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EVALUACIÓN DE LA PRODUCCIÓN DE TRAMPAS EXTRACELULARES DE NEUTRÓFILOS (NETs) EN POBLACIÓN ADULTA EXPUESTA A PLOMO

TESIS QUE PRESENTA

L.C.A.S. LUIS ANTONIO RICO GUERRERO

PARA OBTENER EL GRADO DE MAESTRO EN CIENCIAS BIOMÉDICAS BÁSICAS

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Assessment of neutrophil extracellular trap (NETs) production in a lead-exposed adult population

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Abstract

Lead is a heavy metal that has been widely used due to its physical and chemical properties. Although its use has been restricted, sources of exposure have been identified to date in Mexico, with lead-glazed earthenware being a common source. One of the body systems primarily affected is the immune system, and the main mechanism involved is the production of oxidative stress. Currently there are few reports regarding the effects of lead on neutrophils, especially on NETs, which are web-shaped structures composed of DNA and proteins. It is suggested that lead can favour the production of NETs by the generation of reactive oxygen species (ROS), which are necessary for its formation. Therefore, the aim of this study was to evaluate the plasma levels of NETs markers in a population occupationally exposed to lead. To achieve this, 50 potters from the community of San Pablo del Monte in the State of Tlaxcala, Mexico, were studied. A sample of 10.0 mL of whole blood was collected and plasma was isolated. The blood lead levels (BLL) were measured by graphite furnace atomic absorption spectrophotometry (GFAAS) and plasma was used to evaluate malondialdehyde (MDA) levels by the 1M2F assay, myeloperoxidase (MPO) and histone H3 citrullinated (CitH3) levels by ELISA and cell-free DNA (cfDNA) levels by a fluorescence assay. In addition, plasma inflammatory cytokines levels were quantified using a CBAs kit via flow cytometry. As results, we found a mean BLL of 12.04 ± 6.11 μg/dL. Potter population was divided into two groups: a high-exposure group (BLL ≥ 10 μg/dL, n=30) and a low-exposure group (BLL <10 μg/dL, n=20). Higher plasma levels of cfDNA were found in the high-exposure group compared to the low-exposure group (408.9 ± 90.99 vs 345.8 ± 89.46 ng/mL, $p<0.001$). Notably, a significant positive correlation between BLL and cfDNA ($r = 0.31$, $p=0.04$) was found in the potter's population. Regarding NET markers, a positive correlation was found between cfDNA/citH3 ($r =0.38$, $p=0.037$), and cfDNA/MPO ($r =0.37$, $p=0.04$) in the high-exposure group. Noteworthy, IL-8 was detected in seven potters from the high-exposure group (25.91 ± 14.08 pg/mL). In conclusion, potters with blood lead levels ≥10 μg/dL produce and release NETs into the bloodstream. The potential clinical implications of these findings are an issue that deserves further investigation.

Keywords: blood lead levels; occupational toxicology; neutrophil extracellular traps; pottery; innate immune system

1. Introduction

Lead is a heavy metal that has been widely used in various domestic and industrial activities due to its physical and chemical properties. Although its use has been restricted since it is highly toxic to human health, lead and its compounds are still used in many activities such as the production of paints, the manufacture of motor and electric vehicles, ceramics, among others (1). In Mexico, sources of exposure have been identified as industrial areas, mining areas, battery and electronic waste recycling areas (2). Another well-identified source in Mexican population is the consumption of food in lead-glazed earthenware (3). Despite recommendations to decrease the use of lead in pottery due to its toxicity, some lead compounds such as lead oxide (PbO2) or "greta" continue to be used to produce pieces that will later be used to prepare, serve or store food. Lead is used in the form of sand dissolved in enamels and paints that are used to decorate the pieces of clay and give them a glazed tone after two firings in artisanal kilns to provide with resistance to the pieces. Because the production of these pieces is carried out in low temperature furnaces (850 – 1,000°C) lead cannot be fixed well and becomes bioavailable on contact with acidic substances such as coffee, chili, lemon, etc. (4). In a recent study that measured blood lead concentrations in individuals from urban and rural locations, the major sources of exposure that were identified were mining, smelting and, to a greater extent, pottery. (5). Accordingly, several studies have been conducted to evaluate the levels of this metal in pottery communities. For instance, in workers belonging to the pottery community of San Pablo del Monte in Tlaxcala, a median blood lead of 32 µg/dL was found, as well as a significant correlation with lead levels in contaminated food. (6). In another pottery community located in Oaxaca, the average blood lead level was 43.5 µg/dL and potters were found to have a higher risk of high blood lead levels compared to non-pottery workers (7). In another more recent study in which 46 potters were evaluated, an average blood lead of 13.6 µg/dL was found (5). The reference value in Mexico for whole blood lead levels in an occupationally exposed population (including potters) is 10 µg/dL for women and 30 µg/dL for men (NOM-047-SSA1, 2011) (8). Potters tend to have a higher risk of exposure because, in addition to the possible consumption of food cooked in glazed ceramics, they have an occupational exposure to lead dusts.

Lead can induce various biological effects in humans, depending on the level and duration of exposure. The systems primarily affected by chronic exposure to lead are hematologic, cardiovascular, renal, reproductive, nervous, endocrine, and immune systems (9-11). One of the

main mechanisms of toxicity described for lead is oxidative stress (1). The mechanisms by which lead can induce oxidative stress is through inhibition of the enzyme delta-aminolevulinic acid dehydratase (δ-ALAD) and through the direct effect of lead on the lipid composition of the cell membrane. In addition, lead is classified as a possible carcinogen (2B) by the International Agency for Research on Cancer (IARC) (12). According to the WHO, in adult populations with blood lead levels below 10 μg/dL, clinical and subclinical effects have been reported, such as reduced synthesis of δ-ALAD, decreased cognitive function, and hypertension (13).

Several studies have described that exposure to Pb can exert various effects which can alter the number and/or function of immune system cells. Lower levels of $CD4^+$ T cells have been observed in preschool children exposed to lead, as well as higher levels of $CD8^+$ T cells compared to the control group (14). Another study that analysed the effect of lead on lymphocytes from occupationally exposed workers to this metal showed a decrease in the proliferation capacity of lymphocytes, as well as an increase in the production of IFN-γ in response to stimulation with mitogens (15). In contrast, a study that included shipyard workers exposed to high levels of lead (37.1 μg/dL) showed decreased phagocytic activity, an altered profile in cytokine production induced by mitogens (increase in IL-4 and decrease in IFN-γ), as well as an increase in the number of regulatory T cells, which may favour a suppression of the cellular immune response (16). On the other hand, in workers in a lead stearate manufacturing industry, a decrease in the number and percentage of NK cells was reported in the group with high blood lead levels (≥20 μg/dL) compared to the group with low lead levels (<20 μg/dL) and the control group (17).

Currently there are few reports regarding the effects of lead on neutrophils. Neutrophils are part of the effector cells of innate immunity, which represents the immune system's first line of defence against foreign agents (18). Occupational lead exposure has been associated with decreased chemotaxis and random neutrophil migration in workers with blood lead concentrations of 74.8±17.8 μg/dL (19). In addition, a dose-dependent effect has been observed between lead and increased absolute neutrophil count (20), as well as an increase in the neutrophil-lymphocyte ratio (21). Neutrophils can carry out three main defence mechanisms: phagocytosis, degranulation, and the production and release of extracellular traps (NETs) (22).

