alveoli, which documented the rapid solubility of $Pb(NO_3)_2$ NPs during transport through respiratory passages. However, it is necessary to mention that we are only able to distinguish larger particles or aggregates in TEM; small single NPs cannot be unambiguously identified because of their similarity to ribosomes so that we cannot exclude their presence in pneumocytes using this morphological approach.

The lumens of bronchioles and alveoli contained inflammatory cells, cellular debris and accumulated surfactant; membranes of alveolar epithelial cells were severely damaged (**Fig.**

3B). We also found majority of lamellar bodies being ejected from alveolar type II cells into the alveolar airspaces after 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation (**Fig. 3B**). It seems plausible that abundant surfactant seen by TEM may contribute to solubility of NPs in lower respiratory passages.

Collagen fibres were detected in the wall of blood vessels and bronchioles in the control animals. After 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation, there were ectopic collagen fibres in the alveolar regions of lungs (**Fig. 3C**). TEM also confirmed the presence of higher amount of collagen fibrils in the alveolar septa (**Fig. 3B**). Augmented collagen production and induction of fibrosis can be associated with activation of myofibroblasts [27, 28] and their expression of α -smooth muscle actin (α -SMA) [29]. Previous studies have reported the upregulation of α -SMA expression as a progressive fibrosis marker in the lungs after metal NP exposure [30, 31]. We observed α -SMA-positive cells located in the walls of larger bronchioles or blood vessels in the control animals and $\text{Pb}(\text{NO}_3)_2$ NP inhalation increased their appearance in the walls of small bronchioles and aggregation in alveolar regions in the $\text{Pb}(\text{NO}_3)_2$ NP and Pb/cl groups (**Fig. 3D,E**). Therefore, interstitial fibrosis was induced after 6-week of $\text{Pb}(\text{NO}_3)_2$ NP inhalation and was still active after a 5-weeks clearance period.

Inhalation of $\text{Pb}(\text{NO}_3)_2$ NPs alters number of lung macrophages

Particles $\leq 0.5 \mu\text{m}$ can accumulate in the alveolar parts of the lung and induce an inflammatory response [15]. Although inflammation is usually protective, it can cause serious injury to the organ in the case of prolonged persistence. We found a reduced number of lung macrophages (detected as CD68-positive cells) after 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation ($p < 0.01$, compared with the corresponding control group; **Fig. 4A,B, Table S2**). A similar decrease of CD68-positive cells was noticed in Pb/cl animals ($p < 0.05$) compared with the corresponding control animals (**Fig. 4A,B, Table S2**).

Figure 4: The effect of $Pb(NO_3)_2$ NP inhalation and its clearance on the lung inflammation. **A, B)** Detection of CD68-positive cells (marker of macrophages) in lungs. The number of macrophages was decreased in $Pb(NO_3)_2$ NPs and Pb/cl groups. The difference in number of CD68-positive cells was statistically significant compared with the control group. The graphs values indicate average $\pm$ SD; * $p < 0.05$, ** $p < 0.01$. **C, D)** Detection of Toluidine Blue-positive cells (marker of mastocytes) in lungs (arrows). The number of mastocytes was increased in $Pb(NO_3)_2$ NP and Pb/cl groups and the difference was statistically significant compared with the controls after 11-week $Pb(NO_3)_2$ NP inhalation. The graphs values indicate average $\pm$ SD. **E)** Detection of MPO-positive cells (myeloperoxidase, marker of neutrophils) in blood vessels or in lung infiltrates in lung samples (arrows).

Especially in lung infiltrates, the number of mastocytes (as visualized by Toluidine Blue) and the presence of neutrophils (as visualised by immunolabeling of myeloperoxidase, MPO) was instead increased (**Fig. 4C–E**). Taken together, soluble engineered $Pb(NO_3)_2$ NPs used at a very low concentration – corresponding to environmental levels of metal NPs – induced abnormalities in lung structures that persisted even after a 5-week clearance period.

Concentration of Pb in blood decreased after a 5-week clearance period

The blood Pb level is a sensitive indicator of Pb exposure. Over recent decades, the United States Centers for Disease Control and Prevention (USCDC) has gradually lowered the blood Pb level at which medical intervention is necessary: from 60 µg/dl before 1975 to 10 µg/dl in 1991 [19]. The current limit recommended by the WHO is 5 µg/dl. In our experimental animals, the blood Pb concentration continuously increased as the exposure time to Pb(NO₃)₂ NPs increased (Fig. 5, Table 3).

Figure 5: The concentrations of selected elements in the blood and bone after Pb(NO₃)₂ NP inhalation.

The graphs of selected elements (Pb, Ca, P, alkaline phosphatase – ALP, Mg, Na and K) in the blood and/or in the bone tissue. The graphs values indicate average $\pm$ SD for 5 mice/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the corresponding control group (ctr), and † $p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$ compared with the corresponding Pb(NO₃)₂ NP group by unpaired t -test. The concentrations of Pb in controls were below limit of detection (LOD). LOD for Pb in the blood was 6, 11, 4 and 3 ng/g Pb at individual time points, for Pb in the bone 21 ng/g Pb.

The concentration of Pb in blood increased slightly from 31 ng/g (3.1 µg/dl) after 3-day Pb(NO₃)₂ NP exposure, to 47 ng/g (4.7 µg/dl) after 6-week exposure to 85 ng/g (8.5 µg/dl) after 11-week exposure. Following a 5-week clearance period, such Pb concentration decreased approximately fivefold compared with the level in the 6-week Pb(NO₃)₂ NP exposure group

(10 ng/g or 1 µg/dl). Only the animals exposed to Pb(NO₃)₂ NPs for 11 weeks exceeded the WHO recommended blood Pb limit (5 µg/dl).

A 5-week clearance period did not affect the Pb level in bones

Based on previous studies, the most of total body Pb burden occurs in the skeleton as the bone is the major target tissue for Pb storage [32, 33]. The affinity of Pb for bone has been described predominantly after gastrointestinal exposure to Pb in solutions [34, 35]. However, Pb can also persist for prolonged periods in the atmosphere due to its nature thus representing the exposure route for humans [32]. Here, we analysed mouse femurs as models of the axial skeleton. Only a few studies have analysed the effect of inhaled Pb in the form of submicrometric particles on bone [36, 37].

The concentration of Pb in femur was the highest among the studied organs (lung, liver, kidney, spleen and brain) at all designated Pb(NO₃)₂ NP exposure times except for 3 days (**Fig. 5, Table 2**). At this time, the Pb concentration was the highest in the kidney, followed by the femur (**Table 2**). The pattern of bone Pb content contrasted with the blood Pb level during Pb(NO₃)₂ NP inhalation. The relative increase of the Pb concentration in the inhaled air between 6 and 11 weeks corresponded with a rapid Pb increase in the blood, lungs, liver and kidneys (**Table 2**) but not in the bones.

Inhalation of Pb(NO₃)₂ NPs diminished the levels of blood phosphorus (P) and alkaline phosphatase (ALP)

Exposure to inhaled Pb(NO₃)₂ NPs did not influence the blood level of Ca at any time point (**Fig. 5**); however, the blood level of P was affected (**Fig. 5**). After 2 and 11 weeks of exposure to Pb(NO₃)₂ NP, the blood concentration of P was significantly decreased ($p < 0.01$) compared with control animals. The blood level of P in Pb/cl mice was similar to control animals.

The primary repository of phosphate is the bone and intracellular environment [38]. Phosphate is first absorbed in the intestine, circulates through the body by blood transport, is freely filtered at the glomeruli of kidney and then reabsorbed in the proximal renal tubules. Kidney renal tubules are target tissue during metal exposure including Pb, and thus phosphate reabsorption is usually affected and hypophosphatemia develops [39].