NETs are web-shaped structures composed mainly of DNA, citrullinated histones (such as histone H3), as well as components of the cytoplasm and granules, including peptides with antimicrobial properties and enzymes such as elastase (NE) and myeloperoxidase (MPO) (23,

24). These traps act as physical and chemical barriers that help prevent the spread of pathogens and increase the action of antimicrobial molecules locally. Several inducers of NETs, such as PMA, stimulate the production of reactive oxygen species (ROS) through the activation of NADPH oxidase (24, 25). Studies have shown that ROS promote the release of MPO and NE from azurophilic granules and their translocation to the nucleus (26).

NETs are an important defence mechanism of innate immunity; however, excessive release of these webs can contribute to a chronic inflammatory process (24). It has been described that the exposure of epithelial cells to NETs leads to the release of IL-8, a key chemokine in the recruitment of neutrophils (27). Other studies have reported that NETs promote an inflammatory state through their interaction with other cells of the immune system, such as plasmacytoid dendritic cells, in which they induce the production of type I IFNs (28); while in macrophages, NETs facilitate the secretion of IL-1β and IL-18 in a process that involves the activation of the inflammasome and induce the secretion of other proinflammatory cytokines such as IL-8, IL-6 and TNF-α (29, 30). NETs can also induce the formation of more NETs, it has been observed that proinflammatory cytokines released by neutrophils such as IL-8, TNF-α and IL-1β can induce the formation of NETs and free radicals, amplifying the inflammatory process. (31, 32). While NETs play an important role in the mechanisms of innate immunity, excessive production or poor elimination constitutes a risk factor for the development of several diseases. Studies in animal models have provided evidence of the potential role of NETs in the development of a spectrum of pathologies including cardiovascular, inflammatory, autoimmune, metabolic, infectious and cancer diseases (33).

It has been suggested that lead may promote the formation of NETs through the production of ROS, which are necessary for the formation of NETs, and that chronic exposure to lead may induce a pro-inflammatory state due to excessive production of NETs, which may ultimately promote the appearance of some diseases. Although several effects of lead on the adaptive branch of the immune system have been described, the reports about the possible effects of this toxic on the function of cells of innate immunity are scarce. Furthermore, to date, no studies have evaluated the effect of lead on NET production. Therefore, the aim of this study was to evaluate the plasma levels of NET markers in an adult population occupationally exposed to lead.

2. Materials and methods

2.1 Study population

Fifty potters from the community of San Pablo del Monte in the State of Tlaxcala, Mexico, were eligible for the study. All of them met the inclusion criteria: age of eighteen years or older, both sexes, a minimum time of residence of two years in the community and having worked pottery continuously at least 2 years. The exclusion criteria were previous diagnosis of cardiovascular, autoimmune, or infectious diseases. All volunteers completed a questionnaire to known about their lead exposure history and signed an informed consent. The study was approved by the Ethics Committee (CEI.FM-UASLP/02-2023) of the School of Medicine from the Autonomous University of San Luis Potosi, Mexico. Based on blood lead levels and according to NOM-047-SSA1,2011 individuals were divided into two groups: low-exposure group (<10 µg/dL) and high-exposure group (≥10 µg/dL).

In addition, eleven students from the Red Cross School of Paramedics, San Luis Potosi, Mexico, with no history of lead exposure, were studied as a control group. They were all above eighteen years old (mean age 24.4 years) and had no previous diagnosis of cardiovascular, autoimmune, or infectious diseases.

2.2 Sampling and plasma isolation

A 10 mL blood sample was drawn from each volunteer and collected in BD Vacutainer™ K2 EDTA tubes. A volume of 1 mL of each blood sample was stored at 4°C for determination of lead levels. The rest of the sample was centrifuged at 1000 x g for 15 minutes to obtain the plasma, which were separated in several aliquots and stored at -80°C for further analysis.

2.3 Determination of blood lead levels (BLL)

Blood lead levels were measured according to Subramanian's method (34) by graphite furnace atomic absorption spectrophotometry (GFAAS), with a Pb hollow cathode lamp (Lumina™) using PinAAcle 900T and AS900 autosampler, both from PerkinElmer™. As quality standard Whole Blood Control Level III by ClinChek™ (Ref. 8842, Lot. 445; Munich, Germany) was used, obtaining a recovery rate of 90%. A calibration curve was performed with the following concentrations 0, 1, 5, 10, 20, 40 and 80 µg/L. In brief, in an autosampler cup of 1.2 mL (Part No: B 0510397, PerkinElmer™) 100 µL of sample were added with 400 µl of a Triton-modifier

solution ((NH4)2HPO4 0.5% (Merck™; Art. 201207), Triton-X 100 0.5% (Sigma™; Lot. 67H2502) and HNO3 0.2% (Analytyka™ Lot. C29ACF2201) for a 1:5 dilution. The autosampler cup was then placed in the autosampler for analysis. Concentrations are expressed in microgram/dL ± standard deviation of the mean. All samples were analysed in duplicate.

2.4 Lipid peroxidation colorimetric assay

Malondialdehyde (MDA) levels were determined as a marker of oxidative stress by the 1-Methyl-2-Phenylindole (1M2F; Sigma-Aldrich™) assay (35). In brief, a solution of 1M2F 15.4 mM in acetonitrile (TEDIA™ Catalog No. UN1648) and methanol (TEDIA™ Catalog No. UN1230) was prepared in a 3:1 ratio. From this solution 325 µL were added to 100 µL of sample; subsequently, 75 µL of concentrated hydrochloric acid (HCl) was added for a final volume of 500 µL. The samples were then incubated at 45°C for 60 minutes, and then centrifuged at 10000 x g for 8 minutes. Finally, the Optical Density was measured at 586 nm on a microplate reader (Synergy™ H1 BioTek). A standard curve with 1,1,3,3-tetramethoxypropane (TMPO; Sigma-Aldrich™) 73.86 µM was used to quantify MDA concentration (ranging from 0 µM to 23 µM MDA). Concentrations are expressed in nanomol/mL ± standard deviation of the mean. All samples were analysed in duplicate.

2.5 Myeloperoxidase and citrullinated histone H3 ELISA assays

Commercial ELISA kits (R&D Systems™ Quantikine ELISA Human Myeloperoxidase Immunoassay Catalog No. DMYE00B and MyBiosource Human Citrullinated Histone H3 ELISA kit Catalog No. MBS7254090) were used to analyse the concentrations of myeloperoxidase and histone H3 citrullinated, respectively, according to manufacturer's instructions. Optical Density was determined using a microplate reader (Thermo Scientific™ Multiskan FC) and concentrations were extrapolated from the standard curves values and expressed in nanogram/mL ± standard deviation of the mean. Samples in both assays were run in duplicate.

2.6 Cell-free DNA fluorescent assay

The Quant-iT™ PicoGreen™ dsDNA Catalog No. P11496 kit was used to evaluate cell-free DNA concentrations according to the manufacturer's instructions. Each sample was diluted ten-fold in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH =7.5) with DNase-free buffer, and loaded into a 96 well plate. Fluorescent signal of the plasma and the DNA standard samples were

acquired at excitation of 485 nm and emission of 538 nm using fluorescent microplate reader (Synergy™ H1 BioTek). The plasma cfDNA concentration was extrapolated from the standard DNA values and expressed in nanogram/mL ± standard deviation of the mean. Samples were analysed in duplicate.

2.7 CBA Inflammatory Cytokines assay

Plasma concentrations of IL-1β, IL-6, IL-8, IL10, IL-12p70 and TNF-α were measured using the Human Inflammatory Cytokine Cytometric Bead Array Kit from Becton Dickinson (BDTM Catalog No. 551811), according to the manufacturer's instructions. Samples were acquired on a BD FACS Canto™ II Flow Cytometer and data analysis was performed using FCAP Array software. Cytokines concentration was expressed in picogram/mL ± standard deviation of the mean.

2.8 Statistical analysis

All data sets were tested for normal Gaussian distribution using the Kolmogorov–Smirnov test. Comparisons between two groups were analysed with two-tailed T-test, whilst comparisons among three groups were done with one-way ANOVA and Tukey's post-hoc test. Pearson correlation test was used to analyse association between variables. Qualitative variables were compared using Pearson Chi-square for categorical variables. Statistical analysis was performed using GraphPad Prism™ 8 (San Francisco, CA) software. Results are expressed as the mean ± standard deviation. A p value < 0.05 was considered statistically significant.

3. Results

3.1 Blood lead levels in Tlaxcala potters

A mean blood lead level of 12.04 ± 6.11 μg/dL (mean ± SD) was found in 50 potters from Tlaxcala included in this study (**Table 1**). Potter population was divided into two groups based on the criterion value of 10 μg/dL reported by NOM-047-SSA1: a high-exposure group (blood lead levels ≥ 10 μg/dL) and a low-exposure group (blood lead level <10 μg/dL). Thirty potters with blood lead levels of 15.17 ± 5.97 μg/dL (mean ± SD) were assigned to the high-exposure group, whereas twenty potter with blood lead levels of 7.36 ± 1.87 μg/dL (mean ± SD) were assigned to the low-exposure group (**Figure 1**). In contrast, blood lead levels of the control group were 1.74 ± 0.99 μg/dL (mean ± SD). The difference of blood lead levels between both exposed groups was statistically significant ($p<0.0001$), as well as the difference of both groups, high-exposure ($p<0.0001$) and low-exposure ($p<0.005$), compared to the control group.

Furthermore, when compared the entire population of Tlaxcala potters, a statistically significant positive correlation was found between blood lead levels and the number of working years in pottery ($r = 0.29$, $p<0.05$) (**Figure 2A**), as well as between blood lead levels and the number of hours worked per day by potters ($r = 0.33$, $p<0.05$) (**Figure 2B**).

In addition, based on the information obtained from the questionnaires, data from 50 potters divided into high- and low-exposure groups, are summarised in **Table 2**. The quantitative variables were analysed using the independent *t*-test and the qualitative variables were analysed using the Chi-square test. Not statistically significant differences were found between the groups.

3.2 Malondialdehyde levels in Tlaxcala potters

Regarding plasma MDA levels, a higher concentration was found in the high-exposure group (5.00 ± 1.64 nmol/mL, mean ± SD) compared to the low-exposure (4.20 ± 1.07 nm/mL, mean ± SD) and the control groups (3.69 ± 0.82 nmol/mL, mean ± SD); however, only a statistically significant difference was observed when compared to the control group ($p<0.05$) (**Figure 3**). This would suggest a higher oxidative damage in the high lead exposure group compared to the control group.

3.3 Levels of NETs markers in Tlaxcala potters

Plasma levels of MPO tended to be higher in the high-exposure group (90.15 ± 52.60 ng/mL, mean ± SD) compared to the low-exposure (74.91 ± 33.68 ng/mL) and the control group (63.91 ± 16.69 ng/mL); however, no statistically significant differences were found (**Figure 4A**). Regarding the citrullinated histone H3, the control group showed the highest plasma levels (31.06 ± 16.75 ng/mL, mean ± SD) compared to high- and low-exposure groups (24.11 ± 7.79; 27.00 ± 9.93 ng/mL, respectively) (**Figure 4B**). However, no statistically significant differences were found. On the other hand, the entire population of Tlaxcala potters showed higher levels of circulating free DNA compared to the control group. Regarding potters in the high-exposure group, plasma concentration of this NET marker (408.9 ± 90.99 ng/mL, mean ± SD) was significantly higher ($p<0.001$) compared to the levels found in the low-exposed group of potters (345.8 ± 89.46 ng/mL). In addition, high- and low-exposure groups presented a statistically significant difference ($p<0.0001$ and $p<0.05$, respectively) when comparing to the control group (226.2 ± 42.53 ng/mL) (**Figure 4C**).

Although not significant differences were found when compared MPO levels among groups (**Figure 4A**), the higher concentration of this marker in potters' plasma with blood lead levels above 10 μg/dL would suggest that they are in a higher oxidative stress state due to the role of MPO in the production of ROS. In this regard, a significant positive association was found between the levels of myeloperoxidase and malondialdehyde in the high-exposure group, whilst this association was not significant in the low-exposure group (**Figure 5A, 5B**). In addition, a significant positive association was found between the proteinic markers (MPO and CitH3) in the high-exposure group, whereas in the low-exposure group the association was not significant (**Figures 5C, 5D**). These results suggest that the production of NETs in potters occurs mainly in a CitH3-independent mechanism, except in the high-exposure group where it seems that the mechanism by which NETs are being produced is dependent on the citrullination of histone H3 (**Figure 5C**), through the possible activation of PAD4 by reactive oxygen species.

Furthermore, a significant positive correlation was found between blood lead levels and circulating free DNA concentration ($r = 0.31$, $p=0.04$) in potter population (**Figure 6**); however, the significance was not maintained when correlating blood lead levels and proteinic NET markers. These results would indicate that lead exposure is triggering some cellular process by which potters' cells are releasing high concentrations of DNA into circulation.

In order to validate the presence of cfDNA as a NETosis surrogate, the correlation between cfDNA levels and protein markers involved in the mechanism of NETs such as MPO or citrullinated histone H3 levels was sought, as has been previously done in other studies (36, 37). Interestingly, in the high-exposure group, a positive correlation was found between cfDNA and MPO ($r = 0.37$, $p=0.041$), as well as between cfDNA and citrullinated histone H3 ($r = 0.38$, $p=0.037$) (**Figures 7A, 7B**).

3.4 Inflammatory state in Tlaxcala potters

Cytokine levels above the detection limit were found in only 8 of the 50 potters studied (16%), and it is worth mentioning that these eight potters belong to the high lead-exposure group. Seven of them presented detectable levels of IL-8 (mean 25.91 ± 14.08 pg/mL), one of them presented detectable levels of IL-8 and IL-12p70 (5.66 pg/mL), and only one presented detectable levels of IL-6 (93.46 pg/mL) (**Figure 8**). The rest of the cytokines evaluated (IL-1β, IL-10, IL-12p70 and TNF-α) were not detected in any of the potters.