ALP is an enzyme required to generate free P for hydroxyapatite formation during bone mineralisation [40]. During hypophosphatemia (in our experiment, predominantly after 2 and 11 weeks of Pb(NO₃)₂ NP exposure), ALP activity could increase in order to compensate mineral dysregulation and to provide more phosphate to the bones [38]. However, the ALP level in mice exposed to Pb(NO₃)₂ NPs was decreased compared with control animals at all analysed time points; this difference was only significant after 2 weeks of exposure due to high variability among animals. Nevertheless, the biological significance and consequences of low P levels accompanied by low ALP levels will require additional research.

Deregulation of the storage of basic elements (Na, K, Ca and Mg) in the bone

The femur concentrations of Ca ($p < 0.01$) and Mg ($p < 0.001$) were significantly reduced after 2-week Pb(NO₃)₂ NP inhalation. Following 11-week Pb(NO₃)₂ NP inhalation, Ca ($p < 0.01$), Mg ($p < 0.01$) and K ($p < 0.001$) concentrations were significantly diminished in the femur compared with control animals (**Fig. 5**). On the other hand, the Na level significantly increased in bones after 2- and 11-week Pb(NO₃)₂ NP exposure. This finding is in agreement with our previous study [18], which revealed the same trends in bone mineral content changes after inhalation of PbO NPs: Ca, Mg and K concentrations were significantly decreased while Na was increased (not significant). A 5-week clearance period increased the concentrations of Mg (not significant), Ca ($p < 0.01$) and K ($p < 0.001$) in the bone relative to the corresponding control animal values. The effect of Pb(NO₃)₂ NP inhalation on femur weight (**Fig. S1**) was

similar to their alteration after exposure to PbO NPs [18]. There was only a small effect (slight reduction) in the Pb level in bones (femurs) after a 5-week clearance period (**Fig. 5**).

Lead is stored in ionic forms in the kidney cortex after Pb(NO₃)₂ NP exposure

Next, we studied the kidneys because Pb is primarily excreted by this organ [41, 42]. The concentration of Pb in kidneys continuously increased with increasing length of exposure to Pb(NO₃)₂ NP ($p < 0.001$ at all time points; **Fig. 6A,D, Table 2**).

Figure 6: The distribution of selected metals at designated time points after Pb(NO₃)₂ NP inhalation in the kidney.

A) The graph of Pb level in the kidney at designated time points. The graphs values denote average $\pm$ SD for 5 mice/group, *** $p < 0.001$ compared with the corresponding control group, and ††† $p < 0.001$ compared with the corresponding Pb(NO₃)₂ NP group by unpaired t-test. Limit of detection (LOD) for Pb in the kidney was 9 ng/g. **B)** Time-resolved signal originated

from kidney tissue exposed to $Pb(NO_3)_2$ NPs. **C)** Distribution of Pb signal in the kidney cortex (1.4 x 1.0 mm) exposed to $Pb(NO_3)_2$ NPs. **D)** Distribution of selected elements (Pb, Na, K, Ca and Fe) in kidney samples using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) after $Pb(NO_3)_2$ NP inhalation. Control kidney was without Pb positivity, Pb was detected in kidney cortex (co) after $Pb(NO_3)_2$ NP inhalation but not in the medulla (me). After a 5-week clearance period, Pb was still present in kidney cortex. Na and K were observed in similar manner in control and $Pb(NO_3)_2$ NP-exposed kidneys. The map of Ca exhibited higher distribution in kidney in Pb/cl group compared with the control and $Pb(NO_3)_2$ NP-group. The extent of the Fe was slightly increased after $Pb(NO_3)_2$ NP inhalation compared to the control. Scale bar in all panels = 3 mm.

As determined by both chemical and elemental analysis, upon 5-week clearance period, the level of Pb in kidneys was much lower compared to the state after 6 weeks of inhalation of $Pb(NO_3)_2$ NPs ($p < 0.001$, **Fig. 6**). Specifically, the level of Pb in kidneys decreased by about eightfold after 5-week inhalation of clean air (**Table 2**).

Elemental imaging revealed high Pb concentrations in the kidney cortex at all analysed time points (**Fig. 6**). The kidney medulla exhibited no evident presence of Pb. Togao et al. (2020) reported a similar inhomogeneous distribution of Pb in the kidney; however, the area with the highest Pb concentration was located more deeply in the kidney parenchyma (at the cortico-medullary junction). The levels of Pb in kidneys in that study [43] were, however, much higher both in so-called low (about eightfold) and high (fortyfold) conditions compared with our experiment, which could affect the Pb distribution in kidney tissue.

As the kidney cortex is the part of the imaged organs with the highest concentration of lead, we selected this area to determine the form of lead in tissues (ionic, NPs or both). For this purpose, different imaging mode was used (laser beam diameter was diminished, the signal of lead was monitored with the shortened integration time) and a small region of kidney cortex was analyzed (1.4 x 1.0 mm). The lead in form NP can be identified in this analysis by the presence of a high signal observed as yellow or red color in color-coded map, which

corresponds to sharp peaks on the graph displaying time-resolved signal. However, there were no $\text{Pb}(\text{NO}_3)_2$ NPs found in analyzed kidney cortex (**Fig. 6B, C**) and the lead was present only in the ionic form, which was different from our previously published PbO NPs study, where distinct PbO NPs were identified [18].

Exposure to $\text{Pb}(\text{NO}_3)_2$ NPs caused only minor changes to kidney ultrastructural

The nephrotoxic effects of Pb are usually noted as high blood Pb concentrations $> 50 \mu\text{g/dl}$ in children and $> 40 \mu\text{g/dl}$ in adults. Here, the blood Pb concentration reached rather low level of $8.5 \mu\text{g/dl}$ after 11-week $\text{Pb}(\text{NO}_3)_2$ NP exposure but we still observed morphological changes in kidney tissue at the ultrastructural level following the treatment. There were differences in the arrangement of filtration barrier in glomeruli of animals exposed to $\text{Pb}(\text{NO}_3)_2$ NPs (**Fig. 7A**). The filtration membrane was diffusely thickened with intramembranous electron-dense deposits. Both *laminae rarae* were filled by electron-dense material, with a thicker *lamina densa* compared with controls. The typical electron-density-based contrast among these three layers (*laminae*) disappeared. Pedicles (foot processes) of podocytes were irregularly arranged or lost after 11-week $\text{Pb}(\text{NO}_3)_2$ NP exposure.

Renal tubules (proximal and distal) displayed the typical physiological appearance even after 6-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation; however, after 11 weeks, there were obvious morphological alterations of the epithelial cells in proximal tubules. These findings are consistent with previously detected Pb nephrotoxicity that predominantly resulted in the damage of proximal renal tubules [41]. The apical parts of the epithelial cells were dilated, brush border was absent and cell organelles were randomly dispersed in the cytoplasm of some proximal tubules (**Fig. 7A**). The cell nuclei were altered to euchromatin-rich structures, a change indicative of augmented transcriptional activity both in glomerular and tubular parts of

the kidney after 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation (**Fig. 7A**). It is of note that there was evident regeneration of glomerular and tubular kidney tissue after the 5-week clearance period.

Figure 7: The effect of $\text{Pb}(\text{NO}_3)_2$ NP inhalation and its clearance on the kidney.