4. Discussion

WHO considers lead one of the chemicals of greatest public health concern (13). Although in Mexico its use has been decreasing, it can still be found in the manufacture of pottery pieces. This is evidenced by studies that have reported high blood lead levels in potters, whose exposure is exacerbated when both occupational and environmental exposures are considered. These levels range from 13 - 43 μg/dL on average (5 – 7). In our study, mean lead levels were 12.04 μg/dL with a peak level of 30.22 μg/dL. In our study, 96% of the potter population had levels above 5 μg/dL, and 60% levels above 10 μg/dL. The American College of Occupational and Environmental Medicine (ACOEM) recommends that lead-exposed workers not return to work until their blood lead levels are < 15 μg/dL and states that ideally, workers should maintain levels < 10 μg/dL or < 3.5 μg/dL for pregnant women (38). Interestingly, no statistically significant differences were found between quantitative and qualitative variables when the population was divided into high-exposure and low-exposure groups, suggesting that the population was homogeneous and the difference between blood lead levels is due to other factors not addressed in this study; for instance, consumption of food rich in iron, calcium and vitamin C, hygiene and decontamination procedures in workplaces and personal hygiene habits (39 – 41).

Owing to the fact that the main mechanism of lead toxicity is through the generation of oxidative stress, being lipid peroxidation a mechanism already studied for lead, we decided to evaluate the plasma concentrations of MDA in both groups of potters. In this regard, a study of 40 lead-exposed workers with a BLL mean of 33.65 μg/dL and MDA concentration of 11.82 nmol/mL were found (42). In another study, lead-exposed workers were found to have a mean BLL of 14.5 μg/dL and MDA of 4.4 nmol/mL (43). Accordingly, our study found a mean BLL in the high-exposure group of 15.17 μg/dL and a plasma MDA concentration of 5.00 nmol/mL, the latter showed a statistically significant difference compared to the control group (3.69 nmol/mL). Although no correlation was found between blood lead levels and MDA levels in either potter group, there is evidence that the high-exposure group showed a higher oxidative stress. Additional studies will be needed to evaluate the contribution of lead exposure to increased oxidative damage.

Regarding the protein markers of NETs selected in this study, to date their concentrations have not been evaluated in potters or people occupationally exposed to lead; however, there are a few studies where they have been evaluated in *in vitro* or *in vivo* exposure to lead. In rats exposed to

300 ppm of lead acetate in drinking water, astrocytes were isolated and a significant increase in MPO and MDA levels, as well as in the release of TNF-α and IL-1β was observed, suggesting that lead exposure boosted an inflammatory state and oxidative stress (44). Regarding the effect of lead on histone citrullination, there is no information yet available. In our study, higher levels of MPO were observed in the high exposure group (90.15 ng/mL) compared to the other groups; however, the difference was not statistically significant. MPO is a heme peroxidase found predominantly in neutrophils and monocytes, and whose catalytic cycle requires the presence of H_2O_2. The end-product of MPO activity is hypochlorous acid (HOCl) which acts on microbicidal mechanisms. It is well known that MPO plays a crucial role in the defence mechanisms of neutrophils (NETs, microbial digestion, etc.), however, several studies have found an association between MPO levels and the development of diseases, especially cardiovascular diseases (CVD) (45). It is worth mentioning that the angiogenic response induced by NETs is highly dependent on H_2O_2 and MPO activity. One of the mechanisms attributed to MPO in the development of CVD is the ability of its reactive species to oxidize lipoproteins such as LDL and HDL (46, 47). On this regard, there is strong evidence that support the association between lead exposure and cardiovascular diseases like hypertension, even at concentrations below 5 μg/dL (48). It is interesting to note that in our study a significant association was found between the levels of MPO and MDA in the high-exposure group, therefore, it is evident the presence of oxidative stress in these potters in which it would be of interest to analyse the levels of oxidized lipoproteins.

During the release of NETs, one of the major structural components is DNA, which is released by neutrophils along with a set of various antimicrobial proteins and lipids. To date, there are no studies that have evaluated cfDNA levels in people exposed to lead. Interestingly, the highest levels of cfDNA were found in potters belonging to the high-exposure group which were significantly different from the levels found in the control group. A significant difference was also found between the cfDNA levels of the low-exposure group with respect to the control group. The presence of cfDNA in plasma has been seen to be a pathological sign. For instance, a study found higher levels of cfDNA in COVID-19 patients who developed acute kidney injury (AKI) compared to patients who did not develop AKI (164.8 vs. 123.0 ng/mL) (36). Authors of this study suggest that NETs could be an important contributing factor in the development of AKI associated with COVID-19. On this regard, is noteworthy to mention that lead exposure has also been associated with decreased kidney function (49). Interestingly, in our study we

observed a positive correlation between blood lead levels (BLL) in pottery population and plasma cfDNA levels, suggesting the likely involvement of lead in the release of genetic material into circulation.

In this study, our main objective was to evaluate the levels of NET markers in potters occupationally exposed to lead. To accomplish this, we looked for any correlation between cfDNA levels and MPO or CitH3 levels, according to previous studies (36, 37). We found that cfDNA levels were positively correlated with MPO and CitH3 levels in the high-exposure group. These results showed that potters with BLL above or equal to 10 μg/dL are probably producing NADPH oxidase (NOX)-independent NETs since a correlation between cfDNA and CitH3 was found. Originally, two mechanisms for NETs production have been proposed, the NOX-dependent NET formation where the assembly of the NADPH oxidase is necessary and ROS are key players during the first steps of NETs production, and the NOX-independent pathway where there is an increased influx of Ca^{2+} and the enzyme PAD4, which citrullinates histones for DNA decondensation, is activated by ROS derived from the mitochondria (mROS). Then, it seems that ROS play a crucial role in the production of NETs and a pathway ROS-independent remains unknown. Moreover, the role of mROS in the activation of NOX complex was already demonstrated (50). Nevertheless, it has been shown that specific stimuli can induce varying proportions of both citrullinated and uncitrullinated NETs with different requirements for PAD4, highlighting the complexity of NET formation (51). Additionally, it has been demonstrated recently that the suppression of ROS attenuates the citrullination of H3 in neutrophils, confirming the role of ROS in the production of CitH3 NETs independently of the stimuli (52).

In addition, it is interesting to note that only in the high exposure group detectable levels of proinflammatory cytokines were found, with IL-8 being the most prevalent. IL-8, also known as CXCL8, is a chemokine that acts on CXCR1 and CXCR2 receptors to promote the migration of neutrophils and other leukocytes to the inflammatory focus (53). There is evidence that support the involvement of IL-8 and its receptors in the induction of NETs (54). For instance, in an animal model, it was found that IL-8 interacts with its CXCR2 receptor on neutrophils to promote the release of NETs through the Src/Erk pathway (55).

The results of the study support the idea that in potters with high lead-exposure, neutrophil activation is being carrying out for the release of NETs. Several perspectives emerge from these

results. First, to better establish the relationship between blood lead and oxidative damage in the lead-exposed pottery population studied, potential candidates to be considered are the use of another biomarker of oxidative stress (e.g., 8-Hydroxydeoxyguanosine) and the evaluation of ROS. On the other hand, the employment of additional methods to evaluate NET production, such as the assessment of MPO-DNA or NE-DNA complexes and the use of fluorescence microscopy or flow cytometry to visualize the presence of NETs might reflect more consistent results. Finally, since the ERK signalling pathway is essential for the formation of NOX-dependent NETs, it would be interesting to evaluate the activation of this pathway to confirm the mechanism by which NETs are released.