A) Analysis of kidney ultrastructure in the transmission electron microscope (TEM). Proximal (pt) and distal tubules (dt) were without pathological changes up to 6-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation. After 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation, the proximal tubules of kidney exhibited changes both in nuclear (n) and cytoplasmic (c) architecture. A 5-week clearance period conducted to rescue of proximal tubule cells. The glomerular filtration membrane (GFM, arrowheads) was altered after 6-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation. The membrane was diffusely thickened with intramembranous electron dense deposits, and the typical electron contrast among three layers of GFM disappeared. The thicker GFM was observed still after a 5-week clearance period. The nuclear alterations were observed also in nuclei (n) of podocyte (po) after 11-week $\text{Pb}(\text{NO}_3)_2$ NP inhalation. **B)** The graphs of blood urea and creatinine. The graphs values denote average $\pm$ SD; * $p < 0.05$ and ** $p < 0.01$ compared with the corresponding control group (ctr), and † $p < 0.05$ compared with the corresponding $\text{Pb}(\text{NO}_3)_2$ NP group. **C)** The graphs of Na, K and Ca in the blood. Blood levels of these elements were without alterations during experiment among individual groups. **D)** The graphs of Na, K and Ca in kidney. Kidney

Ca was significantly decreased after 2-week Pb(NO₃)₂ NP inhalation (* $p < 0.05$) compared with the corresponding control group (ctr) and significantly increased in Pb/cl group ($\dagger p < 0.05$) compared with the corresponding Pb(NO₃)₂ NP group.

Alteration of blood-contained biochemical markers of kidney function

The biochemical markers of kidney function that are typically examined in the blood are urea and creatinine. High plasma urea and/or creatinine concentrations indicate the inability of the kidney to excrete these products. The majority of urea produced in the body is the final product of exogenous and endogenous protein metabolism in the liver [44]. Urea is transported through the blood to the kidney, from where it should be excreted. Thus, the blood urea level reflects the function of two vital organs: liver (production of urea) and kidney (excretion of urea). Here, the animals that were exposed for 2 and 11 weeks to Pb(NO₃)₂ NPs had decreased level of blood urea ($p < 0.05$) and this corresponded to overall lower levels of blood proteins (**Fig. 7B, Table 4**).

The blood creatinine level has been commonly used as a simple test of kidney function [45]. Creatinine is a breakdown product of muscle creatine; the kidneys excrete it with an unchanged structure. Creatinine is an organic compound that is endogenously produced primarily in the liver, but it can also be obtained through the diet. Thus, the blood creatinine level can be influenced by various factors (protein intake, muscle breakdown, synthesis of creatinine in the liver). There was reduced blood creatinine in animals exposed for 11 weeks to Pb(NO₃)₂ NPs ($p < 0.05$) compared with the control animals. This decrement may also be related to reduced blood level of proteins after sub-chronic exposure similar to decreased level of urea in blood (**Fig. 7B, Table 4**). On the other hand, it is necessary to mention that the reduced blood urea ($p < 0.01$) and creatinine levels observed after 3 days of Pb(NO₃)₂ NP inhalation were not associated with a decreased protein level in the blood at that time point. We

519 assume that reduced protein synthesis in the liver was not yet reflected in the blood; thus, other
 520 factors may have influenced its level.

521

522 **Table 4: Blood biochemical analysis following Pb(NO₃)₂ NP inhalation**

523

		ctr/2w	Pb/2w	ctr/6w	Pb/6w	ctr/11w	Pb/11w	Pb/cl	CD-1 (ICR)
TP (g/l)	range	56-66	46-59	58-67	63-69	57-62	54-58	56-60	53-60
	mean	61	53*	63	66	60	56*	59	
	SD	5	5	4	3	3	2	2	
Alb (g/l)	range	29-32	25-31	32-34	32-34	31-35	29-33	31-33	36-43
	mean	31	29	33	33	33	31	32	
	SD	1	2	1	1	2	2	1	
Tbil (μmol/l)	range	3.2-3.6	2.4-2.6	2.6-3.7	2.1-3.3	3.3-4.3	2.9-5.4	3.4-3.9	2.7-4.6
	mean	3.4	3.9	3.3	2.7	3.8	3.9	3.7	
	SD	0.2	0.6	0.5	0.5	0.4	0.9	0.2	
Ca (mmol/l)	range	2.44-2.74	2.35-2.56	2.30-2.47	2.31-2.59	2.50-2.80	2.51-2.68	2.54-2.67	2.48-2.88
	mean	2.56	2.46	2.39	2.48	2.68	2.58	2.61	
	SD	0.14	0.08	0.06	0.11	0.13	0.06	0.06	
P (mmol/l)	range	2.31-2.96	1.74-2.13	1.75-2.66	1.73-2.61	1.82-2.95	1.00-1.40	1.37-2.25	2.30-4.52
	mean	2.56	1.92**	2.16	2.10	2.40	1.18**	1.74†	
	SD	0.28	0.15	0.40	0.37	0.62	0.17	0.35	
ALP (μkat/l)	range	1.8-2.9	1.1-1.8	0.6-2.7	0.8-2.0	1.17-2.27	0.7-1.4	1.0-1.1	0.8-2.2
	mean	2.1	1.4*	1.6	1.5	1.5	1.1	1.0	
	SD	0.5	0.3	0.8	0.5	0.5	0.2	0.1	
ALT (μkat/l)	range	0.6-3.3	0.7-4.1	0.3-1.4	0.3-0.5	0.27-0.40	0.3-0.6	0.2-0.5	0.3-0.7
	mean	1.4	1.5	0.6	0.5	0.3	0.4	0.4	
	SD	1.3	1.4	0.4	0.1	0.1	0.1	0.1	
AST (μkat/l)	range	3.7-14.6	6.6-12.1	2.5-9.4	1.7-5.5	1.80-3.13	1.1-3.4	2.2-3.0	0.8-1.3
	mean	8.2	8.4	4.9	3.4	2.3	2.2	2.5	
	SD	4.6	2.3	3.1	1.4	0.6	0.9	0.3	
urea (mmol/l)	range	6.9-8.5	5.8-7.7	6.2-7.7	6.1-13.0	7.6-10.0	4.0-7.5	6.6-8.6	4.6-7.5
	mean	7.8	6.9	7.2	9.2	8.6	5.9*	7.5†	
	SD	0.7	0.8	0.6	3.0	1.0	1.3	0.8	
crea (mmol/l)	range	33.7-40.3	35.1-40.4	29.5-36.5	31.9-32.9	33.5-39.5	28.8-35.4	33.1-34.8	26.5-35.4
	mean	36.7	36.2	32.2	32.5	37.0	32.4*	34.1*	
	SD	2.8	3.8	2.8	0.4	2.5	2.8	0.6	
chol (mmol/l)	range	1.76-2.76	1.87-2.26	1.83-2.54	1.92-2.54	1.88-3.39	1.75-1.93	1.48-2.35	1.84-3.81
	mean	2.27	2.07	2.23	2.16	2.66	1.94*	2.00	
	SD	0.42	0.16	0.27	0.26	0.62	0.19	0.34	
TAG (mmol/l)	range	1.55-3.18	1.48-2.09	1.41-1.99	1.73-3.11	1.05-2.38	1.14-2.49	0.94-1.34	0.27-2.44
	mean	2.07	1.82	1.72	2.24	1.67	1.84	1.18†	
	SD	0.77	0.24	0.21	0.51	0.64	0.52	0.16	
Na (mmol/l)	range	146-149	140-148	141-149	138-146	146-148	143-146	140-147	151-161
	mean	147	145	144	142	147	144	144	
	SD	1	3	3	3	1	1	3	
K (mmol/l)	range	6.1-8.2	7.0-10.8	5.8-8.9	6.6-9.2	5.2-6.9	5.1-7.4	6.7-9.5	8.1-12.2
	mean	7.3	8.0	7.2	8.1	6.2	6.3	7.9	
	SD	0.9	1.6	1.6	1.2	0.9	1.0	1.3	
Cl (mmol/l)	range	115-125	114-121	115-121	112-120	112-120	116-121	117-122	112-124
	mean	119	117	118	116	116	118	119	
	SD	4	2	2	3	3	2	3	
Glu (mmol/l)	range	8.2-11.3	8.8-11.3	8.4-13.8	7.3-14.0	8.0-12.0	8.1-11.6	10.1-12.1	8.0-17.8
	mean	10.2	10.7	11.0	11.0	9.7	10.0	11.0	

	SD	1.4	1.2	2.1	2.8	1.7	1.5	0.9	
GGT (μ kat/l)	range	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07	< 0.07

Data were obtained from five animals per every group. As reference values were used values of female mice *crl:CD-1 (ICR) BR* of different strains of female mice according to Serfilippi *et al.* (2003). Reference biochemical values were count to our used units. * $p < 0.05$, ** $p < 0.01$ compared with the corresponding control group (ctr) by unpaired *t*-test, † $p < 0.05$ compared with the corresponding $Pb(NO_3)_2$ NP group by unpaired *t*-test.