Among the limitations of this study, we can mention that, although the number of individuals participating in this study is considered adequate for a first approach for the elucidation of the presence of NET markers in a lead-exposed population, a larger number of participants would provide greater robustness of the results. On the other hand, the self-selected sample of volunteers who agreed to participate in the study may not be representative of the population. Likewise, clinical variables of the population, such as hematic biometry, lipid profile, or blood pressure measurement, which may help to better understand the health status of the participants, were not evaluated.

Taken together, these results provide evidence that potters with blood lead levels ≥10 μg/dL produce and release NETs into the circulation. Follow-up of the lead-exposed population could help to elucidate whether the release of NETs contributes to the development of pathological processes. In addition, these results provide further evidence of the impact of lead on potters' health and may help strengthen regulations limiting the use of lead in pottery and encourage the adoption of lead-free methods. In this regard, there are programs that seek to reduce the use of lead in pottery and monitor workers' blood lead levels, such as Pure Earth's Approved Clay and the National Fund for the Promotion of Handicrafts (FONART). The gradual replacement of lead in glazes would improve the working and health conditions of potters. To date, this is the first study evaluating the production of NETs associated with environmental and occupational exposure to lead.

5. References

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Figure legends

Figure 1. Blood lead levels (BLL) from Tlaxcala potters and control group. Based on blood lead levels and according to NOM-047-SSA1-2011, Tlaxcala potters were divided into two groups: low-exposure group (<10 μg/dL) and high-exposure group (≥10 μg/dL). Data correspond to the mean ± standard deviation. One-way ANOVA with Tukey's post hoc test was used. **p<0.0001; *p<0.005.

Figure 2. Correlation analysis of quantitative variables and blood lead levels in Tlaxcala potters. *A*) Positive correlation between Daily working hours (number of hours worked in pottery daily) and BLL from the entire population of Tlaxcala potters. *B*) Positive correlation between the Employment years (number of years worked in pottery) and BLL from the entire population of Tlaxcala potters. Data were analysed by using Pearson correlation test. Values of *r* and *p* are indicated. A p value <0.05 was considered statistically significant.

Figure 3. Malondialdehyde (MDA) levels from Tlaxcala potters. MDA levels were determined using the 1M2F assay. MDA levels from controls as well as high and low lead-exposure groups are shown. Data correspond to the mean ± standard deviation. Data were analysed by using One-way ANOVA with Tukey's post hoc test. *p<0.05.

Figure 4. Myeloperoxidase, citrullinated histone H3 and cfDNA plasma levels from Tlaxcala potters. Plasma levels of *A*) MPO, *B)* citrullinated histone H3 and *C*) cfDNA from controls as well as high and low lead-exposure groups are shown. Data correspond to the mean ± standard deviation. Data were analysed by using One-way ANOVA with Tukey's post hoc test. *p<0.05, **p = 0.001, ***p<0.0001.

Figure 5. **Correlation analysis between malondialdehyde and myeloperoxidase, and between proteinic NET markers**. Correlation analysis was performed in both lead-exposure groups. A positive correlation between MDA and MPO plasma levels was observed in the *A*) high-exposure and *B*) low-exposure groups. A positive correlation between CitH3 and MPO plasma levels was observed in the *C*) high-exposure and *D*) low-exposure groups. Data were analysed by using Pearson correlation test. Values of *r* and *p* are indicated. A p value <0.05 was considered statistically significant.

Figure 6. **Correlation analysis between cell-free DNA and blood lead levels in potters.** A positive correlation between cfDNA levels and BLL from the entire population of Tlaxcala potters. Data were analysed by using Pearson correlation test. Values of *r* and *p* are indicated. A p value <0.05 was considered statistically significant.

Figure 7. Correlation analysis between cfDNA levels and proteinic NET markers in Tlaxcala Potters. In order to validate the presence of cfDNA as a NETosis surrogate, the correlation between cfDNA levels and proteinic NET marker (MPO, CitH3) levels were assessed. A positive correlation between *A)* cfDNA and MPO plasma levels, and between B) cfDNA and CitH3 plasma levels were found in the high lead-exposure group. Data were analysed by using Pearson correlation test. Values of *r* and *p* are indicated. A p value <0.05 was considered statistically significant.

Figure 8. Cytokines profile from Tlaxcala potters. Plasma concentrations of IL-1β, IL-6, IL-8, IL10, IL-12p70 and TNF-α were measured using the Human Inflammatory Cytokine Cytometric Bead Array Kit. Three different cytokines (IL-8, IL-6 and IL-12p70) were found above the limits of detection in eight potters belonging to the high lead-exposure group.

Table 1. Blood lead levels (BLL) in 50 potters from Tlaxcala.

				μg/dL			
N	**Mean**	**Minimum**	**Maximum**	**>5**	**>10**	**>15**	**>20**
50	12.04	2.95	30.22	96%	60%	18%	12%

Table 2. Descriptive statistics for the study population.

Variable	High-exposure group (>10 μg/dL) (n = 30)	Low-exposure group (≤10 μg/dL) (n = 20)	Relative Risk	p value
Age	42.67 ± 16.35	43.53 ± 14.33	N/A	0.8518[a]
Employment years	18.99 ± 12.47	18.19 ± 8.34	N/A	0.8079[a]
Years living in SPM	40.67 ± 17.79	43.50 ± 14.74	N/A	0.5727[a]
Daily working hours	8.72 ± 4.07	7.89 ± 2.64	N/A	0.6213[a]
Working days per week	5.86 ± 1.27	6.11 ± 0.85	N/A	0.7873[a]
Sex				
M	15	10	1.029	0.9055[b]
F	14	10		
Workshop location				
Inside	16	13	0.7882	0.2951[b]
Outside	14	6		
Potter parents				
Yes	13	7	1.147	0.5557[b]
No	17	13		
Use of lead				
Yes	13	7	1.170	0.5142[b]
No	15	12		
Use of PPE				
Yes	10	7	0.9907	0.9702[b]
No	19	13		
Smoker				
Yes	5	4	0.8889	0.6993[b]
No	25	15		
Cooking with lead-glazed earthenware				
Yes	16	13	0.8276	0.4129[b]
No	14	7		
Food storage in lead-glazed earthenware				
Yes	14	9	1.055	0.8213[b]
No	15	11		

[a]Analysis by independent t-test.

[b]Analysis by Chi-square test.

SPM: San Pablo del Monte.

PPE: Personal Protective Equipment.