$Pb(NO_3)_2$ NP exposure caused Ca disbalance in the kidney

The kidney participates in the maintenance of physiological homeostasis via regulation of the extracellular fluid volume, acid-base balance, and electrolyte blood concentrations. Sodium is the main cation of the extracellular fluid; it is involved in the regulation of blood pressure and is necessary for the excitability of muscles and neurons [46]. On the other hand, potassium exists predominantly in the intracellular fluid and, together with Na, regulates fluid balance and controls the electrical activity of the heart and other muscles [47]. Calcium is the most abundant mineral element in the body; the majority of Ca resides in the form of hydroxyapatite in the bones and the rest is located in the extracellular fluid or tissues [48]. Na, K and Ca regulation in the body relies on key physiological processes in the kidney. Blood concentrations of Na and K were not altered in any animals that were exposed to $Pb(NO_3)_2$ NPs. The concentrations of Na and K in kidneys were not statistically significantly changed (**Fig. 7C**), the finding that documents the capability of kidney to maintain both the blood and kidney levels of these fundamental minerals in the physiological limits even after $Pb(NO_3)_2$ NP inhalation.

The sodium and potassium kidney concentrations determined by LA-ICP-MS were similar to those determined by atomic absorption spectrometry (AAS) (**Figs. 6 and 7**). Our results are consistent with the previously published data on element distribution in mouse organs [49]. The potassium distribution was similar in the kidney parenchyma of the control, $Pb(NO_3)_2$ NP-treated and Pb/cl animals (**Figs. 6 and 7**), with the strongest K positivity in the

kidney medulla. Elemental sodium imaging was rather problematic (**Fig. 6**), presumably due to its high content in phosphate-buffered saline (PBS), which we used for tissue processing before LA-ICP-MS analysis.

The blood level of Ca is typically maintained within a relatively narrow range. There are three main processes involved in the control of its level: intestinal absorption, renal reabsorption and/or excretion and interchange between the bones and the blood [50]. Here, the blood levels of Ca were maintained constant in all experimental groups at each time points (**Fig. 7C**). This finding demonstrates the ability of kidney to maintain blood level of Ca level in physiological limits. Nevertheless, the levels of Ca in kidneys were changed upon Pb(NO₃)₂ NP inhalation. Previous studies have demonstrated an elevated Ca concentration in the kidney after exposure to Pb and/or Cd [51, 52]. On the other hand, we previously reported decreased Ca levels after exposure to PbO NP [18]. In the present study, the Ca concentrations in kidneys (**Fig. 7D**) also significantly decreased in Pb(NO₃)₂ NP–exposed animals in comparison to the controls, with a significant reduction after 2 weeks ($p < 0.05$). The lead may compete with Ca in kidney tubules and impaired Ca reabsorption can decrease the kidney Ca level; however, the precise mechanism that explains Ca behaviour after metal exposure has not been uncovered yet. Here, in the Pb/cl animals, the Ca levels measured by AAS were significantly higher ($p < 0.05$) compared with the corresponding animals exposed to Pb(NO₃)₂ NPs. LA-IC-MS imaging in kidney cortex also revealed the increased Ca level in the Pb/cl animals (**Fig. 6D**).

The Ca distribution pattern resembled that of Na in the kidney. The Ca images obtained by LA-ICP-MS displayed calcium in the kidney parenchyma and surrounding areas. Togao et al. (2020) reported similar observations with LA-ICP-MS analysis. The reason for such Ca distribution is not clear but is probably associated with tissue processing through the Optimal Cutting Temperature (O.C.T.) solution. The concentration of Fe in kidneys increased after 11

weeks of Pb(NO₃)₂ NP inhalation. This finding corresponds with higher blood circulation in analysed tissues [49].

Severe pathological changes are visible in liver even after a 5-week clearance period

The liver represents the central organ involved in the metabolic processes leading to Pb intoxication. Our previous analyses uncovered toxic effects of insoluble metal NPs on liver cells [13, 53]. The inhalation of highly soluble Pb(NO₃)₂ NPs caused serious remodelling of the liver parenchyma, hydropic degeneration of some hepatocytes, focal necrosis, haemostasis in small veins and liver sinusoids and resulted in fewer small vessels (**Fig. 8A**).

Figure 8: The effect of Pb(NO₃)₂ NP inhalation and its clearance on the liver.

A) The liver in control animals were without serious pathological changes. The liver after 2, 6, and 11-week Pb(NO₃)₂ NP inhalation exhibited hepatocyte dystrophy (dy), remodeling in liver parenchyma (re) and expressive hemostasis (he). We observed inflammatory infiltrates (in), focal necrosis, and numerous megakaryocytes (me) in liver. Remodeling of liver tissue and large inflammatory infiltrates were still detected after a 5-week clearance period. The graphs values denote average $\pm$ SD; * $p < 0.05$ compared with the corresponding control group (ctr) by unpaired *t*-test. **B)** Ultrastructure of liver in the TEM. Binucleated hepatocyte with regularly distributed organelles (mitochondria, mi) and glycogen (gl) in 3-day Pb(NO₃)₂NP group. Enlarged binucleated hepatocyte with altered nuclei (nu), randomly distributed organelles, and several free erythrocytes (arrows) in cytoplasm in 11-week Pb(NO₃)₂NP group. Hepatocyte with physiological morphology of nucleus and regularly arranged organelles in Pb/cl; however, lipid droplets were present in increased number. Perisinusoidal fibroblast (fi) with dilated endoplasmic reticulum, and accumulated collagen fibrils (arrowhead) in space of Disse in Pb/cl group. **C)** The blood levels of albumin, total protein, cholesterol, and triacylglycerols after Pb(NO₃)₂NP inhalation. The graphs values denote average $\pm$ SD for 5 mice/group; * $p < 0.05$ compared with the corresponding control group (ctr), † $p < 0.05$ compared with the corresponding Pb(NO₃)₂ NP group by unpaired *t*-test. **D)** Sirius Red staining of liver samples to detect collagen fibrils. After 11-week Pb(NO₃)₂ NP inhalation and in Pb/cl group, the collagen fibrils were observed around small blood vessels and inside liver parenchyma (arrowheads). Similarly, inflammatory infiltrates contained some scattered collagen fibrils (arrowhead). Polynucleated hepatocytes were observed in Pb/cl group (arrows show 3, 3, 6 and 6 nuclei inside hepatocytes). Scale bar in panels **A**, and **D** = 100 μ m.

We observed megakaryoblasts and megakaryocytes in the liver parenchyma. There were large inflammatory infiltrates in the parenchyma and the portal areas. There were also more hypertrophic hepatocytes and binucleated and polynucleated hepatocytes (up to seven nuclei) after Pb(NO₃)₂ NP inhalation; some hepatocyte nuclei contained large vacuoles. These histopathological changes in liver were statistically significant after 6 ($p < 0.05$) and 11 weeks ($p < 0.05$) of inhalation. There were also pathological changes in liver parenchyma after a 5-week clearance period; their number and extent were significantly greater in Pb/cl animals

compared with the corresponding control group, and $\dagger\dagger\dagger p < 0.001$ compared with the corresponding $\text{Pb}(\text{NO}_3)_2$ NP group by unpaired *t*-test. LOD for Pb in the liver was 3 ng/g. **D)** Immunohistochemical detection of α -SMA signal was located in the walls of blood vessels in control (arrowhead), and additionally α -SMA-positive cells were observed in liver parenchyma after $\text{Pb}(\text{NO}_3)_2$ NP inhalation (arrowheads), megakaryocyte (*m*) and focal infiltrate (*in*). **E)** Statistical evaluation of the number of CD68-positive cells after $\text{Pb}(\text{NO}_3)_2$ NP inhalation. The graphs values denote average $\pm$ SD; $***p < 0.001$ compared with the corresponding controls, and $\dagger p < 0.05$ compared with the $\text{Pb}(\text{NO}_3)_2$ NP group by unpaired *t*-test. **F)** Detection of CD68-positive cells (marker of macrophages) in liver (arrowheads). Scale bar in panels D, and F = 100 μm .

Ultramicroscopic analyses uncovered the presence of numerous erythrocytes inside hepatocyte cytoplasm, alteration of hepatocyte nuclei to euchromatin rich, indicating increased transcriptional activity, and randomly dispersed cell organelles in the cytoplasm after 11 weeks $\text{Pb}(\text{NO}_3)_2$ NP inhalation (**Fig. 8B**). There was microvesicular steatosis in the $\text{Pb}(\text{NO}_3)_2$ NP-exposed and Pb/cl mice. Perisinusoidal fibroblasts exhibited high protein synthesis activity, and perisinusoidal space (of Disse) contained accumulated collagen fibrils (**Fig. 8B**). Sirius Red staining revealed the presence of scattered collagen fibres in liver parenchyma (**Fig. 8D**), predominantly after 6 and 11 weeks of $\text{Pb}(\text{NO}_3)_2$ NP exposure as well as in the clearance animals.

There were α -SMA-positive cells (**Fig. 9D**) in the walls of hepatic blood vessels in the controls. In $\text{Pb}(\text{NO}_3)_2$ NP-exposed and Pb/cl mice, α -SMA-positive cells were also scattered in parenchyma or they were associated with cellular infiltrates. The number of hepatic macrophages (detected as CD-68-positive cells) was significantly decreased ($p < 0.001$) compared with the corresponding control animals after 11 weeks of $\text{Pb}(\text{NO}_3)_2$ NP inhalation (**Fig. 9E,F; Table S4**). By contrast, there were more ($p < 0.05$) hepatic macrophages in Pb/cl animals compared with the corresponding $\text{Pb}(\text{NO}_3)_2$ NP animals.

Pb(NO₃)₂ NP inhalation also caused expressive changes in blood biochemical parameters that mainly manifested as altered hepatic function (**Fig. 8C**). The level of total protein was significantly decreased after 2 ($p < 0.05$) and 11 ($p < 0.05$) weeks of Pb(NO₃)₂ NP inhalation. The albumin blood level, which also reflects kidney function, was not significantly altered during the inhalation experiment (**Fig. 8C**). The association of the liver as the main organ involved in lipid metabolism was manifested in altered blood lipid levels. Total cholesterol (TCH) was significantly reduced ($p < 0.05$) in blood after 11 weeks of Pb(NO₃)₂ NP inhalation. Blood triacylglycerols (TAG) levels were increased after 6 and 11 weeks of Pb(NO₃)₂ NP inhalation compared with control animals. The TAG decrease was significant even after the clearance period ($p < 0.05$). Consistently, increased TAG levels after Pb exposure have been reported in rats that received Pb intragastrically [54] and in mice that received Pb orally [55]. However, not all reports have demonstrated the same blood TCH after Pb exposure [56]. One previous study described reduced TCH [54] similar to our findings, while other study described increased TCH levels [55]. Based on these findings, we conclude that sub-chronic exposure to a low level of Pb alters lipid homeostasis and thus affects blood lipid levels. This phenomenon, in turn, may disturb other tissues/organs in the organism.

The values of bilirubin and specific hepatic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) were in the physiological range after exposure to Pb (**Table 4**). Taken together, these data indicate that engineered highly soluble Pb(NO₃)₂ NPs used at a very low concentration cause persistent inflammatory changes in liver with impaired hepatic function.

Pb(NO₃)₂ NP inhalation caused calcium disbalance in the liver

The contents of Na and K in liver, as determined by AAS, were not altered at the analysed time points (**Fig. 9C**). Interestingly, the Ca level after 11 weeks of Pb(NO₃)₂ NP

inhalation was significantly increased ($p < 0.05$) compared with the corresponding control animals, while there was only small difference in the Pb/cl animals ($p < 0.05$). We previously found a similar increase in Ca after 11 weeks of PbO NP inhalation [18]. Elemental imaging of liver samples revealed elevated Ca levels in Pb(NO₃)₂ NP-exposed and Pb/cl animals correspondingly to the quantitative AAS data (**Fig. 9A,C**).

Comparison of effects of Pb(NO₃)₂ NP and PbO NP inhalation on liver parenchyma

The concentration of Pb in liver parenchyma continuously increased in parallel with lengthening of Pb(NO₃)₂ NP inhalation ($p < 0.001$, **Fig. 9, Table 2**) and then decreased practically to the LOD after a 5-week clearance period (3 ng/g). The distribution of Pb in the liver parenchyma was practically homogeneous (**Fig. 9**).

When we compared the effects of highly soluble Pb(NO₃)₂ NPs applied in this study with the analogous effects observed previously upon application of less soluble PbO NPs [18] we came to the following conclusions: 1) While the total Pb concentrations in analysed secondary organs in animals exposed to Pb(NO₃)₂ NPs were almost twofold lower compared with less soluble PbO NPs, the histopathological changes in liver were more serious than those caused by PbO NP inhalation. 2) A 5-week clearance period following Pb(NO₃)₂ NP exposure hardly promoted a positive effect on reparative processes in liver, although there was significant improvement and enhanced regeneration for a clearance period after PbO NP exposure. Obviously, higher solubility of Pb(NO₃)₂ NPs results in additional serious alteration of liver parenchyma compared to less soluble PbO NPs.

While the total Pb concentrations in lungs, kidney and spleen in animals exposed to Pb(NO₃)₂ NPs was nearly twofold lower (corresponding with the inhaled mass concentration of Pb NPs) compared with less soluble PbO NPs, the liver Pb concentration was nearly fivefold lower compared with less soluble PbO NPs. Thus, the lead from inhaled Pb(NO₃)₂ NPs

exhibited much lower propensity for liver accumulation than Pb contained in PbO NPs. This effect indeed requires further investigation since it will shed light on molecular processes involved in the initiation of reparative processes by more stable PbO NPs and/or their inhibition in the case of more soluble Pb particles.

Expression of factor- κ B (NF- κ B) gene changes in the lung and liver of animals exposed to Pb(NO₃)₂ NPs

NF- κ B is a family of proteins that act as transcription factors; they regulate genes involved in different processes of the immune responses [57]. NF- κ B belongs to the first responders to variable harmful stimuli, such as bacterial and viral antigens, oxidants, ionising radiation or cytokines such as tumour necrosis factor (TNF) or interleukin (1) IL-1 [58]. NF- κ B also regulates genes that control cell proliferation, cell survival and angiogenesis [57]. Exposure to heavy metal compounds can inhibit binding of NF- κ B to DNA and block this signalling pathway. The administration of gold (Au), Zn, Cu and/or mercury (Hg) compounds was shown to inhibit NF- κ B activity [58, 59]. Another study confirmed the inhibitory effects of Zn (in NP and aerosol forms) on NF- κ B activity in *in vitro* cultured cells: NF- κ B activity was upregulated after Cu administration (NP and aerosol forms) [60]. Thus, the effects of metal compounds on NF- κ B messenger RNA (mRNA) expression vary and can be influenced by the exposure time, dose and type of cell population analysed in *in vitro* experiments, among other factors.