Figure 1

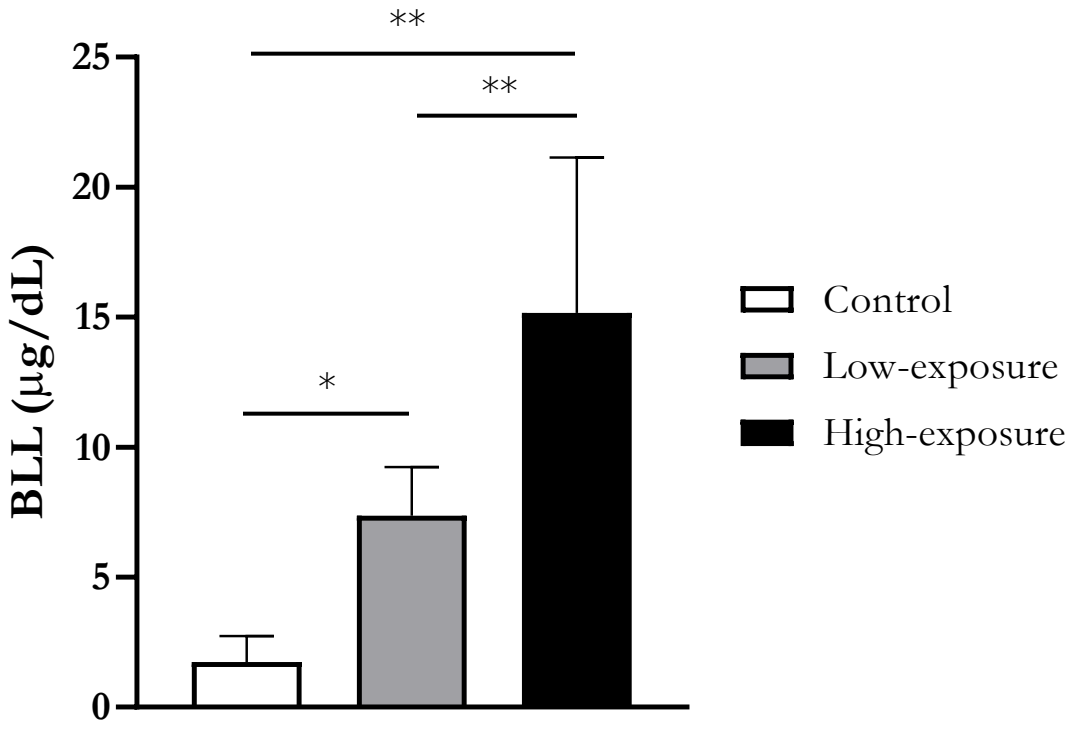
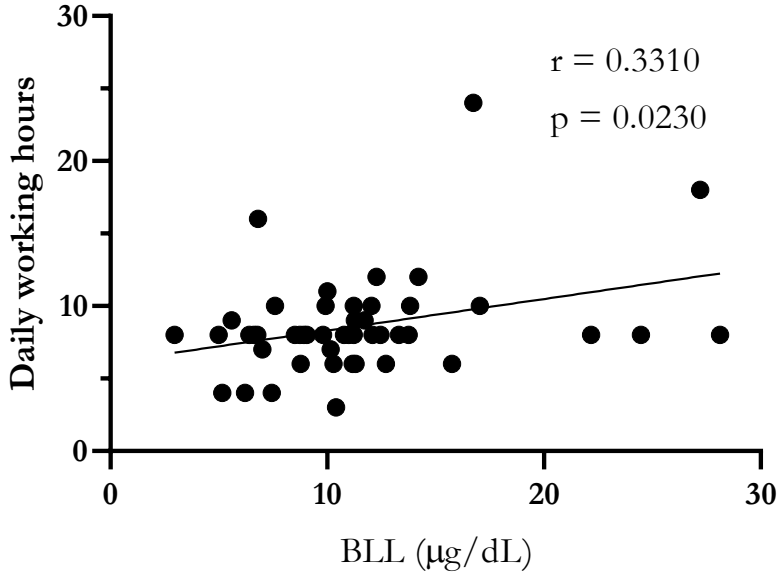


Figure 2

A


Daily working hours
30
20
10
0
0
10
20
30
BLL (μg/dL)
r = 0.3310
p = 0.0230

B

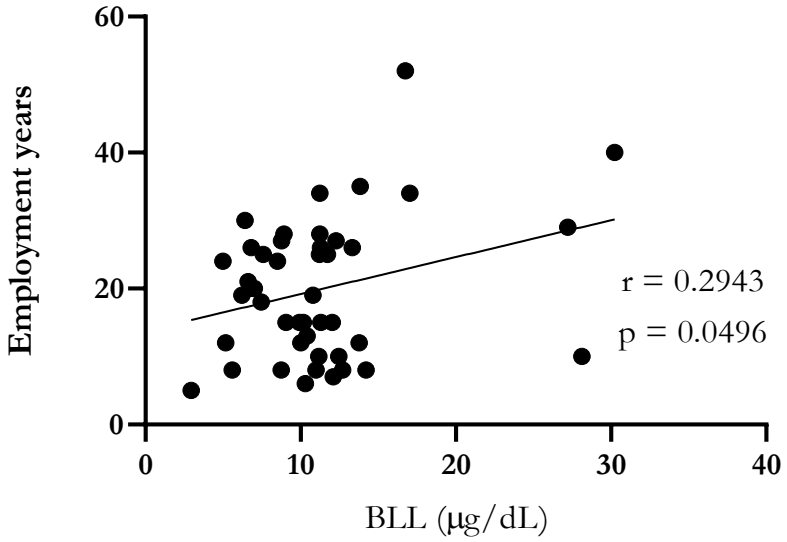

Employment years
60
40
20
0
0
10
20
30
40
BLL (μg/dL)
r = 0.2943
p = 0.0496

Figure 3

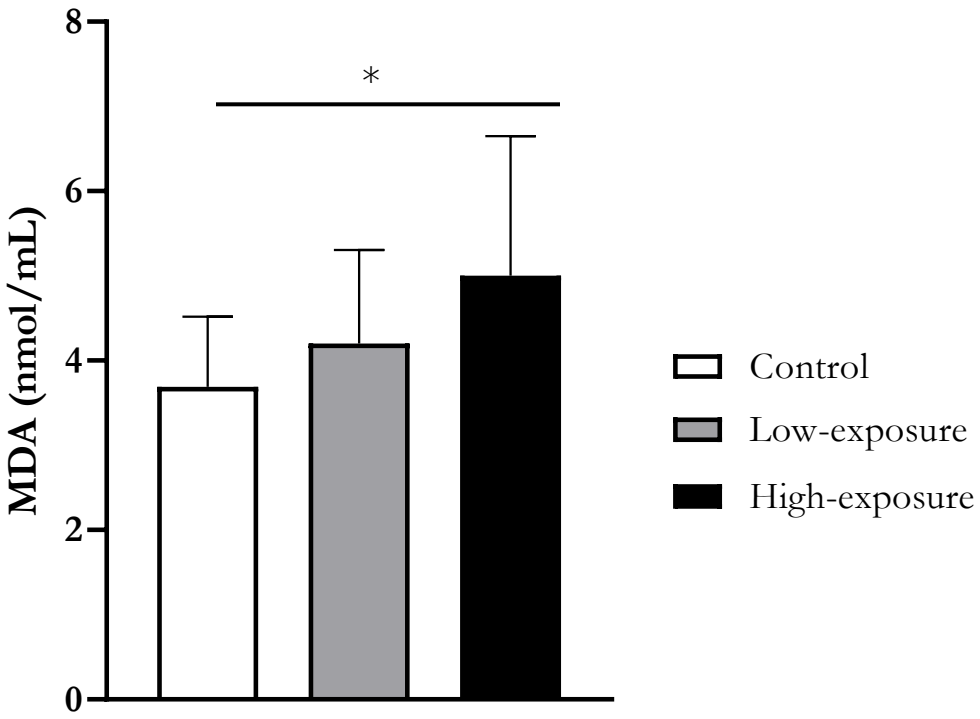


Figure 4

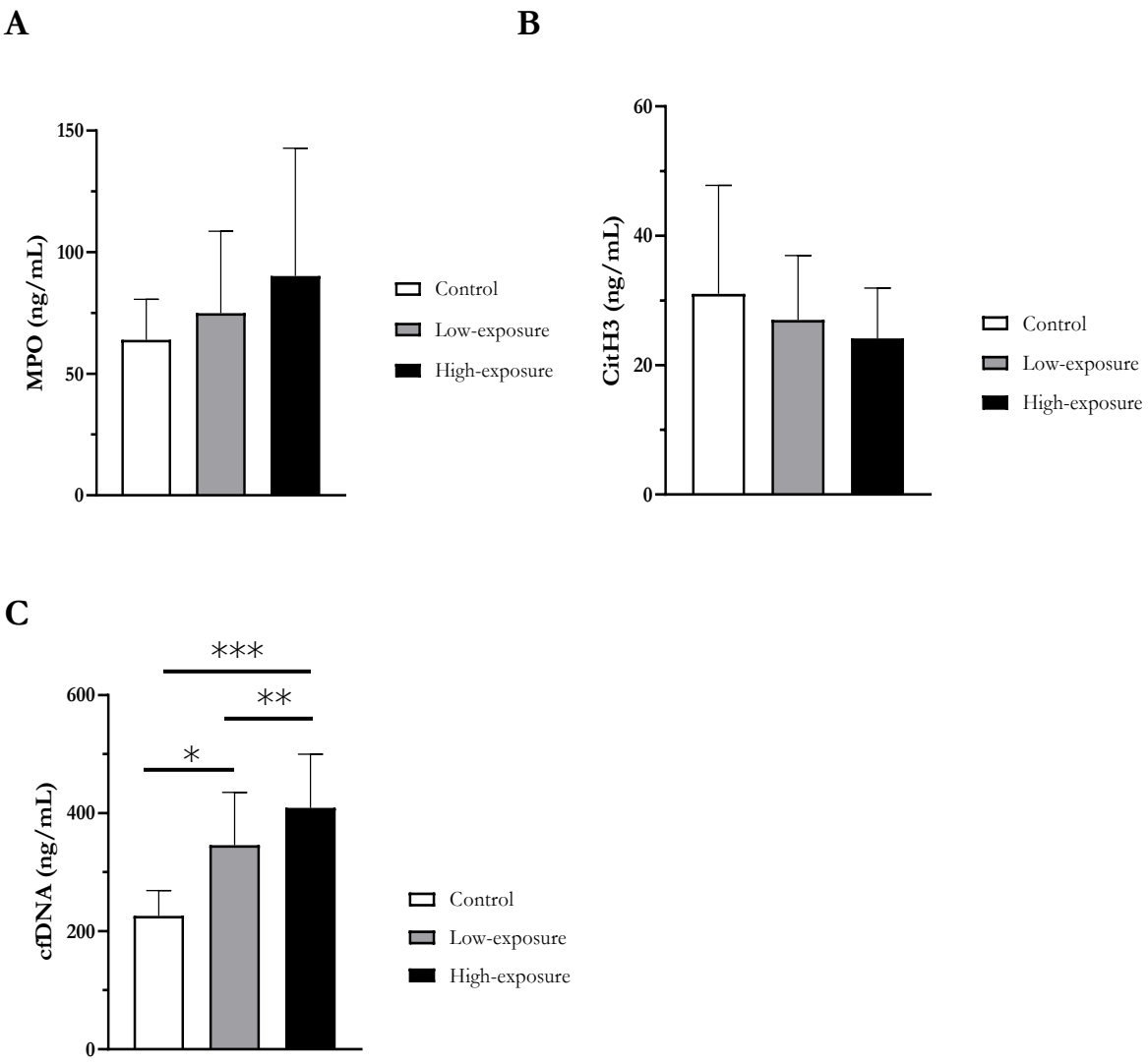

A
B
C
MPO (ng/mL)
150
100
50
0
Control
Low-exposure
High-exposure
CitH3 (ng/mL)
60
40
20
0
Control
Low-exposure
High-exposure
cfDNA (ng/mL)
600
400
200
0
***
**
*
Control
Low-exposure
High-exposure

Figure 5

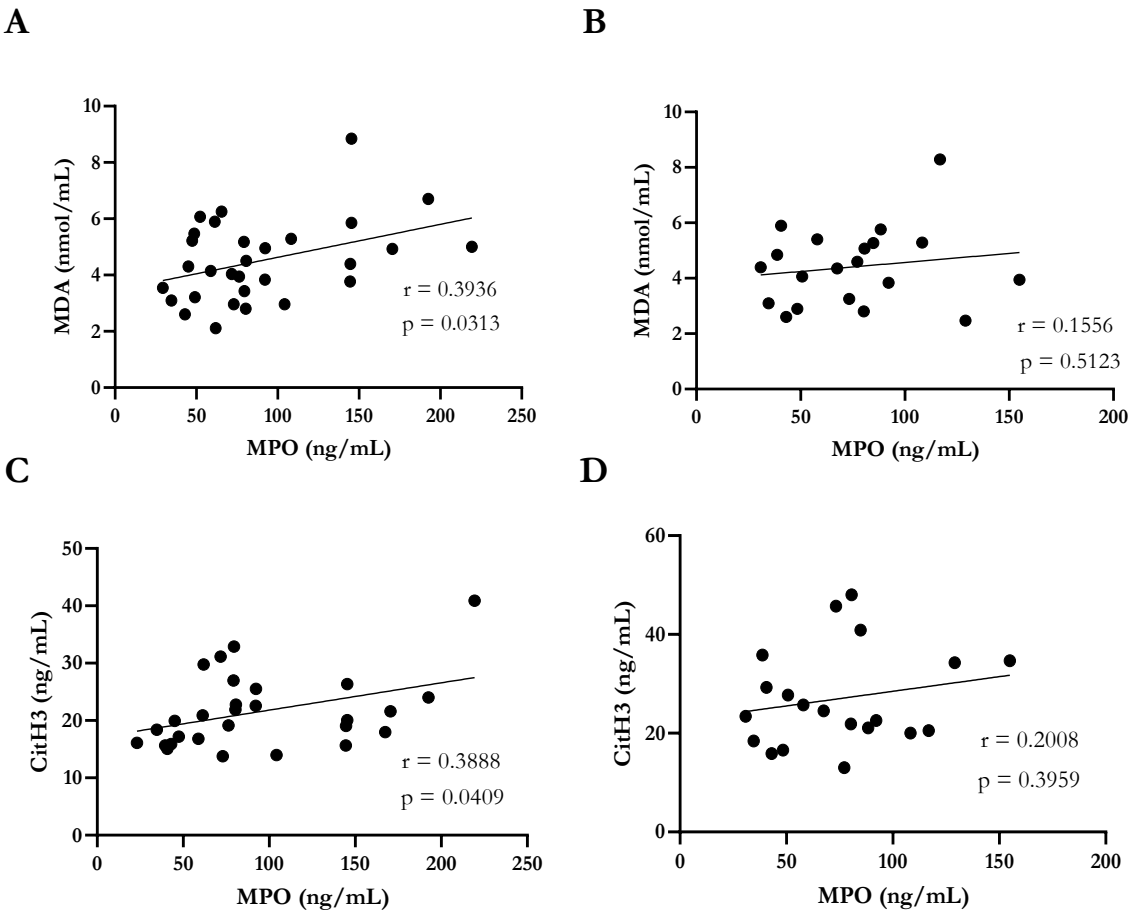

A
MDA (nmol/mL)
MPO (ng/mL)
r = 0.3936
p = 0.0313
B
MDA (nmol/mL)
MPO (ng/mL)
r = 0.1556
p = 0.5123
C
CitH3 (ng/mL)
MPO (ng/mL)
r = 0.3888
p = 0.0409
D
CitH3 (ng/mL)
MPO (ng/mL)
r = 0.2008
p = 0.3959