Here, both 3-week and 11-week exposures to Pb(NO₃)₂ NP inhalation has led to significant downregulation of NF- κ B in the lungs (**Fig. 10**). The level of NF- κ B in kidneys remained unaffected even after 11-week exposure to Pb(NO₃)₂ NPs. In liver, downregulation NF- κ B was detectable already after 2 and 6 weeks (not at day3) and became statistically significant after 11-week exposure to Pb(NO₃)₂ NPs. In both lungs and liver, the clearance period enabled for return of NF- κ B expression close to its normal level (**Fig. 10**).

Figure 10: Gene expression of NFκB and cytokines at selected time points after Pb(NO₃)₂ NP inhalation in the lung, kidney and liver.

Expression of TNFα, IL-1 and IL-6 was altered in the lung and liver of animals exposed to Pb(NO₃)₂ NPs

Cytokines are small proteins or glycoproteins secreted by certain cells; they act as key modulators of the immune response to harmful stimuli such as microorganisms or irritants [61]. TNFα and IL-6 are major proinflammatory cytokines, while the IL-1 family includes 11

members expressed by numerous cell types, which comprises both proinflammatory and anti-inflammatory responses [61]. Previously, the exposure to NPs was found to deregulate many cytokines, including several IL family members or TNF α [62]. Interestingly, the same types of metal NPs can exhibit opposite effects on immune system [63].

First, we focused on TNF signalling, which was previously linked to NF- κ B activation [61]. TNF α is primarily secreted by activated macrophages and T cells, although other immune cells such as B cells, natural killer (NK) cells, monocytes, dendritic cells and neutrophils also produce this cytokine [64]. Moreover, non-immune cell types, such as endothelial cells, epithelial cells, muscle cells, fibroblasts and adipocytes, have been reported to produce TNF α , findings that document the pleiotropic role of this cytokine in cell-to-cell communication [65]. Here, we observed downregulation of TNF α in the lungs and liver after the shortest (3 day) and the longest (11 weeks) exposures (**Fig. 10**). Interestingly, in the liver, the level of TNF α further decreased after a 5-week clearance period below its level at 11-week Pb(NO₃)₂ NP exposure ($p < 0.01$).

IL-1 family members are expressed by numerous cell types, both immune and non-immune. Among the family members are IL-1 α and IL-1 β , both of which are potent acute-phase pro-inflammatory molecules [61]. IL-1 α and IL-1 β activate mitogen-activated protein kinase (MAPK), activator protein 1 (AP-1), bone morphogenetic protein (BMP/Smad) and NF- κ B signalling pathways [66, 67]. Here, we found that both IL-1 α and IL-1 β were downregulated in the lungs after the longest (11 weeks) exposure to Pb(NO₃)₂ NPs (**Fig. 10**), with both IL-1 mRNA levels becoming even lower after a 5-week clearance period (**Fig. 10**). In the liver, IL-1 α and IL-1 β mRNA expressions were significantly downregulated after 3-day ($p < 0.01$ for IL-1 β) and 11-week ($p < 0.05$ for both) exposures to Pb(NO₃)₂ NPs. In the liver, in contrast to the lungs, the levels of IL-1 α and IL-1 β increased upon the 5-week clearance.

IL-6 also evinces proinflammatory properties. It is produced by a wide range of cells, including macrophages, T cells, B cells, endothelial cells, fibroblasts, epithelial cells and hepatocytes in response to tissue injuries [68]. Signalling is achieved through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway or via the soluble IL-6 receptor (so-called trans-signalling pathway), not via NF- κ B activation [64]. While the IL-6 trans-signalling pathway is rather pro-inflammatory, the classic IL-6 signalling through the membrane-bound receptor is mainly involved in regenerative processes [69]. Here, IL-6 was downregulated in the lungs after the shortest (3 days) and the longest (11 weeks) ($p < 0.01$) exposures to Pb(NO₃)₂ NPs. A 5-week clearance then have led to upregulation of IL-6 mRNA in the lungs ($p < 0.05$ compared with the 11-week exposure). The changes in the level of IL-6 in the liver upon exposure to Pb(NO₃)₂ NPs were minimal and statistically insignificant.

Taken together, exposure of animals to Pb(NO₃)₂ NPs dramatically reduces expression of TNF α , IL-1 α , IL-1 β and IL-6 in the lungs and liver. Still, except for TNF α in the liver, all molecules return at least partially to their original levels upon the 5-week clearance period.

Transforming growth factor beta 1 (TGF β 1) gene expression changes in the lung and liver of animals exposed to Pb(NO₃)₂ NPs

TGF β 1 is an inflammatory cytokine secreted predominantly by leukocytes (main sources are T cells and B cells) but also by other cell types, including macrophages [61]. This protein regulates cell proliferation and differentiation of T cells and B cells and can play both anti- and pro-inflammatory roles in the immune system; however, immunosuppressive properties are considered predominant among the systemic effects [70, 71, 72]. TGF β 1 also stimulates the growth of cells of mesenchymal origin, including fibroblasts and smooth muscle cells, and enhances the production of extracellular matrix components [72]. In some conditions, these effects may increase the risk of pathologic fibrosis so that TGF β 1 is considered to be a

potent profibrotic mediator [27]. Activated macrophages are divided into two subpopulations: classical (M1, pro-inflammatory) that predominantly produce IL-1 β , IL-5 and TNF α ; and alternative (M2, anti-inflammatory) that produce TGF β 1, vascular endothelial growth factor (VEGF), IL-10 and IL-12 [63]. Thus, TNF α , and TGF β are cytokines produced by different population of macrophages with distinct final effects.

The shortest (3 days) and the longest (11 weeks) exposures to Pb(NO₃)₂ NPs were here associated with significant downregulation in TGF β 1 mRNA in the lungs and liver ($p < 0.05$) compared with the corresponding control animals (**Fig. 10**). The 5-week clearance allowed for some return of the TGF β 1 mRNA towards its original level in the lungs but not in the liver, when compared to the corresponding Pb(NO₃)₂ NP-exposed animals. It is of note that our findings on the effects of Pb(NO₃)₂ NPs on TNF α and TGF β mRNA levels are similar to those seen in animals exposed to metal NPs [73], which also revealed that M1 and M2 populations of macrophages remained unaffected.

CONCLUSIONS

Most importantly, by combining the current data with previously published findings, we demonstrate that form (solubility) of metal nanoparticles decides about the final effects on the individual organs. Furthermore, we also newly demonstrate that exposure to soluble Pb(NO₃)₂ NPs impairs functionality of immune system in both lungs and liver, and that many aspects of this loss prevent tissue recovery even after 5 weeks long clearance.

METHODS

Animals

Adult female mice (CD-1(ICR) BR strain) were obtained from the Animal Facility of the Masaryk University (Brno, Czech Republic) and were allowed to acclimatize to laboratory conditions for at least one week before the inhalation experiments. Commercial feeding and

drinking water were provided *ad libitum*. The experiment was approved by the Ethical Board of the Institute of Analytical Chemistry, v.v.i., Czech Academy of Sciences, Brno (Approval No. 64/2016).

Generation of Pb(NO₃)₂ nanoparticles

Lead(II) nitrate nanoparticles, (Pb(NO₃)₂ NPs), were generated continuously *in situ* by pneumatic atomization of lead(II) nitrate solution by high-velocity air stream (the air flow rate of 12 L/min) in a home-made generator [74]. Produced Pb(NO₃)₂ NPs were at the output of generator diluted with a stream of air (3 L/min). Finally, Pb(NO₃)₂ NPs were diluted in the second step by a stream of air (20 L/min) and used for whole-body inhalation experiments.