Figure 6

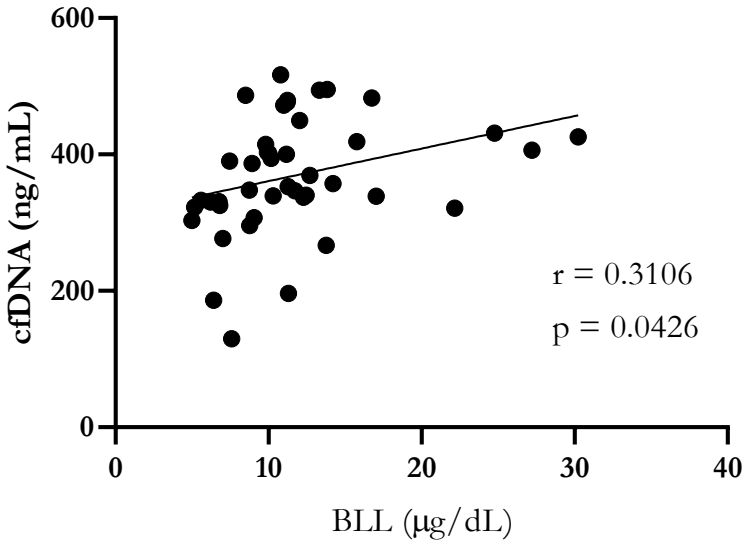
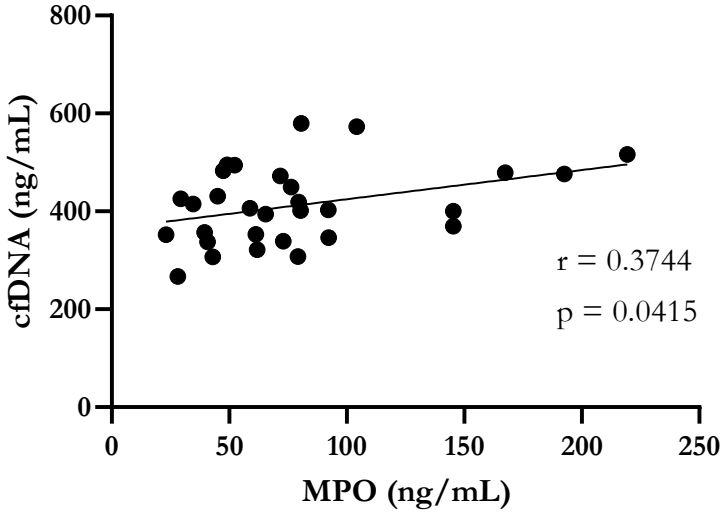


Figure 7

A


800
600
400
200
0
cfDNA (ng/mL)
0
50
100
150
200
250
MPO (ng/mL)
r = 0.3744
p = 0.0415

B

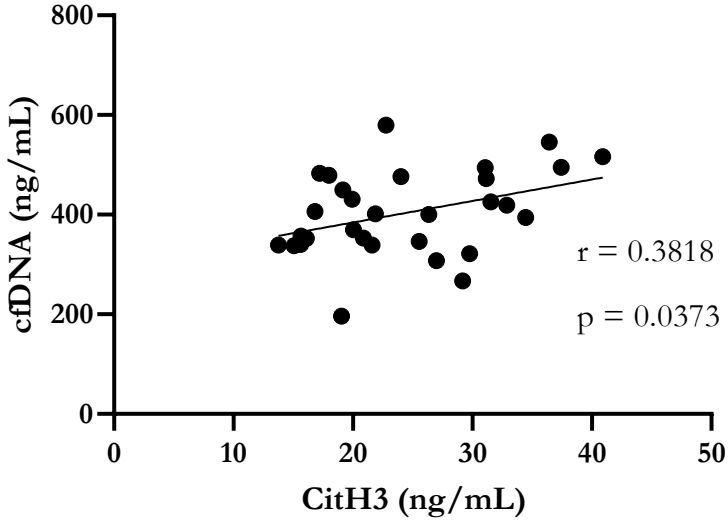
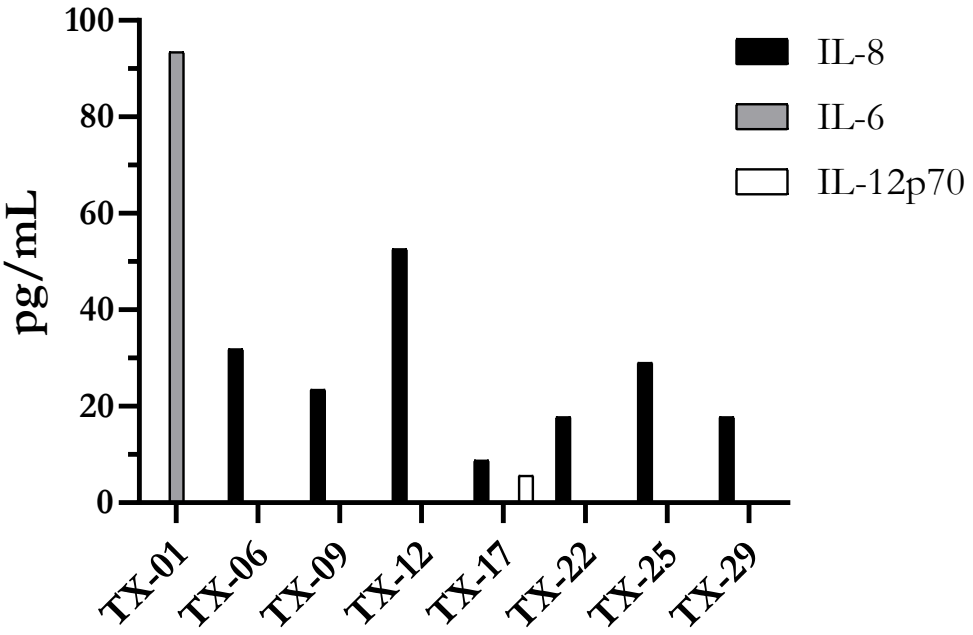

800
600
400
200
0
cfDNA (ng/mL)
0
10
20
30
40
50
CitH3 (ng/mL)
r = 0.3818
p = 0.0373

Figure 8


100
80
60
40
20
0
pg/mL
IL-8
IL-6
IL-12p70
TX-01
TX-06
TX-09
TX-12
TX-17
TX-22
TX-25
TX-29