Exposure to Pb(NO₃)₂ nanoparticles

Mice treatment was performed in same inhalation chambers, which main characteristics have been described previously by us [75]. Adult female mice with an average weight of approximately 24 g at the beginning of the inhalation experiment were continuously exposed to Pb(NO₃)₂ nanoparticles for 11 weeks (24 h/day, 7 days/week). Control animals were exposed to the same air as treated animals without the addition of nanoparticles.

The samples from the controls and the Pb(NO₃)₂ NPs inhaled group of mice were collected at the designated time points of the inhalation period (three days, two weeks, six weeks). Ten biological replicates were harvested from the control (ctr/3d, ctr/2w, ctr/6w) and inhaled groups for each organ (Pb/3d, Pb/2w, Pb/6w). The lung, liver, kidney, spleen, femur and blood were collected and processed for chemical, biochemical, histopathological, ultramicroscopic, immunohistochemical, histochemical and immunofluorescent analyses, elemental imaging and to study the gene expression of selected markers.

Additionally, one group of mice inhaled air with $\text{Pb}(\text{NO}_3)_2$ NPs for six weeks (24 h/day, 7 days/week) and then inhaled clean air for the next five weeks (this group is annotated as the clearance group Pb/cl). At the end of the inhalation period (11 weeks), ten biological replicates were harvested from the control (ctr/11w), $\text{Pb}(\text{NO}_3)_2$ NPs exposed (Pb/11w) and the clearance groups (Pb/cl), and selected organs were examined in the same manner as in previous samples collected in shorter time points.

Characterization of generated $\text{Pb}(\text{NO}_3)_2$ nanoparticles

Generated $\text{Pb}(\text{NO}_3)_2$ NPs were first characterized to obtain information about total future mice exposure (**Table 1**).

The distribution of NP concerning particle number concentration was continuously measured directly inside the exposure cages using a Scanning Mobility Particle Sizer (SMPS; model 3936L72, TSI Inc., Shoreview, USA). The average mass concentration of $\text{Pb}(\text{NO}_3)_2$ NPs was $68.6 \mu\text{g Pb}(\text{NO}_3)_2/\text{m}^3$ (i.e., $42.9 \mu\text{g Pb}/\text{m}^3$) during the inhalation experiment. Mass concentration of generated $\text{Pb}(\text{NO}_3)_2$ NPs was calculated by dividing the mass of $\text{Pb}(\text{NO}_3)_2$ NPs collected on the filter by the volume of the air sample that passed through the filter. Generated $\text{Pb}(\text{NO}_3)_2$ NPs were sampled on nitrocellulose filters (pore size $0.45 \mu\text{m}$, diameter 25 mm, Millipore, Bedford, USA) for 24 hours, one filter per day. Filters were dissolved in HNO_3 using a UniClever microwave mineralizer (Plazmatronika, Wroclaw, Poland) and Pb content in the sample was determined using AAS (AAnalyst 600, PerkinElmer Inc., Shelton, CT, USA).

Size and shape of generated $\text{Pb}(\text{NO}_3)_2$ NPs were characterized by electron microscopy (EM). Immediately after generation at the generator output, $\text{Pb}(\text{NO}_3)_2$ NPs were collected by electrostatic precipitation using a Nanometer aerosol sampler (model 3089, TSI) on EM grids (copper S160-4, 3 mm in diameter, 400 mesh grids, Agar Scientific, Electron Technology, Stansted, Essex, UK). The samples were analyzed using a Magellan 400 L XHR microscope

(FEI Company, Hillsboro, OR, USA), operating in the scanning transmission electron microscope (STEM) mode. The STEM analyses revealed that the $\text{Pb}(\text{NO}_3)_2$ NPs consist of spherical and irregular shaped bean-like formations without clear internal structure whose size correlates with the size obtained by SMPS.

The estimated deposited dose corresponding to $\text{Pb}(\text{NO}_3)_2$ NPs over the 11 weeks inhalation period was 0.774 μg of $\text{Pb}(\text{NO}_3)_2$ NPs (or 0.484 μg Pb) per gram of mouse body weight, and 0.422 μg of $\text{Pb}(\text{NO}_3)_2$ NPs (or 0.264 μg Pb) per gram of mouse body weight for the clearance group.

Histological analysis

Samples of organs (lung, liver, kidney and spleen) designated for histological analyses were fixed overnight in 10% buffered neutral formaldehyde in a fridge and after that immersed in series of increasing concentrations of ethanol, xylene and paraffin wax. Serial histological sections of 5 μm thickness were prepared, and selected slices were stained by Hematoxylin–Eosin, and Sirius Red–Alcian Blue for analysis of collagen fibers. Toluidine Blue was used to detect mastocytes.

Selected samples of lung, liver, and kidney were also immersed in 10% sucrose in PBS in a fridge overnight. Next day, samples were embedded into O.C.T. Compound (Agar Scientific Gardena, CA, USA), and stored at $-25\text{ }^\circ\text{C}$ for subsequent analysis. Further, the cryosections of 20 μm thickness of lung, liver, and kidney samples were prepared, and used for LA-ICP-MS imaging.

Photos of the slices were taken by light microscope (Leica DM5000 B, Leica Microsystem GmbH, Vienna, Austria) and a digital color camera (Leica DFC480, Leica Microsystem GmbH, Vienna, Austria).

Immunohistochemistry

After deparaffinization and rehydration of the sections, citrate buffer (pH = 6) was used as a pre-treatment in a 97 °C water bath. For inhibition of non-specific secondary antibody binding, sections were incubated with a blocking serum (VECTASTAIN ABC Kit, Rabbit IgG, PK-4001, Vector Laboratories, USA; VECTASTAIN ABC Kit, Mouse IgG, PK-4002, Vector Laboratories, USA) for at least 20 min at room temperature (RT) and then, they were incubated with the primary antibody (α -SMA, CD68, MPO; detailed information **Table S5**).

After application of biotinylated secondary antibody (VECTASTAIN ABC Kit, Rabbit IgG, PK-4001, Vector Laboratories, USA; VECTASTAIN ABC Kit, Mouse IgG, PK-4002, Vector Laboratories, USA) for 40 min at RT, slices were incubated with the peroxidase-conjugated avidin-biotin complex (VECTASTAIN ABC Kit, Rabbit IgG, PK-4001, Vector Laboratories, USA; VECTASTAIN ABC Kit, Mouse IgG, PK-4002, Vector Laboratories, USA) for 30 min at RT. Chromogen substrate diaminobenzidine (Liquid DAB+ Substrate Chromogen System, K3468, DAKO, USA) was used for visualization of positive cells. Counterstaining of slices was performed by Hematoxylin. A negative control of the sample was obtained by omitting the primary antibody from the labeling protocol.

Photos were taken by light microscope (Leica DM5000 B, Leica Microsystem GmbH, Vienna, Austria) and a digital color camera (Leica DFC480, Leica Microsystem GmbH, Vienna, Austria).

Immunofluorescence

After deparaffinization and rehydration, samples were pre-treated in citrate buffer (pH = 6) in a 97 °C water bath. Blocking serum (VECTASTAIN ABC Kit, Rabbit IgG, PK-4001, Vector Laboratories, USA) was applied for at least 20 min at RT to inhibit non-specific secondary antibody bindings.

Then, samples were incubated with primary antibody for 1 hour at RT (α -SMA; detailed information **Table S5**). Secondary antibody (Goat anti-Rabbit IgG (H+L), Alexa Fluor 488, R37116, Invitrogen, USA) was applied for 40 min at RT. Sections were covered with a coverslip in reagent containing DAPI (Fluoroshield with DAPI, D9564, Sigma-Aldrich, USA). Photos were taken under a fluorescence microscope (Leica DM LB2, Leica Microsystems GmbH, Vienna, Austria) and a digital color camera (Leica DFC480, Leica Microsystems GmbH, Vienna, Austria).

Transmission electron microscopy (TEM)

Samples of lungs, livers and kidneys were fixed in 3% glutaraldehyde for 24 h, washed three times in 0.1 M cacodylate buffer and post-fixed in 1% OsO₄ solution for 1.5 h. After washing in cacodylate buffer, all samples were dehydrated in a series of increasing concentrations of ethanol, followed by acetone and embedded in the epoxy resin Durcupan ACM followed by 3 days polymerization. The ultrathin sections (about 60 nm thick) were prepared for TEM analysis. The sections were cut using an ultramicrotome Leica EM UC6 (Leica Microsystem GmbH, Vienna, Austria) and placed on formvar-coated nickel grids. Some sections were without further contrasting for analysis of nanoparticles in electron microscope, the others were contrasted with uranyl citrate and lead acetate. All sections were observed using Morgagni™ 268 TEM (FEI Company, Eindhoven, Netherlands) working at 80 kV, designated structures were measured using iTEM software. The photographs were taken using a *Veleta* CCD camera (Olympus, Münster, Germany).

Biochemical analysis of blood

Blood samples (5 animals for each group: the control group, the experimental group exposed to the Pb(NO₃)₂ NPs and the clearance group at each selected time points) were

collected by cardiac puncture into 1 ml lithium heparin tubes (TAPVAL, Dispolab, Czech Republic). The blood samples were centrifuged at 1000 x g for 15 min. Biochemical parameters were determined using a clinical chemistry analyzer, Abbott Architect c4000 (Abbott Laboratories, IL, USA), wet chemistry system.

Determination of lead in mouse blood and organs

Blood samples (5 animals from each analyzed group at each time point) were collected by cardiac puncture into 1 ml plastic Eppendorf tubes containing a small amount of heparin. Whole blood was divided into three fractions by centrifugation of blood cells, precipitation of proteins using methanol (300 µl) and separation of the remaining supernatant. Samples were stored at 5 °C for subsequent analysis.

The weights of individual organs were determined, and the values were recorded for later quantitative evaluation.

The individual organs and blood fractions were decomposed by microwave (MW) assisted digestion in concentrated subboil grade (quartz distillation system model MSBQ 2, Maasen, Eningen, Germany) nitric acid, *i.e.* liver in 5 mL, lung, spleen and kidney in 3 mL, blood cells in 2 mL, femur and other blood fractions in 1 mL of acid. The samples were treated in pre-cleaned quartz tubes of a closed pressurized autoclave system (UltraWave, Milestone s.r.l., Italy). The decomposition program consisted of four steps: 1st step – 10 min with temperature ramp between 100 and 120 °C; 2nd step – 5 min with temperature ramp between 120 and 200 °C; 3rd step – 3 min with temperature ramp between 200 and 250 °C; 4th step – 5 min at 250 °C. After cooling down (a duration of approx. 10 min), digests were quantitatively transferred to high-density polyethylene vials, diluted and adjusted with ultrapure water (Ultra Clear system, SB Barsbüttel, Germany) to the final mass of 10 g for organs, 4 g for blood cells

and 3 g for femur and other blood components, respectively. Simultaneously, blank samples (typically n = 30 per sampling series) were processed analogously.

The content of lead in the digests was determined by electrothermal atomic absorption spectrometry (ET AAS) employing AAnalyst 600 PerkinElmer (USA) instrumentation under recommended conditions. A mixture of ammonium phosphate and magnesium nitrate was used as a combined chemical modifier. The method of standard addition calibration was applied for quantitation.

Determination of element content (Na, K, Mg, Ca) in mouse organs

Content of basic element components (Na, K, and Ca) in mouse organs was determined in the same sample solution prepared for determination of lead concentration. After dilution of samples, the concentrations of Na, K, Mg and Ca were determined by flame atomic absorption spectrometry employing ContrAA 300 Analytik Jena (Germany) High Resolution Continuum Source AAS instrumentation under recommended conditions, using acetylene-air flame (Na, K, Mg) and acetylene-nitrous oxide flame (Ca), respectively. Measurement was performed at prominent analytical lines (Na 589.0 nm, K 766.5 nm, Mg 285.2 nm and Ca 422.7 nm). Calibration was based on certified analyte standard solutions Astasol® (1 ± 0.002 g/L) (Analytika Ltd. Prague, Czech Republic).

LA-ICP-MS analysis

The cryosections of lungs, kidneys and livers were analyzed by Laser ablation with Mass spectrometry of inductively coupled plasma (LA-ICP-MS) to obtain elemental distribution in these sections. The LA-ICP-MS setup consists of a laser ablation system UP213 (NewWave Research, USA), and ICP-MS Agilent 7500ce (Agilent Technologies, Japan). The laser ablation of the samples was done with a laser spot diameter of 100 μ m, scan speed of 200

µm/s, repetition rate of 10 Hz and a laser beam fluence of 3 J/cm². The content of lead (Pb), sodium (Na), potassium (K), calcium (Ca), iron (Fe), and zinc (Zn) was quantified using a series of agarose gels doped with a known amount of Pb, Na, K, Ca, Fe, and Zn.

Imaging of Pb in kidney tissue sections was performed under different LA-ICP-MS parameters than elemental imaging of tissues. For this purpose, the laser beam diameter was diminished to 20 µm and the scan speed was 200 µm/s. During this imaging, isotope ²⁰⁸Pb with the integration time of 1 ms was measured. The size of exposed kidney tissue samples was 1.0 x 1.4 mm.

qRT-PCR analysis

Total RNA was extracted using the RNeasy Plus Mini Kit (Ca. No. 74136, Qiagen, USA). Complementary DNA was synthesized according to the manufacturer's instructions using a gb Elite Reverse Transcription Kit (cat. No. 3012, Genex Biotech, CR). qRT-PCR was analyzed with a LightCycler® 480 (Roche). The number of analyzed cDNA samples was n = 3–5 for each group.

TaqMan Gene Expression Assay (cat. No. 4351372, Applied Biosystems, USA) for *Nfkb1* (ID: Mm00476361_m1), *Tgfb1* (ID: Mm03024053_m1), *Il-6* (ID: Mm99999064_m1), *Il1α* (ID: Mm00439620_m1), *Il1β* (ID: Mm01336189_m1), *Tnf α* (Mm_00443258_m1) were used and gene expression was analyzed with the following program: initial activation step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, annealing temperature at 62 °C for 60 s. Gene expression values for each sample were expressed in terms of the threshold cycle normalized to beta-actin (*Actb*; ID: Mm00607939_s1) expression.

DECLARATIONS:

Ethics approval and consent to participate

1012 The experiments were performed in accordance with the ethics policy of the Institute of Animal
1013 Physiology and Genetics (no. 081/2010).

1014

1015 **Consent for publication**

1016 The manuscript as submitted has been read and approved by all authors.

1017

1018 **Availability of data and Material**

1019 This manuscript has not been submitted simultaneously for publication in any other journal, nor
1020 have the findings been partially disclosed in any other publication.

1021

1022 **Competing interests**

1023 The authors declared that there is no conflict of interest.

1024

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1027

1028 **Author's contributions**

1029 M.B., and P.M. conceived and designed the experiments. T.S., L.V., H.K., V.P., V.J., B.D.,
1030 P.M., K.K., L.C., A.J., Z.H., D.K., M.T., and P.C. performed experiments. J.D., T.S., M.T.,
1031 T.V., V.K., and M.B. analyzed the data. M.B., V.K., and A.H. contributed reagents and
1032 materials. J.D., T.S., M.B., P.M. and A.H. wrote the paper. All authors read and approved the
1033 final manuscript.

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